INTERNATIONAL JOURNAL OF HEALTH & ANIMAL SCIENCE & FOOD SAFETY

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE PER LA SALUTE, LA PRODUZIONE ANIMALE E LA SICUREZZA ALIMENTARE

Ammonia concentration and bacterial evaluation of feline whole blood and packed red blood cell units stored for transfusion

Eva Spada[1,*], Daniela Proverbio[1], Piera Anna Martino[2], Roberta Perego, Luciana Baggiani[1], Nora Roggero[1]

[1] Veterinary Transfusion unit (REV), Department of Health, Animal Science and Food Safety (VESPA), University of Milan, Milan, Italy

[2] Microbiology and Immunology Unit, Department of Veterinary Science and Public Health (DIVET), University of Milan, Milan, Italy

KEYWORDS

Feline; Transfusion medicine; Whole blood unit; Packed red blood cell unit; Ammonia; Bacterial contamination.

PAGES

15 – 23

REFERENCES

Vol. 1 No. 2 (2014)

ARTICLE HISTORY

Submitted: September 23, 2013
Revised: October 20, 2014
Accepted: October 21, 2014
Published: October 23, 2014

CORRESPONDING AUTHOR

Eva Spada,
Veterinary Transfusion unit (REV) - Department of Health, Animal Science and Food Safety (VESPA) - University of Milan

Via Celoria 10, 20133 Milan, Italy

e-mail: eva.spada@unimi.it
phone: +39 02 50318188
fax: +39 0250318171

JOURNAL HOME PAGE

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ABSTRACT.

Ammonia concentrations increase in human, canine and equine whole blood (WB) and packed red blood cell (PRBC) units during storage. The aim of this study was to determine the effect of storage on ammonia concentration in feline WB and PRBC units stored in a veterinary blood bank and to evaluate possible correlations with bacterial contamination. Ammonia concentration was evaluated in 15 WB units and 2 PRBC units on day 1 and at the end of storage after 35 and 42 days, respectively. In an additional 5 WB units and 4 PRBC units ammonia concentrations were determined daily until the day the normal reference range was exceeded and then weekly to the end of storage. All units were evaluated for bacterial contamination. Ammonia increased markedly during storage as a linear function over time. On the 35th and 42th day of storage at 4±2°C (mean±SD) ammonia concentration reached 909±158 µg/dl and 1058±212 µg/dl in WB and PRBC units, respectively. Bacterial culture was negative in all units. High ammonia concentrations in stored WB and PRBC units could result in toxicity, particularly in feline recipients with liver failure, portosystemic shunts or those receiving large transfusion volumes. Clinical in vivo studies evaluating the effects on recipients should be performed.

1 Introduction

In stored units containing RBCs as whole blood (WB) or packed red blood cell (PRBC) units, deamination of adenine, and plasma and intra-erythrocytic protein leads to ammonia formation (Conn, 1962). Ammonia concentration has been shown to increase during storage of human (Barta & Babusikova, 1982), canine (Waddell et al, 2001) and equine (Mudge *et al*, 2004) WB, PRBC and plasma units collected for transfusion purposes. The increased ammonia concentration may result in ammonia toxicity in the recipient of stored units, particularly in patients with liver failure, portosystemic shunts or in recipients receiving large transfusion volumes.

Ammonia concentration (AC) may increase because of contamination or deterioration of blood components during storage. Ammonia is unstable in blood samples. In addition, after blood sample collection urea is exposed to air and begins to break down to ammonia (Conn, 1966). Certain aerobic bacteria such as *Escherichia coli*, *Klebsiella*, *Proteus*, and *Pseudomonas* spp. are known to be potent ammonia producers (Washabau & Day, 2013).

In recent years feline transfusion medicine has become increasingly important with more cats receiving blood or blood components and more veterinary blood banks preparing and storing feline WB or PRBC units worldwide for transfusion purposes. It is therefore useful to have information about the quality and characteristics of feline blood components. To the authors' knowledge AC has not been evaluated in units of feline WB or PRBC stored for transfusion purposes.

The aim of this study was to investigate the effect of storage on AC in feline WB and PRBC units prepared and stored for transfusion use, and to evaluate the influence of bacterial contamination on AC in WB and PRBC units. Our hypothesis was that ammonia concentration increases much more during storage in feline units containing RBCs than in the canine units due to the different blood collection method in cats (open system), that exposes blood to air during collection and different processing that increases the risk of bacterial contamination.

