Infant Rhesus Macaque Brain $\alpha$-Tocopherol Stereosomer Profile Is Differentially Impacted by the Source of $\alpha$-Tocopherol in Infant Formula

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ABSTRACT

Background: $\alpha$-Tocopherol ($\alpha$T) in its natural form [2'R, 4'R, 8'R $\alpha$T (RRR-$\alpha$T)] is more bioactive than synthetic $\alpha$-tocopherol (all-rac-$\alpha$T). All-rac-$\alpha$T is widely used in infant formulas, but its accretion in formula-fed infant brain is unknown.

Objective: We sought to compare $\alpha$T and stereoisomer status in infant rhesus macaques (Macaca mulatta) fed infant formula (RRR-$\alpha$T or all-rac-$\alpha$T) with a reference group fed a mixed diet of breast milk and maternal diet.

Methods: From day 1 after birth until 6 mo of age, infants ($n = 23$) were either nursery reared and exclusively fed 1 of 2 formulas by staff personnel or were community housed with their mothers and consumed a mixed reference diet of breast milk at 69 mL/d at 6 mo transitioning to monkey diet at ~2 mo (MF; $n = 8$). Formulas contained either 21 $\mu$mol RRR-$\alpha$T/L (NAT-F; $n = 8$) or 30 $\mu$mol all rac-$\alpha$T/L (SYN-F; $n = 7$). Total $\alpha$T and $\alpha$T stereoisomers were analyzed in breast milk at 2, 4, and 6 mo and in monkey plasma and liver and 6 brain regions at 6 mo of age. $\alpha$-Tocopherol transfer protein ($\alpha$-TTP), lipoprotein $\alpha$T, and urinary $\alpha$-carboxyethyl-hydroxycroman ($\alpha$-CEHC) were measured. One-way ANOVA with Tukey’s post-hoc test was used for analysis.

Results: At study termination, plasma, liver, lipoprotein, and brain total $\alpha$T did not differ between groups. However, the NAT-F-fed group had higher RRR-$\alpha$T than the SYN-F-fed group ($P < 0.01$) and the MF group ($P < 0.0001$) in plasma (1.7- and 2.7-fold) and brain (1.5- and 2.5-fold). Synthetic $\alpha$T 2R stereoisomers (SYNTH-2R) were generally 3- and 7-fold lower in brain regions of the NAT-F group compared with those of the SYN-F and MF groups ($P < 0.05$). SYNTH-2R stereoisomers were 2-fold higher in MF than SYN-F ($P < 0.0001$). The plasma percentage of SYNTH-2R was negatively correlated with the brain percentage of RRR-$\alpha$T ($r = -0.99, P < 0.0001$). Brain $\alpha$T profiles were not explained by $\alpha$-TTP mRNA or protein expression. Urine $\alpha$-CEHC was 3 times higher in the NAT-F than in the MF group ($P < 0.01$).

Conclusions: Consumption of infant formulas with natural (NAT-F) compared with synthetic (SYN-F) $\alpha$T differentially impacted brain $\alpha$T stereoisomer profiles in infant rhesus macaques. Future studies should assess the functional implications of $\alpha$T stereoisomer profiles on brain health. J Nutr 2020;00:1–9.

Keywords: infant, rhesus macaque, Macaca mulatta, breast milk, lactation, vitamin E, $\alpha$-tocopherol, stereoisomer, RRR-$\alpha$-tocopherol, all rac-$\alpha$-tocopherol

Introduction

Vitamin E is an essential nutrient for vertebrates and therefore must be acquired through the diet. Although the term vitamin E describes 4 tocopherols ($\alpha$, $\beta$, $\gamma$, and $\delta$) and 4 corresponding tocotrienols, only $\alpha$-tocopherol ($\alpha$T) meets vitamin E requirements (1). Plants synthesize only “natural” or 2R, 4'R, 8'R $\alpha$T (RRR-$\alpha$T), whereas dietary supplements and fortified foods typically contain a “synthetic” all-racemic mixture of 8 stereoisomers, RRR, RRS, RSR, RSS, SSS, SSR, SRS, and SSR (all rac-$\alpha$T). Only the 4 stereoisomers with the 2R configuration are recognized by the hepatic $\alpha$-tocopherol transfer protein ($\alpha$-TTP), are bioavailable, and contribute to dietary vitamin E requirements (2). For these reasons, RRR-$\alpha$T is thought to have between 1.36 and 2 times more vitamin E activity than an equal mass of all rac-$\alpha$T (2,3), perhaps depending on dose (4–6).
Methods

