

Determination of tonicity effects of ketoacids and lactate by use of two canine red blood cell assays

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OBJECTIVE

To determine the tonicity effects of β -hydroxybutyrate, acetoacetate, and lactate in canine RBCs.

SAMPLE

RBCs from approximately 40 dogs.

PROCEDURES

2 in vitro methods were used to conduct 4 experiments. The modified osmotic fragility assay was used to measure the ability of ketoacid salts added to serial sucrose dilutions to protect RBCs from osmotic hemolysis. In a second assay, a handheld cell counting device was used to measure changes in RBC diameter to assess the tonicity effect of solutions of ketoacid and lactate salts.

RESULTS

For the modified osmotic fragility assay, all ketoacid salts had an osmoprotective effect, but the effect was determined to be completely attributable to the tonicity effect of added cations (sodium and lithium) and not the ketoacid moieties. However, both the sodium and lithium lactate salts provided osmoprotection attributable to both the cation and lactate anion. For the second assay, RBC diameter was significantly increased with the addition of urea (an ineffective osmole) but did not change with the addition of glucose (an effective osmole), which established the behaviors of ineffective and effective osmoles in this assay. The RBC diameter was significantly increased over that of control samples by the addition of sodium β -hydroxybutyrate, lithium acetoacetate, and lithium lactate but was decreased by the addition of sodium lactate.

CONCLUSIONS AND CLINICAL RELEVANCE

For both assays, β -hydroxybutyrate and acetoacetate acted as ineffective osmoles, whereas lactate acted as an effective osmole in 3 of 4 experiments. (*Am J Vet Res* 2015;76:77–83)

The terms osmolality and tonicity are often used interchangeably; however, these 2 concepts differ in subtle yet important ways. Osmolality describes the total solute concentration but does not distinguish between solutes that exert osmotic pressure (effective osmoles) and those that do not (ineffective osmoles). The barrier function of a 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 a cell membrane to a given osmole. Tonicity, also called effective osmolality, is the effective osmoles per volume of solvent.^{1,2} When ineffective osmoles are added to the solution that surrounds cells, the cells undergo a rapid increase in volume 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 semipermeable cell membrane via osmosis. Hypertonicity can lead to serious cellular dysfunction. Loss of intracellular water causes macromolecular crowding within a cell and dysregulation of intracellular proteins.³ Changes in cell size are a physiologic part of cell signaling in many tissues, and thus shrinking induced by

pathological hypertonicity will disrupt these pathways.⁴ Finally, cells acclimatized to chronic hypertonicity can swell during rapid correction of serum tonicity, which leads to tissue edema.⁵

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, which results from hyperglycemia, in humans and domestic animals. The prevalence of hypertonicity in human diabetics differs depending on the cutoff used to define hypertonicity. In a cross-sectional study,⁶ the prevalence in human diabetics was reported as 35% to 78%; a similar range has been reported in diabetic dogs and cats (33% to 81%).⁷

In addition to the glucose concentration, the sodium concentration also appears to be an important contributor to hypertonicity in human and veterinary patients and may independently impact diabetic progression.⁷⁻⁹ Diabetic patients may also have excess accumulation of endogenous osmoles that typically are maintained at minute concentrations in healthy patients. Examples of these solutes are the ketoacids β -hydroxybutyrate and acetoacetate, which are overproduced in unregulated

ABBREVIATION

H50 Sucrose concentration at which 50% hemolysis is detected

diabetes mellitus by the liver as an indirect consequence of relative or absolute insulin deficiency.¹⁰⁻¹² Serum lactate concentration can also increase, particularly during diabetic ketoacidosis, owing to hypoperfusion and resultant anaerobic glycolysis.¹³ These solutes increase serum osmolality, but it is unclear whether they increase serum tonicity by acting as effective osmoles.

Investigators in 1 study¹⁴ evaluated the effects of glucose, with or without ketoacids, on a number of canine RBC variables, including size and osmotic fragility. They found that changes in these variables are significantly different when both glucose and sodium β -hydroxybutyrate are added, compared with changes after the addition of glucose alone.¹⁴ By use of an osmotic fragility assay with human RBCs, investigators in another study¹⁵ concluded that acetoacetate is an effective osmole because lithium acetoacetate protects RBCs from hypotonic-induced lysis in a manner similar to that of the known effective osmoles NaCl and glucose. However, the methods used in previous investigations involved the addition of ketoacids to solutions in the form of sodium or lithium salts of the weak organic acid, and tonicity effects of added cations may have confounded the analysis. Thus, the tonicity effects of organic anions remain to be firmly established.

