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In Vitro Increase of Mean Corpuscular Volume Difference (dMCV) as a Marker for Serum Hypertonicity in Dogs

Jennifer M. Reinhart, DVM; Misty R. Yancey, BS; Lisa M. Pohlman, DVM, MS, DACVP; Thomas Schermerhorn, VMD, DACVIM (SAIM)

From the Departments of Clinical Sciences (Reinhart, Yancey, Schermerhorn) and Diagnostic Medicine and Pathobiology (Pohlman), College of Veterinary Medicine, Kansas State University, Manhattan, KS.

Address correspondence to Dr. Jennifer M. Reinhart:

E-mail: jmreinha@vet.k-state.edu

Telephone number: 785-532-5690

Fax number: 785-532-2252

Abstract

Spurious increase in erythrocyte mean corpuscular volume (MCV) on automated cell analyzers is a well-characterized lab error in hypertonic patients. A difference between automated and manual MCV (dMCV) greater than 2fl has been shown to predict hypertonicity in humans. The purpose of this study was to investigate dMCV as a marker for serum hypertonicity in dogs and

to examine the relationship between dMCV and three methods of estimating serum tonicity: measured (OsM_M), calculated (OsM_C), and calculated effective (OsM_{CE}) osmolalities. OsM_C, OsM_{CE}, and dMCV were calculated from routine blood values and OsM_M was directly measured in 121 dogs. The dMCV of hypertonic dogs was significantly larger than that of normotonic dogs for all three osmolality methods. dMCV predicted hypertonicity as estimated by OsM_M better than it predicted hypertonicity as estimated by OsM_C and OsM_{CE}. A cut-off of 2.96fl yielded the best sensitivity (76%) and specificity (71%) for hypertonicity estimated by OsM_M.

Keywords: tonicity; osmolality; endocrinology; metabolism; sodium

Introduction

Serum hypertonicity is an important clinical problem in both human and veterinary medicine. Hypertonicity, also known as effective hyperosmolality, is defined as an elevated serum concentration of solutes that draw fluid out of cells by osmosis (i.e. effective osmoles or osmoles with tonic effect) (Wellman et al. 2012). At a tissue level, hypertonicity can lead to cellular dysfunction by altered function of the protein macromolecular apparatus, decreased protein synthesis, changes in cell membrane function, and breaks in nucleic acid strands (Garner and Berg 1994; Lang et al. 1998; Alfieri and Petronini 2007). At a systemic level, hypertonicity is associated with a variety of clinical conditions and has been investigated as an early indicator for progression of several disease states (Stookey et al. 2007a; Stookey 2005; Clegg et al. 2013; Stookey et al. 2004a; Stookey et al. 2004b; Schemerhorn and Barr 2006; Kotas et al. 2008).

In the Third National Health and Nutrition Examination Survey (NHANES III), a cross-sectional survey of non-hospitalized individuals, the prevalence of hypertonicity (OsM $\geq$ 300 mOsM) in human adults in the U.S. is estimated at 20% with borderline hypertonicity (OsM = 295-300 mOsM) affecting an additional 40% of the population (Stookey 2005). Higher prevalence has been associated with age, obesity, race, and diabetic

status (Stookey et al. 2007a; Stookey 2005). Hypertonicity may also be an early indicator for frailty, a condition of decreased functional reserve of the body and vulnerability to systemic morbidity, especially common in the elderly (Stookey et al. 2004b, Clegg et al. 2013). A longitudinal study of older adults found that plasma hypertonicity was an independent predictor for the onset of disability within a four-year period and mortality within an eight-year period (Stookey et al. 2004b). Hypertonicity is particularly important clinical problem for insulin-resistant and diabetic patients. In NHANES III, the prevalence of hypertonicity in human diabetics (identified by fasting glucose or oral glucose tolerance test or by self-reporting by the subject) was reported as 35-78% (Stookey 2005). In human hyperglycemic, pre-diabetic patients, hypertonicity has been shown to be a risk factor for progression to diabetes mellitus, with hypernatremia having an independent effect on disease progression (Stookey et al. 2004a). In diabetic dogs and cats, the prevalence of effective hyperosmolality was reported as 81% (Schemerhorn and Barr 2006). Interestingly, in diabetic animals, sodium (Na) concentrations appear to be highly associated with osmolality (tonicity), emphasizing the importance of this solute in diabetic patients (Schemerhorn and Barr 2006, Kotas et al. 2008).

