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A Study of the Treatment of Rett Syndrome With Folate and Betaine

Daniel G. Glaze, MD, Alan K. Percy, MD, Kathleen J. Motil, MD, Jane B. Lane, RN, BSN, Janet S. Isaacs, PhD, RD, Rebecca J. Schultz, RN, MSN, CPNP, Judy O. Barrish, RN, BSN, Jeffrey L. Neul, MD, PhD, William E. O'Brien, PhD, and E. O'Brian Smith, PhD

Baylor College of Medicine, Houston, Texas (DGG, KJM, RJS, JOB, JLN, WEO, EOS); University of Alabama at Birmingham, Birmingham, Alabama (AKP, JBL, JSI)

Abstract

We tested the hypothesis that increasing methyl-group pools might promote transcriptional repression by other methyl-binding proteins or by mutant methyl-CpG-binding protein 2 with altered affinity, ameliorating the clinical features of Rett syndrome. A 12-month, double-blind, placebo-controlled folate–betaine trial enrolled 73 methyl-CpG-binding protein 2 mutation positive female participants meeting consensus criteria for Rett syndrome. Participants were randomized as young (< age 5 years) or old ($\geq$ age 5 years). Structured clinical assessments occurred at baseline, 3, 6, and 12 months. Primary outcome measures included quantitative evaluation of breathing and hand movements during wakefulness, growth, anthropometry, motor/behavioral function, and qualitative evaluations from electroencephalograms and parent questionnaires. In all, 68 participants completed the study. Objective evidence of improvement was not found. Subjective improvement from parent questionnaires was noted for the <5 years group. This study should inform future treatment trials regarding balancing participants with specific mutations and comparable severity to minimize selection bias.

Keywords

Rett syndrome; *MECP2*; methylation; folate; betaine; vitamin B₁₂

Rett syndrome is a neurodevelopmental disorder that occurs almost exclusively in females following apparently normal psychomotor development during the first 6 months of life. The principal features of Rett syndrome include loss of speech and purposeful hand use, stereotypic hand movements, gait dyspraxia, and deceleration in the rate of head growth.¹ Seizures, abnormal breathing (apnea and hyperventilation) during wakefulness, autonomic nervous system dysfunction, and growth failure may be noted.

Amir et al² reported several mutations in the methyl-CpG-binding protein 2 gene in individuals with Rett syndrome. At present, >95% of those meeting clinical criteria for Rett syndrome have 1 of more than 200 mutations of which 8 common mutations account for about 65% of the total. Methyl-CpG-binding protein 2 gene encodes methyl-CpG-binding protein 2, a member of a family of proteins known to bind specifically to methylated DNA CpGs and to be capable of repressing transcription.^{3–5} Methyl-CpG-binding protein 2 is expressed in all human tissues but is more abundant in brain. The binding site of methyl-CpG-binding protein

Address correspondence to: Alan K. Percy, MD, Civitan International Research Center, University of Alabama at Birmingham, 1530 3rd Avenue South, Birmingham, AL 35294; apercy@uab.edu.

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2 requires only a single methylated CpG dinucleotide. We hypothesized that by increasing the available pool of methyl groups, it might be possible to increase the degree of methylation of some CpG sites and promote transcriptional repression either by other methyl-binding proteins or by a mutant methyl-CpG-binding protein 2 with altered affinity, thereby ameliorating the clinical features of Rett syndrome. These other methyl-binding domain proteins, unlike methyl-CpG-binding protein 2, typically require more than one 5-methyl CpG to bind. Observations in humans, laboratory animals, and cell culture systems support the notion that increasing promoter-region cytosine residue methylation with methyl donors can lead to transcriptional repression.⁶⁻⁸ Others proposed that increasing CpG methylation at gene promoter sites may confer transcriptional repression independent of methyl-CpG-binding protein 2 function.⁸ Methyl groups are provided in vivo by 2 pathways: (1) the methyl group of methyltetrahydrofolic acid can be synthesized completely from CO₂; or (2) methionine is a significant source of active methyl groups via S-adenosylmethionine. The “methyl group pool” requires the vitamin cofactors, vitamin B₁₂, and folic acid. Low levels of either vitamin may result in significant clinical disease thought to be due, at least in part, to abnormal DNA metabolism. High-dose folic acid (5-120 mg/d) and methyl group donors such as betaine (6-20 g/d), a derivative of glycine, have been used safely to treat methyltetrahydrofolate reductase and cobalamin cofactor deficiencies and homocystinuria.⁹⁻¹² Therefore, to test this hypothesis, we conducted a double-blind, placebo-controlled, 1-year study of folate–betaine therapy in girls with Rett syndrome with a methyl-CpG-binding protein 2 gene mutation. Parents of participants < age 5 years receiving folate–betaine reported significant subjective improvement, but we were unable to detect objective evidence of improvement.