The purpose of the study reported here was to determine whether organic anions are effective osmoles. We hypothesized that β -hydroxybutyrate, acetoacetate, and lactate would be ineffective osmoles and would not exert a tonicity effect on canine RBCs.

Materials and Methods

SAMPLES

Surplus EDTA-anticoagulated blood from approximately 40 dogs, stored at 4°C, was obtained within 48 hours after collection; samples were obtained from the Kansas State University Veterinary Diagnostic Laboratory Clinical Pathology Laboratory after routine hematologic analysis. No samples were collected specifically for the purposes of this study, and the samples that were used would have otherwise been discarded. Samples were acquired and used in accordance with institutional guidelines.

Blood samples were used to investigate the hypothesis by use of 2 methods (4 experiments) to assess tonicity effects of various organic osmoles while excluding the effects of accompanying cations. First, a modified RBC osmotic fragility assay was used to compare tonicity effects of organic salts of ketoacids (sodium β -hydroxybutyrate and lithium acetoacetate) and lactate (sodium lactate and lithium lactate) with tonicity effects of inorganic salts (NaCl and LiCl). Second, a novel canine RBC assay was used to compare changes in RBC diameter after the addition of osmoles with known (glucose and urea) and unknown (β -hydroxybutyrate, acetoacetate, and lactate) effects.

MODIFIED OSMOTIC FRAGILITY ASSAY

Samples of RBCs were centrifuged at 264 X g for 10 minutes, followed by 3 serial washings with

300 mOsm NaCl (1 part RBCs to 3 parts NaCl). The final supernatant was discarded, and 100 μ L of pelleted cells was resuspended in 500 μ L of each test or control solution. Cells were incubated for 10 minutes at 22°C and then centrifuged at 435 X g for 3 minutes, and the pellet was discarded. As a marker for cell lysis, the free hemoglobin content of 100 μ L of each solution was measured by spectral absorbance at 450 nm with a 96-well plate spectrophotometer.^a Samples were analyzed in duplicate, and values were expressed as percentage hemolysis, using RBCs incubated in double-distilled water to represent 100% hemolysis. Percentage hemolysis was determined by use of the following equation:

$$\text{Percentage hemolysis} = (\text{OD}/\text{OD}_{\text{ddH}_2\text{O}}) \times 100\%$$

where OD is the optical density of the sample or control solution, and OD_{ddH₂O} is the optical density of double-distilled water and was used to represent 100% hemolysis.

First, relative tonicities of the ketoacid salts (sodium β -hydroxybutyrate^b and lithium acetoacetate^b) were compared with those of their respective chloride salts (NaCl^b and LiCl^b) by assessing their ability to protect RBCs from hypotonic-induced hemolysis. A series of sucrose solutions were prepared in 50mM increments (0 to 300mM; in triplicate). Then, 50mM sodium β -hydroxybutyrate or 50mM NaCl was added to each series. Results were determined for each series, and the unadulterated sucrose series was used as a control series. An identical procedure was performed with lithium acetoacetate and LiCl. The control series (sucrose) was replicated 4 times, and each sample series (sodium β -hydroxybutyrate, NaCl, lithium acetoacetate, and LiCl) was replicated 8 times; the mean for each data point was calculated. From these means, hemolysis protection curves for each series were generated by plotting the percentage hemolysis of each sample or control series against the sucrose concentration of each solution. For each curve, the H50 was visually determined; the H50 was compared between sodium β -hydroxybutyrate and NaCl, between lithium acetoacetate and LiCl, and between each sample series and the control (sucrose) solution. An H50 lower than that of the control series indicated an osmoprotective (tonicity) effect of the added (nonsucrose) solutes.