Animals and diets

Other results from this cohort of infant monkeys have previously been reported, and additional details are available (17). All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. On the day after birth, rhesus macaques (Macaca mulatta) of the Indian-origin subspecies were randomized to groups that received 1 of 3 diets: infant formula containing natural $\alpha$-Tocopherol [Similac Advance with OptiGRO (NAT-F group, $n = 8$)]; infant formula with synthetic all $\text{rac}-\alpha$-tocopherol [Similac Advance base formulation (SYN-F group, $n = 7$)]; and a combination of breast milk and Fiber-Balanced Monkey Diet 5000 (LabDiet) (MF group, $n = 8$). Formula-fed infants were nursed reared from 1 d after birth and were fed by bottle. MF infants were housed with their dam and breastfed. Formulas were labeled with a numerical code by Abbott Nutrition, to which investigators were blinded. All staff were blinded until sample analyses were complete. Randomization was stratified by gender and birth weight as previously described (17). The health of all infant monkeys was continuously monitored by veterinary staff.

For this study, infants were randomly assigned to 1 of 3 infant formula groups: synthetic all $\text{rac}-\alpha$-tocopherol (SYN-F); infant formula with synthetic 2R stereoisomers (SYNTH-2R); i.e., RSS, RSR, and RSR; and a combination of breast milk and Fiber-Balanced Monkey Diet 5000 (LabDiet) (MF group, $n = 8$). Formula-fed infants were nursed reared from 1 d after birth and were fed by bottle. MF infants were housed with their dam and breastfed. Formulas were labeled with a numerical code by Abbott Nutrition, to which investigators were blinded. All staff were blinded until sample analyses were complete. Randomization was stratified by gender and birth weight as previously described (17). The health of all infant monkeys was continuously monitored by veterinary staff.

Details of the $\alpha$-T composition of the diets are presented in Table 1. Dam breast milk (31.4 $\mu$mol $\alpha$-T/L) and SYN-F (29.7 $\mu$mol $\alpha$-T/L) contained similar concentrations of $\alpha$-T, and NAT-F contained 20.9 $\mu$mol $\alpha$-T/L. Formula concentrations of $\alpha$-T were normalized on an international unit (IU) per liter basis; 1 IU of vitamin E equals 1 mg all $\text{rac}-\alpha$-T, or 0.74 mg RRR-$\alpha$-T. The $\alpha$-T in the formulas was added as the acetate derivative. The concentration of $\alpha$-T in SYN-F formula was approximately 1.4 times that of NAT-F, consistent with documented differences in bioactivity between natural and synthetic sources of $\alpha$-T (3). RRR-$\alpha$-T constituted 100% of the $\alpha$-T in NAT-F, but was only 12.5% in SYN-F. The remaining $\alpha$-T composition of SYN-F was synthetic 2R stereoisomers (37.5%) and $\beta$-tocopherol (50%). Although dam breast milk contained all stereoisomers, RRR and SYNTH-2R stereoisomers comprised $\approx$92% of the total $\alpha$-T. Like SYN-F, the Fiber-Balanced Monkey Diet 5000 contained the acetate derivative of all $\text{rac}-\alpha$-T (44 $\mu$mol $\alpha$-T/L) in the diet.

A more complete composition of each diet is provided in Supplemental Table 1. Briefly, NAT-F was supplemented with a carotenoid blend (lutein, 0.135; zeaxanthin, 0.011; $\beta$-carotene, 0.0397, and lycopene, 0.182 $\mu$g/mL). In contrast, SYNF was not supplemented with the carotenoid blend but contained inherent concentrations of lutein (0.022 $\mu$g/mL), zeaxanthin (0.001 $\mu$g/mL), and $\beta$-carotene (0.012 $\mu$g/mL). Lycopene was not detected in SYN-F. Concentrations of the other nutrients and compounds, including docosahexaenoic acid/L and arachidonic acid, were consistent between NAT-F and SYN-F, as they came from the same formula batch.
Breast milk, plasma, urine, and tissue collection

Breast milk samples were collected while the animals were under ketamine sedation (5–10 mg/kg intramuscular) from 5 of the 8 dams, at 4 and 6 mo for 1 of the dams, but milk collection was not possible for 2 of the dams. Samples were collected directly into cryotubes, frozen on dry ice, and stored at −80 °C until analysis. Fasting blood samples (1 mL) were collected in EDTA-containing blood collection tubes and were processed for plasma, placed in cryotubes, frozen in liquid nitrogen, and then stored at −80 °C until analysis. Urine samples collected from the bladder were frozen in liquid nitrogen and stored at −80 °C until analysis. Liver and brain samples (−0.5–1 g each) were flash frozen in liquid nitrogen and then stored at −80 °C until analysis. Brain samples were dissected from the dorsolateral prefrontal cortex, occipital cortex, superior temporal cortex, striatum, cerebellum, and motor cortex.