Clinically, tonicity can be difficult to quantify because this value not only includes solute concentrations but encompasses the physiologic effects of multiple solutes on the cell. Tonicity can be approximated by osmolality (OsM), the number of total solutes or osmoles per kilogram solvent. In biologic (i.e. aqueous) solutions, osmolality is equivalent to osmolarity (osmoles/L solvent), which can be measured directly (measured osmolality, OsM_M) or calculated using commonly measured laboratory values (calculated osmolality OsM_C, Eq. 1). However, OsM_M is a quantification of all osmoles in solution, both effective and ineffective, without regard to tonic effect and thus may overestimate tonicity. An alternative approach is to calculate the calculated effective osmolality, OsM_{CE}, (Eq. 2) from commonly measured solutes known to be effective osmoles (sodium [Na], potassium [K], glucose). However, OsM_{CE} may underestimate tonicity because some serum osmoles that are not measured or included in the calculation may have a tonic effect. Calculated total osmolality (OsM_C) may fall prey to both these limitations because ineffective osmoles are included in its calculation (i.e. blood urea nitrogen [BUN]) and because some effective unmeasured osmoles may not be included in the calculated estimate (Wellman et al. 2012). Because it is impractical to measure every solute with known or possible tonic effect, true tonicity is almost impossible to

rigorously quantify. However, because tonicity exerts a physiologic effect (changes in cell size), a physiologic measurement of tonicity would be preferable to absolute quantification.

Spurious increase in red blood cell (RBC) mean corpuscular volume (MCV) is a well-characterized lab error that occurs when blood from hypertonic patients is assessed by an automated cell analyzer. In vivo, RBCs are acclimated to the hypertonic patient serum. In vitro, when RBCs from hypertonic patients are placed into the isotonic analyzer media, they swell because the analyzer solution is *relatively* hypotonic to the intracellular environment causing an intracellular fluid shift (Stockham and Scott 2008a). Stookey et al. (2007a) exploited this "lab error" to develop a new index for plasma hypertonicity. The MCV difference (dMCV) is the difference between the MCV as measured by an automated cell analyzer (MCV_M) and the MCV calculated from a spun hematocrit (hct), which is performed with RBCs in the original patient plasma (Eq. 3). A cutoff value of only 2 fl or greater for dMCV performed well as an indicator for hypertonicity and, when combined with elevated plasma vasopressin levels, yielded 100% sensitivity and specificity (Stookey et al. 2007b).

Use of dMCV to detect hypertonicity has not been investigated in veterinary patients. The purpose of the present study was to investigate dMCV as a marker for serum hypertonicity in dogs and to examine the relationship between dMCV and three methods of estimating serum tonicity: OsM_M, OsM_C, and OsM_{CE}. It was hypothesized that dMCV would be a useful marker for hypertonicity and that an elevated dMCV would predict hypertonicity estimated by OsM_{CE} better than it would predict hypertonicity estimated by OsM_M or OsM_C.

Materials and Methods

Cases – Patient records at the Kansas State University Veterinary Health Center were searched to identify all animals admitted to the small animal intensive care unit (SA-ICU) between February 1, 2012 and May 16, 2012. Records were identified using a SA-ICU charge as a search criterion for the hospital practice management software.[a] In addition, certain SA-ICU admissions from November 1, 2011 to January 31, 2012 had been identified for inclusion; on several days during this time period, the medical record numbers of all dogs present in the SA-ICU were recorded by hand and subsequently screened for study inclusion. Cases were included if the patient was a dog, if a complete blood

[a] Vetstar, Advanced Technology Corp.; Oak Ridge, TN

count (CBC) and a biochemistry profile were performed during the hospital visit, and if stored serum from the biochemistry profile was available for further analysis. Dogs were excluded if anemia was present. For the purposes of this study, anemia was defined as a low RBC concentration ([RBC] < 5.5 M/μl) on the CBC. This study was performed in accordance with the Kansas State University guidelines for animal research.