Next, the H50s of the control solution, NaCl, and sodium β -hydroxybutyrate were compared to assess whether the β -hydroxybutyrate anion exerts a tonicity effect independent of the sodium cation. The number of nonsucrose effective osmoles added to the NaCl-containing series, as determined by results for the osmotic fragility assay, was calculated by subtracting the H50 of the NaCl solution from the H50 of the control solution. This difference was divided by the nonsucrose effective osmoles known to be added to each solution (ie, 50mM NaCl = 100 mOsm) to establish a correction factor for the assay by use of the following equation:

$$\text{Assay correction factor} = \frac{(\text{H50}_{\text{control}} - \text{H50}_{\text{NaCl}}) / 100 \text{ mOsM}}$$

The difference between the H50 of the control series and the H50 of the sodium β -hydroxybutyrate-containing series was divided by the assay correction factor to determine the number of nonsucrose effective osmoles that 50mM sodium β -hydroxybutyrate added to the solution. It was assumed that if β -hydroxybutyrate were an effective osmole, then sodium β -hydroxybutyrate would contribute 2 effective OsM/mole of salt (50mM sodium β -hydroxybutyrate = 100 mOsM), which represented the tonicity contribution of both sodium and β -hydroxybutyrate. However, if β -hydroxybutyrate were an ineffective osmole, then sodium β -hydroxybutyrate would contribute only 1 effective OsM/mole of salt (50mM sodium β -hydroxybutyrate = 50 mOsM), which represented only the tonicity contribution of sodium. A similar analysis was performed for lithium acetoacetate and LiCl. To assess the tonicity effect of lactate, these experiments were repeated by comparing the H50 of sodium lactate against that of NaCl and the H50 of lithium lactate against that of LiCl.

CANINE RBC DIAMETER ASSAY

Samples of RBCs were prepared by washing in a dilution with 0.01M PBS solution (1 part RBCs to 5 parts PBS solution). The cells were mixed and centrifuged at 202 X g for 5 minutes, and the supernatant was discarded. The wash step was repeated, and the cell pellet was then resuspended in a volume of 300 mOsM NaCl^c (1 part RBCs to 1 part NaCl). An aliquot of suspended RBCs was diluted in 300 mOsM NaCl (1 part suspended RBCs to 20 parts NaCl) and allowed to acclimate for at least 10 minutes at 22°C.

An aliquot (1 μ L) of acclimated RBCs was added to 500 μ L of each control or test solution and incubated for 30 minutes at 22°C. The RBC diameters in each solution were measured with a handheld automated cell counting device.^d After gentle mixing, a 100- μ L sample containing RBCs was aspirated into a disposable 40- μ m sensor attached to the cell counting device; at least 2.02×10^3 RBCs were counted with the cell counter for each analysis. 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,^e and the histograms were gated. Gating was performed manually by selecting the first histogram column from both ends of the range that was taller than it was wide (**Figure 1**). Mean RBC diameter was then calculated by the software and exported to a spreadsheet^f for data analysis.

First, osmoles of known tonicity effect were assayed to define the behavior of effective and ineffective osmoles in the canine RBC diameter assay. A 50mM solution of a known effective osmole (glucose) was prepared in 300 mOsM NaCl.^{1,2} Similarly, a 50mM solution of a known ineffective osmole (urea) was

prepared in 300 mOsM NaCl.^{1,2} The RBC diameters for these solutions, as measured by the canine RBC assay, were compared against the RBC diameters for a 300 mOsM NaCl control solution.

Next, the tonicity effects of β -hydroxybutyrate, acetoacetate, and lactate were examined. The effects on RBC diameter of 300 mOsM solutions of sodium β -hydroxybutyrate and sodium lactate were compared with the effects of a 300 mOsM NaCl control solution. Because sodium acetoacetate was not readily available, acetoacetate was tested by use of the lithium salt. Solutions of 300 mOsM lithium acetoacetate and lithium lactate were prepared, and results for those solutions were compared against results for a 300 mOsM LiCl control solution. All experiments were performed in 15 to 18 replicates.

STATISTICAL ANALYSIS

The RBC diameter for all solutions was expressed as mean \pm SD. The Wilcoxon rank test was used to compare RBC diameter between groups. Values of $P \leq 0.05$ were considered significant.

Results

MODIFIED OSMOTIC FRAGILITY ASSAY

Hemolysis protection curves for sodium β -hydroxybutyrate and NaCl were plotted (**Figure 2**). All curves revealed a clear progression from 0% hemolysis (300mM sucrose) to 100% hemolysis (0mM sucrose). The H50 for both sodium β -hydroxybutyrate (78mM sucrose) and NaCl (31mM sucrose) was lower than that for the sucrose control solution (130mM sucrose); however, the H50 for NaCl was lower than that for sodium β -hydroxybutyrate. Similar relationships were evident between the H50 for lithium acetoacetate (87mM sucrose), LiCl (31mM sucrose), and the control solution (130mM sucrose).

The correction factor for the osmotic fragility assay with sodium salts was determined to be 0.99.