Analysis of αT and its stereoisomers

αT and its stereoisomers were assessed as we described previously (16). In brief, samples were extracted with hexane following saponification as described (18). A portion of the hexane was dried and reconstituted to measure αT by HPLC with electrochemical chemical detection (ECD) as described (16). αT was quantified at the dominant oxidation potential relative to external αT standard (Sigma) that was validated against certified reference material (NIST SRM 968f). To assess αT stereoisomers, the remaining portion of the aforementioned hexane extract was used to measure the percentage distribution of αT stereoisomers as we described (16). In brief, the hexane extract was dried under nitrogen gas and resolubilized, and the reconstructed sample was methylated under basic conditions prior to extracting with hexane. Samples were then separated and detected by HPLC with fluorescence detection using a chiral separation column and excitation/emission settings of (290 nm/330 nm). Under these conditions, each specific 2R stereoisomer of αT (RRR-, RSR-, RSS-, and RSS-αT) was determined along with a single peak for total 2S stereoisomers. The peak area of each stereoisomer was calculated to determine the percentage distribution, and their molar concentrations were determined based on the concentration of total αT obtained by HPLC-ECD.

Urinary α-CEHC analysis

Urinary α-CEHC was extracted and analyzed as described previously (19), with minor modifications. Urine diluted with water was mixed with ascorbic acid (2%, wt/vol) and 6N hydrochloric acid, incubated in a shaking water bath (1 h, 60 °C), extracted with diethyl ether, dried under nitrogen, and reconstituted in 30% acetonitrile/0.05% acetic acid. Samples were analyzed as described previously (19), except that nebulizing and drying gases were supplied at 1.5 L/min and 15 L/min, respectively, and heating block and desolvation temperatures were set to 400 °C and 200 °C, respectively. Urinary α-CEHC concentrations were normalized to urinary creatinine measured using a clinical assay (Pointe Scientific).

TABLE 1 Total α-Tocopherol and α-tocopherol stereoisomer profiles of monkey breast milk, monkey diet, and infant formulas1

| Formulas       | NAT-F | SYN-F | MIXED DIET | MONKEY DIET |
|----------------|-------|-------|------------|-------------|
| αT (μmol/L)    |       |       |            |             |
| RRR            | 20.9  | 29.7  | 31.4 ± 5.22| 44.0        |
| RRS            | 0     | 3.70  | 10.7 ± 2.00| 5.50        |
| RSR            | 0     | 3.70  | 7.84 ± 1.57| 5.50        |
| RSS            | 0     | 3.70  | 3.76 ± 0.37| 5.50        |
| 2S             | 0     | 14.9  | 7.09 ± 1.32| 5.50        |
|                |       |       | 2.03 ± 0.27| 22.0        |
|                |       |       | 8.20        | 50.0        |

Lipoprotein fractions

Serum apoA-I, apoB-48, and apoB-100 levels were assessed with commercial ELISA kits (MyBioSource) according to the manufacturer’s instructions.

Western blotting

Procedures were conducted as previously described (20). Briefly, protein was extracted from frozen brain tissues, separated on SDS-PAGE, and transferred onto a membrane. Membranes were blocked (5% fat-free milk) and probed with polyclonal rabbit anti-α-TTP antibody (1:10000) overnight at 4 °C. (LifeSpan Biosciences, Inc). The secondary antibody was horseradish peroxidase–linked anti-rabbit IgG, 1:30,000 (Cell Signaling). GAPDH monoclonal antibody (1:3000) was used as a loading control (Cell Signaling). Liver was used as a positive control for α-TTP. Immunoblots were visualized with an ImageQuant LAS 4000 (GE Healthcare) and quantified via Quantity One (Bio-Rad). Samples from each of the 3 diet groups and 2 brain regions were evenly distributed between immunoblots to allow for direct comparisons between groups and to prevent bias between batches. One cerebellum sample was included in every batch to normalize band intensity values between blots.