Clinical Pathology – All CBCs (ethylenediaminetetraacetic acid anticoagulated blood) and serum biochemistry profiles were performed by the Kansas State Veterinary Diagnostic Lab Clinical Pathology Laboratory within 24 hours of collection. All laboratory tests were performed by certified medical technologists. CBCs were performed using the Advia 2120 Hematology System;[b] spun hct using a microcentrifuge and card hct reader was included as a standard part of the CBC. Biochemistry profiles were performed using the COBAS C501 Chemistry Analyzer.[c] Serum samples were then frozen at –20°C until identified for use in the study at which point they were transferred to a -80°C freezer where they were stored until batch osmometry measurements could be made.

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[b] Siemens Medical Solutions, Inc.; Malvern, PA. OsM of the RBC diluent is 280 mOsM

[c] Roche Diagnostics; Indianapolis, IN

Serum OsM_M was measured in duplicate by freezing-point depression using the Micro-OSMETTE osmometer.[d]

Calculations – The following values measured as part of the CBC were included in calculation of dMCV: measured mean corpuscular volume (MCV_M), spun hct, and RBC. The following values measured as part of the serum biochemistry profile were included in serum osmolality calculations: Na concentration, K concentration, glucose concentration, and BUN concentration. OsM_C (Eq. 1) and OsM_{CE} (Eq. 2) were determined using standard clinical formulas (Wellman et al. 2012).

Eq. 1 $$OsM_C = 2(Na + K) + \frac{BUN}{2.8} + \frac{glucose}{18}$$

Eq. 2 $$OsM_{CE} = 2(Na + K) + \frac{glucose}{18}$$

The dMCV (Eq. 3) was calculated by as previously reported (Stookey et al. 2007b).

Eq. 3 $$dMCV = MCV_M - \frac{hct \times 10}{RBC}$$

Data Analysis – Continuous data are represented as median and range. dMCV data were assessed for normality using the Shapiro-Wilk test. For each method of estimating tonicity (OsM_M, OsM_C, and OsM_{CE}), dogs were categorized into a normosmolar group (OsM < 320 mOsM) and a hyperosmolar group (OsM ≥ 320 mOsM). Because only one dog would have been considered

[d] Precision Systems, Inc.; Natick, MA

hypoosmolar (OsM < 280mOsM) using measured osmolality (OsM_M = 279mOsM) and no dogs were hypoosmolar using OsM_C or OsM_{CE}, a hypoosmolar group was not included in analysis; the aforementioned dog was included in the normosmolar group for OsM_M analysis. For each method of estimating tonicity, the dMCV of the normosmolar group and the dMCV of the hyperosmolar group were compared using a Student's t-test. The ability of dMCV to predict hypertonicity, as estimated by each osmolality method, was determined using a receiver operator curve (ROC) curve; cut-off values to maximize sensitivity and specificity were visually determined. Area under the ROC curve (AUROC) was calculated for each osmolality method and these were compared.

A post hoc analysis was performed to establish a dMCV cut-off for mild hypertonicity, defined as $OsM_M \geq 300$ mOsM. The dMCV of normosmolar ($OsM_M < 300$ mOsM) and hyperosmolar ($OsM_M \geq 300$ mOsM) dogs were compared using a Student's t-test; an ROC curve was generated and a cut-off value maximizing sensitivity and specificity was visually determined. All statistical analyses were performed using commercial software.[e] A p-value less than 0.05 was considered statistically significant.