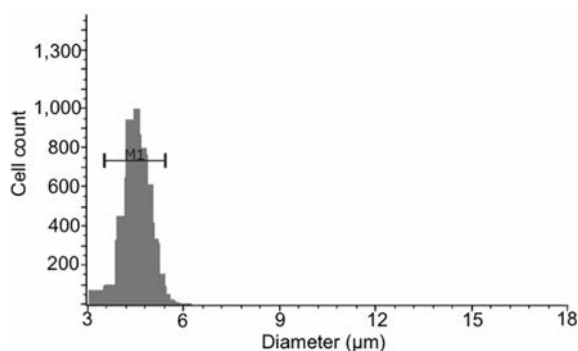


Figure 1—Representative histogram of the output of a handheld cell counting device used to measure canine RBC diameter in a variety of test solutions to assess the tonicity effect of solutions of ketoacid and lactate salts. Gating was performed manually by selecting the first histogram column from both 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 1 histogram column higher. M1 = Automatic gating set by the computer.

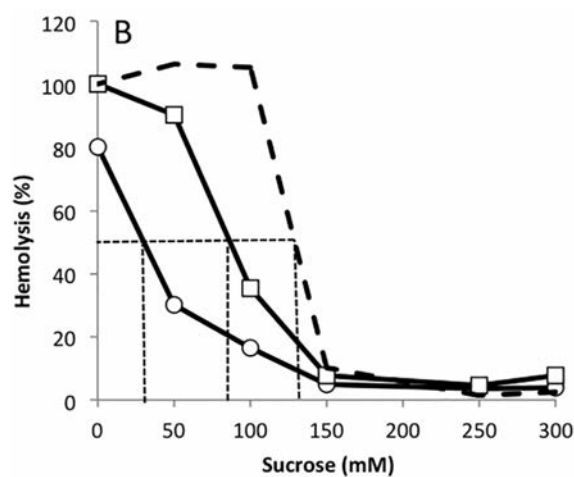
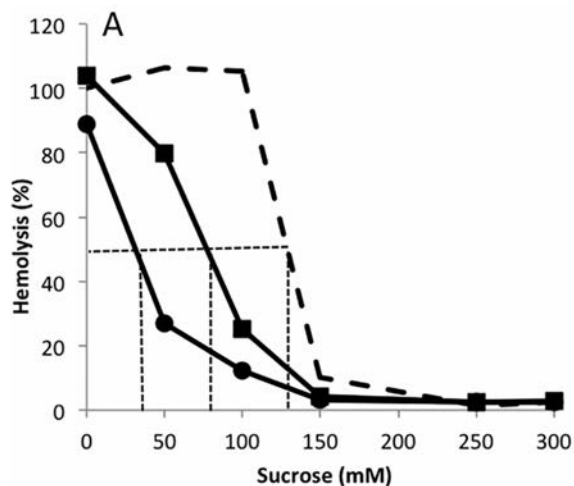


Figure 2—Hemolysis protection curves for ketoacids. In panel A, hemolysis is reported for a series of solutions of NaCl (black circles) and sodium β -hydroxybutyrate (black squares) and control sucrose solutions (dashed line). In panel B, hemolysis is reported for a series of solutions of LiCl (white circles), and lithium acetoacetate (white squares) and control sucrose solutions (dashed line). Dotted vertical lines represent the H50 for each series. Dotted horizontal lines represent the differences between the H50 of the chloride salt, ketoacid salt, and control sucrose solution and thus the nonsucrose effective osmoles added to each series.

Therefore, the corrected nonsucrose effective osmoles from 50mM sodium β -hydroxybutyrate added to a solution was calculated as 51 mOsM $([130\text{mM} - 78\text{mM}]/0.99)$. This was almost exactly the effective osmoles expected to be added in accordance with the assumption that sodium β -hydroxybutyrate contributed only 1 effective OsM/mole of salt (50 mOsM). The correction factor for the osmotic fragility assay with lithium salts was also 0.99. Therefore, the corrected nonsucrose effective osmoles from 50mM lithium acetoacetate added to a solution was calculated as 42 mOsM $([130\text{mM} - 87\text{mM}]/0.99)$. This was closest to the effective osmoles expected to be added in accordance with the assumption that lithium acetoacetate contributed only 1 effective OsM/mole of salt (50 mOsM), as opposed to the value for the assump-