Study Design

We enrolled 73 female participants with Rett syndrome. To be eligible for enrollment, participants were required to meet the consensus criteria for Rett syndrome and to have a methyl-CpG-binding protein 2 gene mutation. They were categorized as young if less than (<) 5 years of age or old if equal to or greater than (≥) 5 years of age. All participants were stable clinically. We selected this age stratification scheme based on experience with a previous trial.¹³ Prior to age 5 years is the period of rapid regression or dynamic changes. The period after age 5 years is associated with stabilization and improved interaction and attentiveness. Failure to stratify in the previous study skewed the data and complicated analysis. This protocol was approved by the Institutional Review Boards of Baylor College of Medicine and affiliated hospitals and the University of Alabama at Birmingham School of Medicine.

The study treatment trial was 12-month, double-blind, placebo-controlled with participants randomized to the folate–betaine combination or placebo. In each age group, equal numbers of participants should receive either drug or placebo. Randomization, packaging, labeling, and dispensing of folate, betaine, and placebos were done by the Alabama or Texas Children's Hospital Pharmacies. Once inclusion criteria were met, randomization was performed and medication dispensed.

Each participant was admitted to the respective Children's Hospital General Clinical Research Centers at baseline, 6 months, and 12 months. Two outpatient visits were completed, the first at 1 month at an outpatient facility convenient for the family and the second at 3 months at Baylor or University of Alabama at Birmingham. Families were contacted monthly by phone or at the scheduled visits to review clinical response and medication compliance.

Dosages

Betaine dosages were 6 g/d for participants <30 kg weight (given 2 g three times a day [tid]) and 12 g/d for participants >30 kg weight (given 3 g four times a day [qid]). Folate dosage was 15 mg/d. Both folate–betaine and placebo were taken by mouth.

In addition, all participants received a Flintstones Complete vitamin tablet daily to ensure adequate oral vitamin B₁₂ intake.

Clinical laboratory testing included complete blood count, blood urea nitrogen, creatinine, urinalysis, and anticonvulsant levels, if applicable, at baseline, 3 months, 6 months, and 12 months. A standard electrocardiogram and 24-hour Holter monitoring was performed at baseline and 12 months. Biochemical testing included plasma homocysteine, methionine, betaine, dimethylglycine, creatine, and guanidinoacetate using electrospray tandem mass spectrometry at baseline, 1, 3, 6, and 12 months. The biochemical tests were performed at the Baylor College of Medicine Biochemical Genetics Laboratory. DNA analyses for methyl-CpG-binding protein 2 gene mutations were performed in the Baylor molecular testing laboratory using established methodologies.

Polygraphic recording captured breathing pattern, frequency of repetitive stereotypic hand movements, and electroencephalogram qualitative characteristics including background and epileptiform activity at baseline, 6 months, and 12 months for each participant during a 1-hour electroencephalogram polygraph study previously developed for evaluation of Rett syndrome.¹³ These monitoring sessions were time synchronized and videotaped to allow for precise characterization of the electroencephalogram and quantification of the breathing patterns and hand movements. The monitoring protocol included 10 channels of electroencephalogram, 1 channel of electrooculogram, 1 channel of electrocardiogram, 1 channel electromyogram, and 1 channel body movement via a triaxial accelerometer. Additional parameters included monitoring of respiratory effort (abdominal and thoracic strain gauges), airflow (thermocouple) end tidal CO₂ (nasal catheter), and oxygen saturation (pulse oximeter).

