

DERANGEMENTS OF TONICITY AND IMPLICATIONS FOR VETERINARY PATIENTS

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## **Abstract**

Tonicity is property of a solution that is defined as the total effective (impermeable) osmole concentration that drives fluid movement across a semipermeable membrane via osmosis. Tonicity is related to but distinct from solution osmolality, which is a summation of all solute concentrations, regardless of the solute membrane permeability. In the mammalian body, tonicity is tightly regulated at both a cellular and systemic level; tonic derangements cause rapid change in cell and tissue volume leading to significant dysfunction. Input from the central nervous, circulatory, endocrine, gastrointestinal, and urinary systems are integral to osmoregulation, so many diseases in veterinary medicine are associated with tonicity disorders. However, because the homeostatic mechanisms that control tonicity overlap with those regulating electrolyte and acid-base balance as well as hydration and vascular volume, tonic consequences of disease can be difficult to isolate. Understanding of disease-associated changes in tonicity is further complicated by the fact that the tonic contributions of many solutes that accumulate in disease are unknown. Additionally, direct assessment of tonicity is difficult because tonicity is not just a physiochemical property, but it implies a physiologic effect. Thus, simple summation of osmole concentrations is an inadequate measurement of tonicity.

The following report includes three studies investigating various aspects of tonicity as it applies to veterinary patients. Chapter 2 reports a study that examines the tonic effects of ketoacids and lactate using two different in vitro red blood cell assays. Results demonstrated that the ketoacids, beta-hydroxybutyrate and acetoacetate, behave as ineffective osmoles while the tonic behavior of lactate is variable, implying a more complex cellular handling of this anion. Two additional studies examine whether the mean corpuscular volume difference (dMCV) is a novel clinical marker for hypertonicity in dogs. Results of separate retrospective (Chapter 3) and prospective (Chapter 4) studies provide evidence that dMCV is a useful clinical marker for hypertonicity in dogs.

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# **Chapter 1 - Physiology and Pathophysiology of Tonicity**

## **Osmolality and Tonicity**

The terms osmolality and tonicity are often used interchangeably, however, these two concepts differ in subtle yet important ways. In an aqueous solution, solutes can be classified as either effective osmoles or ineffective osmoles. Effective osmoles are solutes that are impermeable to the cell membrane. Because effective osmoles do not readily cross the cell membrane, they exert osmotic pressure causing fluid shifts between compartments that are separated by the semi-permeable plasma membrane. Ineffective osmoles are solutes that are permeable to the cell membrane and so do not exert osmotic pressure. Although the chemical properties of a solute have some influence, cell permeability is largely determined by the properties of the cell membrane. The expression and concentration of various ion channels and substrate transporters in the cell membrane, both specific for the solute of interest and general non-specific transporters, determine the relative “effectiveness” of that solute as an osmole.

Osmolality of a solution is the term used to describe the total number of solutes (osmoles) per kilogram of solvent (i.e. water in biologic systems). Osmolality is a measure of total solute concentration, but it does not distinguish between effective and ineffective osmoles; therefore, osmolality cannot accurately predict fluid shifts between the intra- and extracellular spaces. Tonicity, also called effective osmolality, is the number of *effective* osmoles per unit of solvent. Thus a change in tonicity of one fluid compartment implies that a fluid shift will occur across the cell membrane. Tonicity of solutions is usually discussed in relative terms. By convention, extracellular fluid (ECF) tonicity is expressed relative to that of the intracellular fluid. Hypotonicity causes fluid to shift inward from the extracellular to the intracellular space; hypertonicity causes the opposite to occur. In both situations, the net result is equilibration of tonicity between compartments with a change in the volume of both spaces.<sup>1</sup>

## **Components of Extracellular Tonicity**

The major components of extracellular osmolality and tonicity include electrolytes and non-ionic substances (glucose and urea). Because the intravascular space is a sub-compartment

of the extracellular space and because small solutes can easily traverse the vascular wall (in most situations), serum solute concentrations are essentially equivalent to ECF concentrations.

### *Electrolytes*

Electrolytes are charged solutes (cations and anions) that cannot diffuse through the lipophilic cell membrane. Instead, transport of electrolytes into and out of the cell is carrier mediated, either by ion channels or transporter proteins, which are often ATP-dependent. Intra- and extracellular ion concentrations are tightly regulated so that net ion flux is zero under steady state conditions. Thus ions are impermeable solutes, or effective osmoles, and, as such, contribute to both osmolality and tonicity.<sup>2</sup> Sodium is the most abundant serum cation and is the largest contributor to serum osmolality. Serum sodium concentration and ECF volume are intimately related; these values can be used to classify dehydration and hypovolemia (hypo-, iso-, or hypertonic) and predict the direction of volume shift (intra- vs. extracellular).<sup>3</sup> Osmosensing by the JGA cells in the kidneys, by the central osmoreceptors in the hypothalamus, and peripheral osmoreceptors in the GIT and splanchnic vasculature is largely dependent on sodium concentrations and determines the systemic response to changes in volume and tonicity.<sup>4,5</sup> Potassium also contributes to serum osmolality, but to a much smaller degree than sodium. Disease states characterized by severe hyperkalemia such as Addison's disease, urethral obstruction, and acute kidney injury, only raise serum potassium concentration a few milliequivalents above normal and minimally contribute to serum osmolality. Hypokalemia also has a minor effect on serum osmolality because extracellular potassium is normally maintained at a low concentration. Even severe hypokalemia (2.0 mM) only lowers serum osmolality by approximately 4 mOsM (accounting for accompanying anions). Other serum cations (e.g.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) also have a relatively small impact on osmolality and tonicity because they are maintained at low concentrations in ECF. The effect of serum anions on serum osmolality and tonicity is similar to that of cations, with those of higher concentration (e.g.  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ) having a greater impact than those of lower concentrations (e.g. phosphates, sulfates). According to the principles of electroneutrality, the number of anions in solution is always equal to the number of cations. Therefore, the total contribution of electrolytes to total serum osmolality is usually expressed as double the concentration of the major cations:  $2([\text{Na}^+] + [\text{K}^+])$ .<sup>1</sup>

### ***Nonionic Osmolytes***

Nonionic solutes such as glucose and urea also contribute to serum osmolality in health, although their contribution is small compared to the major ionic osmoles. Glucose and urea are relatively large molecules compared to the electrolytes. Osmolality is a colligative property and is measured in number of *particles* per unit volume. Therefore, if glucose and urea nitrogen are not reported in SI units, then serum concentrations must be converted using molecular weight to determine their osmolar contribution.<sup>1</sup> For example, normal canine serum glucose concentration is approximately 100 mg/dl; this is equivalent to only 5.56 mOsM, a relatively small contribution when compared to normal canine sodium concentrations (150 mOsM). However, in certain disease states, glucose and urea concentrations can rise several-fold and lead to significant hyperosmolality. In diabetic ketoacidosis (DKA) and hyperglycemic, hyperosmolar syndrome (HHS), glucose concentrations are often greater than 500 mg/dl (27.8 mOsM) and can sometimes reach as high as >1000 mg/dl (>55.6 mOsM) in severe cases. Cellular glucose uptake is dependent on various glucose transporters (GLUT); although the rate of glucose transport is proportional to the expression of GLUT at the cell surface, glucose remains a relatively impermeable solute and an effective osmole.<sup>6</sup> Thus hyperglycemia contributes both to hyperosmolality and hypertonicity. Severe azotemia is characterized by large elevations in serum urea nitrogen concentrations (>200 mg/dl) and contributes significantly to serum hyperosmolality (>71 mOsM). Most mammalian cells are relatively permeable to urea, due to the presence of a facilitated urea transporter (UT-B), making urea an ineffective osmole.<sup>7</sup> Therefore, azotemia does not usually contribute significantly to hypertonicity. However, in the kidney, the distribution of the renal urea transporter (UT-A) varies along the different tubular segments. For example, UT-A expression in inner medullary collecting duct cells is ADH dependent. Without ADH, urea can act as an effective osmole in this renal tubular segment.<sup>8</sup> Similarly, in human patients with dialysis disequilibrium syndrome, an abrupt drop in plasma urea during dialysis sets up an osmolar gradient between brain cells and the interstitium, because of decreased UT-B expression, leading to cerebral edema and neurologic dysfunction.<sup>9</sup>

### ***Osmolytes of Unknown Tonic Effect***

Other endogenous organic substances, such as lactate and the ketoacids, are produced in minimal quantities in health, but can increase significantly in certain disease states. Lactate is

produced by the enzymatic conversion of pyruvate by lactate dehydrogenase. This represents an alternate pathway for the end products of glycolysis, when the Krebs's cycle and the electron transport chain are unavailable. Systemically, lactate is a biologic marker for anaerobic metabolism, due to either inadequate oxygen delivery (hypoperfusion) or inadequate oxygen utilization (cellular dysfunction).<sup>10</sup> The ketoacids,  $\beta$ -hydroxybutyrate (BHB) and acetoacetate (AA), along with acetone, are synthesized from free fatty-acids via  $\beta$ -oxidation. In DKA and starvation, ketoacids are considered to be mainly metabolic byproducts, but represent a global shift from predominantly carbohydrate to lipid metabolism.<sup>11</sup> Accumulation of lactate and ketoacids causes a metabolic acidosis and, by definition, addition of these solutes to serum increases osmolality. Although the ketoacids and lactate can permeate the cell membrane to some degree via facilitated diffusion through the various monocarboxylate transporters (MCT 1-4), it is unclear whether these substances contribute significantly to serum tonicity.<sup>12</sup> There are few studies that address the question of tonic influences of lactate and ketoacids. Puliyel (2003) demonstrated, using a RBC osmotic fragility model, that LiAA and NaBHB prevent hemolysis in a manner similar to glucose at concentrations approximating physiologic serum osmolality, suggesting that AA and BHB act as effective osmoles.<sup>13</sup> However, it remains unclear whether the ketoacids are truly effective or ineffective osmoles because the Puliyel study did not account for possible tonic effect of the accompanying cations ( $\text{Li}^+$  and  $\text{Na}^+$ ) in solution.

All solutes not typically measured on a routine biochemistry panel are classified as unmeasured osmoles. Unmeasured osmoles can be endogenous such as lactate, ketoacids, uremic acids, and ammonia, or can be exogenous such as ethylene glycol, propylene glycol, ethanol, mannitol and various drugs. These unmeasured osmoles constitute the osmolar gap, which is the difference between the measured osmolality (via osmometry) and the calculated osmolality (derived from serum biochemistry values). An elevation in the osmole gap can be clinically useful in detecting toxicities, but can also occur in metabolic conditions.<sup>1</sup> However, it is important to note that an elevation in the osmolar gap is not equivalent to serum hypertonicity and does not imply that significant fluid shift will occur because the true tonic nature (effective or ineffective osmole) of many of the unmeasured osmoles remains unknown.

## **Tonic Stress and Mechanisms of Cellular Osmoregulation**

Virtually instantaneously after acute exposure to hypertonicity, fluid is drawn out of the cell and leading to cell shrinkage; conversely, acute hypotonicity causes fluid to shift inwards and leads to cell swelling. In extreme situations, severe, acute hyper- and hypotonicity can lead to apoptosis and osmotic lysis, respectively. However, more mild derangements of tonicity and cell size cause cellular dysfunction and trigger a series of compensatory changes in the cell leading to a regulatory volume decrease (RVD) in the case of hypotonicity, or a regulatory volume increase (RVI) in the case of hypertonicity.<sup>14</sup>

Intracellular protein function is altered by hypertonicity and the resultant cell size decrease. Hypertonicity causes an increase in intracellular ionic and non-ionic solute concentrations (e.g. urea), which can negatively impact protein function. Similarly, the concentrations of intracellular proteins increase in a hypertonic state and cause macromolecular crowding, which alters the thermodynamic behavior of individual enzymes and transporters as well as molecular interactions between various proteins.<sup>15</sup> Changes in cell size can also disrupt the delicate balance of the highly-organized eukaryotic cytoskeleton by inducing depolymerization and repolymerization of actin filaments in response to mechanical stress.<sup>2</sup> Protein synthesis is suppressed under hypertonic conditions through inhibition of initiation and elongation of the polypeptide strand. Double-stranded DNA breaks also occur under hypertonic stress, which trigger the DNA damage-response systems leading to cell cycle arrest, a protective mechanism common to many cellular stressors.<sup>14</sup> Finally, modest changes in cell size are an integral part of various hormone-cell signaling mechanisms; therefore pathologic tonic change may disrupt such mechanisms leading to cellular dysfunction.<sup>16</sup>

Whether physiologic or pathologic, the cellular response to hypotonicity is cell swelling, which triggers mechanisms of regulatory volume decrease (RVD). The initiators of RVD are not completely understood and likely differ between cell types. In enterocytes, tyrosine cross-phosphorylation has been identified as an essential step in RVD, whereas membrane stretch in hepatocytes activates  $\text{Ca}^{2+}$  channels, increasing intracellular  $\text{Ca}^{2+}$  concentration; both of these steps likely initiate signal transduction and activate the effectors of RVD. Efflux of ionic solutes, mainly  $\text{K}^+$ ,  $\text{Cl}^-$ , and bicarbonate, cause osmotic shift of water extracellularly and a subsequent decrease in cell volume. Within minutes of hypotonic stress, all mammalian cells are capable of RVD resulting in complete or near-complete restoration of the original cell size.<sup>17</sup>