Total RNA isolation and RT-qPCR analysis

Procedures were conducted as reported previously for brain tissues (20). After isolating total RNA from each sample, RNA purities and concentrations were determined and cDNA was synthesized. RT-qPCR was performed on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) following the manufacturer’s protocol for SYBR® Green ROX qPCR Mastermix (Qiagen). Actin γ 1 (ACTG1) was chosen as the reference gene due to its low variability between the occipital cortex and the cerebellum samples. Relative quantitation of gene expression was calculated using the 2−ΔΔCt method. The following primer sequences were used: TTP (designation of gene for α-TTP) forward: 5′-CAGAAATCGCACTGTTGGACC-3′; TTP reverse: 5′-GGCAGCCCTCTACGATCCGAAAG-3′; ACTG1 forward: 5′-GCTCC TGAACACAGTTCGTGC-3′; ACTG1 reverse: 5′-AGTAAACAGCCCA CGGTGTTTC-3′.

Statistical analysis

All data, except Western blot and RT-qPCR data, were analyzed with GraphPad Prism version 7.01 for Windows, GraphPad Software, www. graphpad.com. Dietary effects on total αT and αT stereoisomers (RRR, SYNTH-2R, and 2S) were analyzed using 1-way ANOVA followed by Tukey’s Multiple Comparison Tests. Pearson’s correlation was used to test for significant correlations between study variables. Unless indicated otherwise, data are expressed as mean ± standard deviation of the mean. Western blot and RT-qPCR data were analyzed using SPSS Statistics for Windows version 25 (IBM Corp.). Shapiro-Wilk and Levene’s tests were used to evaluate normality and homogeneity of variance, respectively. When necessary, data were transformed to

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**Results**

**Breast milk, monkey diet, and αT intake**

We estimated the daily intake of αT for each dietary group at 6 mo of age based on calorie intake calculated from week 24 formula intake data previously reported for these monkeys (15) (Table 2). Daily formula intake was very similar between NAT-F and SYN-F, resulting in a combined average intake of 294 kcal/d during the last week of the study. Breast milk intake values were not available for the MF infants, but from 2 mo of age MF infants consumed increasing amounts of monkey diet, culminating in an estimated intake of approximately 235 kcal/d.

We assumed that MF infants had the same daily energy intake as the formula-fed infants since there were no differences between the dietary groups for either growth velocity or final body weight (15). Based on this assumption, monkey diet provided most of the energy for the MF group at 6 mo of age, accompanied by a modest intake of breast milk of approximately 69 mL per d. The estimated intakes of plasma and liver αT concentrations were significantly lower in the NAT-F group than in the SYN-F group (P < 0.05). In contrast, plasma αT accumulation pattern in liver was similar, but not identical, to that in plasma. There were no differences among groups for total αT. Liver RRαT in the NAT-F group was not different from that in the SYN-F group (P = 0.064) but was significantly higher than in the MF group (P < 0.0001). The SYN-F group had significantly higher RRαT than the MF group (P < 0.001). The pattern of liver SYNTH-2R αT stereoisomer profiles

There were no differences in total plasma αT concentration between the 3 dietary groups (Table 3). However, the proportions of plasma αT stereoisomers differed among the groups. Plasma RRαT concentrations were significantly higher in the NAT-F group than both the SYN-F group (P < 0.0001) and the MF group (P < 0.0001), and the SYN-F group was significantly higher than the MF group (P < 0.05). In contrast, plasma SYNTH-2R concentrations were significantly lower in the NAT-F group compared with those in both the SYN-F group (P < 0.001) and the MF group (P < 0.0001) and were significantly lower in the SYN-F group compared with the MF group (P < 0.001). The sum of the 2 αT stereoisomers in plasma was not different between groups.

The αT accumulation pattern in liver was similar, but not identical, to that in plasma. There were no differences among groups for total αT. Liver RRαT in the NAT-F group was not different from that in the SYN-F group (P = 0.064) but was significantly higher than in the MF group (P < 0.0001). The SYN-F group had significantly higher RRαT than the MF group (P < 0.001). The pattern of liver SYNTH-2R

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TABLE 4  Concentration of α-CEHC in urine samples from 6-mo old infant rhesus macaques that were fed infant formulas with different sources of α-tocopherol or a mixed diet

| Urine creatinine (g/L) | Urine α-CEHC (μmol/L) | Urine α-CEHC/creatinine (μmol/g) |
|------------------------|------------------------|----------------------------------|
| NAT-F                  | 0.382 ± 0.339          | 2.21 ± 1.368                     |
| SYN-F                  | 0.428 ± 0.523          | 1.32 ± 1.049                     |
| MF                     | 0.384 ± 0.178          | 0.501 ± 0.410                     |