Clinical evaluation included a history at baseline and a nutritional evaluation at baseline, 3, 6, and 12 months. The nutritional status of the girls was determined using standard stadiometric (height, weight) and anthropometric (multiple skin-fold thicknesses and body circumferences) techniques. These measurements were converted to z scores using the National Center for Health Statistics database,¹⁴ degrees of malnutrition using Waterlow's criteria,¹⁵ and body fatness using Durnin's criteria.¹⁶ The clinical evaluation also included physical and neurological examinations, and motor-behavioral assessment at baseline, 3, 6, and 12 months. A previously developed motor-behavioral assessment provided objective numerical ratings in 3 areas: behavioral/social, orofacial/respiratory, and motor assessment/physical signs using the following rating scale: 0 = *absent or normal*; 1 = *mild, rare*; 2 = *moderate, occasional*; 3 = *marked, frequent*; and 4 = *very severe, constant*.¹⁴

A parent questionnaire was completed at the end of each study month either in person or by phone interview with the study coordinator for each site. The parents were asked to indicate whether their child had experienced worsening, no change, or improvement (0%-25%, 25%-50%, 50%-75%, and 75%-100%), in each of the following: sleep, breathing problems (awake), hand stereotypies, feeding, walking, hand use, communication, and mood.

Data Analysis

Primary outcome measures included quantitative evaluation of breathing and hand movements during wakefulness, growth parameters, motor function, and qualitative evaluations from the electroencephalogram and parent questionnaire.

As this was a double-blind, placebo-controlled study, data analyses were performed using the *t* test or its nonparametric equivalent, the Kruskal-Wallis test for continuous variables and the chi-square test for categorical variables. For each variable, analysis of covariance was used to compare placebo and folate–betaine groups with the measurement of the variable obtained at

the 12-month completion of the trial as the outcome and the baseline values of the same variable used as a covariate.

Results

Seventy-three participants were enrolled in this study. Ages ranged from 1.2 to 28.9 years. The mean age was 7.4 years for the placebo group and 7.1 years for the folate–betaine group. These did not differ by *t* test ($P = .94$) or Kruskal-Wallis ($P = .84$). Of these, 34 participants were randomized to placebo (19 < age 5 years and 15 $\geq$ age 5 years) and 39 to folate–betaine (18 < age 5 years and 21 $\geq$ age 5 years). No difference in race or ethnicity was noted. The majority of participants were white (82% in the placebo group and 90% in the folate–betaine group). Thirty-seven participants were < age 5 years. Of these, 19 were randomized to placebo and 18 to folate–betaine. The ages were also similar between the 2 groups. Thirty-six participants were older than age 5 years. Of these, 15 were randomized to placebo and 21 to folate–betaine. Their ages were also similar. In all, 68 participants completed the full 12-month study, 37 in the < age 5 years group and 31 in the $\geq$ age 5 years group. Participants not completing the study were in the $\geq$ age 5 years group. Four were on placebo: 1 stopped because of increased seizure activity, 1 expired suddenly, and 2 stopped because of the travel requirements. The single participant on folate–betaine stopped the study because of vomiting associated with the folate–betaine.

The distribution of participants by mutation for placebo and folate–betaine is depicted in Table 1. Although the numbers with specific mutations are too small to analyze statistically, in the < age 5 years group, 3 mutations appear to be overrepresented in the placebo or folate–betaine groups. T158M is seen more frequently in the folate–betaine group and R168X and R270X in the placebo group. In the $\geq$ age 5 years group, R168X and R306C appear to be overrepresented in the folate–betaine group. Smaller differences can also be seen in each age group. We considered an analysis of participants with missense and nonsense mutations in the methyl-binding domain of methyl-CpG-binding protein 2. Technically, R168X is just outside the methyl-binding domain. However, we had insufficient numbers in each group to conduct this analysis with sufficient power. We also considered an analysis of individuals < age 3 years, based on the premise that younger participants might have a more robust response. Again, as most participants were > age 3 years, the numbers were insufficient to conduct this analysis.

The results of biochemical testing are displayed for the folate–betaine (Table 2 [panels a and c]) and placebo (Table 2 [panels b and d]) panels within each age grouping. In as much as dimethylglycine and betaine would be very responsive to treatment, it was possible to monitor compliance with study medication requirements through these results. For both folate–betaine groups, dimethylglycine and betaine increased substantially. Smaller changes were noted for methionine and homocysteine, the former rising, the latter declining. Creatine and guanidinoacetate levels changed inconsistently. Among the individual participants, in the < age 5 years group, 2 in the folate–betaine panel were noncompliant at the time of their 12-month samples and 1 in the placebo panel had a spike in dimethylglycine, betaine, and methionine values at 12 months, suggesting that this individual was receiving active agents either purchased by the parents or as a result of a pharmacy error. Review of pharmacy logs failed to corroborate the latter possibility. In the $\geq$ age 5 years group, 1 participant on active agents had a decline in dimethylglycine, betaine, and methionine levels, suggesting incomplete compliance. No unusual values were noted in the placebo group for this age category.

