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Cognitive Dysfunction Associated with Diabetic Ketoacidosis in Rats

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Abstract

Background—Type 1 diabetes mellitus in children may be associated with neurocognitive deficits of unclear cause. A recent retrospective study in children suggested possible associations between diabetic ketoacidosis (DKA) and decreased memory. The current investigation was undertaken to determine whether cognitive deficits could be detected after a single episode of DKA in an animal model.

Methods—Streptozotocin was used to induce diabetes in juvenile rats, and rats were then treated with subcutaneous insulin injections. In one group, insulin was subsequently withdrawn to allow development of DKA, which was then treated with insulin and saline. After recovery from DKA, subcutaneous insulin injections were re-started. In the diabetes control group, rats continued to receive subcutaneous insulin and underwent sham procedures identical to the DKA group. One week after recovery, cognitive function was tested using the Morris Water Maze, a procedure that requires rats to locate a hidden platform in a water pool using visual cues. During a 10 day period, mean time to locate the platform (latency) during 4 trials per day was recorded.

Results—Comparison of latency curves demonstrated longer mean latency times on days 7 and 8 in the DKA group indicating delayed learning compared to diabetic controls.

Conclusions—These data demonstrate that a single DKA episode results in measurable deficits in learning in rats, consistent with findings that DKA may contribute to neurocognitive deficits in children with type 1 diabetes.

Keywords

brain injury; diabetic ketoacidosis; neurocognitive; pediatric

Introduction

Cerebral injury may occur as a complication of diabetic ketoacidosis (DKA) in children. Severe, clinically-apparent cerebral injuries occur in approximately 1% of DKA episodes,

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frequently resulting in death or permanent neurological disability.[4, 8] Although cerebral complications of DKA were previously thought to be limited to only this small subset, recent studies clearly demonstrate that subtle cerebral edema and cerebral metabolic alterations characteristic of ischemia can be demonstrated on brain MRI in most children with DKA, even in the absence of alterations in mental status or other neurological symptoms.[9–10] Furthermore, one recent study demonstrated memory deficits in children with diabetes and a history of DKA when compared with children with diabetes of similar duration and similar glycemic control but without DKA history.[7] These data suggest that subtle neurological injury may result from DKA, even in the absence of obvious mental status abnormalities during DKA treatment. Data from human studies of DKA, however, are by necessity retrospective and not randomized, raising the possibility that confounding factors might be responsible for the apparent association between DKA and memory dysfunction. We undertook the current study to determine whether subtle neurocognitive deficits could be demonstrated after a single episode of DKA using a juvenile rat diabetes model.

Methods

This study was conducted in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health and was approved by the Animal Use and Care Committee at University of California Davis. Four-week old Sprague Dawley rats (n=19, Charles River Laboratories, Wilmington, MA) were given an intraperitoneal injection of streptozotocin (STZ, 150 mg/kg) to induce diabetes as described previously.[26] Rats' drinking water was replaced with water with 10% dextrose for 24-hours after STZ to prevent hypoglycemia. Urine glucose and ketoacids (acetoacetate) were measured daily using Multistix urinalysis strips (BAYER, Fisher Scientific, Santa Clara, CA). Beginning 24 hours after STZ, rats received 4 units of Novolin 70/30 insulin (Novo Nordisk, Princeton, NJ) subcutaneously daily in the evening to coincide with the rats' nocturnal feeding behavior.