The regulatory volume increase (RVI), the cellular response triggered by hypertonicity and cell shrinkage, is a complex, multi-stage process. The early response consists of ion influx ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ) mediated by pre-existing membrane transporters including the  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  co-transporter, the  $\text{Na}^+ / \text{H}^+$  exchanger, and the  $\text{Cl}^- / \text{HCO}_3^-$  exchanger. Here again, the initiators of the RVI are not completely understood, but likely are similar to those of RVD including membrane stretch, changes in cytoskeletal tension, adjustment of intracellular  $\text{Ca}^{2+}$  concentration, and phosphorylation/dephosphorylation leading to activation of signal transduction.<sup>14</sup> These early changes occur within seconds to minutes of tonic insult, but only produce partial restoration of cell size. Not only is this early response incomplete but it results in an increased intracellular concentration of salts which, as previously discussed, adversely affects protein function. The late response to hypertonicity involves replacement of excess intracellular ions with *compatible osmolytes*, non-ionic small molecules that do not interfere with protein function even at high concentrations but maintain intracellular tonicity. These osmolytes are either taken up from the extracellular environment via  $\text{Na}^+$ -coupled transporters (e.g. taurine, glutamate, GABA) or are produced endogenously via hypertonic-induced upregulation of the enzymatic systems required for their synthesis (e.g. glycerophosphorylcholine, sorbitol). Because genomic mechanisms are involved, this late response takes hours to days and, although it is more effective at restoring cell volume than the early response, the late response of the RVI does not usually result in complete volume restoration.<sup>2</sup>

### **Extracellular Fluid Tonicity and Mechanisms of Systemic Osmoregulation**

Although individual cells have the ability to adapt to moderate degrees of tonic stress, systemic regulation of the extracellular fluid composition constitutes a second level of defense against tissue dehydration and edema. These mechanisms are particularly important for organs such as the brain and spinal cord, which are encased within rigid bony structures. Cellular swelling in these tissues leads to increased tissue hydrostatic pressure, decreased tissue perfusion, and risk of brain herniation during acute hypotonic insult.<sup>18</sup> The overarching control system for systemic osmoregulation is, like many regulatory systems in the body, a negative feedback loop. Changes in ECF tonicity are detected by specialized receptors (osmoreceptors) in the central nervous system and the periphery, which send neuronal impulses to the osmoregulatory control centers. This input is integrated with other visceral signals, in particular

those detecting changes in vascular volume, and induce homeostatic adjustments by the major systemic effectors of osmoregulation: the ADH-pituitary-renal axis, thirst and salt appetite.<sup>5</sup>

### ***Osmoregulatory Sensors: Osmoreceptors***

The central osmoreceptors are specialized neurons located in the circumventricular organs of brain, which are devoid of the blood-brain-barrier. Thus, the central osmoreceptors are subjected to the same tonic variation experienced by the rest of the body and undergo obligatory changes in cell volume. Shrinking in response to hypertonicity increases the basal firing rate of osmoreceptor neurons, which send projections to other areas of the brain, communicating the ECF tonic status to neural control centers for the osmoregulatory effectors, including the supraoptic nucleus of the hypothalamus, anterior cingulate and insular cortices, and the parabrachial and paraventricular nuclei. Conversely, swelling of the osmoreceptor in response to hypotonicity decreases the neuron firing rate. The mechanism by which changes in cell size are sensed and translated into action-potentials is incompletely understood. However, it is believed that the pathways involved are similar to those controlling cellular osmoregulation and initiation of regulatory volume changes (RVD and RVI). Alteration of cell membrane stretch or cytoskeletal components in response to changes in cell size likely activate or inactivate sensitive ion channels. However, instead of altering the intracellular solute load and correcting cell volume, as occurs during regulatory volume changes, these channels alter the resting membrane potential of the neuron. Because of cation influx, the cell membrane becomes less negative in response to hypertonicity, increasing the likelihood that an action potential will form and thus increasing the firing rate. Hypotonicity causes hyperpolarization of the cell membrane, therefore suppressing action potential formation and decreasing the neuron firing rate.<sup>5</sup>

Osmoreceptors in the periphery are mostly associated with the gastrointestinal tract. Peripheral osmoreceptors in the oropharynx and the duodenum allow the body to sense the tonicity of ingesta; similarly, osmoreceptors in the splanchnic and portal circulation detect the osmotic content of nutrients absorbed by the intestines. These signals are carried by vagal fibers to the brain and modulate the homeostatic response to body tonicity. The net effect is that, rather than waiting for the absorbed fluid to equilibrate with the ECF and be detected by the central osmoreceptors, thirst and ADH secretion are modified in anticipation of absorption. This

circumvents the several-minute delay in adjustment that would otherwise occur and buffers large fluctuations that might otherwise occur in response to an ingested solute load.<sup>5</sup>

### ***Osmoregulatory Effectors: ADH, Thirst, and Salt Appetite***

The major effector of systemic osmoregulation is the release of ADH and the pituitary-renal axis. ADH is a nine amino-acid polypeptide hormone produced by magnocellular neurosecretory cells (MNCs) in the supraoptic and paraventricular nuclei of the hypothalamus and released into the circulation from these neurons' axon tips in the posterior pituitary gland. Hormone release occurs in response to hypertonicity, as sensed by the MNCs and other osmoreceptive neurons in the brain, and is modulated by peripheral input from GI and portal osmosensors via vagal afferents and the premotor cortex. The plasma threshold for ADH release is ~280 mOsM in humans, below which ADH secretion is inhibited; this threshold is likely similar or slightly higher in dogs but has not been extensively studied. ADH acts on V<sub>2</sub> receptors present on medullary collecting duct epithelial cells in the kidney to induce fusion of endosomes carrying the aquaporin-2 (AQP-2) water channel with the apical cell membrane through a G<sub>s</sub>-coupled signal transduction mechanism. The apical AQP-2 and basolateral AQP-3 and AQP-4 channels make the tubular epithelium permeable to water and, in the presence of an adequate medullary interstitial gradient, lead to concentration of the tubular fluid and reclamation of free water. Excretion of concentrated urine is equivalent to a decrease in solute (effective osmoles) load in excess of total body water, leading to a decrease in ECF tonicity, the desired compensatory response to hypertonicity. In hypotonic conditions, ADH secretion is partially or completely inhibited. In the absence of ADH, the medullary collecting ducts are impermeable to water so no water reabsorption occurs and hypotonic urine is excreted; this causes a net loss of free-water and a compensatory increase in ECF tonicity.<sup>5,19</sup>

Thirst and salt appetite are important but opposing mechanisms involved in regulation of systemic tonicity. Thirst is activated and salt appetite inhibited in hypertonic states whereas the opposite occurs under hypotonic conditions. Although the central regulators of these mechanisms in the dog are not well established, functional imaging studies have disclosed that the anterior cingulate and insular cortices as well as the parabrachial and paraventricular nuclei appear to at least partially regulate thirst and salt appetite in humans. As previously discussed, peripheral osmoreceptors, along with other visceral afferents associated with the gastrointestinal



tract, monitor pre-systemic changes in tonicity and modulate thirst and salt appetite in anticipation of salt and water absorption.<sup>5</sup>

### ***Osmoregulatory and Vascular Control Integration***

Systemic mechanisms of osmoregulation are intimately associated with vascular volume regulatory mechanisms. In many cases, these responses overlap using the same regulatory effector systems or employing a mechanism that influences both volume and tonicity. For example, non-osmotic ADH secretion can be induced by afferent signals from baroreceptors in response to vascular under-filling. ADH release leads to water retention and thus expansion of the vascular space. In contrast, release of the natriuretic peptides (atrial natriuretic peptide [ANP], brain natriuretic peptide [BNP], and C-type natriuretic peptide [CNP]) from stretched myocardial cells during volume expansion stimulates a primary increase glomerular filtration as well as inhibition of sodium resorption in the collecting ducts; the resultant natriuresis and diuresis decrease vascular volume. The renin-angiotensin-aldosterone system (RAAS) also figures prominently in both tonic and volume regulation. Decreased sodium delivery to the distal tubules stimulates renin release and subsequent angiotensin activation and aldosterone secretion, leading to sodium and water retention and subsequent volume expansion and increased plasma sodium load.<sup>3</sup>

The net effect of these combined volume regulatory mechanisms on plasma tonicity is determined by the proportion of water excretion/conservation and changes in the absolute plasma solute load. If water is excreted in excess of solute, plasma tonicity increases whereas if solute is lost in excess or water, plasma tonicity decreases. The opposite occurs in states of fluid or solute retention. Oftentimes osmoregulatory and volume-regulatory mechanisms work in concert to correct homeostatic perturbances. For example, in an animal with hypovolemia and hypertonicity, the compensatory release of ADH benefits both conditions by retaining water in excess of solute, increasing plasma volume and diluting out the high concentrations of plasma solutes. However, an animal that has lost solute in excess of fluid, such as with severe gastroenteritis, becomes hypovolemic and hypotonic. In these situations, physiologic responses prioritize plasma volume at the expense of plasma tonicity – non-osmotic stimulation of ADH release restores plasma volume, but may temporarily exacerbate plasma hypotonicity. However,

if all osmoregulatory and volume regulatory mechanisms are intact, with appropriate treatment, both vascular and tonic homeostasis will eventually be reached.<sup>3</sup>

## **Hypotonicity in Clinical Disease**

At a fundamental level, hypotonicity of plasma (and the ECF compartment) occurs by two basic mechanisms: solute loss or water gain. The mammalian body is an aqueous system, so to excrete solute from the body some water must also be excreted. Likewise, almost all water ingested contains some solute. Therefore, it is more appropriate to say that the two basic mechanisms by which hypotonicity develops are solute loss *in excess* of water loss (a net solute loss), and water gain *in excess* of solute gain (a net water gain). A variety of canine diseases are associated with hypotonicity; in particular, hyponatremia is often a prominent feature, given that sodium concentration is major determinant of plasma tonicity.<sup>3</sup>

### ***Hypotonic Diseases with Solute Loss***

Solute loss leading to hypotonicity can be classified as renal in origin or extrarenal. A classic example of renal solute loss is hypoadrenocorticism in which mineralocorticoid deficiency prevents sodium reabsorption by the principle cells of the distal tubule and cortical collecting ducts of the kidney. Hypoadrenocorticism is characterized by hyponatremia and hyperkalemia, reported in 82% and 85%, respectively, of patients in one study.<sup>20</sup> Although hyperkalemia would tend to increase plasma tonicity, the relative magnitude compared to the concurrent low sodium concentration is such that there is a net decrease in ion concentration and thus in plasma tonicity. Patients with hypoadrenocorticism also develop elevations in the BUN concentration, which contributes to an increased total plasma osmolality; however, as urea is an ineffective osmole, it will not contribute to plasma tonicity. Without aldosterone, sodium resorption is impaired in the distal tubule as well as in the colon, increasing renal and extrarenal water loss. This, along with poor vascular tone due to glucocorticoid deficit, results in a decreased effective circulating volume. Decreased effective circulating volume is a potent non-osmotic stimulus for ADH secretion (see below), which causes renal water conservation in excess of solute retention and may further exacerbate plasma hypotonicity.<sup>3,20-22</sup> Similarly, plasma hypotonicity may occur during diuretic administration, particularly loop and thiazide diuretics. Loop diuretics inhibit the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  symporter in the thick ascending limb of the loop of Henle and thiazide diuretics inhibit the  $\text{Na}^+/\text{Cl}^-$  symporter in the distal tubules, impairing

tubular electrolyte reabsorption leading to salt wasting. Diuresis invariably ensues and, although compensation for this water loss may occur (through non-osmotic ADH stimulation), the kidney's inability to conserve electrolytes leads to a hypotonic state.<sup>19,23</sup>

Extracellular solutes can also be lost through non-renal means. Although hypotonic fluid loss is common during vomiting and diarrhea, hypertonic fluid can be lost through the GIT leading a net decrease in total body solute concentration. Even if gastrointestinal fluid is hypotonic, substantial volume loss will cause vascular hypoperfusion leading to non-osmotic ADH secretion and hypotonic fluid retention by the collecting ducts. Thus, either hypertonic or hypotonic GIT losses may lead to plasma hypotonicity.<sup>3</sup> Although not a true total body loss of solute, third-spacing can compartmentalize fluids of a high solute concentration away from vascular and extracellular spaces leading to plasma hypotonicity. In particular, exudates, such as those found in septic peritonitis and pancreatitis, can have high solute loads as does the abdominal effusion found during uroabdomen.<sup>3</sup>

### ***Hypotonic Diseases with Water Gain***

Hypotonicity may also occur in patients with a normal or even increased total body solute load, when water is conserved in excess of solute. Although very uncommon, acute water intoxication causing hyponatremia and hypotonicity has been reported in a dog.<sup>24</sup> More commonly, diseases associated with decreased effective circulating volume cause water conservation and solute dilution in veterinary patients. In patients with heart disease, a decreased cardiac output is sensed by the arterial baroreceptors leading to non-osmotic stimulation of ADH release. Poor renal perfusion leads to a decrease in GFR and distal tubular sodium delivery causing activation of the RAAS. Both of these mechanisms induce net water retention, which has a dilutional effect on plasma solutes and causes hypotonicity.<sup>25</sup> The same responses occur during hypoalbuminemia in patients with liver failure and nephrotic syndrome. Low oncotic pressure leads to edema and third-spacing of fluid and ultimately a decrease in vascular volume. Hypoalbuminemic hypovolemia triggers the normal compensatory responses (non-osmotic stimulation of ADH and RAAS activation) leading to water retention and extracellular fluid dilution.<sup>25</sup>

Although rarely reported in veterinary patients, the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) is the most frequent cause of hyponatremia in human patients

and, as sodium is the most important determinant of plasma tonicity, SIADH represents an important cause of hypotonicity in humans.<sup>26</sup> The syndrome is characterized by hyponatremia, hypotonicity, and concentrated urine in a euvolemic patient. The establishment of euvoolemia is an important diagnostic criterion as elevated plasma ADH concentration is an appropriate response in a hypovolemic patient. Several patterns of ADH secretion have been documented in SIADH including: erratic secretion of ADH; constitutively increased ADH secretion; normal secretion of ADH in response to an inappropriately low plasma osmolality (reset osmostat syndrome); and low or even undetectable concentrations of ADH with inappropriate urine concentration due to mutations of the V2 receptor in the renal collecting ducts.<sup>3,26</sup> In dogs, SIADH has been reported both as a primary, idiopathic condition and as a sequel to other diseases such as heartworm infection, amebic meningoencephalitis, and congenital hydrocephalus.<sup>27-33</sup> A variety of drugs are also reported to cause inappropriate release of ADH from the neurohypophysis (e.g. barbiturates, beta-adrenergic drugs, cholinergics, tricyclic antidepressants) or potentiate the response to ADH at the level of the kidney (e.g. alpha-adrenergic drugs, glucocorticoids) causing a temporary, SIADH-like state.<sup>3</sup>

### ***Treatment of Hypotonicity***

As with all metabolic derangements, the primary treatment goal of hypotonicity is amelioration of the underlying disease process. In particular, alterations in volume status or oncotic pressure must be addressed, as these are often the driving force behind systemic hypotonicity. In many cases, plasma hypotonicity is mild and requires no specific treatment. However, in cases of acute or severe hypotonicity, clinical signs associated with cerebral edema may occur and include lethargy, nausea, and weakness, ataxia, seizures, and coma. In such cases, specific treatment to correct hypotonicity is warranted and, as hypotonicity is almost always accompanied by hyponatremia, is directed at correcting plasma sodium concentration. No optimal treatment regimen for sodium replacement has been established in the human or veterinary literature. However, it is generally recommended that plasma sodium concentration not rise faster than 8-12 mmol/L per day, particularly in patients in whom hypotonicity is suspected to be chronic (>48-72 hours in duration) and are expected to have undergone complete cellular adaptation (RVD) to hypotonicity. Rapid correction or overcorrection can lead to

myelinosis in which acute cellular dehydration causes oligodendrocyte injury and demyelination of axons in the pons and other areas of the brain.<sup>3,26,34</sup>

### **Hypertonicity in Clinical Disease**

Similar to hypotonicity, the mechanisms causing plasma hypertonicity can be divided into two major categories: water loss in excess of solute loss and solute gain in excess of water gain.