2 Materials and Methods

2.1 Blood collection

This prospective *in vitro* study was performed as internal quality control at the Veterinary Transfusion Unit (REV) of University of Milan. For this reason ethics approval by a specific committee is not required.

Informed owner consent was obtained to collect 10 ml/kg (with a maximum of 60 ml/cat) of blood from anesthetized (5 mg/kg tiletamine and zolazepam intramuscolarly, ZOLETIL 100, VIRBAC, Milan, Italy) healthy adult feline blood donors at the Veterinary Transfusion Unit (REV) of University of Milan.

Blood (10 ml/kg to a maximum volume of 60 ml) was collected, via a 19G butterfly needle, into 20-ml syringes containing citrate-phosphate-dextrose-adenine-1 (CPDA-1) anticoagulant in a ratio CPDA1 : blood of 1 : 7 (Spada *et al*, 2014).

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CPDA1 anticoagulant was taken from human sterile 450 ml blood bags (TERUMO CPDA-1 single blood bag, TERUMO EUROPE, Leuven, Belgium).

2.2 Production and storage of WB and PRBC units

For WB units the anticoagulated blood was transferred to a 150 ml transfer bag (TERUFLEX® Transfer Bag, TERUMO EUROPE, Leuven, Belgium).

PRBC units were obtained after sedimentation of the RBCs in the three syringes, removal of plasma and transfer of RBCs into a 150 ml transfer bag filled with 10 ml of the nutritive additive solution contain saline, adenine, glucose and mannitol (SAGM), taken from human sterile bags (TERUMO TRIPLE BLOOD BAG, TERUMO EUROPE, Leuven, Belgium).

Blood units were stored in a controlled-temperature blood bank refrigerator (EMOTECA 250, FIOCCHETTI & CO, Luzzara, Italy), where the temperature was consistently maintained at 4±2°C. WB and PRBC units were stored horizontally and mixed gently 2-3 times a week to maximize exposure of cells to the preservative solution. Blood samples for all analyses were taken from segments attached to the blood bags.

2.3 Ammonia Concentration evaluation

Firstly, in order to confirm the hypothesis that ammonia increases in WB and PRBC feline units at the end storage, AC was determined in 15 WB units (approx. 60 ml) and in 2 PRBC units (approx. 40 ml) on the day of preparation (D1) and after 35 (D35) and 42 days of storage (D42), respectively.

In a second study that aimed to verify the trend in ammonia increases, AC was measured in 5 new WB units and in 4 new PRBC units on D1 (within 12 hours of donation) and daily until AC exceeded the feline (normal) reference range (100 µg/dl) (Willard & Twedt, 2012), and then weekly (at D7, D14, D21, D28) until D35 for WB units and D42 for PRBC units. The products considered in the first and second part of the study were different and collected from different feline blood donors. The number of units tested depended on the units available during the study.

AC was measured with a point-of-care portable analyzer (Ammonia Checker II, Menarini, Florence, Italy) based on a microdiffusion method. Except for the first 15 WB units, all blood samples in which AC exceeded the upper limit of the assay (465 µg/dl) were diluted with bi-distilled water and retested to obtain an AC end point value. Internal validation of the point-of-care portable analyzer for measurement of ammonia on feline whole blood was performed (data not shown).

2.4 Bacterial evaluation

To evaluate bacterial contamination microbiological analysis was performed on all WB and PRBC units on D1, and on D35 and D42 of storage, respectively. From 200 to 500 microliters of blood samples were seeded aseptically in Tryptic Soy Broth (Oxoid, Italy) with a ratio of 1:10. Then tubes were incubated at 37 °C for 24 hours under aerobic atmosphere. After the

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incubation time, if the broth-culture was limpid (negative) it was incubated again at the same conditions for 24 hours (until 72 hours). Otherwise, if the culture was positive (turbid), 100 microliters of the broth was plated onto blood-agar plates (Oxoid, Italy) and incubated at 37 °C for 24 hours under aerobic condition. Then colonies, eventually grown on plates, were identified by macroscopic and microscopic evaluation (e.g. Gram stain), biochemical tests and using selective media (e.g. MacConkey Agar for Enterobacteriaceae, Mannitol Salt Agar for Staphylococcaceae).