Growth parameters including weight, height, body mass index, and head circumference were assessed at baseline and throughout the study (Table 3 [panels a–c]). No differences were noted between the placebo and the folate–betaine groups for weight, height, or body mass index. However, head circumference did show a significant difference between the overall placebo

and the folate–betaine groups. By both the 2 sample *t* test ($P = .027$) and the Kruskal-Wallis ($P = .024$), the folate–betaine group had a smaller increment in head circumference growth than the placebo group (mean increase being 0.74 vs 1.51 cm, respectively) during the 12-month study period. Analysis by the general linear model failed to show a difference between the panels in the overall group ($P = .88$) but did support the difference in the $< \text{age } 5 \text{ years}$ group ($P = .028$). In the $< \text{age } 5 \text{ years}$ group, the mean increase in head circumference was 0.66 cm in the folate–betaine panel and 1.72 cm in the placebo panel. No difference was noted in the $\geq \text{age } 5 \text{ years}$ group ($P = .37$ for the 2 sample *t* tests).

The 3 principal elements of the motor behavioral assessment (behavioral/social, oromotor/respiratory, and motor assessment/physical signs) assessing periodic breathing, hand stereotypies, and overall performance were analyzed for differences between the placebo and the folate–betaine panels for the overall group, the $< \text{age } 5 \text{ years}$ group, and the $\geq \text{age } 5 \text{ years}$ group. No difference was noted for any of these quantitative measures (Table 3 [panels a-c]). Similarly, no differences were noted between placebo and folate–betaine panels in any of these groups for any of the neurophysiological assessments, namely, for electroencephalographic qualitative background assessment, percentage time awake breathing irregularities, change in O_2 saturation, corrected QT (QTc) interval, or hand stereotypies while awake.

Analysis of the parental questionnaires that assessed whether their child experienced worsening, no change, or improvement with respect to sleep, breathing problems while awake, hand stereotypies, feeding, walking, hand use, alertness, communication, and mood did reveal differences (Table 4) by ordinal logistic regression between the placebo and the folate–betaine panels in the overall group ($P = .036$) and the $< \text{age } 5 \text{ years}$ group ($P = .012$) but not the $\geq \text{age } 5 \text{ years}$ group ($P = .98$).

Discussion

This study examined the hypothesis that increasing the available pool of methyl groups through intermediate metabolites (folate and betaine) known to be involved in the DNA methylation pathway might increase the degree of methylation of some CpG sites and lead to transcriptional repression either by other methyl-binding proteins or by a mutant methyl-CpG-binding protein 2 with altered affinity. In this manner, the clinical features of Rett syndrome might thereby be ameliorated. In all, 68 participants composed of 2 groups ($< \text{age } 5 \text{ years}$ and $\geq \text{age } 5 \text{ years}$) completed the double-blind, placebo-controlled study. Objective evidence of improvement was not obtained. However, subjective improvement based on a parent questionnaire was noted among the $< \text{age } 5 \text{ years}$ group.

To participate in the study, participants were required to have a mutation in the gene, methyl-CpG-binding protein 2 gene, associated with Rett syndrome. We did not restrict the study to specific mutations and did not account for mutations in the randomization scheme that would have ensured balance in the treatment arms with respect to mutation status. However, it is questionable whether the imbalance greatly affected the results as differences occurred in both arms. We now know that specific mutations may confer milder (eg, R133C, R294X, R306C, and C terminal truncations) or greater (eg, R168X, R255X, and R270X) severity.¹⁵⁻¹⁷ In reviewing Table 2, the $< \text{age } 5 \text{ years}$ group had an excess of R168X and R255X in the placebo arm and slightly more individuals with R306C and C terminal truncations. Similarly, in the $> \text{age } 5 \text{ years}$ group, the folate–betaine arm had an excess of R168X but also greater numbers of R133C, R306C, and C terminal truncations. As such, the impact of the differences should be interpreted cautiously. Overall, no dramatic improvements were noted.

This study has been instructive for the development of future treatment trials. Care must be given not only to stratification by age but also to inclusion of appropriate numbers of participants with specific mutations to minimize the introduction of undue sample bias.

