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Blood D-(–)-3-Hydroxybutyrate Concentrations after Oral Administration of Trioctanoin, Trinonanoin, or Tridecanoin to Newborn Rhesus Monkeys (*Macaca mulatta*)

Mark A Tetrick,[1] Frank R Greer,[2] and Norlin J Benevenga[1,3,*]

Premature newborn infants are born with limited stores of glycogen and fat. Energy, such as medium-chain triglycerides (MCT), which can spare the use of body protein as metabolic energy, may be beneficial. This study compares MCT containing C8, C9, or C10 fatty acids as oral sources of energy for newborn rhesus monkeys (*Macaca mulatta*). On day 1 of life, 4 groups of 5 monkeys were given a single dose of water or MCT by nasogastric tube. The dose provided approximately 80% of the expected energy requirement. Plasma C8:0, C9:0, and C10:0 fatty acids and whole-blood D-(–)-3-hydroxybutyrate (3HB) concentrations were measured at 0, 1, and 3 h after dosing. Concentrations of free fatty acids (C8, C9, or C10) and ketone (3HB) increased with time after the dose. At 1 and 3 h, concentrations of C8 and C9 did not differ, but C9 was greater than C10. At 1 h, blood 3HB concentrations due to C8 triglyceride were higher than C9 or C10 (503 versus 174 and 225 μmol/L respectively). As MCT chain length increased from C8 to C10, blood concentration of 3HB decreased. Odd-chain MCT (C9 versus C8) resulted in lower whole-blood ketone (3HB), perhaps due to C9 metabolism or the rate of release or uptake of fatty acids. These results have implications for the use of MCT in nutritional supplements for preterm infants.

Abbreviations: MCT, medium-chain triglycerides; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids; 3HB, D-3-hydroxybutyrate.

The mammalian fetus is supplied by the placenta with a constant supply of energy substrates: glucose, lactate, and amino acids, as well as fatty acids in those species with placenta tissues that allow fatty acid transport.[3,10] At birth the placental source of energy substrates is interrupted, and the newborn animal must rely on endogenous substrate reserves, glycogen, lipid, and amino acids from body protein until feeding is established. Liver glycogen is the primary direct source of glucose for circulation in the unfed human newborn but may be depleted within 24 h postpartum.[32]

Human term and preterm infants as well as newborns of other species vary in the amounts of glycogen and endogenous lipid available for energy postpartum. Compared with term infants, premature infants have less than half of the amounts of liver and skeletal muscle glycogen.[31,32] Term human infants have 11% to 16% body fat at birth, the majority of which is available for oxidation. In contrast, premature human infants, as well as newborn piglets and rhesus monkeys, have only 1% to 2% body fat at birth.[9,17] Therefore, in premature infants and newborn piglets and monkeys, glycogen and body protein are considered to be the primary endogenous fuels oxidized after birth. However, piglets starved from 12 to 72 h of age were shown to derive 25.7% of energy from lipid, 36.8% from glycogen, and 37.6% from protein.[18] Apparently, body fat can provide a source of energy even though it comprises only 1% to 2% of the newborn body. Because premature human infants have relatively less glycogen storage than do term infants, premature infants are at risk of hypoglycemia if glucose or another fuel is not exogenously supplied. Although glucose often is provided by parenteral means, the energy requirement of premature infants cannot be met by glucose infusion alone, due to insulin resistance and osmotic constraints on the rate of glucose infusion. Alternate energy sources that can meet the energy requirement of the newborn premature infant are needed.