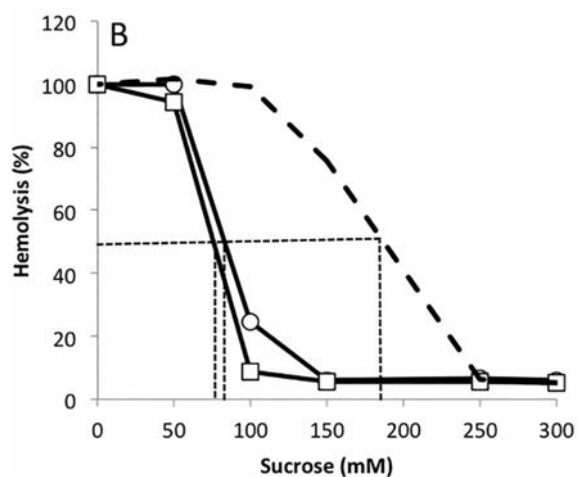
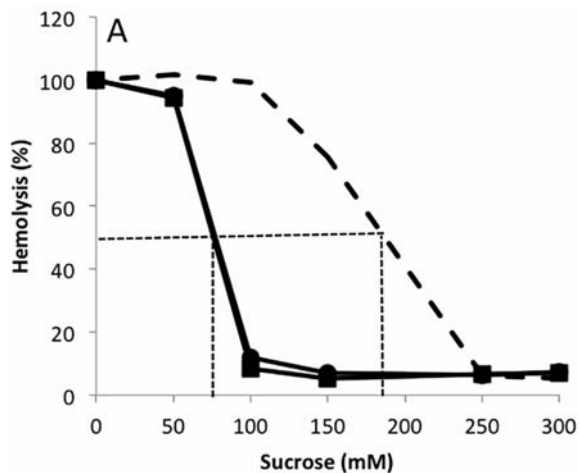


Figure 3—Hemolysis protection curves for lactate. In panel A, hemolysis is reported for a series of solutions of NaCl (black circles) and sodium lactate (black squares) and control sucrose solutions (dashed line). In panel B, hemolysis is reported for a series of solutions of LiCl (white circles) and lithium lactate (white squares) and control sucrose solutions (dashed line). See Figure 2 for remainder of key.

tion that lithium acetoacetate contributed 2 effective OsM/mole of salt (100 mOsM).

Hemolysis protection curves for sodium lactate and NaCl were plotted (**Figure 3**). All curves revealed a clear progression from 0% hemolysis (300mM sucrose) to 100% hemolysis (0mM sucrose). The H50 for both sodium lactate and NaCl was 80mM sucrose and lower than that for the sucrose control solution (185mM sucrose). The H50 was similar for lithium lactate (75mM sucrose) and LiCl (80mM sucrose), and both were lower than that for the sucrose control solution (185mM).

The correction factor for the osmotic fragility assay with sodium salts was determined to be 1.05. Therefore, the corrected nonsucrose effective osmoles from 50mM sodium lactate added to a solution was calculated as 100 mOsM $([185\text{mM} - 80\text{mM}]/1.05)$. This was exactly the effective osmoles expected to be added in accordance with the

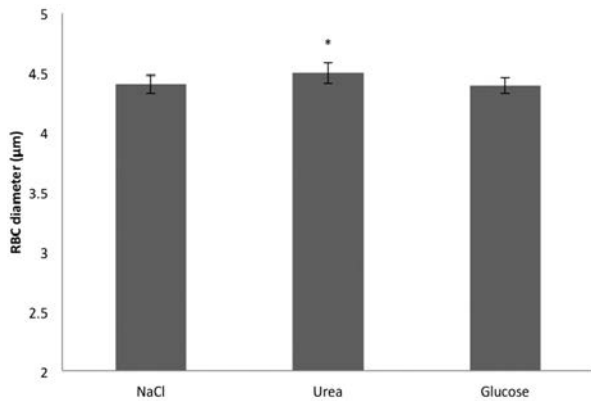


Figure 4—Mean \pm SD RBC diameter for solutions containing osmoles of known tonicity effect (50mM urea or glucose in 300 mOsM NaCl). Notice that addition of the ineffective osmole urea caused a significant $^*(P = 0.014)$ increase in RBC diameter, compared with results for the NaCl control solution, whereas addition of the effective osmole glucose did not significantly ($P = 0.56$) change RBC diameter, compared with results for the NaCl control solution.

assumption that sodium lactate contributed 2 effective OsM/mole of salt (100 mOsM). The correction factor for the osmotic fragility assay with lithium salts was 1.10. Therefore, the corrected nonsucrose effective osmoles from 50mM lithium lactate added to a solution was calculated as 95 mOsM [(185 - 80mM)/1.10]. This was closest to the effective osmoles expected to be added in accordance with the assumption that lithium lactate contributed 2 effective OsM/mole of salt (100 mOsM), as opposed to the value for the assumption that lithium lactate contributed only 1 effective OsM/mole of salt (50 mOsM).