2.5 Statistical analysis

Results have been expressed as mean ± SD. Data were tested for normality using the Kolmogorov-Smirnov test. In the first study on 15 WB units and 2 PRBC units, mean ACs was compared using a Mann Whitney Rank Sum test. In the second study (on 5 WB units and 4 PRBC units), mean ACs at each sampling point was compared using one way repeated measures ANOVA for multiple comparisons. Correlation and regression of AC and storage time were evaluated by Pearson correlation and simple linear regression, respectively. Mean AC values between WB and PRBC units were compared using t-test. $P<0.05$ was considered statistically significant. Data analysis was performed using statistical software (MEDCALC® software, version 12.7.0, Mariakerke, Belgium).

3 Results

In the first study, in 15 evaluated WB units AC increased progressively from 99±78 µg/dl (range 28-251 µg/dl) on D1 to over 465 µg/dl on D35 in all units.

ACs, expressed as mean ± SD and range (min-max) in 5 WB units on different days of storage are reported in Table 1. In these WB units AC exceeded the normal range on D1 in one unit, on D2 in three units and on D3 in one unit. There was a significant difference in the median AC measured every 7 days between D1 and D14 ($P = 0.009$), D21 ($P = 0.004$), D28 ($P = 0.001$), and D35 ($P = 0.023$), between D7 and D35 ($P = 0.009$), and between D14 and D28 ($P=0.002$), but not between other comparisons ($P > 0.05$).

AC in WB units was highly correlated with storage time ($R2 = 0.96$, $P < 0.0001$, 95% CI 0.92 - 0.98) and increased over time in a linear fashion ($y = 25X + 86$, $P < 0.0001$). Relative to the first 2 PRBC units evaluated, AC increased from 87±2 µg/dl (range 86-89 µg/dl) on D1 to 898±210 µg/dl (range 750-1047 µg/dl) on D42 ($P < 0.001$).

ACs, mean ± SD and range in 4 PRBC units on different days of storage are reported in Table 2. In the PRBC units considered in the second study, AC exceeded the normal range on D2 in three units and on D3 in one unit, and was not determined in two units. There was significant difference in the median AC measured every 7 days between D1 and D7 ($P = 0.029$), D14 ($P = 0.002$), D21 ($P = 0.041$), D28 ($P = 0.007$), D35 ($P = 0.011$), and D42 ($P = 0.0002$); between D7 and D14 ($P = 0.015$), D21 ($P = 0.037$), D28 ($P = 0.002$), D35 ($P = 0.005$), and D42 ($P = 0.003$); D14 and D28 ($P = 0.018$), and D42 ($P = 0.001$); D28 and D42 ($P = 0.022$), but not between other comparisons ($P > 0.05$).

AC in PRBC units was highly correlated with storage time ($R2 = 0.93$, $P < 0.0001$, 95% CI 0.86 - 0.97) and increased over time in a linear fashion ($y = 25X + 98$, $P < 0.0001$).

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Table 1. Mean ± standard deviation (SD), range (min-max) of ammonia concentration (µg/dl) in 5 feline WB units on different days of storage in a veterinary blood bank refrigerator

Days of storage (D)	AMMONIA (µg/dl)		
	Mean	±SD	Range (min-max)
D1	64	24	34-102
D2	121	30	77-158
D3	171	68	108-265
D7	296	135	177-288
D14	490	71	364-560
D21	532	67	471-613
D28	833	96	674-919
D35	909	158	738-1151

There was no significant difference ($P > 0.05$) between mean AC in WB and PRBC at different points during storage (Figure 1). No aerobic bacterial growth occurred in any WB and PRBC unit analyzed on D1 and D35 and D42 of storage.

Table 2. Mean ± standard deviation (SD), range (min-max) of ammonia concentration (AC, µg/dl) in 4 feline PRBC units on different days of storage in a veterinary blood bank refrigerator**.** Values at D1 and D42 were calculated considering also the 2 PRBC units used in the first study.