Lipids have advantages as an energy source for newborn infants, especially those born prematurely. Lipids are energy-dense and can reduce the use of other body substances as sources of energy. Premature infants rapidly develop an essential fatty acid deficiency if these are not included in parenteral solutions after birth.[11]

Of the lipids considered for supplementation of newborn infants, medium-chain fatty acids (C6:0 to C12:0; MCFA) have characteristics that are advantageous for energy metabolism. MCFA are more water-soluble than longer chain fatty acids, and medium-chain triacylglycerols (MCT) and the MCFA released during digestion or infusion (if given parenterally) may be more rapidly absorbed and oxidized.[1] MCFA transport into mitochondria may not be as carnitine-dependent as that for longer chain fatty acids. This difference could be important for newborn infants with either a limited ability to synthesize carnitine, low body carnitine

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Departments of [1]Nutritional Sciences, [2]Pediatrics, and [3]Animal Sciences, University of Wisconsin, Madison, Wisconsin.
[*]Corresponding author. Email: njbeneve@wisc.edu

concentrations, or low or no intake of dietary carnitine.[2,5,6,25] In circulation, MCFA are not as tightly bound to albumin as are long-chain fatty acids (LCFA, carbon chain length greater than 14), a theoretical benefit in newborns given that LCFA can displace bilirubin from albumin, thereby exacerbating neonatal hyperbilirubinemia.[20]

The rapid uptake and metabolism of MCFA results in increased production of acetyl-CoA and subsequently the ketone bodies acetoacetate and D-(–)-3-hydroxybutyrate (3HB). Ketonemia in newborn infants (blood concentrations of 3HB up to approximately 0.5 mmol/L) during fasting is common if endogenous fat is available. Higher concentrations may be detrimental, causing metabolic acidosis.[26,28,37,40] Although 3HB was shown to contribute less than 5% of the energy requirement of newborn piglets,[35] the estimated contribution of 3HB and acetoacetate to the energy requirement of term newborn human infants is approximately 25%.[7,8] Ketone bodies generated from MCFA metabolism also may be beneficial in that they can be used instead of glucose by neural tissue. Furthermore, they serve as a substrate for lipid synthesis supporting myelination in neural tissue and phospholipid (surfactant) production in the developing lung.[24,38]

Using LCFA and MCFA triacylglycerol supplements in newborns can have drawbacks. In a single study of extremely low-birthweight newborn human infants (birth weight of 600 to 800 g), those receiving LCFA lipid emulsion had a 50% mortality rate compared with 30% in unsupplemented control infants. However, in the same study, this effect was not seen in a group of infants of 801 to 1000 g birthweight.[33] In neonatal piglets, administration of MCT did not increase and perhaps even decreased survival.[15] MCFA can be toxic. Parenteral octanoate and trioctanoin have been observed to have narcotic effects in mice, guinea pigs, dogs, chicks, rats, and adult rhesus monekys.[19,27,29] Oral doses of MCT have caused periods of narcolepsy or coma in piglets.[16] In mice, LD_{50} doses are 0.12, 0.32, 3.7 g/kg for MCFA of C6:0, C7:0, and C8:0, respectively, and greater than 10 g/kg for C9:0, C10:0, and C11:0.[39]

Piglets dosed with odd-chain MCT had lower 3HB concentrations than did those dosed with even-chain MCT.[21] If MCT supplementation with even-chain fatty acid tiracylglycerols (trioctanoin) increases ketone body production to pathologic concentrations, could the use of odd-chain fatty acid triacylglycerols (trinonanoin), which provide propionyl-CoA as a result of their catabolism, reduce the level of ketone bodies and avoid pathologically high levels of ketone bodies while providing a source of metabolic energy?

The present experiment was conducted to examine chain length and even-chain compared with odd-chain fatty acid effect on toxicity, plasma fatty acid, and whole-blood 3HB concentrations as a first step in evaluating the suitability of newborn rhesus monkeys as a model for newborn human infants. This study examined the potential for acute toxicity of a single oral MCT dose composed of different MCFA in newborn rhesus monkeys. This assessment included evaluation of ketone body production from MCFA metabolism, which would potentially result in pathologic hyperketonemia or induce metabolic acidosis.