CANINE RBC DIAMETER ASSAY

Results of experiments for osmoles of known tonicity effect were plotted (**Figure 4**). Mean \pm SD RBC diameter in the glucose-containing solution ($4.39 \pm 0.067 \mu\text{m}$) was smaller than, but not significantly ($P = 0.56$) different from, the mean RBC diameter in the NaCl control solution ($4.40 \pm 0.076 \mu\text{m}$). However, the mean RBC diameter in the urea-containing solution ($4.50 \pm 0.088 \mu\text{m}$) was significantly ($P = 0.014$) larger, compared with the mean RBC diameter in the NaCl control solution.

Results of experiments for osmoles of unknown tonicity effect (β -hydroxybutyrate, acetoacetate, and lactate) were plotted (**Figure 5**). Mean \pm SD RBC diameter in the sodium β -hydroxybutyrate solution ($5.43 \pm 0.394 \mu\text{m}$) was significantly ($P < 0.001$) larger, compared with the mean RBC diameter in the NaCl control solution ($4.52 \pm 0.190 \mu\text{m}$). In contrast, mean RBC diameter in the sodium lactate solution ($4.08 \pm 0.122 \mu\text{m}$) was significantly ($P < 0.001$) smaller, compared with the mean RBC diameter in the NaCl control solution. Mean RBC diameter was significantly larger in both the lithium acetoacetate solution ($5.09 \pm 0.194 \mu\text{m}$; $P < 0.001$) and lithium lactate solution ($4.33 \pm 0.335 \mu\text{m}$; $P = 0.003$), compared with

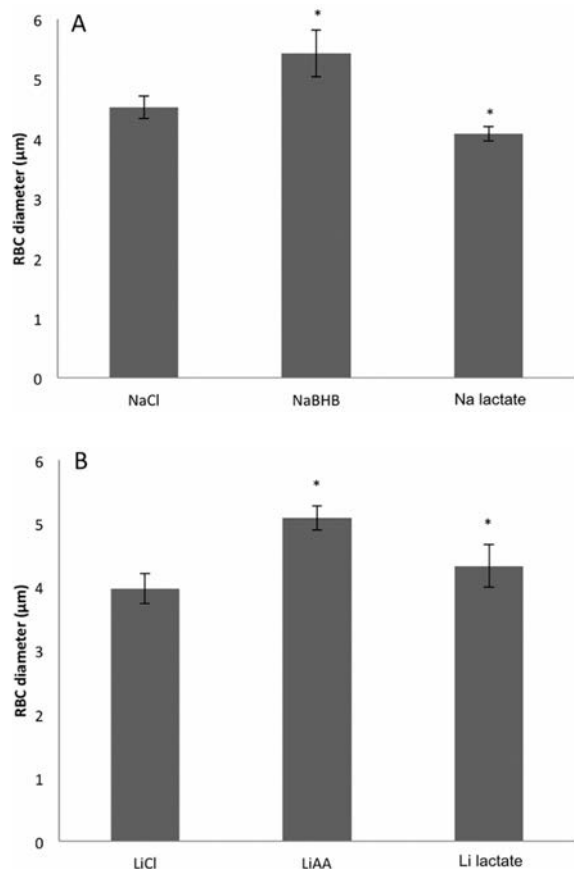


Figure 5—Tonicity effects of unknown osmoles on mean \pm SD RBC diameter in sodium salts (A) and lithium salts (B). All solutions had concentrations of 150mM (300 mOsM). Notice in panel A that the mean RBC diameter for both sodium β -hydroxybutyrate (NaBHB) and sodium lactate was significantly $^*(P < 0.001)$ different from that for the NaCl control solution. In panel B, notice that mean RBC diameter was significantly greater for lithium lactate $^*(P = 0.003)$ and lithium acetoacetate (LiAA; $^*P < 0.001$) than that for the LiCl control solution.

the mean RBC diameter in the LiCl control solution ($3.97 \pm 0.239 \mu\text{m}$).