Rats were randomly assigned to either the DKA group or the diabetes mellitus control group (DM). The DM group (n=8) continued to receive subcutaneous insulin to treat diabetes throughout the study. For the DKA group (n=11), subcutaneous insulin was administered for 5 days after induction of diabetes, after which insulin was withdrawn to allow DKA to develop. Ketosis developed four to five days after insulin withdrawal. Once ketosis was detected, standard rat chow was replaced with a 60% high fat diet (Research Diets, Inc., OpenSource Diets #D12492) and water was withdrawn for 15 hours to promote ketogenesis and increase dehydration. These procedures increased the similarities between human DKA and the rat DKA model as both ketosis and dehydration in humans are typically more severe than that which develops after insulin withdrawal in rats. Rats were identified as having developed DKA when urine glucose and acetoacetate concentrations were ≥ 2000 mg/dL and 160 mg/dL, respectively. DKA was confirmed by blood assays (serum glucose ≥ 300 mg/dL and β -hydroxybutyrate ≥ 3 mmol/L). Blood samples during DKA and during sham procedures were obtained via lancet puncture of the saphenous vein and were collected into heparinized capillary tubes.

After establishment of DKA, rats were treated with an intraperitoneal (IP) injection of 0.9% saline (8 ml/100gm body weight), followed by subcutaneous Regular human insulin (0.5 units/100 gm). After one hour, 0.45% saline (8 ml/100 gm, IP) and insulin (0.5 units/ 100 gm, SC) were administered. IP 0.45% saline (8 ml/100 gm) was then administered every 2 hours until resolution of DKA (see criteria below). Subcutaneous insulin (0.5 units/ 100 gm) was given every 1–2 hours with the frequency of administration based on the rates of decline of serum glucose and resolution of ketosis. Serum glucose, electrolytes, pH and beta-hydroxybutyrate were measured every 1.5 hours from venous samples (I-STAT Portable

Clinical Analyzer; Sensor Devices, Waukesha, WI, U.S.A.). DKA treatment was continued until glucose was below 300 mg/dL, venous pH was above 7.30 and β -hydroxybutyrate was below 0.5 mmol/L (range 4–8 hours). After DKA resolution, long-acting insulin (Novolin 70/30, 4 units) was administered subcutaneously immediately and daily thereafter and the rat was allowed unlimited access to water and standard rat chow. Blood glucose concentrations were tested daily using Precision Xtra test strips (Abbott Laboratories, Alameda, CA) with samples obtained via lancet puncture of the saphenous vein. The DM group underwent identical sham procedures (IP puncture, venipuncture), but continued to receive insulin to treat diabetes throughout the study.

Neurocognitive evaluation began 14 days after induction of diabetes (7 days after DKA treatment for rats in the DKA group), using the Morris Water Maze, a procedure requiring rats to locate a hidden platform in a water tank using visual cues.[12] The maze is a circular pool 157 cm in diameter filled to a depth of 29 cm with 23 ± 1 °C water. A clear Plexiglas pedestal (27 cm high, 9.7 cm diameter) was submerged in the water and the water was made opaque by addition of white tempera paint. Mean time to locate the platform during 4 trials per day was recorded. Blood glucose was measured before the first trial each day. To avoid alterations in performance resulting from hypoglycemia, rats with glucose levels below 70 mg/dL were given oral carbohydrate (sugar coated breakfast cereal) and maze testing was delayed for 30 minutes to allow glucose concentrations to rise. For each trial, the rat was placed in the maze facing the pool wall at a point corresponding to North, South, East or West on the compass. The order of entry location was randomized and changed daily. After being placed in the maze, rats were allowed to swim for a maximum allotted time (originally 120 seconds; later changed to 90 seconds) or until they located the pedestal. If the rat did not locate the pedestal within the allotted time, the rat was guided to the pedestal and allowed to remain on it for 30 seconds. Rats that located the pedestal were also allowed to stay on the pedestal for 30 seconds. Rats were then towel dried and allowed to rest for 4 min before the next trial.