#### ***Hypertonic Diseases with Water Loss***

Water loss in excess of solute loss is caused by excretion or sequestration of fluid that is hypotonic to the ECF fluid and can be further divided into renal or extrarenal loss.

Gastrointestinal losses are a common form of extrarenal hypotonic fluid loss and are caused by vomiting, diarrhea or in fluid sequestration that occurs during upper gastrointestinal obstruction. Third-spacing of hypotonic fluid can also cause plasma hypertonicity as can cutaneous losses such as those that occur in burn victims. Although these hypotonic losses initially will cause plasma hypertonicity, if a significant enough drop in vascular volume is present, then non-osmotic stimulation of vasopressin will occur which has a dilutional effect on plasma and may actually cause hypotonicity (see above).<sup>3</sup>

Water can also be lost by renal means. Although patients with intrinsic kidney disease usually have normal sodium concentrations, fluid loss in excess of sodium can occur during dehydration, which would lead to hypertonicity.<sup>3</sup> This net fluid loss may occur in patients with impaired sodium excretion due to tubular dysfunction and/or increased sodium retention due to RAAS activation. Patients with kidney disease treated with intravenous fluids containing a high concentration of sodium (e.g. 0.9% NaCl) are also at risk for hypernatremia and hypertonicity, again, likely caused by impaired sodium excretion.<sup>3</sup> It is important to note that azotemia can cause an increase in plasma osmolality due to an increased plasma urea concentration, but azotemia does not imply hypertonicity, as urea is an ineffective osmole. Other solute concentrations that rise during uremia, such as phosphates and sulfates, may add to plasma tonicity, but likely do not accumulate in sufficient quantities to greatly impact total effective osmole concentration. The tonic effects of creatinine have not been directly studied; it is known that creatinine does diffuse across cell membranes, albeit at a slower rate than urea due to its higher molecular weight.<sup>35,36</sup> However, even if creatinine does exert a tonic effect, the total

molar concentration is unlikely to be high enough to cause significant hypertonicity, even in severe azotemia (e.g. [creatinine] = 20 mg/dl = 1.7 mOsM).

Potent renal water loss and plasma hypertonicity also occur in patients with diabetes insipidus. Central diabetes insipidus (CDI), an inability to release ADH from the neurohypophysis, can occur both as an idiopathic or congenital disorder or secondary to neural damage from trauma, neoplasms, inflammatory disease, or iatrogenic damage during adenohipophysectomy for treatment of pituitary-dependent hyperadrenocorticism.<sup>37-41</sup> The severity of CDI ranges from inadequate ADH release or appropriate ADH release at a higher than normal set-point (reset osmostat) to complete lack of ADH secretion. Nephrogenic diabetes insipidus (NDI) has rarely been reported in dogs as a primary disease entity in which mutation of the V2 receptor blunts or obliterates the normal tubular response to ADH.<sup>42,43</sup> However, secondary NDI, in which the action of ADH on the V2 receptor and AQP-2 channel is impaired by extrinsic means, occurs commonly in a variety of conditions affecting veterinary patients including sepsis, hyperadrenocorticism, and hypercalcemia.<sup>37</sup> Many animals with DI are otherwise healthy except for severe polyuria, but are often normo- or only mildly hypertonic. A compensatory polydipsia, induced by activation of the osmoregulatory thirst mechanism, negates the effects of this diuresis. However, when water is withheld or the thirst mechanism is suppressed by concurrent disease, hypernatremia and hypertonicity rapidly ensue reaching concentrations in excess of 170 mmol/L and 375 mOsM, respectively. Such severe, acute hypertonicity induces myelinosis, which, if not corrected, can cause ataxia, stupor, seizures, coma, and death.<sup>37</sup>

### ***Hypertonic Diseases with Solute Gain***

Hypertonicity can occur by a primary increase in solute load through exogenous intake or endogenous production of effective osmoles. Such solutes are either measured osmoles (most commonly sodium or glucose), or are unmeasured osmoles of which detection requires either solute-specific assays or calculation of the osmole gap. Excess ingestion of table salt or salt-containing products can cause significant, even fatal, hypernatremia and hypertonicity.<sup>44,45</sup> Similarly, iatrogenic hypertonicity can occur with administration of hypertonic saline during which sodium concentration must be carefully monitored.<sup>3</sup>

In contrast to sodium, exogenous glucose administration rarely causes significant hyperglycemia and hypertonicity except in animals with glucose intolerance. However, poorly controlled diabetes mellitus (DM) often results in clinically significant hypertonicity, particularly during DKA and HHS. In one study of diabetic dogs and cats, the prevalence of effective hyperosmolality was 81%, with 33% exhibiting marked effective hyperosmolality ( $\geq 330$  mOsM).<sup>46</sup> A second study reported mean calculated effective osmolality for cats with DM, DKA, and HHS as 326.5 mOsM, 316.1 mOsM, and 344.1 mOsM, respectively.<sup>47</sup> In diabetic patients, an absolute or relative insulin deficiency causes poor tissue uptake of plasma glucose leading to profound plasma hyperglycemia. Unconstrained by insulin and under the influence of counter-regulatory hormones (e.g. glucagon, cortisol, epinephrine), the liver synthesizes additional endogenous glucose via gluconeogenesis and glycogenolysis, further exacerbating hypertonicity.<sup>48</sup>

The pathophysiology of DM is complex and solute concentrations other than glucose can become abnormal which may influence plasma osmolality and tonicity.<sup>49</sup> When insulin concentrations are very low and glucagon and other counter-regulatory hormone concentrations predominate, fatty acids are liberated from adipose tissue and become substrate for ketogenesis in the liver. Synthesis of the ketoacids, AA and BHB, (along with small amounts of acetone) represents the addition of unmeasured anions to plasma causing a normochloremic or high anion gap metabolic acidosis. The presence of plasma ketoacids and metabolic acidosis are the hallmark features of DKA.<sup>48,50</sup> As discussed previously, the addition of the ketoacids increases plasma osmolality, but the tonic effects of AA and BHB have not been well established; thus, the influence of the ketoacids on plasma tonicity is unknown. Increased lactate production is also reported in DM and DKA, which contributes to an increased anion gap and metabolic acidosis.<sup>50,51</sup> The cause of diabetic lactic acidosis is likely multifactorial. Hypoperfusion and increased circulating catecholamines induce anaerobic metabolism in peripheral tissues which increases lactate production.<sup>10,51</sup> Severe hyperglycemia and ketosis may also alter glucose metabolism, shunting glucose into the glyoxalase pathway leading to the production of D-lactate, the enantiomer of L-lactate, which is the normal product of anaerobic metabolism. Increased D-lactate concentrations have been documented in cats and humans with DM and DKA.<sup>52,53</sup> Here again, this increase in lactate production can be expected to increase plasma osmolality, but the tonic effect of lactate is unknown; therefore, the effects of lactate on plasma tonicity are yet to be

determined. In many diseases, sodium concentration is the driving force behind tonic derangements, however, in DM alterations in sodium may actually play a compensatory role.<sup>46</sup> Mild to moderate hyponatremia is relatively common in diabetic patients, due to plasma dilution by fluid shift into the ECF caused by the hyperglycemic osmotic gradient.<sup>48,50</sup> One study documented a rise in plasma sodium concentration as hyperglycemia was corrected during DKA in cats. The authors suspected that this increase in sodium was likely due to the common practice of using 0.9% NaCl in treatment of DKA, but noted that the increase in sodium appeared to mitigate rapid changes in serum tonicity that might otherwise have occurred with correction of hyperglycemia.<sup>54</sup>

Hypertonicity also occurs with the addition of exogenous unmeasured effective osmoles to the ECF. Perhaps the best-known example of this is ethylene glycol (EG) intoxication. EG is rapidly absorbed from the GI tract and as the plasma concentration increases, so does measured osmolality and the osmolar gap.<sup>55</sup> Although the high mortality rate associated with EG toxicity is often attributed to the acute kidney injury induced by EG metabolites later in the course of the disease (>48-72 hours post-ingestion), significant morbidity can occur early on in the form of neurologic signs caused by the ethanol-like effect of EG on the CNS as well as early and profuse polyuria and polydipsia secondary to hypertonicity.<sup>55,56</sup> Propylene glycol is considered a safer alternative to EG as it does not cause oxalate formation and AKI. However, at least one case of propylene glycol toxicity accompanied by hypertonicity and an increased osmolar gap was reported in a dog.<sup>57</sup> Hypertonicity caused by addition of exogenous, unmeasured osmoles can also be iatrogenic and even therapeutic. Mannitol is commonly used in both human and veterinary medicine to treat cerebral edema by establishing an osmotic gradient between the vascular space and cerebral parenchyma, drawing water out of the brain tissues, thus reducing intracranial pressure and the risk for brain herniation. By the same mechanism, mannitol causes transient vascular volume expansion and acts as an osmotic diuretic; thus it can be used in the treatment of oliguric acute kidney injury.<sup>58,59</sup>

### ***Hypertonicity in Human Medicine***

Thus far, hypertonicity has been discussed as a consequence of a variety of diseases. However, several studies in humans have investigated hypertonicity as a distinct syndrome and have examined the relationship between hypertonicity and a variety of clinical parameters and



disease states. In one large cross-sectional study of the general population, mild ( $> 295$  mOsM) and overt ( $> 300$  mOsM) hypertonicity were found to be relatively common in adults in the United States with an overall prevalence of 40% and 20%, respectively.<sup>60</sup> The risk of hypertonicity was significantly and positively associated with age as well as impaired glucose tolerance. It was noted that hypertonicity in younger adults was associated with increased water intake, whereas hypertonicity in older adults was associated with decreased water intake, which is a known phenomenon among the elderly. Thus, the authors suggested that there may be mechanisms resulting in hypertonicity in the elderly independent of diabetic status.<sup>60</sup> Another study investigated the relationship between hypertonicity and obesity and found a higher prevalence of hypertonicity in obese adults than in adults of normal body mass. Based on bioelectrical impedance data, obese individuals had a significantly higher ECF/ICF ratio, which was correlated with plasma sodium concentrations. It was suggested that this altered fluid distribution might be caused by hypertonicity in obese adults.<sup>61</sup> Hypertonicity may also be an early indicator for frailty, a condition of decreased functional reserve and vulnerability to morbidity, especially common in the elderly.<sup>62</sup> 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.<sup>63</sup> Finally, hypertonicity is a particularly important clinical problem for insulin-resistant and diabetic patients. In one cross-sectional study, the prevalence of hypertonicity in self-reported human diabetics ( $n = 1,239$ ) was reported as 35-74%.<sup>60</sup> 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.<sup>64</sup> Similar large investigations of hypertonicity in veterinary patients are lacking, but could provide valuable information regarding the significance of hypertonicity in the general canine and feline populations.

### ***Treatment of Hypertonicity***

The principles of therapy for hypertonicity are very similar to those underlying treatment of hypotonicity. In cases of mild hypertonicity, no specific therapy is necessary and treatment should be directed at correcting the underlying disease process. In patients with severe hypertonicity, solute concentrations may be directly manipulated via calculated intravenous fluid therapy. A free-water deficit may be calculated and replaced using 5% dextrose in sterile water

(D5W).<sup>3</sup> However, rapid correction of hypertonicity, particularly in cases where the hypertonicity is of long duration, carries the risk of cerebral edema, increased intracranial pressure, and brain herniation due to osmotic swelling of neurons in a closed space.<sup>18</sup> In cases of hypertonicity due to hypernatremia, it is recommended that free-water replacement occur slowly over 48 hours and plasma sodium concentrations not decrease by more than 0.5 mmol/L/hr to decrease the risk of neurologic events.<sup>3</sup> Some clinicians prefer to use low-sodium fluids such as 2.5% dextrose in 0.45% NaCl ( $[\text{Na}^+] = 77$  mmol/L) or LRS ( $[\text{Na}^+] = 128$  mmol/L) rather than D5W for a gradual effect. Regardless of the specific fluid chosen, serum electrolytes must be monitored carefully during treatment. Correction of hypertonicity in diabetic patients requires fluid therapy for rehydration and volume expansion as well as insulin administration to correct hyperglycemia. In human diabetics, children appear to be at much higher risk for osmotic and neurologic complications than adults during DKA treatment.<sup>65,66</sup> In veterinary patients, the risk for cerebral edema during DKA treatment also appears low, although this may be due in part to the common clinical practice of treating diabetics with higher-sodium fluids.<sup>54</sup> Normal saline ( $[\text{Na}^+] = 154$  mmol/L) is recommended for patients with DKA due to their severe dehydration and the volume expanding effects of this fluid. Additionally, the higher sodium content may partially compensate for the decrease in plasma glucose concentration. Similar recommendations have been made for the treatment of HHS, although plasma solute concentrations must be even more carefully monitored as many of these patients already have neurologic signs caused by osmotic complications.<sup>67</sup>

### **Clinical Measurement of Osmolality and Tonicity**

Given the importance of tonic regulation in health and the sometimes disastrous consequences of tonicity derangement during disease, accurate assessment of plasma tonicity is an important clinical goal. Unfortunately, there is no direct means to measure tonicity in biologic fluids. By definition, tonicity is the summation of the concentration of all effective osmoles in a solution. However, given the diverse constituents of plasma and ECF, it would be nearly impossible to assess each solute concentration individually. Furthermore, the tonic effects of some solutes, such as ketoacids and lactate, are not well established so it is not known whether those concentrations should be included in the calculation of tonicity.

