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Osmotonicity of acetoacetate: possible implications for cerebral edema in diabetic ketoacidosis

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Summary

Background: Rapid drops in blood glucose and sodium levels during treatment of diabetic ketoacidosis (DKA) can cause a drop in the osmotonicity of plasma, resulting in cerebral edema. Ketone bodies are assumed to move freely in and out of cells, so it is assumed that they do not contribute to the tonicity of plasma or influence fluid shifts. The assumption that ketone bodies do not contribute to osmotonicity has not been tested previously. The experiment described here was done to check if acetoacetate has osmotonicity.

Material/Methods: A modified erythrocyte fragility test was used to check the osmotonic and osmoprotective effects of the ketone body. Red blood cells were suspended in different test tubes containing distilled water, normal saline, glucose, urea and acetoacetic acid (lithium salt $C_4H_3O_3Li$). All solutions (except the tube with distilled water) were made to match the osmolality of plasma. We hypothesized that solutions in which red cell hemolysis does not take place have greater tonicity than the tonicity of 0.45% saline.

Results: Spectrophotometry showed that there was no hemolysis in the solutions of normal saline or solutions containing glucose or acetoacetate. Complete hemolysis was demonstrated in the tube with plain distilled water and also in the solutions containing urea.

Conclusion: This study shows that acetoacetate is functionally similar to glucose in that it contributes to increased osmotonicity. The drop in ketone body levels can produce a drop in the osmolar tonicity of plasma and precipitate cerebral edema.

key words: **osmotic fragility test • osmolality • osmolar gap • ketone bodies • red cell fragility test**

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BACKGROUND

Cerebral edema has been recognized as a devastating and unpredictable complication of diabetic ketoacidosis (DKA). The mortality rate is said to be between 24% and 90% [1]. The pathophysiological mechanism underlying the development of cerebral edema associated with DKA is an enigma. It has been hypothesized that cerebral edema in children with diabetic ketoacidosis may be caused by the exposure of osmolytes in brain cells to hyperosmolar conditions [2]. A rapid decrease in extracellular osmolality during treatment would result in osmotically mediated swelling of the brain [3]. Due to the implied role of changes in plasma osmolality, it is recommended that abrupt alterations in glucose and electrolytes be avoided during therapy of DKA [4]. In DKA there is yet another aspect of osmolality which has received little attention: the osmolality produced by ketoacids. Ketone body levels are not routinely measured in DKA, and so this parameter is not mentioned as a factor in the calculated osmolality (calculated osmolality = 1.86 (Na + K) + Urea + glucose) [5]. The levels of ketone bodies are noticed only when osmolality is measured objectively, for example by the depression of freezing point technique, and there is a large disparity between real osmolality and calculated osmolality. This difference is called the osmolar gap. The osmolar gap is made up of unmeasured compounds.

Davidson [6] reported a large osmolar gap in his series with DKA. He noted that the osmolar gap decreases to insignificant values within 20 hours of starting treatment. In their series, the real osmolality fell by 18.5 mOsm/L within 20 hours of treatment, while the calculated osmolality fell by only 9.5 mOsm/L. A significant drop in osmolality thus goes unnoticed unless real osmolality is measured in all cases of DKA. Davidson found that the osmolar gap could be accounted for almost entirely by an increase in acetone, decrease in plasma water fraction, and smaller increments in aminoacids and glycerol.

We have elsewhere reported [7] that the mean osmolality (depression of freezing point method) of a group of DKA patients was 318 mOsm/kg (SD 12.9, range 297–337) while the calculated osmolality in this group was only 288 mOsm/kg (Range 282–304).) The mean osmolar gap was 29 mOsm/kg (SD 5.3, range 16–48).

Given that ketone bodies are responsible for a large share of the osmolar gap in DKA, it becomes a crucial question whether ketone bodies are osmotonic. Van der Meulen [8] in a paper looking at possible mechanisms of cerebral edema in DKA assumes that ketone bodies are not osmotonic and do not influence fluid shifts. This assumption is not supported, however, either by a statement of theoretical considerations or by experimental evidence. To our knowledge the assumption that ketone bodies are not osmotonic has not previously been tested. We conducted our *in vitro* test in order to determine if the neutral salt of acetoacetic acid, one of the ketone bodies present in DKA, is osmotonic and can influence fluid shifts across the cell membrane.