Rats were studied in the maze for 10 consecutive days, with the first 5 days labeled training days and the last 5 days labeled evaluation days. Visual cues on the walls of the maze room did not move or change throughout the study. The platform remained in the same location for four days. On the fifth day, latency time to the platform was not recorded. Instead, vision tests were conducted. For this test, a flag extending 12 cm above the water surface was attached to the pedestal and the pedestal was placed randomly in one of the four quadrants. Rats were placed in the pool facing the wall in the opposite quadrant and allowed to swim until they found the pedestal. Rats were then dried and allowed to rest for 4 min before the next trial. Between the first and second vision trials the pedestal was randomly moved to one of the three remaining quadrants. Vision testing indicated that the mean times to locate the platform were nearly identical in both groups (mean $\pm$ SD in DKA group= 9.58 ± 0.83 sec vs. 9.88 ± 1.94 sec in the DM group, $p=0.74$). Therefore the vision test was omitted from subsequent studies (see below). On day 6, the pedestal was moved and remained in this second location for the remaining five days.

Initial trials using the protocol described indicated that both groups had similar learning curves during the first five days, after which the curves diverged. We hypothesized that these first five days involved more basic learning processes (i.e. swimming skills, orientation to the task, etc), after which more subtle differences in cognitive or memory function became evident. To explore this hypothesis, we modified the protocol slightly for the second half of the study (5 of 11 DKA rats; 4 of 8 DM rats). In the modified protocol, the pedestal remained in the same location for all 10 days. Pedestal movement was included in the statistical analysis of daily mean latency as a fixed effect in a 2×2 factorial design (see

below). Trial times were trimmed at 90 seconds to minimize the influence of excessive scores.

Statistical Analyses

Between-group comparisons of mean latencies began with graphical and descriptive summaries to assess statistical modeling assumptions. Tukey's boxplot method found extreme outliers at training day 3 and at evaluation days 3 and 5, and possible outliers at evaluation day 4. [23] Because these outliers represent serious violations of the assumptions of the planned analysis of variance and regression analyses for longitudinal data, we report two sets of analyses for the daily mean latency outcome. First, to assess whether DKA vs. DM differences varied by day of testing, we performed a F-test on the DKA * DAY interaction term in a mixed-effects model of the 10-day trajectories of daily log-transformed mean latency scores. This model was estimated with the SAS/Stat PROC MIXED procedure, using the heteroskedasticity-consistent robust variance estimator for fixed effects parameters to account for non-homogeneous residual distributions.[17, 22] Finding significant between-day variation in DKA vs. DM mean differences, the second set of analyses used the outlier-resistant robust regression procedure PROC ROBUSTREG in SAS/Stat software to fit separate models for each trial day.[6, 14, 22, 24] To adjust for possible effects of the protocol change for training day 5, an additional fixed-effects term was specified in models of latency during the five evaluation days. DKA vs. DM contrasts are reported as the adjusted mean difference (AMD) in latency, along with 95% confidence intervals estimated using the bias-corrected percentile bootstrap method.[5]

To determine whether differences in learning curves were more likely related to deficits in reference memory versus working memory, we also conducted sub-analyses evaluating latency scores on only the first trial of those days in which the platform was in the same location as the day before (reference memory) and on the second through fourth trial of each day (working memory).

Results

Biochemical data for DKA rats before and after DKA treatment and for diabetes control (DM) rats measured during the sham procedures are presented in Table 1. Mean blood glucose concentrations (mg/dL) measured before and after the water maze task were DKA=314±147; DM=270±148 (mean±SD; p=0.33) and DKA=319±155; DM=332±148, respectively (p=0.75). Mean blood glucose concentrations (mg/dL) during the week prior to maze testing were 343±80 in the DKA group and 319±120 (mean±SD) in the DM group (p=0.63).

Across the 10 days of testing, significant DKA vs. DM heterogeneity was observed in daily mean latency (F-test for DKA * DAY interaction on 9 numerator and 139 denominator degrees of freedom = 3.44; p<0.001), indicating differences in learning curves. During the first five days of testing (labeled the "training period"), rats in both groups had similar latencies (Figures 1 & 2). During the second five days of testing (labeled the "evaluation period"), rats in the DKA group had significantly longer latencies during days 2 and 3 compared to rats in the DM group (Figure 2). There were no significant differences between rats evaluated with and without pedestal movement on day 6. Mean blood glucose concentrations before maze testing did not differ between groups on any of the test days. When pre-trial glucose concentration was included as a covariate in the regression models for latency, it did not have significant associations on any day of testing, nor did inclusion of glucose as a covariate change the finding that latency was significantly different on days 2 and 3 of the evaluation period.