As initially discussed, osmolality and tonicity are interrelated concepts – osmolality being the sum total concentration of all osmoles in solution and tonicity being the total concentration of all *effective* osmoles. If tonicity were a directly quantifiable property, then the difference between osmolality and tonicity would be the total concentration of all ineffective osmoles in a solution including measured ineffective osmoles such as urea and ineffective osmoles not routinely measured by the biochemistry profile. Osmolality and tonicity also differ in that osmolality is solely dependent on solute concentrations whereas tonicity is contingent upon solute concentrations and the tonic effects of those solutes. Because effectiveness of an osmole is relative to its permeability through the cell membrane, tonicity of a solution may change based on the surrounding tissue even though the solution osmolality remains constant. Therefore, osmolality can be used to estimate tonicity with three caveats: (1) in general, osmolality overestimates tonicity, (2) osmolality may not be an adequate estimate during pathophysiologic states such as uremia in which an increased concentration of ineffective osmoles is expected, and (3) osmolality cannot approximate tonicity in situations where the tonic nature of the major osmoles are unknown.

Osmolality can be inferred from osmotic pressure, which is one of the four colligative properties: vapor pressure, boiling point, freezing point, and osmotic pressure. Colligative properties are properties of a solution that are solely dependent on the total number of solute particles in solution and are independent of the mass of those particles. Thus, information regarding one colligative property of a solution may be inferred by directly measuring another. This is the basis for modern osmometric techniques. Vapor pressure osmometry measures changes in the dew point of a solution and compares it against standards to determine the osmolality of that solution. However, vapor pressure osmometry is technically difficult and cannot be used in the presence of volatile solutes such as ethanol. The alternative technique is freezing point depression osmometry. In osmometry by freezing point depression, the sample is supercooled and stirred to initiate crystallization. The temperature then rises due to release of latent heat from crystallization and this temperature is compared against a set of standards. Freezing point depression osmometry is considered to be more precise than vapor pressure osmometry and is thus the osmometric test of choice in most modern clinical pathology laboratories.<sup>68</sup>

Direct measurement of total osmolality requires specialized equipment, which may not be readily accessible in all clinical situations. Therefore, a variety of equations have been proposed to estimate total osmolar concentration in the form of calculated osmolality. Most formulas for calculated osmolality ( $OsM_C$ ) incorporate the major cations ( $Na^+$  and  $K^+$ , or  $Na^+$  alone) multiplied by a coefficient, usually 2.0, to account for accompanying anions; those formulas that use a lower coefficient (e.g. 1.86) do so to represent incomplete dissociation of salts. Glucose and BUN are usually included and divided by a conversion factor (18 and 2.8, respectively) to convert from mass to molar units. A constant is sometimes added to account for unmeasured osmoles such as  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $PO_4^{3-}$ , and lactate in solution. Some published equations also divide the entire summation by a factor (usually 0.92 – 0.93), as this is the percentage of plasma that is aqueous, the rest of the plasma suspension being comprised of protein and lipids. Several studies in humans and animals have compared calculated osmolality formulas to determine which best approximates measured osmolality and minimizes osmole gap.<sup>69-72</sup> For dogs, Barr and Pesillo-Crosby (2008) recommended the traditional formula:  $OsM_C = 2(Na^+ + K^+) + BUN/2.8 + glucose/18$ , for ease of clinical use and because its performance for estimating measured osmolality was second best among formulas investigated, closely following a much more complex equation.<sup>71</sup> In a similar study in cats, exclusion of potassium concentration from the formula ( $OsM_C = 2(Na^+) + BUN/2.8 + glucose/18$ ) yielded superior results.<sup>72</sup> Although formulas for calculated osmolality may estimate measured osmolality well, it is difficult to predict how they may perform for estimating tonicity. The formulas recommended for use in cats and dogs do not include terms to account for osmoles not traditionally measured including those that may have tonic effect. In this way, calculated osmolality may underestimate tonicity. However, by including measured ineffective osmoles (i.e. BUN), calculated osmolality may overestimate tonicity, particularly in conditions associated with increase ineffective osmolar concentrations (e.g. kidney disease). This second limitation may be remedied by excluding the BUN concentration from the estimate, a value known as calculated effective osmolality ( $OsM_{CE} = 2(Na^+ + K^+) + glucose/18$ ). Unfortunately, because of exclusion of unmeasured effective osmoles, calculated effective osmolality likely underestimates true effective osmolality – tonicity. Formulas for calculated effective osmolality are included in many of these osmolality studies, but do not appear to perform as well as those formulas for calculated osmolality.<sup>69,70,72</sup>

However, this is to be expected because the gold standard used in these studies is measured osmolality, not tonicity – for which there is no gold standard of measurement.

By definition, changes in the tonicity of a biologic solution will cause changes in the size of cells immersed in that solution. This physiologic effect of tonicity may open new avenues of investigation for measuring tonicity. Instead of attempting to directly measure the total effective osmole concentration, it might be possible to measure the physiologic effect of those osmoles by detecting changes in cell size and then extrapolating the tonicity of the solution from the behavior of the cells. The mammalian red blood cell (RBC) is an easily harvestable and measureable cell that has been used in many classic fluid physiology experiments.<sup>73-77</sup> RBCs have also been used in osmotic fragility assays specifically investigating the effects of various solutes of importance in diabetes mellitus.<sup>13,78</sup> The endpoint for RBCs in these studies was hemolysis due to cell swelling from hypotonicity. However, hemolysis assays are insensitive to small increases in cell size (mild hypotonicity) and are completely unable to detect cell shrinking due to hypertonicity. With modern techniques, it is possible to detect smaller changes in cell size that could correspond to changes in extracellular tonicity. Spurious elevation of erythrocyte 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 (~300 mOsM), they swell because the analyzer solution is *relatively* hypotonic to the intracellular environment causing an intracellular fluid shift.<sup>79</sup> Stookey et al. (2007) exploited this “lab error” to develop a new index for plasma hypertonicity, the MCV difference (dMCV).<sup>80</sup> The dMCV is the difference between the MCV as measured by an automated cell analyzer and the MCV calculated from a spun hct, which is performed with RBCs in the original patient plasma ( $dMCV = MCV_M - hct \times 10/[RBC]$ ). A cutoff value of only 2 fl or greater for dMCV performed well as an indicator for hypertonicity and, when combined with an elevated plasma vasopressin concentration, yielded 100% sensitivity and specificity.<sup>80</sup>

Use of the RBC as a detector of tonicity may yield valuable information for veterinary practitioners and researchers. However, it does have some potential limitations because RBC size is not solely governed by tonicity. 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 curvature.<sup>81</sup> Therefore, if cell

volume is derived from measurements of cell diameter, volume may be underestimated. This problem is circumvented by a ‘sphering’ treatment to produce homogenous RBC spheres prior to analysis. The sphering technique is standard practice for most RBC analyzers used in clinical pathology laboratories and has been shown to render accurate and repeatable measurements of RBC size.<sup>82</sup> 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.<sup>1</sup> Albumin is the most important plasma determinant of oncotic pressure, contributing approximately 80% of the total plasma colloid osmotic pressure.<sup>1</sup> Finally, and most importantly, all mammalian cells have the ability to regulate volume through the RVD and RVI. Complete regulatory responses take time to occur (hours to days) so studies examining acute RBC volume changes will likely yield valid information about tonic effects. However, incomplete regulatory responses that occur in the acute phase of volume regulation may add unanticipated variability.<sup>17</sup>

## Chapter 2 - Tonic Effects of Ketones and Lactate Assessed Using Two Canine Red Blood Cell Assays

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### Abstract

**Objective:** To establish the tonic effects of  $\beta$ -hydroxybutyrate (BHB), acetoacetate (AA), and lactate.

**Procedures:** Two *in vitro* models were used. The modified osmotic fragility assay measured the ability of ketoacids salts added to serial sucrose dilutions to protect red blood cells (RBCs) from osmotic hemolysis. The sucrose concentration at which 50% hemolysis occurred under control conditions was designated the H50. In a second assay, a handheld cell counting device measured changes in RBC diameter (dRBC) to assess the tonic effect of solutions of ketoacid and lactate salts.

**Results:** In the modified osmotic fragility assay, addition of sodium BHB or lithium AA yielded a lower H50 than the control sucrose series indicating an osmoprotective effect. All ketoacid salts demonstrated an osmoprotective effect; however the effect was determined to be completely attributable to the tonic effect of added cations (sodium and lithium), not the ketoacid moieties. In the dRBC assay, the dRBC was significantly increased with the addition of urea (an ineffective osmole), but did not change with the addition of glucose (an effective osmole). The dRBC was significantly increased over controls by addition of sodium BHB, lithium AA, and lithium lactate but was not increased by sodium lactate.

**Conclusions:** In both assays, BHB and AA act as ineffective osmoles. The tonic effects of lactate were complex and differed with the accompanying cation.

### Introduction

The terms osmolality and tonicity are often used interchangeably; however, these two concepts differ in subtle yet important ways. Osmolality is the term used to describe the total solute concentration, but does not distinguish between solutes that exert osmotic pressure (effective osmoles), those that do not (ineffective osmoles). The barrier function of the cell membrane is an essential component in the generation of an osmotic gradient and the ability to

exert osmotic pressure depends on the permeability of the cell membrane to a given osmole. Tonicity, also called *effective* osmolality, is the number of effective osmoles per unit of solvent.<sup>1</sup> When ineffective osmoles are added to the solution surrounding cells, cells undergo a rapid volume increase as new osmoles diffuse into the cells and water follows. However, when effective osmoles are added, cells shrink as intracellular water is drawn across the semi-permeable cell membrane via osmosis. Hypertonicity can lead to significant cellular dysfunction. Loss of intracellular water causes macromolecular crowding within the cell and dysregulation of intracellular proteins.<sup>15</sup> Changes in cell size are a normal part of cell signaling in many tissues and thus shrinking induced by pathologic hypertonicity will disrupt these pathways.<sup>2</sup> Finally, cells acclimatized to chronic hypertonicity can swell during rapid correction of serum tonicity leading to tissue edema.<sup>18</sup>

In health, the major contributors to serum tonicity include the monovalent ions and glucose. In many disease states, hypertonicity results from an increased serum concentration of endogenous or exogenous effective osmoles. Diabetes mellitus is an important cause of hypertonicity, resulting from hyperglycemia, in both humans and animals. The prevalence of hypertonicity in human diabetics varies depending on the cut-off value used to define hypertonicity. In one cross-sectional study, the prevalence in human diabetics was reported as 35-78%; a similar value has been reported in diabetic dogs and cats (33-81%).<sup>46,60</sup> In addition to glucose, sodium concentration also appears to be an important contributor to hypertonicity in human and veterinary patients and may independently impact diabetic progression.<sup>46,54,64</sup> Diabetic patients may also have excess accumulation of endogenous osmoles that normally are maintained at minute concentrations in healthy patients. Examples of these solutes are the ketoacids, BHB and AA, which are overproduced in unregulated diabetes mellitus by the liver as an indirect consequence of relative or absolute insulin deficiency.<sup>11,48,83</sup> lactate concentration can also increase, particularly, during diabetic ketoacidosis, owing to hypoperfusion and altered glucose metabolism.<sup>51</sup> These solutes increase serum osmolality, but it is unclear whether they increase serum tonicity by acting as effective osmoles.

Comazzi et al (2004) investigated the effects of glucose, with or without ketoacids, on a variety of canine RBC parameters including size and osmotic fragility. They found that changes in these parameters were significantly greater when both glucose and NaBHB were added, compared with the addition of glucose alone.<sup>78</sup> Using an osmotic fragility assay with human



RBCs, Puliyeel (2003) concluded that AA is an effective osmole because LiAA protected RBCs from hypotonic-induced lysis in a manner similar to that of the known effective osmoles, NaCl and glucose.<sup>13</sup> However, the methodologies used in previous investigations added ketoacids to solutions as Na<sup>+</sup> or Li<sup>+</sup> salts of the weak organic acid and tonic effects of added cations may have confounded the analysis. Thus, the tonic effects of the organic anions remain to be firmly established.

The present study examined the hypothesis that organic anions (BHB, AA, lactate) are ineffective osmoles and do not exert a tonic effect. The hypothesis was investigated using two methods to assess tonic effects of various organic osmoles while excluding the effects of accompanying cations. First, a modified RBC osmotic fragility assay was used to compare tonic effects of organic salts of ketoacids (NaBHB and LiAA) with inorganic salts (NaCl and LiCl). Second, a novel canine red blood cell assay was used to compare changes in dRBC in the presence of osmoles of known (glucose, urea) and unknown (BHB, AA, lactate) effect.