1Values are means ± SDs. n = 23; MF, n = 8; SYN-F, n = 7; NAT-F, n = 8. Labeled means in a column without a common lowercase superscript letter differ. a, b P < 0.05; c, d P < 0.01 by 1-way ANOVA and Tukey’s post hoc test. α-CEHC, α-carboxyethylhydroxichroman; all rac T, synthetic α-tocopherol (all-racemic mixture stereoisomers RRR, RRS, RSR, RSS, SSR, SRS, and SSR); αT, α-tocopherol; MF: received breast milk (31.4 μmol αT/L) and Monkey Diet 5000 (44 μmol αT/100 g); NAT-F: formula with 20.9 μmol RRαT/L; RRαT, 2R’, 4R, 8R α-tocopherol (natural); SYN-F: formula with 29.7 μmol all rac αT/L.

steroisomers was reversed compared with that of RRαT. Specifically, the concentration of SYNTH-2R in the NAT-F group was significantly lower than the concentrations in both the SYN-F group (P < 0.002) and the MF group (P < 0.0001). The SYN-F group had significantly lower SYNTH-2R than the MF group (P < 0.001). Mean liver 2S stereoisomer concentrations were significantly lower in the NAT-F group than in the SYN-F group (P < 0.0001), but not the MF group (P = 0.07). The 2S stereoisomer liver concentrations were significantly lower in the SYN-F group than the MF group (P < 0.004).

Urinary α-CEHC concentrations

Concentrations of α-CEHC in urine samples at the time of death are presented in Table 4. The concentration of urinary creatinine was not different between the dietary groups. Infants in the NAT-F group had a significantly higher concentration of α-CEHC than MF infants (P < 0.05). This difference persisted (P = 0.004) when α-CEHC concentration was normalized to creatinine concentration. The α-CEHC concentrations and α-CEHC normalized to creatinine concentration did not differ from those in the NAT-F or MF groups.

Lipoprotein fraction αT concentrations

Approximately two-thirds of plasma αT was found in the HDL apoA-I fraction after a 6-h fast (Table 5). The remaining one-third of plasma αT was found in the apoB fraction (apoB-48 + apoB-100). The mode of feeding did not influence either the concentration of αT in these fractions, or the mol/mol ratio of αT to apo B.

Table 5

|   | NAT-F   | SYN-F   | MF      |
|---|---------|---------|---------|
| αT, μmol/L | 28.9 ± 3.28 | 29.3 ± 7.69 | 33.1 ± 8.46 |
| HDL fraction αT, μmol/L | 18.7 ± 7.24 | 20.5 ± 5.86 | 20.7 ± 5.83 |
| ApoB fraction αT, μmol/L | 10.2 ± 3.86 | 8.80 ± 2.62 | 12.3 ± 3.41 |
| αT/apolB, mol/mol | 10.1 ± 6.91 | 9.80 ± 4.94 | 7.34 ± 3.17 |

1Values are means ± SDs. n = 23; MF, n = 8; SYN-F, n = 7; NAT-F, n = 8. Values were compared using 1-way ANOVA. all rac αT, synthetic α-tocopherol (all-racemic mixture stereoisomers RRR, RRS, RSR, RSS, SSR, SRS, and SSR); αT, α-tocopherol; MF: received breast milk (31.4 μmol αT/L) and Monkey Diet 5000 (44 μmol αT/100 g); NAT-F: formula with 20.9 μmol RRαT/L; RRαT, 2R’, 4R, 8R α-tocopherol (natural); SYN-F: formula with 29.7 μmol all rac αT/L.

2Total αT in HDL fraction (apoA-I) plus that in the apoB (apoB-48 + apoB-100) fraction.

Table 6

| Occipital cortex | Dietary group (μmol αT/g) |
|------------------|---------------------------|
| Total αT         | 28.9 ± 5.488             |
| RRαT             | 27.3 ± 5.128             |
| Synthetic 2RαT   | 1.51 ± 0.529             |
| Σ 2SαT           | 0.113 ± 0.165             |

1Values are means ± SDs. n = 23; MF, n = 8; SYN-F, n = 7; NAT-F, n = 8. Labeled means in a row without a common lowercase superscript letter differ. a, b P < 0.05; c, d P < 0.01 with or P = 0.001 by 1-way ANOVA and Tukey’s post hoc test. Labeled RRαT and synthetic 2R αT or Total αT means in a column without a common uppercase superscript letter differ P < 0.01 by 1-way ANOVA and Tukey’s post hoc test. all rac αT, synthetic α-tocopherol (all-racemic mixture stereoisomers RRR, RRS, RSR, RSS, SSR, SRS, and SSR); αT, α-tocopherol; MF: received breast milk (31.4 μmol αT/L) and Monkey Diet 5000 (44 μmol αT/100 g); NAT-F: formula with 20.9 μmol RRαT/L; RRαT, 2R’, 4R, 8R α-tocopherol (natural); SYN-F: formula with 29.7 μmol all rac αT/L.