Days of storage (D)	AMMONIA (µg/dl)		
	Mean	±SD	Range (min-max)
D1	66	23	35-89
D2	127	32	72-157
D7	243	41	202-293
D14	503	46	456-547
D21	665	234	421-872
D28	914	139	779-1068
D35	1018	65	959-1087
D42	1058	212	750-1412

ISSN: 2283-3927

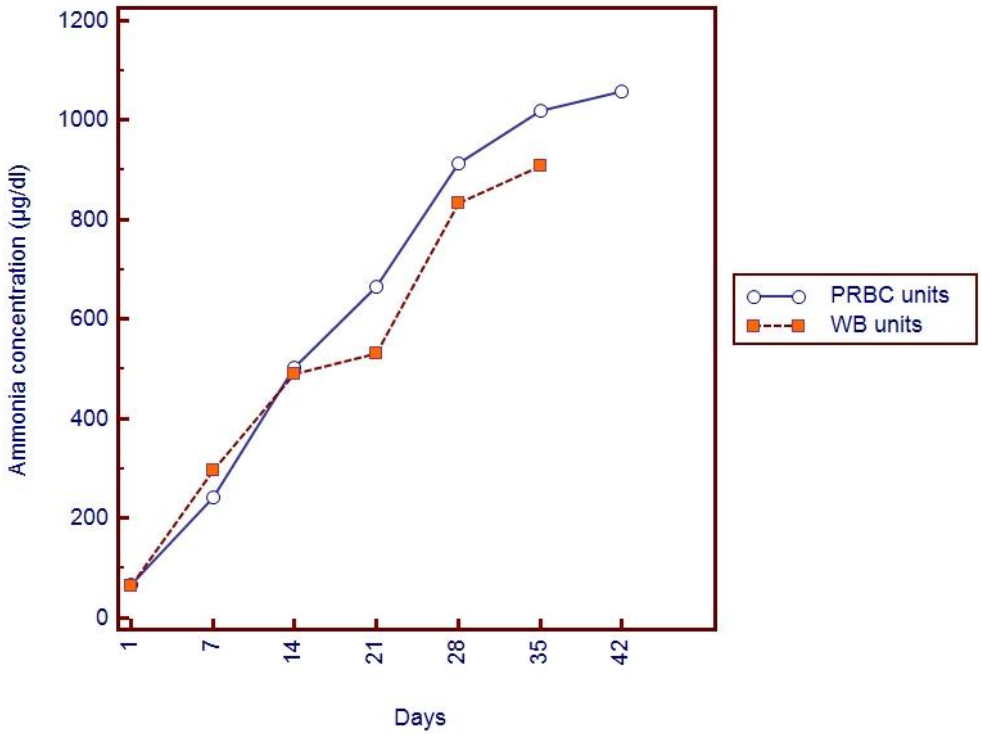
4 Discussion

As in human (Barta & Babusikova, 1982), canine (Waddell *et al*, 2001) and equine (Mudge *et al*, 2004) blood and plasma units, the AC in stored feline WB and PRBC units increased markedly during storage in this study.

We hypothesized that AC in feline WB and PRBC units collected and stored for transfusion purpose would increase much more than in canine blood units due to the open blood collection system used in cats. In an open system collection, blood is in contact to the air during collection and transfer. When urea is in contact with air after sample collection it begins to break down to ammonia. This breakdown is sufficient to elevate the ammonia concentration considerably resulting in spurious hyperammonemia (Conn, 1966). Open system collection may also predispose to bacterial contamination, and Gram-negative enteric bacteria such as *Escherichia coli*, *Klebsiella*, *Proteus*, and *Pseudomonas* spp. are known to be potent ammonia producers (Washabau & Day, 2013).

Contrary to our hypothesis, the increase in AC in feline units was similar to that reported in canine PRBC (Barta & Babusikova, 1982). No significant difference ($P > 0.05$ at t-test) was found between 4 canine PRBC units stored in a similar controlled-temperature blood bank refrigerator in a previous study (Barta & Babusikova, 1982) and the mean values of feline PRBC AC at D1, D7, D14, D21, D28 of our study.

Figure 1. Mean ammonia concentrations (AC) in 5 feline WB units and 6 PRBC units evaluated weekly during storage in a blood bank refrigerator. Mean AC for PRBC units was calculated using the two PRBC units of the first study (D1 and D42) and the four PRBC units of the second study (for the data on D1, D7, d14, d21, D28, D35 and D42).