Discussion

Analysis of results of the present study supported the hypothesis that the ketoacids β -hydroxybutyrate and acetoacetate acted as ineffective osmoles and therefore did not exert a tonicity effect. When tested by use of the modified osmotic fragility assay, both the NaCl and sodium β -hydroxybutyrate series had a higher H50, compared with the H50 for the control (sucrose) series, which indicated that the addition of NaCl or sodium β -hydroxybutyrate exerted an osmoprotective effect. The same effect on H50 was detected in experiments in which LiCl and lithium acetoacetate were compared against sucrose alone. This finding was expected because the addition of 50mM NaCl or LiCl, which are known to be effective osmoles, increases the tonicity of sucrose solutions

and reduces the osmotic gradient across the RBC membrane, which decreases the likelihood of lysis.¹ Comparison of the H50 for the NaCl versus sodium β -hydroxybutyrate and LiCl versus lithium acetoacetate series permitted the specific effect of an anion (β -hydroxybutyrate and acetoacetate) to be determined. Addition of 50mM NaCl or LiCl increased tonicity by 100 mOsM because these salts dissociate completely in aqueous solution. Similar to results for all salts, sodium β -hydroxybutyrate and lithium acetoacetate also dissociate completely in aqueous solution. If β -hydroxybutyrate were an effective osmole, 50mM sodium β -hydroxybutyrate would increase tonicity by 100 mOsM, but if β -hydroxybutyrate behaved as an ineffective osmole, 50mM sodium β -hydroxybutyrate would only increase tonicity by 50 mOsM; the same was true for lithium acetoacetate solutions. In both series, the osmoprotective effect of the ketoacid salt was less than that of the corresponding chloride salt, which suggested that the effective osmoles contributed by each ketoacid salt were less than those contributed by the corresponding chloride salt. Calculation of a correction factor allowed for the tonicity effects of ketoacids to be distinguished from those of the sodium and lithium cations. The concentrations of effective osmoles from the 50mM sodium β -hydroxybutyrate and 50mM lithium acetoacetate were 51 and 42 mOsM, respectively, which were both approximately 50 mOsM, as expected if β -hydroxybutyrate and acetoacetate were ineffective osmoles. Thus, analysis of results of the modified fragility assay supported the conclusion that β -hydroxybutyrate and acetoacetate acted as ineffective osmoles and did not exert tonicity effects.

In contrast to the ketoacids, lactate appeared to act as an effective osmole in the osmotic fragility assay. With sodium or lithium as a cation, the difference in H50 between lactate and chloride was minimal; 50mM of both the chloride salt and lactate salt contributed approximately 100 mOsM to the osmoprotective effect of the solutions, as would be expected when both the cation and anion are effective osmoles. Interestingly, the H50s of NaCl, LiCl, and the sucrose control solution differed between the ketoacid and lactate experiments. Different RBC samples were used in the ketoacid and lactate experiments; therefore, it is possible that differences in intrinsic osmotic fragilities of these samples contributed to the discrepancy. However, the correction factor and comparison of the ketoacids and lactate relative to chloride were used to mathematically account for these differences.

In a second set of experiments, effects of urea and glucose were assessed with a novel assay to determine changes in RBC diameter. The RBC diameter was increased significantly by the addition of urea, an ineffective osmole. The increase in RBC diameter was expected because RBCs are highly permeable to urea.¹⁶ Influx of urea is accompanied by water that causes swelling of the cells and increases RBC diameter. Exposure to glucose, a known effective osmole, was expected to decrease RBC diameter through an opposite mecha-

nism that encouraged water efflux and decreased cell size.¹ In experiments with glucose, the RBC diameter decreased, albeit not significantly, relative to that for the control solution. The reason for the reduced effect of glucose in these test conditions was not clear but could have been caused by partial permeability of the RBC membrane to glucose. In particular, it has been reported that glucose transport into canine RBCs is higher than in many other species.¹⁷ It is possible that a high intracellular glucose concentration opposed the transcellular gradient induced by glucose incubation, which reduced the tonicity effect of the extracellular glucose. Although traditional physiology texts classify glucose as an effective osmole, some authors dispute this classification and argue that the tonicity effect of glucose is tissue and insulin dependent, given that both affect glucose transporter expression.^{1,2} In fact, the tonicity effect of almost all solutes is determined by membrane transporter expression, which changes on the basis of the cell milieu. Thus, it could be argued that effectiveness of a solute should be treated as a relative and malleable property, rather than given a binary classification. However, the urea and glucose experiments yielded distinct responses in RBC diameter. Thus, for the purposes of the present study as well as for simplicity, the behaviors of urea and glucose were considered the standard for ineffective and effective osmoles, respectively.