[e] Excel, Microsoft; Redmond, WA

Results

Two hundred and eighty-nine cases were identified from the records search (n = 255) or previously recorded (n = 34). One hundred and one cases were excluded because they were not dogs, did not have a CBC and biochemistry panel performed during hospitalization, or did not have stored serum available for further analysis. An additional 67 dogs were excluded because they were found to be anemic. One-hundred and twenty-one dogs were included in the final analysis. The median age of all dogs was 7.08 years (range 1 month to 16 years). Fifty-eight dogs were neutered males and 44 were neutered females; 14 dogs were intact males and five were intact females. Pertinent clinicopathologic values, measured osmolality, and calculated values are summarized in Table 1.

Regardless of the method used to estimate tonicity (OsM_M, OsM_C, OsM_{CE}), the dMCV for hyperosmolar dogs was significantly larger than the dMCV for normosmolar dogs (Table 2). ROC curves for dMCV predicting OsM_M, OsM_C, and OsM_{CE} are depicted in Figure 1. A dMCV of 2.96 fl, 2.47 fl, and 2.96 fl provided maximal sensitivity and specificity for predicting hypertonicity as estimated by OsM_M, OsM_C, and OsM_{CE},

respectively. dMCV best predicted hypertonicity as estimated by OsM_M (AUROC = 0.7738, Table 3).

The finding that dMCV could predict hypertonicity defined as $OsM_M \geq 320$ mOsM prompted the question whether dMCV might predict smaller elevations in measured osmolality. A post-hoc analysis was performed using hypertonicity defined as $OsM_M \geq 300$ mOsM. The dMCV for hyperosmolar dogs (2.82 fl; -2.60 – 6.92 fl) was significantly larger than that of normosmolar dogs (0.75 fl; -2.95 – 3.50 fl; $p < 0.001$). An ROC analysis was performed (AUROC = 0.8021; Figure 2); a dMCV cut-off of 1.49 fl yielded a 75% sensitivity and 76% specificity.

Discussion

The results of this study support the hypothesis that dMCV can be used as a physiologic marker for hypertonicity in hospitalized canine patients. This finding was anticipated because red blood cells acclimated in a hypertonic solution (i.e. patient plasma) swell when placed into isotonic solution (i.e. cell analyzer media). Interestingly, dMCV predicted serum hypertonicity best when tonicity was estimated using measured osmolality ($OsM_M \geq 320$ mOsM) rather than calculated effective osmolality (OsM_{CE}). This differs from the original hypothesis: it was suspected that dMCV would be a better marker for OsM_{CE} because, as a

physiologic marker of tonicity, dMCV should only be susceptible to changes in effective osmole concentrations; it should not be affected by changes in ineffective osmole concentrations, such as BUN, which are included in OsM_M and OsM_C. Indeed, dMCV did perform better for OsM_{CE} (AUROC = 0.7337) than it did for OsM_C (AUROC = 0.7063), which includes BUN as well as Na, K, and glucose. Thus, it can be inferred that dMCV is not heavily influenced by BUN and this may hold true for some or all other ineffective osmoles. However, dMCV predicted hypertonicity as estimated by OsM_M better than by either calculated method. The difference between measured and calculated osmolality is known as the osmolar gap and consists of all osmoles, effective and ineffective, not routinely measured. Because unmeasured osmoles are included in OsM_M and because dMCV performs best for predicting OsM_M, it is likely that at least a portion of the unmeasured osmoles act as effective osmoles, influencing dMCV and therefore the physiologic effects of hypertonicity on RBCs.