Sub-analyses indicated that there were no significant differences between the two groups in performance on the first trial of each day, but mean latency scores for trials 2–4 of each day were significantly different between groups on evaluation days 2 (AMD=7.1, 95% CI: 1.6 to 12.8) and 3 (AMD = 6.2, 95% CI: 0.5 to 6.2). These data suggest that differences in learning between groups were more likely related to deficits in working memory than to deficits in reference memory.

Discussion

DKA is a frequent complication of diabetes in children, and severe, clinically-apparent cerebral injuries develop in approximately 1% of episodes. [4, 8] Until recently, children who did not develop obvious signs of cerebral injury during DKA were presumed to have no lasting neurological effects from the episode. One recent retrospective study challenged this assumption, suggesting that subtle memory deficits might be associated with DKA.[7] Although these data raise concern about the effects of DKA on the brain, the study was by necessity retrospective and therefore a causal relationship between DKA and neurocognitive deficits could not be verified. The current study provides prospective results consistent with the human findings and suggests that in rats, a single DKA episode causes measurable deficits in cognitive ability. These findings emphasize the need to study methods of protecting children from brain injury during DKA and suggest that evaluation of maze learning curves in rats should be further explored as a means of evaluating interventions aimed at preventing DKA-related neurological injury.

Several studies demonstrate neurocognitive deficits in children with type 1 diabetes including modest decreases in IQ, lower mental and psychomotor processing speeds, decreased attention and mental flexibility and decreased memory.[3, 13, 19–21] In addition, previous studies documented diminished memory function in rodents with STZ-induced diabetes.[1, 16, 18] Most studies demonstrate greater deficits in children with early onset of diabetes (under 4–7 years), but attempts to correlate neurocognitive deficits with aspects of metabolic control yielded mixed results. Hypoglycemic episodes have been found to increase the likelihood of neurocognitive deficits in some studies, but these results are not consistent.[21] Poorer metabolic control (higher HbA1c) has also been associated with neurocognitive deficits in some but not all studies.[20–21] Importantly, very few studies included DKA history as a covariate in data analyses. It is possible that failure to account for the effects of DKA may partially explain the variability in results observed in these studies.

In a recent study, Ghetti et al evaluated memory and IQ in children with diabetes with and without DKA history.[7] The study groups were nearly identical in mean age, diabetes duration, frequency of hypoglycemia, and glycemic control (HbA1c). Most children (91%) in the DKA group had experienced only one DKA episode, generally at the time of diagnosis of diabetes. Nonetheless, the DKA group had significantly decreased memory performance. Specifically, the DKA group had deficits in memory for item-context associations, a function localized to the hippocampus. [2] Animal data suggest that DKA is associated with cerebral hypoperfusion and that metabolic changes in the brain during DKA are similar to those observed in hypoxia/ischemia.[11, 26] Functional deficits localized to the hippocampus might therefore be explained by the sensitivity of this region to hypoxic/ischemic injury.[2, 15, 25]

Although the study by Ghetti et al supports an association between DKA and cognitive deficits, as in any retrospective study, possible effects of confounding variables could not be ruled out. Available measures of glycemic control such as HbA1c levels do not capture all aspects of diabetes management, particularly daily glucose variability, and patients' recall of episodes of hypoglycemia may not be reliable. The current prospective data are therefore of

importance because they are not subject to the inherent biases of retrospective studies and support the hypothesis that DKA may cause cognitive deficits.