Materials and Methods

Animals. Dams and infant rhesus monkeys (*Macaca mulatta*) negative for SIV and simian retrovirus were obtained from the breeding colony maintained at the Wisconsin National Primate Research Center. This facility has been AAALAC-accredited since 1982. Standard care of the dams included controlled temperature (22 °C), 12:12-h light:dark schedule (0600 to 1800), ad libitum water, and standard primate diet (Monkey diet 5037/5038, PMI Nutrition International, St Louis, MO) supplemented with fresh fruit 2 to 3 times per week. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*[12] and under the approval of the University of Wisconsin Graduate School Animal Care and Use Committee. Infant monkeys were obtained within 15 h of birth, usually the morning after their birth during the previous night. The infants were held in a relatively quiet location, provided with a blanket to cling to and a heat lamp. Infants were excluded from this study if the primate center staff judged them to be at risk of maternal rejection after the 3- to 5-h separation needed for the evaluation.

Chemicals and enzymes. Triacylglycerols composed of octanoate, nonanoate, or decanoate were obtained from Karlshamns USA (Columbus, OH). (D)-β-Hydroxybutyrate dehydrogenase, Type 4 (EC 1.1.1.30); *N*-Methyldibenzopyrazine methyl sulfate salt; 4,7-diphenyl-1,10-phenantroline-disulfonic acid, and Tris were obtained from Sigma Chemical Company (St Louis, MO). [3-^{14}C]-(D)-(-)-3-Hydroxybutyrate ([3-^{14}C]-3HB) potassium salt was from NEN Research Products (EI Du Pont, Boston, MA). [1-^{14}C]Nonanoic acid was synthesized and supplied as a courtesy by Dr Jack Odle.[22]

Treatments and sample collection. A total of 20 rhesus monkey newborns was enrolled in this study. An initial group of 15 was assigned to treatments in a randomized complete-block design. Treatments included oral doses of octanoate (C8:0) or nonanoate (C9:0) or water control. An additional group of 5 monkeys became available later and were treated with the triacylglycerol decanoate (C10:0). This additional group allowed comparison of C8, C9, and C10 MCT. After separation from their mothers, infants were weighed (birthweight approximately 530 g), and 1 mL blood was obtained from a femoral vein by using a heparinized syringe. This sample was considered as time 0. Triacylglycerol or water then was administered by nasogastric tube at a dose of 8.4 mL/kg bodyweight. The triacylglycerol dosage was calculated to provide approximately 272 kJ gross energy per kilogram or 136 kJ for a 500-g newborn rhesus monkey, based on a basal energy requirement of:

$$(292 \text{ kJ } W^{0.75}) \times (0.5 \text{ kg body wt})^{0.75} = 174 \text{ kJ}$$

The triacylglycerol dose was estimated to provide approximately 80% (135 of 174 kJ) of the rhesus infants' daily basal energy requirement. Estimation of resting energy expenditure was based on our observations with newborn piglets, approximately 293 kJ/(kg × d)[17] according to expired CO_2 (367 µmol/[min×kg]$^{0.75}$)[23] and an assumed respiratory quotient of 0.86. Use of 80% of the basal energy requirement was in our judgment a sufficient amount to have an effect, in light of our work with energy metabolism in newborn piglets and their response to MCT.

Additional 1-mL blood samples were drawn from a femoral vein 1 and 3 h after administration of triacylglycerol or water. Rhesus infants were allowed to rest on a cotton diaper and were kept warm with a heat lamp between blood sample collections. All blood samples were held on ice and processed within 30 min of collection. Blood samples were divided and analyzed for 3HB and fatty acid concentration according to the following procedures.

3HB analysis. Perchloric acid (70%, 60 µL) was added to 0.5 mL blood for deproteinization. After centrifugation for 3 min at