Hypertonicity results from an increase in the concentration of solutes that do not cross the cell membrane. Examples of such solutes include mannitol, glucose (in the absence of insulin), and sodium chloride [9]. With an increase in the tonicity of the extracellular fluids (ECF), there is a shift in fluids from the intracellular to the extracellular space, resulting in a reduction in cell size. Conversely, hypotonicity of the extracellular fluid may be associated with movement of fluid into the cell.

On the other hand, solutes such as urea and alcohol are permeant with respect to the membrane [9]. Hence even if they are present in large amounts in the ECF, this results only in hyperosmolality, with no attendant increase in osmotonicity, and there is no change in the size of the intracellular and extracellular spaces. Thus an increase in serum urea concentration results in hyperosmolality without hypertonicity, whereas hypernatremia causes hypertonicity and hyperosmolality [9].

The modified saline fragility test was used in this study. We hypothesized that complete red cell lysis should occur if red cells are suspended in acetoacetate of osmolality 290 mOsm/L, if it produces no osmotonicity. Cells suspended in normal saline (sodium chloride solution of osmolality 290 mOsm/L) and glucose of the same osmolality would show no hemolysis. Cells suspended in urea solution with identical osmolality (osmolality 290 mOsm/L) would show complete hemolysis.

MATERIAL AND METHODS

Stock solutions of normal saline, glucose, urea and acetoacetate (Lithium salt, $C_4H_5O_3Li$, Sigma Chemicals Lot 44H5038, Poole, Dorset, UK) were prepared by adding calculated amounts of the various compounds to distilled water. The amounts of the various substances added to water was derived by theoretical considerations of its molecular weight, and these amounts are noted in Table 1. The osmolality of the solutions formed was tested by the depression of freezing point technique, on an Advanced Micro-Osmometer Model 3 MO Plus (Advanced Instruments, Norwood, Massachusetts, USA), and it was confirmed that the osmolality of each of the solutions closely approximated 290 mOsm/L.

50 μgm of fresh heparinized blood (collected from an individual not known to have any hemolytic diseases) was added to 2 ml of each of the solutions and incubated for 30 minutes at room temperature. The tubes were then spun at 3500 rpm for 10 minutes, and the optical density of the supernatant solution was examined on a Beckman DU-50 spectrophotometer (Beckman Instruments, Irvine, California, USA). The instrument was calibrated with the optical density of saline solution taken as zero.

The osmotic fragility test measures *in vitro* the lysis of red cells suspended in progressively more hypotonic solutions. In the standard test, red cells are incubated for 30 minutes in hypotonic fluid. Since there is almost no exchange of cations during the relatively short duration of the test, osmotic equilibrium is achieved by rapid

Table 1. Modified osmotic fragility test results. (Optical density in 1 normal saline is taken as 0).

	Osmolality mOsm/L	Solute added (mg/ml)	Optical density after incubating with red cells
Distilled water	0	0	2.826
Acetoacetate	285	15.66	0
Sodium chloride	283	9	0
Glucose	292	52.2	0
Urea	286	17.4	2.826

movement of water across the red cell membrane [10]. The normal red cell membrane is unstretchable and is virtually freely permeable to water. Hence the cell behaves as a nearly perfect osmometer, in that it progressively increases its volume in hypotonic solutions until a critical hemolysis volume is reached [11]. At this point the red cell membrane ruptures and hemoglobin escapes into the supernatant fluid [12].

It is customary to note the point at which hemolysis begins and that at which it is complete. The slightest trace of red color in the supernatant fluid indicates destruction of the least resistant cell. Complete hemolysis is indicated by a clear red solution and the absence of a residue at the bottom of the tube or any cloudiness on gently shaking the tube. Normal blood shows slight hemolysis in 0.45 to 0.39 % saline, which becomes complete at 0.33 to 0.30% [13].

Results

Table 1 shows the amounts of solute added to the distilled water and the resultant osmolality of the solution as measured by the osmometer. The spectrometer readings of the supernatant fluid after incubation with red cells and centrifugation are also shown. The tubes with urea and the tube with plain distilled water showed complete hemolysis. The supernatant was uniformly red colored and there was no residue at the bottom of the tube after centrifuging. Both fluids showed an optical density of 2.826. No hemolysis was detected in the tubes with sodium chloride or glucose, or in the solution of acetoacetate. The optical density in each of these tubes was zero. Thus hemolysis was seen only in the tubes containing urea and those with plain distilled water. No hemolysis occurred in the tubes with saline, glucose, or acetoacetate

Discussion

75% to 90% of the ketone bodies in DKA are made up of betahydroxybutyrate and acetoacetate [14]. The pKa of the former is 4.4, while that of the latter is 3.8. Thus at physiological pH these compounds would be completely ionized. In the present experiment we used a modified red cell saline fragility test to deduce the tonicity of acetoacetate, a representative for all the ionized ketoacids. We used a neutral salt of acetoacetic acid to avoid the effect of pH in this in-vivo experiment, where the extracellular fluid has no buffer system added.