## Materials and Methods

### *Modified Osmotic Fragility Assay*

Canine RBCs in EDTA (drawn < 48 hours and kept at 4 °C) were centrifuged at 1200 rpm for 10 minutes, followed by three serial 1:3 washings with 300 mOsM NaCl. The final supernatant was discarded and 100 µl of pelleted cells were resuspended in 500 µl of each test or control solution. Cells were incubated for 10 minutes at 22 °C and then centrifuged at 2000 rpm for three minutes and the pellet was discarded. As a marker for cell lysis, the free hemoglobin content of 100 µl of each solution was measured by spectral absorbance ( $\lambda = 450$  nm) using a 96-well plate spectrophotometer.<sup>a</sup> Samples were analyzed in duplicate and values were expressed as % hemolysis, using RBCs incubated in double-distilled water to represent 100% hemolysis (Eq. 2.1).

$$\text{Eq. 2.1 } \% \text{ hemolysis} = \frac{\text{Sample or Control O.D.}}{\text{adH}_2\text{O O.D.}} \times 100$$

First, the relative tonicities of the ketoacid salts (NaBHB and LiAA) were compared to their respective Cl<sup>-</sup> salts (NaCl and LiCl) by assessing their ability to protect RBCs from

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<sup>a</sup> SpectraMax190; Molecular Devices; Sunnyvale, CA

hypotonic-induced hemolysis.<sup>b</sup> A series of sucrose solutions were prepared in 50mM increments (0 mM – 300 mM) in triplicate. To each series, either 50 mM NaBHB or 50 mM NaCl, or were added and assayed as described above; and unadulterated sucrose series was used as control. An identical procedure was performed using LiAA and LiCl. The control series (sucrose) was performed four times and each non-control series (NaBHB, NaCl, LiAA, LiCl) was performed eight times; the mean for each data point was calculated. From these means, hemolysis protection curves for each series were generated by plotting the % hemolysis (y-axis) of each sample/control against the sucrose concentration of each solution (x-axis). For each curve, the sucrose concentration at which 50% hemolysis occurred (H50) was visually determined and compared between NaBHB and NaCl and between LiAA and LiCl as well as to the H50 for the control (sucrose) solution. An H50 lower than that of the control series indicated an osmoprotective (tonic) effect of the added (non-sucrose) solutes.

Next, the H50s of the control solution, NaCl, and NaBHB were compared to assess whether the BHB anion exerts a tonic effect independent of the sodium cation. The number of non-sucrose, effective osmoles added to the NaCl-containing series as determined by the osmotic fragility assay, was calculated by subtracting the H50 of the NaCl solution from H50 of the control solution. This difference was divided by the number of non-sucrose, effective osmoles known to be added to each solution (i.e. 50 mM NaCl = 100 mOsM) to establish a correction factor for the assay (Eq. 2.2).

$$\text{Eq. 2.2 Assay Correction Factor} = \frac{H50(\text{control}) - H50(\text{NaCl})}{100 \text{ mOsM}}$$

The difference between the H50 of the control series and the H50 of the NaBHB-containing series was multiplied by the assay correction factor to determine the number of non-sucrose, effective osmoles added to solution by 50 mM NaBHB. It was assumed that, if BHB is an effective osmole, then NaBHB would contribute two effective osmoles per mole salt (50 mM NaBHB = 100 mOsM), representing the tonic contribution of both Na<sup>+</sup> and BHB. However, if BHB is an ineffective osmole, then NaBHB would contribute only one effective osmole per mole salt (50 mM NaBHB = 50 mOsM), representing only the tonic contribution of sodium. A similar analysis was performed for LiAA and LiCl.

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<sup>b</sup> NaCl, NaBHB, NaLactate, LiCl, LiAA, LiLactate, sucrose, urea; Sigma-Aldrich; St. Louis, MO

### *Canine dRBC Assay*

Canine RBCs in EDTA (drawn within 36 hours and kept at 4 °C) were prepared by washing in a 1:5 dilution with 0.01 M phosphate buffered saline. The cells were mixed and centrifuged at 1050 rpm for five minutes and the supernatant discarded. The wash step was repeated and the cell pellet was then re-suspended in a 1:1 volume of 300 mOsM NaCl.<sup>c</sup> An aliquot of suspended RBCs was diluted 1:20 into 300 mOsM NaCl and acclimated for at least 10 minutes at 22 °C.

An aliquot (1 µl) of acclimated RBCs was added to 500 µl of control or test solutions and incubated for 30 minutes at 22 °C. The dRBC of cells in each solution was measured using a hand-held automated cell counting device.<sup>d</sup> After gentle mixing, a 100 µl sample containing RBCs was aspirated into a disposable 40 µm sensor attached to the cell counting device. Cell counts stratified by cell diameter in the form of a histogram were automatically generated by the cell counting device. Data were downloaded and imported into the cell counting device software<sup>e</sup> and the histograms were gated. Gating was performed manually by selecting the first bin from both ends of the range that was taller than it was wide (Figure 2.1). Mean cell dRBC was then calculated by the software and exported to a spreadsheet<sup>f</sup> for data analysis.

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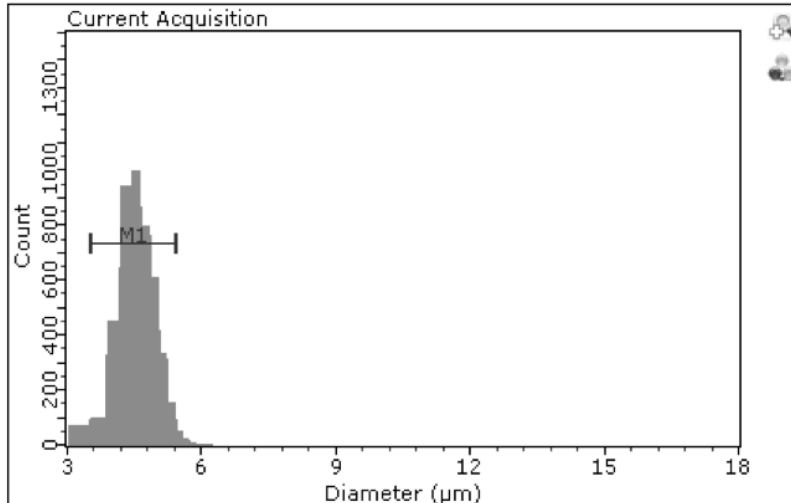
<sup>c</sup> Standard Solution, 300 mOsm/kg H<sub>2</sub>O; Precision Systems; Natick, MA

<sup>d</sup> Scepter 2.0; EMD Millipore; Haywood, CA

<sup>e</sup> Scepter Software Pro 2.0; EMD Millipore; Haywood, CA

<sup>f</sup> Excel; Microsoft; Redmond, WA

**Figure 2.1 Histogram output of the hand-held cell counting device. Gating was performed manually by selecting the first bin from both ends of the range that was taller than it was wide. In this example, the upper gating is appropriate; the lower gate would be manually set to one bin higher.**



First, osmoles of known tonic effect were assayed to define the behavior of effective and ineffective osmoles in the canine dRBC assay. A 50 mM solution of a known effective osmole, glucose, was prepared in 300 mOsM NaCl. Similarly, a 50 mM solution of a known ineffective osmole, urea, was prepared in 300 mOsM NaCl. The dRBCs of these solutions, as measured by the canine RBC assay were, compared to a 300 mOsM NaCl control solution.

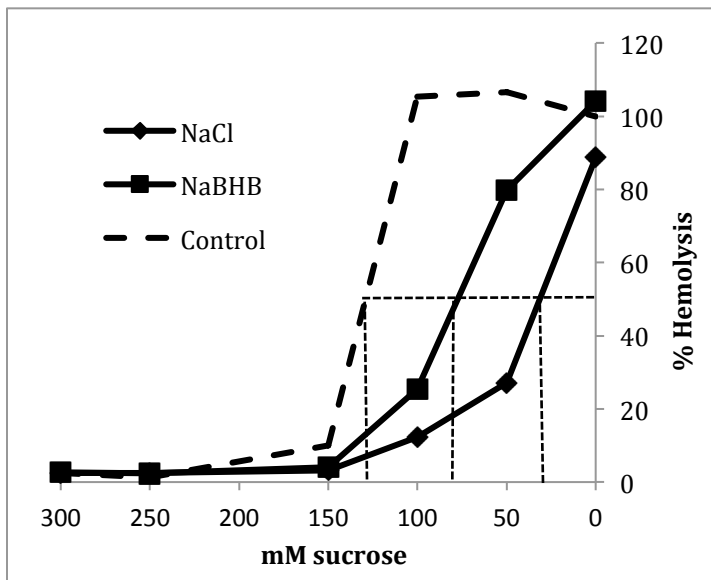
Next, the tonic effects of BHB, AA, and lactate were examined. The effects on dRBC of 300 mOsM solutions of NaBHB and NaLactate were compared with a 300 mOsM NaCl control solution. Because NaAA was not readily available, AA was tested using the Li salt. Three hundred mOsM solutions of LiAA and LiLactate were prepared and compared to a 300 mOsM LiCl control solution. All experiments were performed in replicate (n = 15-18 replicates). The dRBC value for all solutions was expressed as mean  $\pm$  standard deviation. The Wilcoxon rank test was used to compare dRBC between groups. A p-value less than 0.05 was considered significant.

## Results

### *Modified Osmotic Fragility Assay*

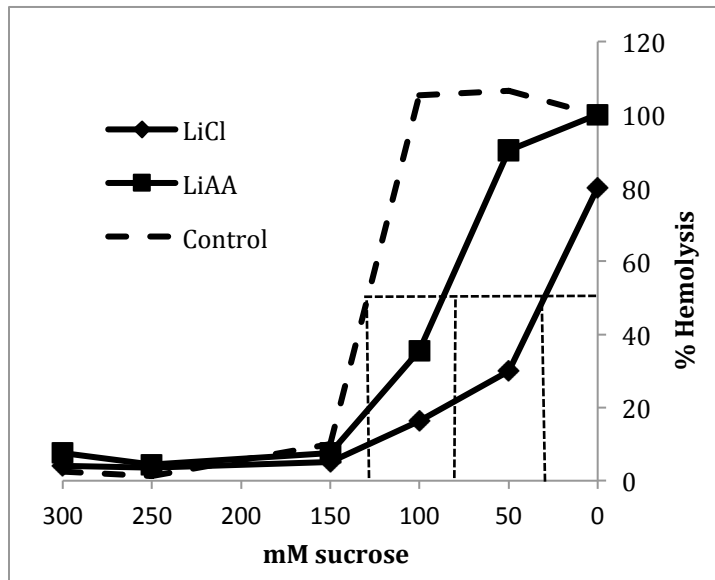
Hemolysis protection curves for NaBHB and NaCl are displayed in Figure 2.2. All curves demonstrated a clear progression from 0% hemolysis (300 mM sucrose) to 100% hemolysis (0 mM sucrose). The H50 for both NaBHB (78 mM sucrose) and NaCl (31 mM sucrose) were lower than that for the sucrose control (130 mM sucrose); however, the H50 for NaCl was lower than that for NaBHB.

**Figure 2.2 Hemolysis protection curves for NaCl and NaBHB containing series relative to control. The vertical thin dotted lines represent the sucrose concentration at which 50% hemolysis occurs (H50) for each series. The horizontal thin dotted lines represent the differences between the H50 of NaCl, NaBHB, and control and thus the amount of non-sucrose, effective osmoles added to each series.**



Similar relationships were evident between the H50s of LiAA (87 mM sucrose), LiCl (31 mM sucrose), and control (130 mM sucrose) as seen in Figure 2.3.

**Figure 2.3 Hemolysis protection curves for LiCl and LiAA containing series relative to control. The vertical thin dotted line represents the sucrose concentration at which 50% hemolysis occurs (H50) for each series. The horizontal thin dotted lines represent the differences between the H50 of LiCl, LiAA, and control and thus the amount of non-sucrose, effective osmoles added to each series.**



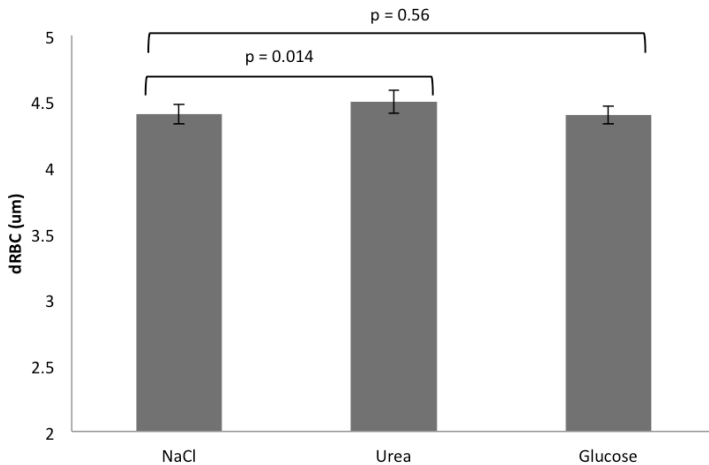
The correction factor for the osmotic fragility assay using sodium salts was determined to be 0.99. Therefore, the corrected number of non-sucrose, effective osmoles added to solution by 50 mM NaBHB was calculated as 51 mOsM ( $0.99 \times [130 \text{ mM} - 78 \text{ mM}]$ ). This value is almost exactly the number of effective osmoles expected to be added when assuming NaBHB only contributes a single effective osmole per mole salt (50 mOsM). The correction factor for the osmotic fragility assay using lithium salts was also 0.99. Therefore, the corrected number of non-sucrose, effective osmoles added to solution by 50 mM of LiAA was calculated as 42 mOsM ( $0.99 \times [130 \text{ mM} - 87 \text{ mM}]$ ). This value is closest to the number of effective osmoles expected to be added when assuming LiAA only contributes a single effective osmole per mole salt (50 mOsM) as opposed to assuming LiAA contributes two effective osmoles per mole salt (100 mOsM).

## Canine dRBC Assay

### Osmoles of Known Tonic Effect

The results of these experiments are displayed in Figure 2.4. The dRBC in the glucose-containing solution ( $4.39 \mu\text{m} \pm 0.067$ ) was smaller than the dRBC in the NaCl control solution ( $4.40 \mu\text{m} \pm 0.076$ ), but this difference was not statistically significant ( $p = 0.56$ ). However, the dRBC in the urea-containing solution ( $4.50 \mu\text{m} \pm 0.088$ ) was significantly larger compared with the NaCl control ( $p = 0.014$ ).