Brain α-tocopherol stereoisomers infant macaques

Brain α-tocopherol concentrations in 6 brain regions (occipital cortex, cerebellum, superior temporal cortex, striatum, motor cortex, and prefrontal cortex) are presented in Table 6. Total αT was not significantly different between the dietary groups for any of the brain regions tested, and generally, αT concentrations did not differ among brain regions. However, within each dietary group, αT concentrations were significantly lower in cerebellum than in occipital, superior temporal, motor, and cortices and striatum (P < 0.01 for each), with the exception that total αT concentrations were not different between the cerebellum and temporal cortex in the SYN-F group.
Brain αT stereoisomer concentrations

αT stereoisomer concentrations are presented by dietary group and brain region in Table 6. In each brain region, infants fed NAT-F had a significantly greater concentration of RRR-αT than both the SYN-F group (occipital, temporal, motor, and prefrontal cortices: P = 0.01; striatum: P < 0.0001) and the MF group (P < 0.0001 all cortices). The SYN-F group had a significantly higher concentrations of RRR-αT than the MF group (P < 0.0001 all cortices). The concentrations of RRR-αT in the cerebellum were not different between the SYN-F and MF groups. Generally, the opposite pattern was found for SYNTH-2R. The NAT-F group had significantly lower concentrations of SYN-2R than the SYN-F group in the temporal cortex, striatum, motor cortex, prefrontal cortex (all P < 0.01), and the occipital cortex (P < 0.05), but not the cerebellum (not significant). Additionally, both the NAT-F and SYN-F groups had significantly lower SYN-2R than the MF group (P < 0.0001 all cortices). The concentrations of the individual SYNTH-2R stereoisomers by brain region and dietary group are presented in Supplemental Table 2. The sum of the 2α stereoisomers was low in all 3 groups for all brain regions tested (1–2% of total αT). In the NAT-F group, RRR-αT was significantly higher (P < 0.01) than the sum of the SYNTH-2R stereoisomers in all cortices. The same was true for SYN-F, except for the cerebellum, where RRR-αT and SYNTH-2R were not different. In contrast, in the MF group RRR-αT values were not different from the SYNTH-2R group values, except that SYNTH-2R was significantly higher than RRR-αT in the cerebellum (P < 0.01) and striatum (P < 0.01). With the 3 diet groups combined, the plasma percentage of RRR-αT positively correlated (r = 0.99, P < 0.0001) with the percentage of RRR-αT in the occipital cortex (Figure 1A) but the plasma percentage of SYNTH-2R was negatively correlated with the percentage of RRR-αT in the occipital cortex (r = −0.99, P < 0.0001) (Figure 1B). Results for the other brain regions were similar.

α-TTP expression in cerebellum and occipital cortex

α-TTP protein expression was compared between the 3 diet groups and between 2 brain regions of interest (cerebellum and occipital cortex). These brain regions were chosen because of their relatively low and high total αT concentrations, respectively; it was hypothesized that α-TTP expression levels may be associated with tissue αT levels. α-TTP protein expression did not significantly differ by diet group in the brain (P = 0.363) but was ~2-fold higher in the cerebellum than the occipital cortex (P < 0.0001) (Figure 2A and B). Analysis of α-TTP mRNA expression corroborated the protein expression findings, as levels were ~10-fold higher in the cerebellum than the occipital cortex (Figure 2C). Hepatic α-TTP protein levels were also determined, but they did not significantly differ between diet groups (P = 0.319, data not shown).

Comparison of diet stereoisomer profile with that of plasma and brain

The αT stereoisomer profiles in infant diet, liver, infant plasma, and occipital cortex (as a representative brain region) are presented by dietary group in Figure 3. RRR-αT constituted 100% of the αT in the NAT-F formula, as well as the majority of αT in the liver (96%), plasma (97%), and occipital cortex (94%) in the NAT-F group. In contrast, RRR-αT constituted only 12.5% of the SYN-F diet, and synthetic αT stereoisomers made up 87.5% (37.5% SYNTH-2R) of the SYN-F diet. SYN-F tissues were appreciably enriched in RRR-αT; liver (61%), plasma (70%), and the occipital cortex (72%), but also each contained about 27% SYNTH-2R. Likewise, MF tissues were enriched in RRR-αT (36–38%) and SYNTH-2R (35–38%) relative to their diet.

