Choline is a Methyl Donor in Dairy Cows: The Proof is in the Label

J. W. McFadden and T. L. France
Department of Animal Science
Cornell University

Introduction

Choline is a quasi-vitamin with unique metabolic fates. In the gastrointestinal tract, unprotected choline may be converted to trimethylamine by the actions of trimethylamine lyase. In turn, trimethylamine may pass into circulation and subsequently transformed into trimethylamine *N*-oxide (TMAO) in the liver by flavin monooxygenase 3. Choline absorbed at the intestines, via choline transporters, is subject to a wide array of metabolic uses. First, choline may be used for the synthesis of complex phospholipids including phosphatidylcholine (PC) and lysophosphatidylcholine (LPC). Phosphatidylcholine is a component of bile, and a cellular and lipoprotein membrane lipid synthesized by the cytidine diphosphate (CDP) choline pathway (i.e., Kennedy pathway). The initial reaction, controlled by choline kinase, produces phosphocholine. Sphingomyelin synthase utilizes ceramide and PC to produce sphingomyelin, which supports brain myelination. The Lands cycle is responsible for the conversion of PC into LPC, which includes the lipolytic enzyme lipoprotein-associated phospholipase A_2 in circulation. Lysophosphatidylcholine has received attention in humans as a bioactive lipid with immunoregulatory properties (Kabarowski, 2009). Moreover, LPC has been implicated in the endotoxin-response in lactating dairy cattle (Javaid et al., 2022). Choline may also be oxidized to betaine, a key osmoregulator, by the actions of the zinc metallo-enzyme betaine-homocysteine methyltransferase.

In humans and cows, choline and betaine have been defined as methyl donors (McFadden et al., 2020). A methyl donor helps produce *S*-adenosylmethionine (SAM), via the actions of the folate and methionine cycles. In turn, SAM is used for various methylation reactions including methyl tagging on histones and deoxyribonucleic acid. Furthermore, the methylation of phosphatidylethanolamine (PE) is controlled by phosphatidylethanolamine *N*-methyltransferase (PEMT), which transforms PE to PC via three sequential methylations using SAM. In non-ruminants, the CDP choline pathway is believed to account for 70% of PC synthesis in the liver (DeLong et al., 1999). The remaining 30% of PC produced by the actions of PEMT (DeLong et al., 1999). These percentages were determined by culturing rat primary hepatocytes with tritium-labeled choline chloride or ethanolamine hydrochloric acid followed by lipid extraction and phospholipid separation. Additional experiments by DeLong and coworkers (1999) demonstrated that the fatty acyl composition is unique for PC produced by PEMT, which has a preference for fatty acyl chains of high unsaturation (e.g., C22:6). As established in choline-deficient rats, PEMT compensates for a lack of dietary choline, which may be critical during periods of inadequate dietary choline supply such as starvation, pregnancy, or lactation (Cui and Vance, 1996).

Choline Biology and Nutrition in the Dairy Cow

In dairy cattle, ruminal degradation of unprotected choline to trimethylamine is rapid (Neill et al., 1978; Sharma and Erdman, 1989). This discovery triggered the development of rumen-protected choline chloride to prevent ruminal degradation and increase dietary choline supply to the small intestines. In situ rumen degradation and in vitro intestinal digestibility methodologies have been used to estimate metabolizable choline; albeit, such an approach has ignored choline degradation by bacterial trimethylamine lyase in the intestines and thus likely overestimates metabolizable choline supply. Moreover, studies have demonstrated lower net choline absorption at the small intestine for cows fed rumen-protected choline chloride, relative to abomasal choline chloride infusion (de Veth et al., 2016), and demonstrated marked increases in plasma TMAO concentrations following the a pulse dose of rumen-protected choline chloride to the rumen (France et al., 2022). Regardless, a plethora of studies have demonstrated positive effects of rumen-protected choline feeding on milk production and composition, measures of hepatic health, disease prevention, and fertility (Humer et al., 2019; Arshad et al., 2020), which suggests the delivery of choline to the intestines for absorption and use by the cow.