3000 × *g*, the supernatant was removed and saved. The acid-insoluble pellet was washed twice by resuspension in 0.25 mL 0.1 M perchloric acid and centrifugation for 3 min at 3000 × *g*. The wash supernatants were added to the primary supernatant. Octanol (1 µL) was added to the combined supernatants to reduce foaming in subsequent steps. Perchloric acid in the combined supernatants was precipitated as potassium perchlorate by addition of 100 µL 2 M potassium carbonate (pH 9.0). The sample was allowed to stand for 30 min after addition of potassium carbonate. The sample was centrifuged for 3 min at 3000 × *g* and the supernatant pH-adjusted to 8.50 ± 0.05 by addition of approximately 100 µL 5.8 M potassium carbonate and HCl. Supernatants were frozen and stored at −20 °C prior to analysis for 3HB. More than 93% of the radioactivity from [3-^{14}C]3HB added to similar blood samples was recovered in the supernatant by using this protocol (data not shown). 3HB was quantitated in duplicate samples of the frozen supernatants by using a dye-linked spectrophotometric method[13,14] with the following modifications. Sample absorbances were read at 40 min and corrected for a blank consisting of sample and all reagents plus 0.1 mol/L Tris buffer without D-β-hydroxybutyrate dehydrogenase (no-enzyme blank). Heat treatment of the sample (56 °C for 15 min) was necessary to reduce the increase in absorbance not associated with enzymatic conversion of 3HB to acetoacetate. The 3HB detection limit in the 0.5-mL blood sample by using this assay was 3 to 5 µM.

MCFA analysis. Fatty acid quantitation in the remaining 0.5 mL blood was corrected for recovery of 1.6 kBq [1-^{14}C]nonanoic acid added to the sample prior to plasma separation. Plasma was separated by centrifugation at 3,000 × *g* for 3 min. Fatty acids were extracted from plasma by using chloroform–methanol extraction.[4] The phenacyl ester of the extracted fatty acids was formed by using phenacyl-8 reagent (bromoacetophenone and crown-8-ether catalyst in acetonitrile; Pierce Chemical Company, Rockford, IL). Formation of the phenacyl ester allowed spectrophotometric detection at 254 nm and quantitation of the phenaycl–fatty acid ester after separation from other plasma components on an HPLC system equipped with a C18 reverse-phase column and an acetonitrile–water gradient as described previously.[21]

Statistics. Plasma fatty acid and blood 3HB concentrations from groups of newborn rhesus monkeys were analyzed by one-way ANOVA[34] by using a General Linear Models procedure of Statistical Analysis System release 6.09 (SAS Institute, Cary, NC).[30] The split-plot model included the main effect of oral dose, with time and time×treatment interaction as a subplot. Inferences regarding oral dose were based on nonorthogonal contrasts based on perceived biologic importance. Nonorthogonal contrasts were used to compare plasma fatty acid concentrations due to treatment: water versus 8:0, 8:0 versus 9:0, and 9:0 versus 10:0. Orthogonal contrasts were used to compare the group mean 3HB concentrations: water-treated group to MCT treatments, 8:0 versus 9:0, 8:0 versus10:0, and 9:0 versus10:0. Fatty acid and 3HB concentrations at 0 versus 1 h, 0 versus 3 h, and 1 versus 3 h were compared for each treatment by using orthogonal contrasts. Outlier values were defined as individual values falling outside a range of 2 SD on either side of the mean for the other animals in the group.

Results

Newborn monkeys dosed with MCT remained alert and active after dosage. No episodes of narcolepsy were noted. Octanoate concentrations for the time 0 sample are reported. Fatty acid concentrations reported for 1 and 3 h samples were 8:0 in animals dosed with water and trioctanoin and 9:0 and 10:0 in animals dosed with trinonanoin or tridecanoin, respectively.

Plasma C8 fatty acid concentrations (Table 1) prior to the MCT or water dose were similar for water-, C8-, and C9-dosed (27 µmol/L) groups. No detectable concentrations of C9 were found at time 0. Plasma C10 concentration at time 0 was measured only in monkeys dosed with C10 and was 4 µmol/L. Plasma fatty acid concentrations were significantly higher ($P < 0.03$) at 1 and 3 h after MCT dose for C8, C9, and C10, when compared with the respective time 0 fatty acid concentration. Plasma fatty acid concentrations were significantly ($P < 0.05$) higher at 1 h after MCT dose in monkeys treated with C8 and C9 compared with those that received water.

Three hours after dosing, the group given C10 had a significantly ($P < 0.05$) lower C10 fatty acid concentration (Table 1) compared with C9 fatty acid concentration in those dosed with C9. No other differences in fatty acid concentrations were detected among treatment groups for samples obtained at 3 h. In summary, fatty acid concentration increased after MCT dose. For the even-chain fatty acids, plasma fatty acid concentrations reflected chain length effects (8:0 > 10:0), a result similar to that observed in piglets.[21,22] However, the chain length effect was not seen when odd versus even chains were compared.