The current study has several limitations. Diabetes management in rodents is difficult due to lack of control over the animals' dietary intake. Blood glucose fluctuations were common among the rats, making the model more similar to poorly controlled human diabetes than well-controlled diabetes. It is likely that many hyperglycemic and/or hypoglycemic episodes were not detected by daily glucose monitoring. Such episodes may have affected the rats' learning capabilities, leading to greater variability in latency measures. We would anticipate, however, that glucose fluctuations would have been equally likely in both groups and therefore, although causing more variability in outcome measures, would not have altered the conclusions. In addition, as this was a pilot study, the sample size was relatively small and the protocol was modified slightly during the second half of the study. The latter issue may have increased variability among rats and the former would decrease our ability to detect differences of smaller magnitude. These issues do not affect the conclusion that DKA appears to cause cognitive deficits, however, replication of the experiment with a larger sample would provide a more precise estimate of the extent of cognitive dysfunction caused by DKA. Furthermore, because of possible interspecies differences in the effects of DKA on the brain, the degree to which our findings in rats could be generalized to humans is unknown. Finally, because this study was an initial exploration to determine whether any neurocognitive deficits could be detected after DKA, our protocol did not include testing to rule out sensory or motor deficits. It seems unlikely that such deficits could have resulted from instrumentation, however, because control animals underwent identical sham procedures. Further, if such deficits resulted from brain injury, this would similarly represent evidence of neurological damage caused by DKA.

Conclusions

Our data suggest that a single episode of DKA in rats may result in measurable decreases in neurocognitive function and that additional larger studies are indicated to further explore the nature of neurocognitive dysfunction in this setting. Evaluations of maze learning curves in rats may possibly be useful as a means of assessing interventions to diminish DKA-related brain injury. Furthermore, our findings are consistent with data from previously published retrospective studies in children. In combination, these data suggest that children's history of DKA should be accounted for in studies aimed at investigating neurocognitive deficits related to diabetes.

Highlights

- Children with diabetes may develop neurocognitive deficits of unclear cause.
- We tested learning in rats after diabetic ketoacidosis (DKA) using the Morris Water Maze.
- Rats exposed to DKA had decreased maze learning compared to diabetic controls.
- These data may partially explain neurocognitive deficits in children with diabetes.
- Increased efforts to diminish DKA frequency in children are necessary.

Abbreviations

DKA	diabetic ketoacidosis
DM	diabetes mellitus
IP	intraperitoneal
STZ	streptozotocin

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Figure 1. Individual daily latency for rats in the DKA group and diabetes control group
Mean escape times are based on four trials per day.

Figure 2. Mean differences in latency (AMD) - DKA group (n=11) vs. diabetes control group (n=8)

Error bars show 95% confidence intervals; $P < 0.05$ for evaluation days 2 and 3.

Table 1

Blood chemistry values in the DKA group (before and after DKA treatment) and in the diabetes control group.

Study Variable	DKA Group		Post vs. Pre	Diabetic Control (DM)	DKA (Pre-treatment) vs. DM	
	Pre-treatment	Post-treatment	P-value		P-value	
Glucose (mmol/L)	19.1 ± 4.4 (343 ± 80 mg/dL)	10.8 ± 7.2 (194 ± 129 mg/dL)	<0.001	17.7 ± 6.7 (319 ± 120 mg/dL)	0.63	
Sodium (mmol/L)	135 ± 6	134 ± 4	0.85	136 ± 4	0.61	
BUN (mmol/L)	19.6 ± 7.8 (55 ± 22 mg/dL)	10.3 ± 4.3 (29 ± 12 mg/dL)	0.01	7.8 ± 2.1 (22 ± 6 mg/dL)	<0.001	
pH	7.04 ± 0.12	7.28 ± 0.06	<0.001	7.39 ± 0.04	<0.001	

Mean ± SD. DKA post- vs. pre-treatment mean comparisons assessed using paired t-test. DKA vs. DM mean comparisons assessed using Student's t-test.