Incubation with sodium β -hydroxybutyrate and lithium acetoacetate significantly increased RBC diameter, compared with RBC diameter for the control solutions of NaCl and LiCl. Changes in RBC diameter caused by solutions of ketoacids were consistent with changes caused by incubation with urea, which suggested these substances act as ineffective osmoles. This is in accordance with the hypothesis and results from the modified fragility assay. However, changes in RBC diameter caused by lactate were discordant. Sodium lactate caused a decrease in RBC diameter relative to that for the NaCl control solution, which was a response expected for an effective osmole and consistent with the osmotic fragility results for lactate. Conversely, lithium lactate caused an increase in RBC diameter, compared with that of the LiCl control solution, which was consistent with action as an ineffective osmole. The reason lactate appeared to function differently when paired with different cations was not clear. The lithium concentration in these *in vitro* solutions exceeded that typical for RBCs and may have affected cell membrane function. For example, lithium could alter the function of the monocarboxylate transporter, which facilitates entry of lactate into cells.¹⁸ If this were the case, then discordant osmotic fragility results between sodium lactate and lithium lactate might also be expected; however, the total lithium concentration used in the RBC diameter assay was much higher (150mM) than that used in the osmotic fragility assay (50mM), which could create a differential effect. The different lithium concentrations may also explain the reason that the RBC diameter of NaCl (4.52 μ m) was higher than that of LiCl (3.97 μ m) but the H50s were the same (80mM su-

crose). The monocarboxylate transporters also mediate transport of ketoacids, so the tonicity effect of acetoacetate, which was measured as a lithium salt, might also be affected by a supranormal lithium concentration.¹⁸ Unfortunately, acetoacetate is not readily available as a sodium salt, so comparisons between the sodium and lithium salts of acetoacetate could not be determined.

A limitation of the present study was that only RBCs were evaluated. Tonicity effects of solutes may differ by tissue type and disease state, so the effects of ketoacids and lactate on RBCs may not represent the response for all cell types. For practical reasons (ie, ease of sample collection and handling) RBCs were used, but RBCs also serve as a classic model for biological osmolality studies.^{19,20} However, other cell lines could be considered in future experiments to assess effects on target tissues that are particularly susceptible to tonicity injury, such as neural tissues.⁵ The test solutions used in these experiments were simple isotonic or hypertonic aqueous solutions, which were needed to isolate the effects of individual osmoles. Ideally, RBC diameter would have been measured in solutions that approximated the composition of serum or interstitial fluid. Serum was not used for RBC diameter experiments because of technical limitations of the cell counting device used to determine RBC diameter. Similarly, technical limitations also precluded evaluation of RBCs in hypotonic solutions. The RBC diameters yielded by the cell counting device were smaller than that reported for typical canine RBCs (7 μm).²¹ This may reflect a technical characteristic of the device used to measure RBC size or may reflect changes caused by the storage and processing of the cells. A methodological difference appears the most likely cause for the discrepancy between previously reported RBC diameters and those in the present study because the mean corpuscular volume of RBCs stored at 4°C can increase over time.²² Regardless, all cells used were directly compared with cells that underwent identical *in vitro* processing and measurement; therefore, conclusions regarding tonicity effects of solutes were likely valid but extrapolations to *in vivo* systems should be made with caution.

In the biological models of the present study, β -hydroxybutyrate and acetoacetate acted as ineffective osmoles, whereas lactate predominantly acted as an effective osmole. On the basis of these findings, it is unlikely that β -hydroxybutyrate and acetoacetate would contribute to tonicity in clinical syndromes, such as diabetes mellitus or ketoacidosis; however, it is possible that severe lactic acidosis could affect tonicity of the milieu.

Acknowledgments

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Footnotes

- a. SpectraMax190, Molecular Devices, Sunnyvale, Calif.
- b. Sigma-Aldrich, St Louis, Mo.
- c. Standard solution, 300 mOsm/kg H₂O, Precision Systems, Natick, Mass.
- d. Scepter, version 2.0, EMD Millipore, Hayward, Calif.
- e. Scepter Software Pro, version 2.0, EMD Millipore, Hayward, Calif.
- f. Excel, Microsoft, Redmond, Wash.

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