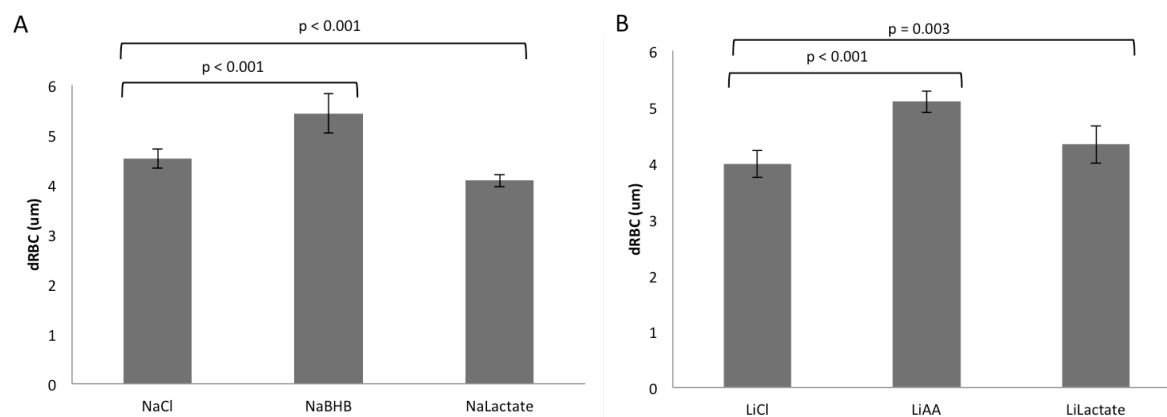
**Figure 2.4** dRBCs for osmoles of known tonic effect (50 mM urea or glucose in 300 mOsm NaCl). Addition of the ineffective osmole, urea, causes a significant increase in dRBC ( $4.50 \mu\text{m} \pm 0.088$ ) compared to saline control ( $4.40 \mu\text{m} \pm 0.076$ ). Addition of the effective osmole, glucose, does not significantly change dRBC ( $4.39 \mu\text{m} \pm 0.067$ ) relative to control.



### Osmoles of Unknown Tonic Effect (BHB, AA, and lactate)

The results of these experiments are displayed in Figure 2.5. The dRBC in the NaBHB solution ( $5.43 \mu\text{m} \pm 0.394$ ) was significantly larger compared with the NaCl control solution ( $4.52 \mu\text{m} \pm 0.190$ ;  $p < 0.001$ ). In contrast, dRBC in the NaLactate solution ( $4.33 \mu\text{m} \pm 0.335$ ) was significantly smaller compared with the NaCl control ( $p < 0.001$ ). The dRBC in both the LiAA solution ( $5.09 \mu\text{m} \pm 0.194$ ) and the LiLactate solution ( $4.33 \mu\text{m} \pm 0.335$ ) were significantly larger compared with LiCl control ( $3.97 \mu\text{m} \pm 0.239$ ;  $p < 0.001$  and  $p = 0.003$ , respectively).

**Figure 2.5 Tonic effects of unknown osmoles on red blood cell diameter (dRBC) in sodium salts (panel A) and lithium salts (panel B); 150 mM (300 mOsM) concentrations for all solutions. dRBC for NaBHB ( $5.43 \mu\text{m} \pm 0.394$ ), LiAA ( $5.09 \mu\text{m} \pm 0.194$ ), and LiLactate ( $5.09 \mu\text{m} \pm 0.194$ ) are statistically greater compared to their relative controls (NaCl  $4.52 \mu\text{m} \pm 0.190$ ; LiCl  $3.97 \mu\text{m} \pm 0.239$ ) in a manner similar to the ineffective osmole, urea. The dRBC for NaLactate ( $4.33 \mu\text{m} \pm 0.335$ ) was not larger than control, in a manner similar to the effective osmole, glucose.**



## Discussion

The results support the hypothesis that the ketoacids (BHB and AA), act as ineffective osmoles and therefore do not exert a tonic effect. When tested using the modified osmotic fragility assay, both the NaCl and the NaBHB series had higher H50 values compared to the control (sucrose) series, indicating that the addition of either NaCl or NaBHB exerted an osmoprotective effect. The same effect on H50 was detected in experiments comparing LiCl and LiAA against sucrose alone. This finding was expected since the addition of 50 mM NaCl or LiCl, which are known to be effective osmoles, increases the tonicity of the sucrose solutions and reduces the osmotic gradient across the RBC membrane, decreasing the likelihood of lysis. Comparison of the H50 value for the NaCl/NaBHB and LiCl/LiAA series permits the specific effect of the anion (BHB and AA) to be determined. Addition of 50 mM NaCl or LiCl increases tonicity by 100 mOsM because these salts dissociate completely in aqueous solution. Like all salts, NaBHB and LiAA also dissociate completely in aqueous solution. If BHB is an effective



osmole, 50 mM NaBHB would increase tonicity by 100 mOsM, but if BHB behaved as an ineffective osmole, 50 mM NaBHB would only increase tonicity by 50 mOsM; the same is true for LiAA solutions. In both series, the osmoprotective effect of the ketoacid salts was less than the corresponding chloride salt, suggesting that each ketoacid salt did not contribute the same number of effective osmoles as the corresponding chloride salt. Calculation of a correction factor allowed for the tonic effects of ketoacids to be distinguished from those of the Na<sup>+</sup> and Li<sup>+</sup> cations. The concentrations of effective osmoles added by 50 mM NaBHB and 50 mM LiAA were 51 mOsM and 42 mOsM, respectively, which are both close to 50 mOsM, the expected value if BHB and AA are ineffective osmoles. Thus, the results of the modified fragility assay support the conclusion that BHB and AA act as ineffective osmoles and do not exert tonic effects.

In a second series of experiments, the effects of urea and glucose were assessed using a novel assay to determine changes in RBC diameter (dRBC). The dRBC was increased significantly by addition of urea, an ineffective osmole. The increase in dRBC was expected because RBCs are highly permeable to urea.<sup>7</sup> Influx of urea is accompanied by water causing swelling of the cells and increasing the dRBC. Exposure to glucose, a known effective osmole, was expected to decrease dRBC through an opposite mechanism that encouraged water efflux and decreased cell size. In experiments using glucose, the dRBC decreased relative to control but the difference did not reach statistical significance. The reason for the reduced effect of glucose under test conditions is not clear but could be related to the complex handling of glucose by canine RBCs. It has been reported that glucose transport into canine RBCs is higher than in many other species.<sup>84</sup> It is possible that a high intracellular glucose concentration opposed the transcellular gradient induced by glucose incubation, reducing the tonic effect of the extracellular glucose. Regardless, the urea and glucose experiments demonstrated the expected changes in dRBC in the presence of ineffective and effective osmoles.

Incubation with NaBHB and LiAA significantly increased dRBC compared with control solutions of NaCl and LiCl. The changes in dRBC caused by solutions of ketoacids are consistent with the changes caused by incubation with urea, suggesting these substances act as ineffective osmoles. This is in accordance with the hypothesis and the results from the modified fragility assay. The changes in dRBC caused by lactate, however, were discordant. NaLactate caused a decrease in dRBC relative to the NaCl control, consistent with action as an effective

osmole, whereas LiLactate caused an increase in dRBC compared with the LiCl control, consistent with action as an ineffective osmole. It is not clear why lactate appears to function differently when paired with different cations. The  $\text{Li}^+$  concentration in these in vitro solutions far exceeds that normally experienced by RBCs and may affect cell membrane function in unpredictable ways. For example,  $\text{Li}^+$  could alter the function of the MCT, which facilitate lactate cell entry.<sup>12</sup> The MCTs also mediate transport of the ketoacids, so the tonic effect of AA, which was measured as a  $\text{Li}^+$  salt, might also be affected by the supranormal  $\text{Li}^+$  concentration. Unfortunately, AA is not readily available as a sodium salt so comparisons between the sodium and lithium salts of AA could not be determined.

Although tonicity is often discussed at a systemic level (e.g. serum tonicity), there can be significant variability in tonic effect when examining individual tissues and cells. The tonic behaviors of effective and ineffective osmoles are not solely dependent on the chemical properties of the solute such as charge, size and hydrophobicity. Tonic behavior is also dependent on tissue characteristics and the properties of the cell membrane. It is apparent that the tonic effects of osmoles can vary in different situations. For example, urea acts as an ineffective osmole in most tissues because most cells have abundant expression of urea transport proteins, which allows for fast equilibration of urea across the cell membrane.<sup>7</sup> However, in the renal tubules there is differential expression of the urea transporter, which is under hormonal regulation. Under certain conditions in the tubule, urea can behave as an effective osmole, which is an important mechanism for tubular fluid dilution.<sup>8</sup> Similarly, in most tissues, especially muscle, membrane localization of the glucose transporter, GLUT 4, is regulated by insulin.<sup>6</sup> In the absence of insulin or in insulin-resistant states (i.e. diabetes mellitus), the cell becomes even less permeable to glucose, which presumably increases the tonic effects of glucose. Cell permeability to ketoacids and lactate may also be modulated by differences in MCT protein expression, especially MCTs 1-4. The various MCT isoforms have different substrate affinities and there is differential expression of these transporters among tissues. Furthermore, MCT expression and activity is regulated by a variety of pathways that may be influenced by the intracellular milieu.<sup>12</sup>

A limitation of this study is that only RBCs were studied. As previously discussed, tonic effects of solutes may vary by tissue type and disease state, so the effects of the ketoacids and lactate on RBCs may not represent the response seen in all cell types. RBCs were used for

practical reasons due to ease of sample collection and handling but RBCs also serve as a classic model for biologic osmolality studies.<sup>73,74</sup> However, other cells line could be considered in future experiments to assess effects on target tissues that are particularly susceptible to tonic injury, such as neural tissues.<sup>18</sup> The test solutions used in these experiments were simple iso- or hypertonic aqueous solutions, which were needed to isolate the effects of individual osmoles. Ideally, dRBC would have been measured in solutions that approximate the composition of serum or interstitial fluid. Serum was not used for dRBC experiments due to technical limitations of the cell counting device used to determine dRBC. Similarly, technical limitations also precluded evaluation of RBCs in hypotonic solutions. Finally, the results reflect the tonic effects on RBCs *in vitro*. Given the variability and diversity of biologic systems, extrapolations to clinical situations should be made with caution.

In conclusion, BHB and AA act as ineffective osmoles in a biologic model. The tonic effects of lactate are complex and may be influenced by the cation concentration. These experiments used ketoacid and lactate concentrations that exceed those found in healthy dogs and reported for various disease states. Based on these findings, it is predicted that BHB or AA do not contribute to serum tonicity in clinical syndromes, such as diabetes or ketoacidosis. Similarly, lactate is unlikely to exert a strong tonic effect but elucidating the mechanism of the lactate effect will require additional investigation.

# **Chapter 3 - In Vitro Increase of Mean Corpuscular Volume Difference (dMCV) as a Marker for Serum Hypertonicity in Dogs**

Jennifer M. Reinhart, Misty R. Yancey, Lisa M. Pohlman, Thomas Schermerhorn

## **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 2 fl 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 cutoff of 2.96 fl yielded the best sensitivity (76%) and specificity (71%) for hypertonicity estimated by  $OsM_M$ .

## **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).<sup>1</sup> 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.<sup>2,14,15</sup> 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.<sup>46,54,60-64</sup>

In the Third National Health and Nutrition Examination Survey (NHANES III), a cross-sectional survey of nonhospitalized 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.<sup>60</sup> Higher prevalence has been associated with age, obesity, race, and diabetic status.<sup>60,61</sup> 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.<sup>62,63</sup> A longitudinal study of older adults found that plasma hypertonicity was an independent predictor for the onset of disability within a 4-year period and mortality within an 8-year period.<sup>63</sup> Hypertonicity is a 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%.<sup>60</sup> 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.<sup>64</sup> In diabetic dogs and cats, the prevalence of effective hyperosmolality was reported as 81%.<sup>46</sup> Interestingly, in diabetic animals, sodium (Na) concentrations appear to be more closely associated with osmolality (tonicity) than glucose, emphasizing the importance of sodium in diabetic patients.<sup>46,54</sup>

Clinically, tonicity can be difficult to quantify because this value not only includes solute concentrations but also 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. 3.1). 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. 3.2) 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.<sup>1</sup> 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.<sup>79</sup> Stookey et al. (2007) 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. 3.3). 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.<sup>80</sup>

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.<sup>§</sup> 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

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<sup>§</sup> Vetstar, Advanced Technology Corp.; Oak Ridge, TN

hand and subsequently screened for study inclusion. Cases were included if the patient was a dog, if a complete blood 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 ( $[RBC] < 5.5 \text{ M}/\mu\text{l}$ ) 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;<sup>h</sup> 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.<sup>i</sup> Serum samples were then frozen at  $-20^{\circ}\text{C}$  until identified for use in the study, at which point they were transferred to a  $-80^{\circ}\text{C}$  freezer where they were stored until batch osmometry measurements could be made. Serum  $\text{OsM}_M$  was measured in duplicate by freezing-point depression using the Micro-OSMETTE osmometer.<sup>j</sup>

### ***Calculations***

The following values measured as part of the CBC were included in the calculation of dMCV: measured mean corpuscular volume ( $\text{MCV}_M$ ), spun hct, and RBC. The following values measured as part of the serum biochemistry profile were included in serum osmolality calculations: Na concentration (mmol/l), K concentration (mmol/l), glucose concentration (mg/dl), and BUN concentration (mg/dl).  $\text{OsM}_C$  (Eq. 3.1) and  $\text{OsM}_{CE}$  (Eq. 3.2) were determined using standard clinical formulas.<sup>1</sup>

$$\text{Eq. 3.1 } \text{OsM}_C = 2(\text{Na} + \text{K}) + \frac{\text{BUN}}{2.8} + \frac{\text{glucose}}{18}$$

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<sup>h</sup> Siemens Medical Solutions, Inc.; Malvern, PA. OsM of the RBC diluent is 280 mOsM

<sup>i</sup> Roche Diagnostics; Indianapolis, IN

<sup>j</sup> Precision Systems, Inc.; Natick, MA

$$\text{Eq. 3.2 } \text{OsM}_{CE} = 2(\text{Na} + \text{K}) + \frac{\text{glucose}}{18}$$

The dMCV (Eq. 3.3) was calculated by as previously reported.<sup>80</sup>

$$\text{Eq. 3.3 } \text{dMCV} = \text{MCV}_M - \frac{\text{hct} \times 10}{\text{RBC}}$$

### ***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 ( $\text{OsM}_M$ ,  $\text{OsM}_C$ , and  $\text{OsM}_{CE}$ ), dogs were categorized into a normosmolar group ( $\text{OsM} < 320 \text{ mOsM}$ ) and a hyperosmolar group ( $\text{OsM} \geq 320 \text{ mOsM}$ ). Because only one dog would have been considered hypoosmolar ( $\text{OsM} < 280 \text{ mOsM}$ ) using measured osmolality ( $\text{OsM}_M = 279 \text{ mOsM}$ ) and no dogs were hypoosmolar using  $\text{OsM}_C$  or  $\text{OsM}_{CE}$ , a hypoosmolar group was not included in analysis; the aforementioned dog was included in the normosmolar group for  $\text{OsM}_M$  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 characteristic (ROC) curve; cutoff values to maximize sensitivity and specificity were visually determined. The 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 cutoff for mild hypertonicity, defined as  $\text{OsM}_M \geq 300 \text{ mOsM}$ . The dMCV of normosmolar ( $\text{OsM}_M < 300 \text{ mOsM}$ ) and hyperosmolar ( $\text{OsM}_M \geq 300 \text{ mOsM}$ ) dogs were compared using a Student's t-test; an ROC curve was generated and a cutoff value maximizing sensitivity and specificity was visually determined. All statistical analyses were performed using commercial software.<sup>k</sup> A p-value less than 0.05 was considered statistically significant.