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Table 1
Distribution of Mutations by Age and Treatment Group

Mutation	<5 years		≥5 years	
	Drug	Placebo	Drug	Placebo
R106W	2	1	1	2
R133C	2	1	2	0
T158M	5	2	3	3
R168X	2	5	3	0
R255X	2	1	1	2
R270X	0	3	0	1
R294X	0	0	2	1
R306C	1	3	4	1
Other point mutation	0	0	1	3
Deletion/insertion	2	1	1	1
C terminal	1	2	3	1
Exon 1	1	0	0	0
Total	18	19	21	15

Table 2

Biochemical Measurements in Plasma^a

Study Period	DMG	Betaine	Methionine	Homocysteine	Creatine	GAA
(a) <5 years old on study drug						
Baseline	7.8	55.6	17.9	8.1	73.7	2.1
3 months	158.6	1172.8	23.9	4.4	77.8	5.7
6 months	120.7	968.3	28.6	6.8	76.1	2.9
12 months	84.9	834.4	22.0	3.1	68.9	2.2
(b) <5 years old on placebo						
Baseline	8.3	48.3	20.0	8.3	86.9	1.9
3 months	12.0	48.9	16.5	7.5	84.8	2.0
6 months	9.6	49.4	20.1	4.7	82.3	2.1
12 months	12.9	63.9	21.9	6.1	78.9	2.5
(c) ≥5 years old on study drug						
Baseline	7.8	60.1	21.7	9.2	97.4	2.3
3 months	108.4	1308.1	27.1	7.1	89.9	6.7
6 months	106.4	1028.7	25.5	4.9	90.7	3.6
12 months	65.2	899.8	30.2	6.2	70.9	4.1
(d) ≥5 years old on placebo						
Baseline	7.0	59.9	20.6	10.6	93.0	2.7
3 months	7.5	53.8	20.2	7.1	92.2	2.9
6 months	6.4	46.7	14.1	3.8	106.4	2.3
12 months	6.9	45.7	21.4	7.7	100.1	2.8

Note: DMG, dimethylglycine; GAA, guanidinoacetate; SD, standard deviation.

^a All values are given in $\mu\text{mol/L}$.

Table 3

Folate–Betaine Trial

Parameter	Drug Mean (SD)	Placebo Mean (SD)	Paired <i>t</i> -Test <i>P</i> Value
(a) Overall cohort objective measures			
Height (z score)	−0.35 (0.61)	0.22 (0.55)	.40
Weight (z score)	0.04 (0.68)	−0.01 (1.12)	.81
FOC (% change)	0.46 (1.92)	1.52 (1.92)	.027
Body mass index (z score)	0.40 (.99)	0.34 (1.40)	.85
MBA change	3.2 (15.5)	2.4 (14.8)	.82
Periodic breathing	0.21 (3.61)	0.67 (5.33)	.68
Hand stereotypies	0.76 (5.26)	−0.70 (5.47)	.28
(b) Age ≥5 years objective measures			
Height (z score)	−0.38 (0.62)	−0.17 (0.63)	.40
Weight (z score)	−0.04 (0.57)	−0.29 (1.39)	.50
FOC (% change)	0.24 (2.53)	1.16 (0.86)	.34
Body mass index (z score)	0.17 (0.65)	0.05 (1.42)	.75
MBA change	8.00 (12.4)	4.7 (11.4)	.49
Periodic breathing	2.13 (2.92)	1.55 (4.13)	.67
Hand stereotypies	1.88 (4.67)	0.91 (4.41)	.59
(c) Age <5 years objective measures			
Height (z score)	−0.31 (0.63)	−0.25 (0.61)	.60
Weight (z score)	0.13 (0.81)	0.13 (0.98)	.99
FOC (% change)	0.66 (1.24)	1.72 (1.23)	.028
Body mass index (z score)	0.67 (1.26)	0.50 (1.40)	.71
MBA change	−1.00 (17.1)	1.00 (16.6)	.72
Periodic breathing	−1.5 (3.35)	0.16 (5.96)	.84
Hand stereotypies	−0.22 (5.68)	−1.63 (5.91)	.47

Note: FOC, frontal–occipital circumference; MBA, motor-behavioral assessment; SD, standard deviation.

Table 4Parental Qualitative Assessment^a

Treatment Group	Worse	No Change	Improved
Overall group			
Placebo	3	11	16
Drug ^b	2	10	25
< Age 5 years			
Placebo	3	5	11
Drug ^c	0	1	16
≥ Age 5 years			
Placebo	0	6	5
Drug ^d	2	9	9

^a One participant in the < age 5 years folate–betaine panel had missing data and could not be included.

^b $P = .036$.

^c $P = .012$.

^d $P = .98$.