In this study, dogs were divided into normosmolar and hyperosmolar groups using 320 mOsM as a cut-off for OsM_M, OsM_C, and OsM_{CE}. This value was initially chosen in order to include only dogs with clinically relevant hypertonicity in the hyperosmolar group. However, in certain situations, it may be useful to identify mild hypertonicity as is the case in humans in

whom it was shown that mild hypertonicity ($OsM_M \geq 300$ mOsM) was a risk factor for frailty in the elderly and for progression of disease in pre-diabetic patients (Stookey et al. 2004b; Stookey et al. 2004a). Therefore, the canine data was reexamined to determine dMCV performance when mild hyperosmolality is present. Using a lower cut-off for dMCV (≥ 1.5 fl) for mild hypertonicity ($OsM_M \geq 300$ mOsM) performed as well as the higher dMCV cut-off (≥ 3 fl) did for overt hypertonicity ($OsM_M \geq 320$ mOsM). This gradated cut-off system may be useful in future longitudinal studies investigating disease progression or markers for various morbidities.

It is important to recognize that RBC size is not solely governed by tonicity, so changes in erythrocyte volume in response to hyper- or hypotonicity are complex and may be different than anticipated. Part of this phenomenon may be due to the biconcave shape of the mammalian erythrocyte. Changes in cell volume alter the conformation of the erythrocyte cytoskeleton leading to a change in cell membrane cell curvature (Wong 2006). Therefore, if cell volume is derived from measurements of cell diameter, volume may be underestimated. In the present study, RBCs were subjected to 'sphering' treatment to produce homogenous spheres by the cell analyzer prior to analysis, so biconcave structural effects were eliminated. The sphering technique is standard

practice for most RBC analyzers and has been shown to render accurate and repeatable measurements of RBC size (Kim and Ornstein 1983). Plasma and intracellular proteins exert an effect on fluid balance through oncotic pressure, which varies according to size, structure, and charge of the protein moieties. Because changes in net oncotic pressure can elicit fluid shifts independently of tonicity, plasma protein concentrations may alter cell size (in either direction) from values predicted based on plasma tonicity alone (Wellman et al. 2012). Albumin is the most important plasma determinant of oncotic pressure, contributing approximately 80% of the total plasma colloid osmotic pressure (Wellman et al. 2012). Finally, and most importantly, all mammalian cells have the ability to regulate volume through a variety of active processes, which allow the cell to acclimate during periods of osmotic stress as well as participate in a variety of metabolic functions (Lang et al. 1998; Alfieri and Petronini 2007; Haussinger 1996; Schliess et al. 2007). The cellular compensation for hypertonic-induced cell shrinkage is termed regulatory volume increase (RVI) and the response to hypotonic-induced cell swelling, regulatory volume decrease (RVD) (Alfieri and Petronini 2007; Schliess et al. 2007). Early responses to cell shrinkage or swelling include increased membrane ion transport followed by transport of small non-ionic organic molecules

(osmolytes) including alcohols, methylamines, and amino acids. These compensatory mechanisms precipitate a partial RVI or RVD within seconds to minutes of initial fluid shifts. The late response to tonic change involves activation (or inhibition) of a variety of cellular pathways that lead to the production of heat shock proteins and to novel synthesis of intracellular osmolytes. These processes require up- or down-regulation of gene expression, so complete volume compensation (RVI or RVD), if it occurs, takes hours to days (Lang et al. 1998). In this study, cell volume (MCV_M) was measured using an automatic cell analyzer, which completes its analysis within seconds. Therefore, it can be expected that any compensatory volume regulatory changes would be incomplete and measured cell volume would still reflect initial fluid shifts cause by tonic insult to the cells.

There are several limitations to the present study. First, the determinants of dMCV (hct, RBC, and MCV_M) were measured in plasma, whereas osmolality, electrolyte, BUN, and glucose measurements were made in serum, which may have affected associations between dMCV and the various methods of estimating tonicity. Ideally, all measurements would have been made in plasma because plasma is a better representation of the in vivo environment; however, serum is the preferred sample type for measurement of electrolytes and plasma samples were not