In the dairy cow, identifying choline as a methyl donor has been debated. Chandler and White (2017) concluded that increasing the supply of choline to bovine neonatal hepatocytes may have supported methionine regeneration from the folate methyl pool; albeit, PEMT expression was reduced by choline chloride supplementation. Feeding cows rumen-protected choline has been shown to increase plasma concentrations of betaine and phosphocholine, which suggests the provision of substrate for transmethylation and activation of the CDP choline pathway, respectively (de Veth et al., 2016; France et al., 2022). However, evidence suggests that stage of lactation uniquely influences the choline metabolite response (i.e., preferred pathway activation) to dietary rumen-protected choline supplementation (de Veth et al., 2016; France et al., 2022). In dairy cow primary liver cells, gene evaluation data has suggested that transmethylation and transsulfuration are more responsive to methionine supplementation; whereas, choline supplementation preferentially actives the CDP-choline pathway (Zhou et al., 2018).

Several gaps in knowledge remained to adequately define choline biology in the pregnant and lactating dairy cow. First, direct evidence to support the ability of dietary choline to support hepatic methylation of PE was needed. Second, we lacked an understanding for the extent of post-ruminal choline degradation to trimethylamine by bacterial trimethylamine lyase, which has implications for defining metabolizable choline supply using in vitro intestinal digestibility assays. Third, our understanding of choline utilization between the CDP choline pathway and PEMT pathway during gestation and lactation required clarity. Fourth, direct evidence to support endogenous recycle of dietary choline, via bile, has received little attention. Fifth, the extent of dietary choline metabolized by the cow versus what is excreted in urine or secreted in milk required clarity. To answer these questions, the use of deuterium-labeled choline chloride is a means to trace choline and methyl group utilization in the cow.

Lessons Learned from Using Deuterium-labeled Choline Chloride in Humans

Deuterium is a stable isotope of hydrogen. Methyl-d9-choline chloride may be used to trace methyl group utilization in mammals. Such an approach allows the investigator to evaluate the partitioning of labeled choline (i.e., d9-choline) and choline metabolites with labeled methyl groups (i.e., d3- or d6-metabolites). The approach allows for assessment of choline and choline methyl group partitioning between the CDP choline pathway (i.e., d9-PC) and PEMT pathway (e.g., d3- or d6-PC), respectively. Since d6-PC is often undetectable or detected at low concentrations, the detection of d9-PC derived from PEMT activation is unlikely because of the greater concentration of unlabeled methyl groups, relative to labeled methyl groups, in the biological system. Figure 1 provides a schematic for methyl group utilization during choline metabolism.

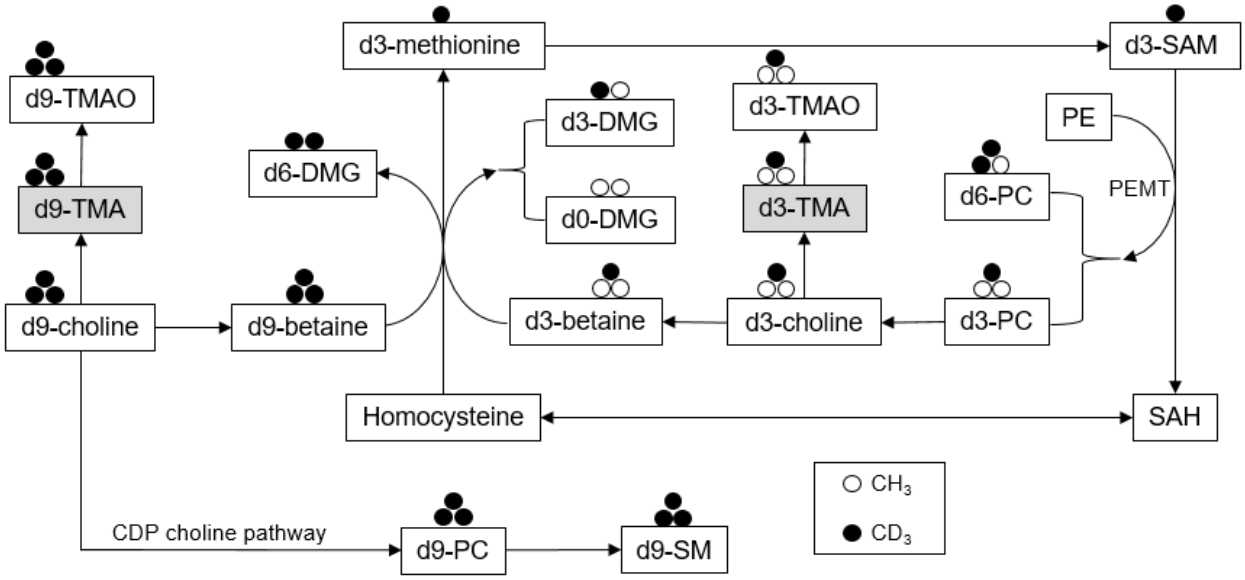

Figure 1. Deuterium-labeled methyl-d9-choline for methyl group tracing. Black circles represent deuterium-labeled methyl groups. White circles represent unlabeled methyl groups. The grey box reflects trimethylamine (TMA) produced from bacterial metabolism in the gut. Abbreviations not found in main body: DMG, dimethylglycine; SAH, *S*-adenosylhomocysteine; SM, sphingomyelin. Adapted from Yan et al. (2013) with modifications.