### **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

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<sup>k</sup> Excel, Microsoft; Redmond, WA



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 5 were intact females. Pertinent clinicopathologic values, measured osmolality, and calculated values are summarized in Table 3.1.

**Table 3.1 Summary statistics of pertinent clinicopathologic values, measured osmolality, and calculated values.**

	<b>Median</b>	<b>Range</b>	<b>Reference Range*</b>	<b>Units</b>
<i>Na</i>	149	118 – 164	147 – 154	mmol/l
<i>K</i>	4.5	3.1 – 6.6	2.6 – 5.3	mmol/l
<i>BUN</i>	15	4 – 212	9 – 33	mg/dl
<i>Glucose</i>	109	30 – 1108	73 – 113	mg/dl
<i>RBC</i>	7.15	5.52 – 9.40	5.5 – 8.5	M/ $\mu$ l
<i>hct</i>	48	35 – 66	35 – 55	%
<i>MCV<sub>M</sub></i>	69	62 – 76	60 – 77	fl
<i>OsM<sub>M</sub></i>	307.0	279.0 – 510.5 <sup>^</sup>	n/a	mOsM
<i>OsM<sub>C</sub></i>	319.4	296.0 – 377.1	n/a	mOsM
<i>OsM<sub>CE</sub></i>	314.4	291.1 – 344.2	n/a	mOsM
<i>dMCV</i>	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.

<sup>^</sup>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 it was included in the data set. Exclusion of this data point does not significantly alter the results of this study.

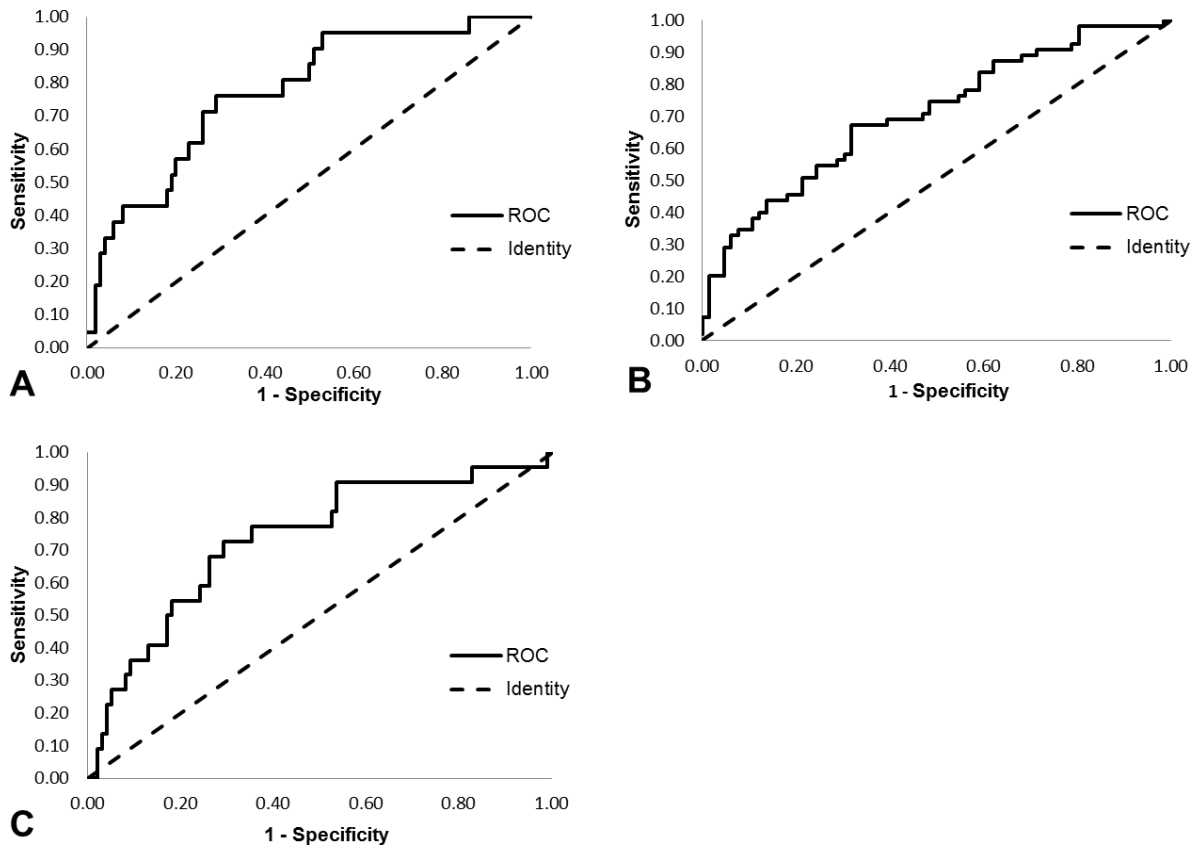
Regardless of the method used to estimate tonicity (*OsM<sub>M</sub>*, *OsM<sub>C</sub>*, *OsM<sub>CE</sub>*), the *dMCV* for hyperosmolar dogs was significantly larger than the *dMCV* for normosmolar dogs (Table 3.2).

**Table 3.2 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

ROC curves for dMCV predicting  $OsM_M$ ,  $OsM_C$ , and  $OsM_{CE}$  are depicted in Figure 3.1.

**Figure 3.1 ROC Curves for dMCV predicting hypertonicity ( $OsM \geq 320$  mOsM) as estimated by  $OsM_M$  (A),  $OsM_C$  (B), and  $OsM_{CE}$  (C).**



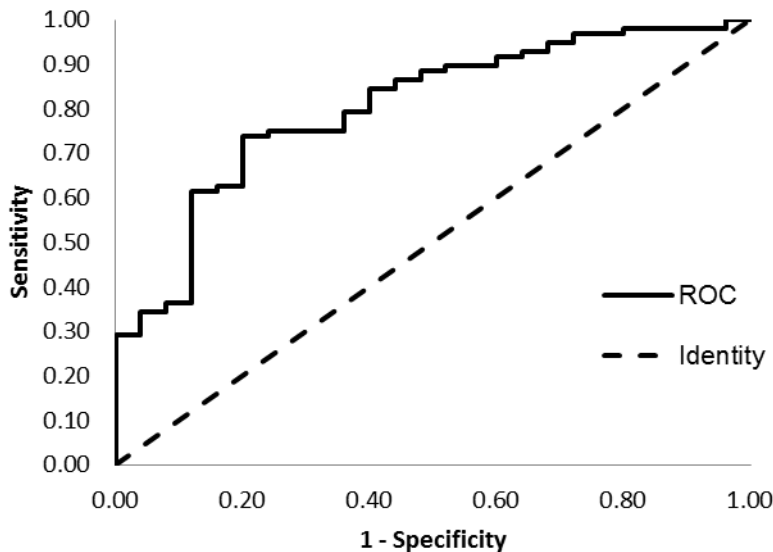
A dMCV of 2.96, 2.47, and 2.96 fl provided maximal sensitivity and specificity for predicting hypertonicity as estimated by  $OsM_M$ ,  $OsM_C$ , and  $OsM_{CE}$ , respectively. dMCV best predicted hypertonicity as estimated by  $OsM_M$  (AUROC = 0.7738; Table 3.3).

**Table 3.3 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

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 3.2); a dMCV cutoff of 1.49 fl yielded a 75% sensitivity and 76% specificity.

**Figure 3.2 ROC curve for dMCV predicting mild hypertonicity ( $OsM_M \geq 300$  mOsM)**



## 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 cutoff 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.<sup>63,64</sup> Therefore, the canine data were reexamined to determine dMCV performance when mild hyperosmolality is present. Using a lower cutoff for dMCV ( $\geq 1.5$  fl) for mild hypertonicity ( $OsM_M \geq 300$  mOsM) performed as well as the higher dMCV cutoff ( $\geq 3$  fl) did for overt hypertonicity ( $OsM_M \geq 320$  mOsM). This graduated cutoff 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.<sup>81</sup> 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.<sup>82</sup> 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.<sup>1</sup> Albumin is the most important plasma determinant of oncotic pressure, contributing approximately 80% of the total plasma colloid osmotic pressure.<sup>1</sup> 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.<sup>2,14,17,85</sup> 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).<sup>14,85</sup> Early responses to cell shrinkage or swelling include increased membrane ion transport followed by transport of small nonionic 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 downregulation of gene expression, so complete volume compensation (RVI or RVD), if it occurs, takes hours to days.<sup>2</sup> 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 caused 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.<sup>25</sup> 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 consistent with those reported here (Reinhart, unpublished), suggesting the effect of storage is minimal. 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 the 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 ( $\geq 3$  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.

# Chapter 4 - Mean Corpuscular Volume Difference (dMCV) as a Marker for Serum Hypertonicity During Water Deprivation in Dogs

Jennifer M. Reinhart, Misty R. Yancey, Lisa M. Pohlman, Thomas Schermerhorn

## Abstract

*Introduction* – The mean corpuscular volume difference (dMCV) is a novel technique that assesses tonicity by measuring the physiologic response of red blood cells to tonic stress. Increased dMCV has been suggested as a marker for hypertonicity in dogs, but has not been evaluated prospectively.

*Objective* – The purpose of this study was to evaluate dMCV as a marker for hypertonicity induced by water deprivation.

*Animals* – Five healthy, research colony, Greyhound dogs.

*Methods* – Water was withheld for 24 hours and blood and urine were collected every six hours. Serum and urine osmolality were measured by freezing point depression and dMCV was calculated from routine hematologic parameters.

*Results* – Serum and urine osmolality both significantly increased and body weight decreased over time as is expected in healthy dogs during water deprivation, although the increase in serum osmolality was mild in most dogs. dMCV also increased over time, but was not statistically different from baseline at 24 hours. However, there was a significant correlation between serum osmolality and dMCV; a dMCV cut-off of 5 fl yields 100% specificity for predicting hypertonicity when hypertonicity is defined as serum osmolality  $\geq 310$  mOsm.

*Conclusions* – dMCV may be a useful marker for detection of mild hypertonicity in dogs and may have clinical and research applications for screening for hypertonicity in canine populations.

## Introduction

Hypertonicity is a physiologic state of increased extracellular solute load causing water to shift out of cells by osmosis, which is known or suspected to have a role in numerous human and veterinary diseases.<sup>1,46,54,61</sup> Hypertonicity negatively impacts cellular function but tonicity is difficult to quantify in a clinical setting because it is the numeric sum of all effective osmole concentrations, not all of which are easily identified and measured.<sup>14,15</sup> Rather than attempting to

quantify individual solute concentrations, it is possible to infer total tonicity from a physiologic effect on cell size.<sup>86</sup> Spurious elevation of erythrocyte mean corpuscular volume (MCV) occurs when blood from hypertonic patients is assessed by an automated cell analyzer. Red blood cells (RBC) acclimated to hypertonic patient plasma, swell due to uptake of extracellular fluid when placed in isotonic media used in automated analyzers because the analyzer solution is relatively hypotonic to the RBC intracellular environment.<sup>79</sup> Stookey et al. (2007) exploited the physiologic basis for the autoanalyzer error to develop the ‘dMCV’ as an index for plasma hypertonicity.<sup>80</sup> The dMCV value is the difference between the MCV as measured by an automated cell analyzer ( $MCV_M$ ) and the MCV calculated using the value for the spun hematocrit (hct), which is performed with RBCs in the original patient plasma.<sup>80</sup>

In a recent retrospective study, we demonstrated that dMCV is a marker for hypertonicity in dogs.<sup>86</sup> We found that  $dMCV \geq 3$  fl predicted hypertonicity with a moderate sensitivity and specificity (76% and 71%, respectively) when total serum osmolality was  $\geq 320$  mOsM.<sup>86</sup> Although serum osmolality is an approximation of tonicity, it likely overestimates tonicity by inclusion of ineffective osmoles such as urea.<sup>1</sup> The relationship between osmolality and tonicity is further obscured by the fact that the tonic nature of many solutes has not been defined. Given these difficulties, an *in vivo* model of induced hypertonicity is needed to further validate dMCV as a hypertonicity marker in dogs. In the normal dog, hypertonicity can be induced by oral fluid restriction.<sup>87</sup> Because hypertonic plasma stimulates vasopressin release, increased plasma vasopressin would verify the presence of hypertonicity. At this time, direct measurement of vasopressin is not possible because a canine assay is not commercially available. However, release of vasopressin can be indirectly assessed by documenting an increase in urine concentration after a hypertonic stimulus. Therefore, production of concentrated urine can be used to verify a hypertonic state during water deprivation and allow for controlled assessment of dMCV as a marker for hypertonicity.

The purpose of the present study was to validate dMCV as a marker for hypertonicity in dogs and to determine a cutoff value for dMCV that indicates hypertonicity. We hypothesized that dMCV would increase with induction of hypertonicity during water restriction and that a dMCV value of  $\sim 3$  fl would be a useful cutoff for identification of serum hypertonicity.