ISSN: 2283-3927

Open system collection does not seem to predispose to bacterial contamination since no bacterial growth was achieved in any of the 26 units tested, as previously documented in feline units collected with an open system at the University of Berlin (Weingart *et al*, 2004), where blood products have been stored successfully without microbial growth if blood banking is done by experienced staff as in our study. High ACs in our study do not appear to be the result of microbiological contamination with ammonia producing bacteria, since aerobic cultures were negative in all 20 feline WB and 6 PRBC units analyzed on day of collection and on the expiration day of the units.

AC may increase because of deamination of proteins such as glutamine, breakdown of adenyl pyrophosphate and/or adenylic acid, or hydrolysis of other ammoniogenic substances (Conway & Cooke, 1939). Adenine, which is added to PRBC in the red cell extender SAGM, and in CPDA anticoagulant as used in our study, may be the major substrate for *in vitro* ammonia formation in the feline WB and PRBC units analyzed. The slightly greater increase in AC in our feline PRBC units (that contain more adenine, which is present either in CPDA-1 anticoagulant than in the nutritive solution SAGM) compared to that in WB units (which contain only CPDA-1) supports the theory that adenine breakdown could be a major factor in increased ACs in stored blood bags.

Toxic ammonia levels are not known in cats (Willard & Twedt, 2012). In the feline WB units analyzed AC rapidly exceeded the normal feline range (within 3 days of storage) in all units evaluated. In this study the highest AC measured in a WB unit was 1151 µg/dl (D35) and 1412 µg/dl in a PRBC unit (D42). Assuming no metabolism or distribution from the intravascular space and a feline blood volume of 62 to 66 ml/kg (median 64 ml/kg) (Wellman et al, 2012), the plasma AC in a 4 kg cat receiving one WB unit (approx. 60 ml) with an AC of 1151 µg/dl or one PRBC unit (approx. 40 ml) with an AC of 1412 µg/dl would be expected to increase by 270 µg/dl and 220 µg/dl, respectively.

Samples for AC evaluation and for bacterial contamination in this study were taken from segments rather than the blood bags itself then that could represent a different microenvironment as demonstrated in a recent human study with different results for hematocrit, hemoglobin content, hemolysis, hematologic indices, and adenosine triphosphate concentration in samples taken from the bags and from the segments (Kurach *et al*, 2014). The bags are gas permeable whereas the segment line is made of a different material. More importantly, if few bacteria made it into a blood unit then they are more likely to be in the bag rather than the line and so lack of contamination in the segment does not indicate lack of bag contamination. However, nondestructive testing offers several advantages, including the possibility of continuing storage of the units after testing without a shortened expiry time. This is a potential limitation of the current study and should be addressed in future studies.

Others limitations of this study were that anaerobic cultures were not performed although some anaerobes can synthesize ammonia. The accuracy of the ammonia analyzer used in this study has only been evaluated in dogs, although in this species results correlated well with the results from the standard enzymatic method in all AC ranges (Sterczer *et al*, 1999). However, internal validation in our laboratory (data not shown) confirmed that the analyzer was also accurate in feline samples. Finally, in our study blood units were stored in a blood-banking refrigerator with a temperature recorder and the refrigerator was not opened more than twice a day for the duration of storage. Temperature has been demonstrated to be one of the most important factors affecting the increase in AC in stored blood bags (Conn, 1962; Waddell

  ISSN: 2283-3927

et al, 2001). This may be important since, in most veterinary private practices, temperature control may not be closely regulated during blood storage. It is possible that temperature fluctuations will be more significant in a small private veterinary practice refrigerator leading to higher AC in stored units.

5 Conclusions

In conclusion, this is the first study that shows that ammonia increases markedly, and linearly, with time of storage in feline WB and PRBC units stored for transfusion purpose. There is a risk of ammonia toxicity in the recipient of stored units, particularly in feline recipients with liver failure, portosystemic shunts or those receiving large transfusion volumes. However, further clinical *in vivo* studies evaluating the effects on recipients need to be performed.

6 Funding acknowledgment

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

7 Conflict of interest

All the authors declare no conflicts of interest.

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  ISSN: 2283-3927