available at the time of sample retrieval for most dogs (Stockham and Scott 2008b). Another concern is that prolonged storage of frozen samples could introduce artifact in the measurement of serum osmolality. To the authors' knowledge, stability of frozen canine serum for osmometric analysis has not been reported. However, a prospective study that examined dMCV and serum osmolality using fresh samples analyzed concurrently showed results similar to those reported here; therefore, the present results are likely valid (Reinhart, unpublished). In this study, all blood samples were collected during SA-ICU hospitalization, but timing of collection was not standardized. It is likely that some patients received treatment prior to sample collection; the number and degree of interventions performed prior to collection also likely varied among patients. This limitation does not weaken the ability of dMCV to predict hypertonicity, but the summary data presented for dMCV, osmolality, and various clinicopathologic values should not be considered representative of a general SA-ICU population at admission. Ideally, all blood collection would have been performed at presentation prior to treatment; however, this was precluded by retrospective nature of the study. Similarly, the dogs in this study are a heterogeneous population of various breeds, ages, and disease processes. Future studies could include assessment of tonicity in specific disease states such as diabetes

mellitus; in general survey populations stratified by breed, sex, or age; in hospitalized populations stratified by disease category or severity; or in longitudinal studies tracking changes in tonicity over time, with disease progression, or during treatment.

In conclusion, the results of this study support the use of dMCV (≥ 3 fl) a physiologic marker for overt serum hypertonicity ($OsM_M \geq 320$ mOsM) in dogs. dMCV predicts hypertonicity estimated by measured osmolality better than by either calculated method, suggesting the influence of unmeasured effective osmoles in serum on dMCV.

Acknowledgements/Conflict of Interests

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Figure Captions

Figure 1 – ROC Curves for dMCV predicting hypertonicity (OsM $\geq$ 320 mOsM) as estimated by OsM_M (A), OsM_C(B), and OsM_{CE} (C)

Figure 2 – ROC curve for dMCV predicting mild hypertonicity ($OsM_M \geq 300$ mOsM)

Tables

Table 1 – Summary statistics of pertinent clinicopathologic values, measured osmolality, and calculated values.

	Median	**Range**	**Reference Range***	**Units**
Na	149	118 – 164	147 – 154	mmol/l
K	4.5	3.1 – 6.6	2.6 – 5.3	mmol/l
BUN	15	4 – 212	9 – 33	mg/dl
Glucose	109	30 – 1108	73 – 113	mg/dl
RBC	7.15	5.52 – 9.40	5.5 – 8.5	M/µl
hct	48	35 – 66	35 – 55	%
MCV_M	69	62 – 76	60 – 77	fl
OsM_M	307.0	279.0 – 510.5^	n/a	mOsM
OsM_C	319.4	296.0 – 377.1	n/a	mOsM
OsM_{CE}	314.4	291.1 – 344.2	n/a	mOsM
dMCV	2.37	-2.94 – 6.92	n/a	fl

*These are the reference ranges reported by the Kansas State Veterinary Diagnostic Lab, Clinical Pathology Laboratory.

^This value is unlikely to be the true osmolality of the sample, as a serum osmolality of 510 mOsM is incompatible with life, and likely represents technical error. However, this value was repeatable using the freezing-point depression osmometer so was included in the data set. Exclusion of this data point does not significantly alter the results of this study.

Table 2 – Comparison of dMCV for normosmolar and hyperosmolar dogs as determined by three osmolality methods.

	Normosmolar			**Hyperosmolar**			**P-value**
	Median	*Range*	*N*	*Median*	*Range*	*N*	
OsM_M	2.13 fl	-2.95 – 6.87	100	3.42 fl	-0.17 – 6.92	21	<0.001
OsM_C	1.79 fl	-2.95 – 6.25	66	3.02 fl	-2.60 – 6.92	55	<0.001
OsM_{CE}	2.20 fl	-2.95 – 6.92	99	3.55 fl	-2.60 – 6.80	22	0.001

Table 3 – dMCV cut-off for predicting hypertonicity

	Cut-off (fl)	**Sensitivity (%)**	**Specificity (%)**	**AUROC**
OsM_M	2.96	76	71	0.7738
OsM_C	2.47	67	68	0.7063
OsM_{CE}	2.96	73	71	0.7337