At time 0 no significant differences in whole-blood 3HB concentrations (Table 1) were seen among groups (water, C8:0, C9:0, C10:0). Blood 3HB concentrations varied with treatment over time, as shown by significant ($P = 0.03$) time×treatment interaction. Therefore, treatment comparisons were made within sampling time. One hour after water or MCT dose, 3HB concentrations were significantly ($P < 0.03$) higher in MCT-dosed animals compared with water-dosed control monkeys. Among groups dosed with MCT, 3HB concentration was higher ($P = 0.07$) 1 h after treatment with 8:0 compared with 9:0 and 10:0. No difference was detected between groups dosed with 9:0 or 10:0 ($P = 0.7$). Three hours after dosing, 3HB concentrations were significantly ($P = 0.04$) lower in macaques given water when compared with those treated with MCT. Monkeys dosed with C8 had significantly higher 3HB concentrations compared to C9- and C10-dosed monkeys 3 h after treatment ($P < 0.03$).

Because macaques dosed with C8:0 MCT tended to have an increased 3HB concentration at time 0, the initial 3HB concentration was examined as a covariable in the analysis of 3HB concentration over the 3 h following the MCT dose. However, inclusion of the initial 3HB concentration as a covariable in the analysis did not alter inferences made about treatment comparisons. Therefore the apparent initial values of 3HB in the 8:0-dosed group did not appear to affect subsequent concentrations.

Discussion

MCFA are absorbed and metabolized from their respective triacylglycerols in newborn rhesus monkeys, as evidenced by the respective increased plasma fatty acid concentration and blood 3HB concentrations after gavage of MCT compared with water. The 3HB concentrations in the newborn rhesus monkeys in the present study (500 µmol/L and greater) are somewhat higher than those previously reported in human infants (approximately 140 µmol/L) fed formula in which 50% of the fat was composed of MCT (weight %: C8, 28%; C10,14%; and C12,10%).[18] Increased 3HB concentrations might be expected in blood samples from

Table 1. Plasma fatty acid and whole-blood 3HB concentrations (μM , mean ± SEM) after oral gavage of water or MCT

Treatment group	Metabolite measured	Concentration (μM) at 0 h	1 h	3 h
water	C8:0	30 ± 8	29 ± 8[f]	64 ± 28
C8	C8:0	21 ± 17[d]	117 ± 28[e,g]	128 ± 8[e]
C9	C9:0	not detected[d]	154 ± 19[e,h]	207 ± 60[e,f]
C10[a]	C10:0	4 ± 1[d]	76 ± 27[e,g]	83 ± 9[e,g]
water	3HB[b]	69 ± 36[d,f]	50 ± 16[d,f]	112 ± 53[d,f]
C8	3HB	130 ± 25[d,g]	503 ± 113[e,g]	585 ± 111[e,g]
C9	3HB	48 ± 12[d,f]	174 ± 42[e,h]	268 ± 62[e,h]
C10[c]	3HB	46 ± 16[d,f]	225 ± 150[d,h]	282 ± 177[d,h]

All treatment groups contained 5 macaques, except for C10 groups, which contained 4 monkeys each due to elimination of an outlier.
[a]Concentrations (μM, mean ± SEM) with outlier included were: 0 h, 13 ± 1; 1 h, 119 ± 47; and 3 h, 107 ± 25.
[b]Due to significant ($P < 0.05$) treatment x time interaction, all treatment comparisons for 3HB were made within time point.
[c]Concentrations (μM, mean ± SEM) with outlier included were: 0 h, 163 ± 117; 1 h, 463 ± 265; and 3 h, 710 ± 449.
[d,e]Values that differ ($P < 0.05$) within treatment groups (rows) are indicated by different superscript letters.
[f,g,h]Values that differ ($P < 0.05$) within time points (columns) are indicated by different superscript letters.