We have demonstrated that acetoacetate is capable of exerting osmotonicity when present in the extracellular fluid. We have demonstrated this using the red cell and red cell membrane, which is considered to behave as a 'nearly perfect osmometer' [12]. In our experiment, complete hemolysis was seen in the tube containing urea and the tube with plain distilled water, so the osmotonicity of those solutions is less than that of 0.33 saline. No hemolysis was seen in the tube with acetoacetic acid, so its osmotonicity must be more than 0.45% saline [13]. Our finding was that acetoacetic acid, like sodium chloride or glucose, is capable of affecting osmotonicity. It is conceivable that a sudden drop in the ketoacid level during treatment may cause a reduction of osmotonicity, and this could theoretically precipitate cerebral edema.

One patient in the series we reported earlier [7] had an osmolar gap of 48 mOsm/kg. The suggestion that a drop of ketoacid levels during treatment of DKA could contribute to the development of cerebral edema is purely speculative. Clinical studies are required to see if cerebral edema is associated with a rapid fall in ketone levels, or more simply whether cerebral edema is associated with a steep fall in measured osmolality and the osmolar gap. The present study is only a preliminary study to suggest that such clinical studies may be useful

The statement that ketone bodies are osmotonic only implies that they do not diffuse freely in and out of cells. This does not imply that the ketone bodies cannot enter a cell by active transport. In fact, cerebral metabolism of ketones particularly in starvation, would necessitate cellular entry. All that the experiment shows is that acetoacetate is osmotonic. Glucose is also osmotonic, but it enters the cell for its metabolism.

One drawback of the present study is that it did not examine the osmotonicity of the individual ketone bodies present in DKA. Acetoacetate is a major component of the ketoacids present in DKA, and for purposes of this experiment we tested acetoacetate only. Ideally we should study the individual compounds, and each of them at different dilutions, to find out at what dilution hemolysis occurs. Such an elaborate procedure using red cells was thought redundant, because the final arbitrator of the clinical significance of this paper will be clinical studies looking at children who develop cerebral edema with DKA. It was felt that there was sufficient evidence available from this simple experiment to prompt such clinical studies.

Very large amounts of the lithium salt of acetoacetic acid were used in the experiment. It is known that a small impurity of silver [15] may act as a hemolytic agent and cause hemolysis in spite of its isotonicity. The compound we used was not toxic to the cell and no hemolysis occurred even at the high concentrations used.

To date ketone bodies have not been thought to be osmotonic, and little attention is paid to the rate of fall in ketone levels or the fall in the osmolar gap. This is

reflected in a recent study looking at the risk factors for the development of cerebral edema in DKA [16] The authors note that since none of the 'relevant variables' – serum glucose concentration at presentation, change in serum glucose concentration during therapy, rate of fluid and sodium administration – were associated with the risk of cerebral edema, their data did not support the theory that a rapid decrease in extracellular osmolality during treatment produces osmotically mediated swelling of the brain. Osmolality and osmolar gap were not measured, nor were ketone body levels (N. Glaser, personal communication). Our study demonstrates that ketone levels are probably a 'relevant variable' that needs to be estimated before one can be certain that a rapid decrease in extracellular osmolality has not occurred.

CONCLUSION

To our knowledge the omotonicity of ketone bodies has not previously been investigated. They have been assumed to be freely permeant across the cell membrane and not capable of influencing fluid shifts across that membrane. This simple experiment tests that assumption in relation to one of the ketoacids. We found that acetoacetate, one of the ketone bodies that accumulate in diabetic ketoacidosis, is indeed osmotonic, and as such can influence fluid shifts across cell membranes. It can be speculated that a rapid fall in the levels of this compound during the treatment of DKA could influence the overall osmotonicity of plasma, which may be one of the causes of cerebral edema seen in this condition. More clinical studies are required to see if cerebral edema is related to the ketone body levels or osmolar gap levels at the start of treatment, or to rate of the fall in measured osmolality during treatment.

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