## Materials and Methods

Water was withheld for 24 hours from five clinically healthy Greyhound dogs (three male, two female), each five years old, with body weights that ranged between 33.4 – 40.7 kg. For each dog, body weight was recorded and blood and urine samples collected prior to water withdrawal (time 0) and every six hours during the 24 hour study period. For each dog, maximum dehydration was limited to 5% (based on 5% loss of body weight). A dog was withdrawn from the study if at any time weight loss exceeded 5% of initial body weight. This study protocol was approved by the Kansas State University Institutional Animal Care and Use Committee.

Routine hematologic parameters were determined using an automated cell analyzer<sup>1</sup> and manual hematocrit using a microcentrifuge and card reader. The dMCV was calculated as previously described:  $dMCV = MCV_M - (hct \times 10) / [RBC]$ .<sup>80,86</sup> Blood urea nitrogen (BUN) and sodium concentrations were determined using a clinical biochemistry analyzer<sup>m</sup>. Serum osmolality (OsM<sub>S</sub>) and urine osmolality (OsM<sub>U</sub>) were measured using a freezing point depression osmometer<sup>n</sup>.

The effect of water deprivation over time on body weight, BUN, sodium, OsM<sub>S</sub>, OsM<sub>U</sub>, and dMCV was assessed with the Friedman's test for repeated measures and Tukey test for post hoc multiple comparisons. Effectiveness of the water deprivation model to induce hypertonicity was determined using body weight, OsM<sub>S</sub>, and OsM<sub>U</sub> trends. To evaluate the validity of OsM<sub>S</sub> as a surrogate standard for tonicity, BUN was monitored to evaluate the contribution of ineffective osmoles to OsM<sub>S</sub> and sodium was monitored to evaluate the contribution of effective osmoles. The Pearson correlation coefficient was calculated to determine the association between OsM<sub>S</sub> and dMCV. A receiver operator characteristic curve (ROC) was generated to assess the ability of dMCV to predict mild hypertonicity and sensitivity, specificity, and area under the curve (AUROC) were calculated. For the purposes of the ROC analysis, mild hypertonicity was

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<sup>1</sup> Advia 2120; Siemens Medical Solutions, Inc.; Malvern, PA

<sup>m</sup> COBAS C501; Roche Diagnostics; Indianapolis, IN

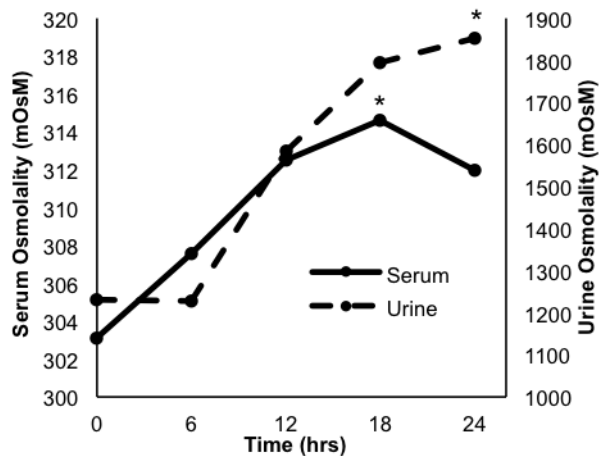
<sup>n</sup> Micro-OSMETTE; Precision Systems, Inc.; Natick, MA

defined as  $OsM_S \geq 310$  mOsM. All statistical analyses were performed using commercial software<sup>o</sup>. A p-value  $\leq 0.05$  was considered significant.

## Results

Body weight ( $p < 0.001$ ) decreased significantly over time, but no dog exceeded 5% loss.  $OsM_S$  ( $p = 0.02$ ) and  $OsM_U$  ( $p = 0.01$ ) increased significantly over time (Figure 4.1).

**Figure 4.1 Changes in mean serum and urine osmolality over time.**



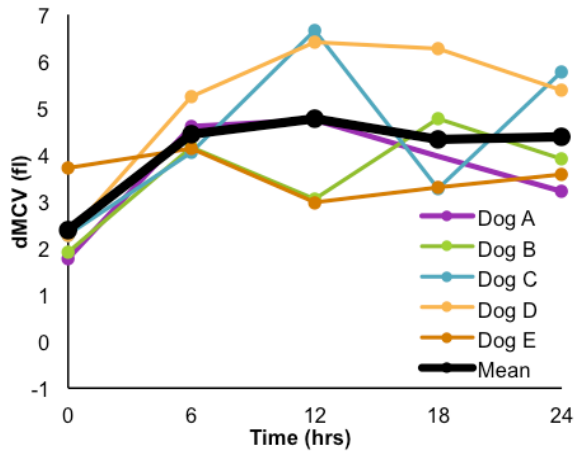
\* denotes osmolality statistically different from baseline (time 0 hrs).

The dMCV increased in 4 of 5 study dogs after 24 hours water deprivation. Mean dMCV also increased over time, nearly doubling in 24 hours, but this increase did not reach statistical significance ( $p = 0.53$ ; Figure 4.2).

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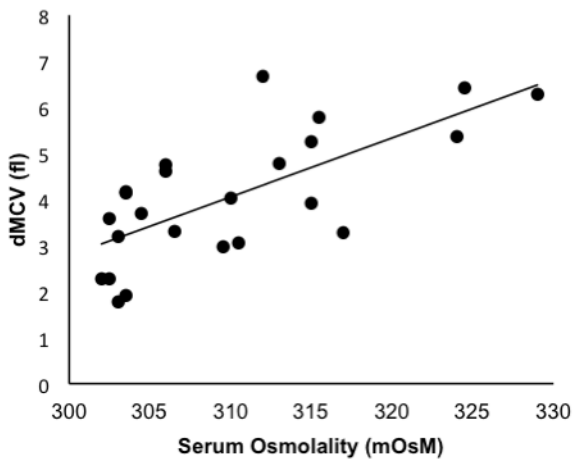
<sup>o</sup> Winks Kwikstat, Texasoft; Cedar Hill, TX and StatPlus:mac, AnalystSoft AnalystSoft;  
<http://www.analystsoft.com/en/>

**Figure 4.2 dMCV change over time in response to water deprivation for individual dogs (A-E) and mean dMCV at each time point.**



However, dMCV was positively correlated with  $OsM_s$  ( $r = 0.71$ ;  $p < 0.001$ ; Figure 4.3).

**Figure 4.3 Scatterplot of  $OsM_s$  compared to dMCV for all dogs at all time points demonstrating a strong correlation ( $r = 0.71$ ;  $p < 0.001$ ).**



The AUROC for dMCV predicting mild hypertonicity ( $OsM_s \geq 310$  mOsM) was 0.84. A dMCV cutoff for dMCV of 3 fl yielded 100% sensitivity and 31% specificity. A cutoff of 5 fl yielded 64% sensitivity and 100% specificity. There was no significant change in BUN during the study ( $p = 0.36$ ), but sodium concentration did increase from time 0 to 24 hours ( $p = 0.001$ ).

## Discussion

The results of this study suggest that dMCV is a useful marker for serum hypertonicity in dogs. Body weight decreased while  $OsM_S$  and  $OsM_U$  increased significantly during water deprivation. The increase in  $OsM_U$  is likely a result of hypertonic stimulation of vasopressin release and can be considered an indirect indicator of plasma hypertonicity. Thus, based on physiologic changes documented in the study dogs, water deprivation is an effective model for induction of hypertonicity in the dog. Although not statistically significant, mean dMCV increased over time (Figure 4.1), which is the expected response of RBCs sampled from hypertonic dogs.<sup>86</sup> Given that dMCV increased in four of five study dogs and the mean dMCV increased by nearly 100% within 24 hours, it is possible that the effect of hypertonicity on dMCV is real but the sample tested (five dogs) was not large enough to detect it. Alternatively, in healthy dogs, the tonic stimulus induced by 24 hours of water deprivation may not be strong enough or persist long enough to elicit a significant dMCV change in healthy dogs. Serum tonicity is tightly regulated by the hypothalamic-renal axis, which appeared intact in these study dogs as evidenced by increasing  $OsM_U$ .<sup>1</sup> Thus, compensatory water resorption by the kidney under the influence of vasopressin may have been adequate to offset hypertonicity induced by water restriction. This is likely the case as the increase in  $OsM_S$ , although significant, was mild in most dogs (Figure 4.1). Many veterinary diseases blunt the tubular response to vasopressin (secondary nephrogenic diabetes insipidus) allowing overt hypertonicity to occur so the effect on dMCV in clinical patients might be easier to observe.<sup>1</sup>

A significant correlation was observed between dMCV and  $OsM_S$ . As previously discussed,  $OsM_S$  is less than ideal as a gold standard for tonicity measurement, but in healthy dogs it may be an appropriate surrogate index. Quantitatively, the difference between osmolality and tonicity is the total concentration of ineffective osmoles. In health, the major ineffective serum osmole is urea and the major effective osmole is sodium. Urea did not significantly change during water deprivation while sodium significantly increased over time, which suggests that the observed  $OsM_S$  increase was caused by increased concentrations of effective osmoles. Tonicity is determined by summation of all effective osmole concentrations; therefore, in normal dogs, it is appropriate to use  $OsM_S$  in lieu of tonicity for validation of dMCV. The ROC analysis yielded two useful dMCV cutoff values. All dogs with a dMCV  $\leq 3$  fl were normotonic at the time of measurement. Conversely, a dMCV  $\geq 5$  fl yielded 100% specificity for hypertonic

serum. dMCV values of 3-5 fl may be difficult to interpret without clinical context. The ROC analysis was designed to detect mild hypertonicity, defined as  $OsM_S \geq 310$  mOsM, which represents an increase in  $OsM_S$  of less than 5% (normal canine  $OsM_S = 300$  mOsM).<sup>1</sup> Thus a dMCV value  $\geq 5$  fl predicts hypertonicity, which can be mild, but mild tonic increases may not always be detected. Ideally, the analysis would have been designed to detect overt hypertonicity ( $OsM_S \geq 320$  mOsM), which is more likely to be clinically relevant; however, hypertonicity of this magnitude was rarely observed in the healthy dogs, presumably because the compensatory response to tonic stimulation was effective.

The results of the present study are similar to those from a recently published study from our group in which  $dMCV \geq 3$  fl predicted hypertonicity with moderate sensitivity (76%) and specificity (71%) in a group of dogs with various clinical illnesses.<sup>86</sup> The difference in sensitivity using a 3 fl cut-off for dMCV between healthy and clinically ill dogs (100% vs. 76%, respectively) may reflect differences in serum solute compositions of the study populations. The previous study evaluated hospitalized canine patients in the intensive care unit, some of which had increased ineffective osmole concentrations (e.g. BUN). Because the presence of ineffective osmoles increased  $OsM_S$  in the canine patients studied,  $OsM_S$  was less likely to be a useful surrogate for tonicity than  $OsM_S$  in the healthy dogs used the present study.

Analysis demonstrated a moderate, but significant, positive correlation between dMCV and  $OsM_S$  in healthy dogs. As previously discussed, dMCV is insensitive to minor or short-term alterations in serum tonicity, which may explain why the correlation between dMCV and  $OsM_S$  was not stronger. However, the dMCV may provide information that other estimates of tonicity do not because not only does dMCV respond to increased effective osmolar concentration but it incorporates the functional effects of hypertonicity on cells. Thus, the dMCV represents the physiologic implications of tonic derangements and provides superior information over purely quantitative estimates, such as the  $OsM_S$  value.

Although hypertonicity develops in many canine diseases, the clinical significance of hypertonicity is unclear in most situations. dMCV is a novel marker for serum hypertonicity that, in conjunction with osmolality and solute concentration assessment, may better elucidate the role of hypertonicity in canine medicine.

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## Appendix A - Abbreviations

AA	acetoacetate
ADH	antidiuretic hormone
AKI	acute kidney injury
ANP	atrial natriuretic peptide
AQP	aquaporin
ATP	adenosine triphosphate
AUROC	area under receiver operator curve
BNP	brain natriuretic peptide
BHB	$\beta$ -hydroxybutyrate
BUN	blood urea nitrogen
Ca <sup>2+</sup>	calcium
CBC	complete blood count
CDI	central diabetes insipidus
CNP	C-type natriuretic peptide
Cl <sup>-</sup>	chloride
CNS	central nervous system
D5W	5% dextrose in sterile water
DKA	diabetic ketoacidosis
DM	diabetes mellitus
dMCV	mean corpuscular volume difference
DNA	deoxyribonucleic acid
dRBC	red blood cell diameter
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
EG	ethylene glycol
fl	femtoliter
GABA	$\gamma$ -aminobutyric acid
GFR	glomerular filtration rate
GIT	gastrointestinal tract

GLUT	glucose transporter
H <sup>+</sup>	hydrogen
H50	50% hemolysis sucrose concentration
HCO <sub>3</sub> <sup>-</sup>	bicarbonate
hct	hematocrit
HHS	hyperglycemic, hyperosmolar syndrome
ICF	intracellular fluid
JGA	juxtaglomerular apparatus
K <sup>+</sup>	potassium
Li <sup>+</sup>	lithium
MCT	monocarboxylate transporter
MCV	mean corpuscular volume
MCV <sub>M</sub>	measured mean corpuscular volume
Mg <sup>2+</sup>	magnesium
MNC	magnocellular neurosecretory cells
Na <sup>+</sup>	sodium
NDI	nephrogenic diabetes insipidus
OsM	osmolality
OsM <sub>C</sub>	calculated osmolality
OsM <sub>CE</sub>	calculated effective osmolality
OsM <sub>M</sub>	measured osmolality
OsM <sub>S</sub>	serum osmolality
OsM <sub>U</sub>	urine osmolality
RAAS	renin-angiotensin-aldosterone system
RBC	red blood cell
ROC	receiver operator characteristic
RVD	regulatory volume decrease
RVI	regulatory volume increase
SA-ICU	small animal intensive care unit
SIADH	syndrome of inappropriate secretion of antidiuretic hormone
UT-A	urea transporter A

UT-B      urea transporter B