the present study if one takes into account that MCT is the only source of exogenous energy provided to the monkeys, whereas the human infants 40 received a complete formula in which half of the fat was MCT.[19]

No narcolepsy was noted in the newborn monkeys dosed with MCT in the present study. The highest measured concentration of nonesterified fatty acid after the MCT dose was 200 μmol/L at 3 h after dosage of monkeys given C9. Signs of narcolepsy or coma were not seen in dogs infused with MCT until octanoate concentrations reached 780 μmol/L or greater.[19] Piglets orally dosed with an emulsified MCT product containing C4:0, C5:0, C6:0, or C7:0 achieved fatty acid concentrations of as high as 12 mmol/L and experienced narcolepsy, coma, and high mortality.[39] Emulsification of the MCT product increased the fatty acid concentration after oral dosage by 1 order of magnitude and the use of shorter chain fatty acids yielded correspondingly lower LD_{50} values.[39] These are plausible explanations for increased incidence of pathology after dosage of the emulsified product.[36] This result raises a point of caution for future studies with MCT products in newborn rhesus monkeys and human infants. The form of the MCT (chain length and emulsification) can have dramatic effects on the rate of absorption and plasma fatty acid concentrations after oral dosage and, therefore, on the product's toxicity.

Piglets dosed with MCT composed of even-chain fatty acids (75% 8:0 and 25% 10:0) had higher 3HB concentrations as long as 4 h after the dose than did piglets given odd-chain (9:0) MCT.[21] The authors[21] speculated that the difference in 3HB concentrations could reflect differences in chain length or odd versus even chain length. The current study supports the conclusion that chain length over the range of 8:0 to 10:0 and even versus odd chain length effect accounts for differences in 3HB concentrations after MCT dose. 3HB concentrations in newborn rhesus monkeys dosed with C10:0 MCT were similar to those given C9:0 MCT (that is, approximately 250 μmol/L), whereas C8:0 MCT increased blood 3HB concentrations (value approximately 550 μmol/L) compared with those after C9:0 or C10:0 MCT. 3HB concentrations in animals treated with C9:0 tended to be lower than the intermediate value expected (approximately 400 μmol/L) from an effect of chain length alone. Perhaps propionyl-CoA due to catabolism of odd-chain MCT affected the rate of catabolism of acetyl-CoA such that the rate of production of 3HB from acetyl-CoA was suppressed.

The observed difference in 3HB concentrations among C8:0, C9:0, and C10:0 MCT may be due to differences in rates of digestion, absorption, and metabolism of MCT and the MCFA released, rather than reflective of a difference in metabolism of odd versus even chain length fatty acids. This idea is supported by earlier work,[23] which showed that 3HB concentrations in piglets infused intravenously with fatty acids C7:0, C8:0, C9:0, or C10:0 could be predicted by a linear function of fatty acid concentration regardless of even versus odd chain length or total carbon number of the fatty acid carbon chain. However, the 3HB concentration response of primates dosed with 10:0 MCT seemed to fall into 2 categories (data not shown) after dosage, compared with 3HB concentrations in rhesus newborns given C8:0 or C9:0. One subset of 10:0-dosed macaques responded with clearly higher concentrations of 3HB after dosage, whereas the other subset did not respond as dramatically despite no apparent difference in the C10:0 concentration in the 2 subsets and no apparent correlation between C10:0 concentration and 3HB level. This observation suggests that other factors play a role in the 3HB response to C10:0 fatty acids. Future studies are needed to determine whether a relationship might be established between C10:0 and 3HB concentrations through more rigorous evaluation by using larger numbers of macaques and by dosing macaques with 7:0 MCT as a short-chain-length, odd-chain fatty acid.

Rhesus monkey newborns appear to be a viable model for studying MCT and 3HB metabolism as it might apply to premature human infants. This model likely will facilitate indepth study of the effects of chain length and even versus odd chain length on MCFA metabolism and its application to supplementing the energy requirements of newborn primates. This knowledge likely would have implications for the use of MCT in pre- and full-term infants.

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