Discussion

Contrary to our hypothesis, neither the infant formula αT source (RRR-αT compared with all rac-αT), nor the mode of feeding impacted total αT concentration in the infant primate brain, plasma, or liver. However, the αT stereoisomer profile in each group was substantially impacted by both the infant formula αT source and by the mode of feeding. Our data reveal for what is to our knowledge the first time that infant formula supplemented with RRR-αT results in higher
accretion of brain RRR-αT and lower accumulation of SYNTH-2R compared with a formula supplemented with all rac-αT. This is the first direct comparison of αT sources in infant formula together with analysis of brain αT. The results clearly indicate that synthetic αT stereoisomers accumulate in the brain despite the selective activity of hepatic α-TTP and reveal an inverse relation between plasma SYNTH-2R and brain RRR-αT.

Surprisingly, our data revealed that MF infant monkeys accreted less brain RRR-αT and more SYNTH-2R stereoisomers than SYN-F infants, possibly due to the consumption of a substantially higher quantity of SYNTH-2R and αT from the dam’s diet. Note that the MF group was a reference group that consumed both breast milk and the maternal monkey diet and therefore inferences from comparisons with this group should be made with caution. This observation is, however, consistent with previous findings in other animals. Increasing doses of all rac-αT in rats and lambs led to decreased RRR-αT and increased SYNTH-2R in most nonbrain tissues analyzed (5, 6, 22). Mink fed all rac-αT had decreased brain RRR-αT and increased SYNTH-2R compared with those fed RRR-αT (4). Stereoisomer differences between the NAT-F and SYN-F groups are also generally consistent with previous reports. Previously, tissues, including brains of piglets (23, 24) and rodents (25, 26), preferentially accumulated RRR-αT over all rac-αT stereoisomers by ~1.8:1 to 2:1 when equimolar labeled quantities of both αT forms were fed. Notably, individual α stereoisomers were not measured in those reports. Thus, our findings indicate that brain from infant monkeys fed infant formula with all rac-αT or an MF diet had lower concentrations of RRR-αT and higher concentrations of SYNTH-2R compared with those fed infant formula containing RRR-αT.

Jeon et al. (17) previously reported higher concentrations of blood apoB and apoAI in the reference MF group in this cohort of infant primates. We sought to determine if differential distribution of αT in the lipoprotein fractions helped to explain our observations. We observed higher apoB fraction αT and lower αT/apoB molar ratios in the MF-fed infants compared with formula-fed infants, but neither was significantly different. Therefore, these findings did not offer mechanistic insight into our observations.

We also measured protein and mRNA concentrations of α−TTP in the brain regions with the highest (occipital cortex) and lowest (cerebellum) αT concentrations to determine if the selective activities of α−TTP toward specific αT stereoisomers could explain our observations. Contrary to our hypothesis, however, we found that α−TTP protein and mRNA expressions were higher in cerebellum than in occipital cortex. Clearly there was not a direct relation between αT concentration in these brain regions and α−TTP expression. This finding is particularly interesting in view of the proposal by Ulatowski.
and Manor (27) that cerebellar \( \alpha^{-} \)-TTP is essential for the coordination of the various brain pools of \( \alpha \)T.

It is noteworthy that brain RR\( \alpha \)-T concentrations in the MF group were significantly lower than those of the SYN-F group. This finding was surprising, as RR\( \alpha \)-T intake was estimated to be higher in the MF infants (5.2 \( \mu \)mol/L/d) than in the SYN-F infants (1.5 \( \mu \)mol/L/d), with similar RR\( \alpha \)/SYNTH-2R ratios (1:2.8 compared with 1:3, respectively). Consistent with this finding, MF SYNTH-2R concentrations in liver were 2-fold higher than those in SYN-F liver and were accompanied by MF brain and plasma SYNTH-2R concentrations that equaled those of RR\( \alpha \)R in most cases and even exceeded those of RR\( \alpha \)R in some cases. Taken together, these findings indicate that SYN-F infants were able to select for RR\( \alpha \)-T and to markedly increase the RR\( \alpha \) to SYNTH-2R ratio in tissues and plasma, but MF infants were not.

Mechanistic studies are needed to explain the inability of MF infants to select for RR\( \alpha \) to the same extent as SYN-F infants, despite the similar dietary \( \alpha \)T stereoisomer profile consumed by the 2 groups. As discussed earlier, a possible explanation is that the elevated intake of all rac-\( \alpha \)T by MF infants, which was obtained from Monkey Diet 5000, saturated stereoisomer-selective mechanisms including hepatic \( \alpha \)-TTP. This explanation is consistent with the elevated hepatic SYNTH-2R and depressed RR\( \alpha \)R that we observed in the MF liver.

With respect to the high accumulation of SYNTH-2R in the MF brain, it is interesting that the \( \alpha \)T stereoisomer profiles of the MF tissues were remarkably similar to those of the dam breast milk. Daily breast milk consumption was estimated to be approximately 69 mL per d at 6 mo of age; therefore, it would be surprising if breast milk \( \alpha \)T, which comprised 5% of total \( \alpha \)T intake, directly impacted tissue profiles. Instead, we speculate that different hepatic chylomicron production driven by breast milk consumption might be consistent with this observation. Human breastfed infants have higher amounts of plasma chylomicrons sooner after a feeding than formula-fed infants (28) and have higher fasting plasma concentrations of apoB (apoB-48 + apoB-100) and HDL (29, 30). Similarly, this cohort of MF infant macaques had higher fasting plasma concentrations of apoB and apoA-I compared with the formula groups at 6 mo of age (17). Like retinol, dietary \( \alpha \)T is incorporated into chylomicrons (31). Chylomicrons directly deliver fatty acids to neonatal brain in hepatotoxicated neonatal rats (32) and have been proposed to deliver an important amount of retinol to neonatal rodent brain (33), suggesting that brain and other tissues have access to chylomicron-associated nutrients prior to their delivery to the liver.

When considered together, the current hepatic \( \alpha \)T stereoisomer and urinary \( \alpha \)-CEHC observations further indicate that \( \alpha \)T was handled differently in the MF group. Despite having the highest hepatic concentration of SYNTH-2R, the MF group had lower urine \( \alpha \)-CEHC concentrations than the NAT-F group and trended toward lower concentrations than the SYN-F group. These findings are consistent with the possibility of chylomicron-dependent delivery of \( \alpha \)T that bypasses hepatic catabolism of synthetic stereoisomers in the MF infant monkey.

Total brain \( \alpha \)T concentrations reported here are higher than those previously reported for autopsied human infant brain (13), and are similar to those reported for an autopsied older adult human (34, 35), rat (36), and piglet (23) brain, but are lower than autopsied human centenarian brain (37). Our results confirmed those of previous reports revealing that cerebellum has lower concentrations of \( \alpha \)T than those measured in other brain regions (38, 39).

One confounding factor of this investigation was that the MF-group infants were housed with their biologic mothers. Thus, rearing conditions for this group differed from those in the nursery-reared formula groups, including differences in feeding patterns; breastfeeding typically involves ingestion of small but frequent volumes of breast milk, while the formula-fed groups were bottle-fed at regular intervals by humans on a predetermined schedule, which generally resulted in larger and less frequent feedings. Findings derived from the MF group should be further tested in controlled studies since the MF group was different from the formula-fed groups in potentially important ways.

In summary, the current data reveal that infant formulas containing RR\( \alpha \)-T or all rac-\( \alpha \)T lead to significant differences in brain \( \alpha \)T stereoisomer profiles. It is unknown whether an increased proportion of RR\( \alpha \)-T is advantageous to the developing brain, or if the accumulation of SYNTH-2R stereoisomers has unfavorable outcomes. However, it is known that RSR- and RSS-\( \alpha \)T have lower vitamin E activity than RR\( \alpha \)-T (14). Further, \( \alpha \)T may modulate gene expression in the brain (40–42), and the \( \alpha \)T stereoisomers may also differentially affect gene expression (15, 16). Thus, future studies should consider whether synthetic \( \alpha \)T stereoisomers differentially affect brain development compared with RR\( \alpha \)-T. Our data indicate that a mixed diet of breast milk and solid foods containing synthetic \( \alpha \)T stereoisomers leads to higher brain SYNTH-2R \( \alpha \)T and lower brain RR\( \alpha \)-T. These findings raise key questions for human translation, as women are routinely provided supplements containing all rac-\( \alpha \)-T, and they accumulate SYNTH-2R \( \alpha \)T in their milk. Importantly, human infants are also commonly transitioned to solid foods that are supplemented with all rac-\( \alpha \)-T. Whether human infants have brain \( \alpha \)T stereoisomer profiles like those of the infant monkeys in this study is an important question raised by the current finding that may yield interesting findings in future research.

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