The use of methyl-d9-choline chloride was used in pregnant and nonpregnant women (Yan et al., 2013) and lactating women (Davenport et al., 2015) to evaluate methyl group utilization. A total of 22% or 20% of total choline intake was provided as isotopically labeled choline tracer (i.e., methyl-d9-choline chloride) for 6 wk (final 6 wk of 12 wk choline supplementation) or 10 wk, respectively. It was concluded that pregnancy increases the demand for choline, which was supported by enhanced use of choline for d9-PC and d3-PC production via the CDP choline and PEMT pathways, respectively. In nonpregnant and pregnant women, additional evidence to support the use of methyl-d9-choline methyl groups for transmethylation including increased blood enrichment of d3-SAM and d3-choline in response to increased dietary choline supplementation levels

(480 to 930 mg/d). Increasing dietary choline supplementation elevated blood enrichment of d9-betaine, suggesting enhanced choline oxidation, and d3-methionine. Yan and coworkers (2013) also observed enhanced use of PEMT-derived PC for fetal use during pregnancy. Davenport and coworkers (2015) were able to demonstrate improvements in breast milk choline supply in women that exceeded current dietary choline recommendations by increasing the production of PEMT-derived choline metabolites. However, increased choline intake also increased plasma and milk concentrations of TMAO as well as urinary TMAO yield (i.e., unlabeled and d9-TMAO).

Abomasal Choline Chloride Infusion and Methyl Group Utilization in Cows

Our objective was to evaluate the effects of abomasal choline chloride infusion on methyl group utilization in pregnant and lactating dairy cows using stable isotope methodology. Six multiparous, rumen-cannulated Holstein dairy cows (779 ± 72.0 kg of body weight) were enrolled in a longitudinal study design following transport from the Cornell Dairy Research Center (Harford, NY) to the Large Animal Research and Teaching Unit (Ithaca, NY). Cows were acclimated to the facility for 1 wk. Both pre- and postpartum diets were formulated using CNCPS v. 6.5 as implemented by AMTS.Cattle.Professional v. 4.14 (AMTS, LLC; Groton, NY). Diets were formulated to contain no supplemental rumen-protected choline chloride and deficient in methionine (< 0.96 g Met / Mcal metabolizable energy). Cows were milked twice daily at 0600 and 1700 h. Cows were fed once daily after morning milking.

Prepartum (wk -3 prior to expected due date) and postpartum (+2 wk relative to parturition) cows received a continuous abomasal infusion of unprotected choline chloride (18 g of choline chloride/d; 13.5 g of choline ion/d; Sigma-Aldrich, St. Louis, MO) for a 5-d infusion period. Methyl-d9-choline chloride (98% purity; Cambridge Isotope Laboratories, Inc., Andover, MA) replaced 20% of unlabeled choline chloride daily. Unlabeled and labeled choline chloride were dissolved in 4.1 L of water per day and infused at a rate of 170 mL/h using a pump. Cows were fed ad libitum during acclimation and between pre- and postpartum infusion periods. During the infusions, cows received their daily feed allotment as equal provisions every 4 h starting at 0 h, relative to the start of infusions.

Milk yields were recorded daily. Milk samples were collected at each milking during the last 2 days of the covariate and infusion periods. Blood samples were collected for plasma separation at 0600 h daily during each infusion period. Urinary catheters were used during the last 2 consecutive days of the infusion periods to collect total urine. Urine pH was measured at the end of each day and maintained at a pH ≤ 2 using hydrochloric acid. Liver tissue (~1 g) was biopsied using a custom fabricated trocar on the final hour of each infusion period. Milk, plasma, urine, and liver samples were frozen at -80°C until analysis.

Choline, betaine, dimethylglycine, methionine, glycerophosphorylcholine, phosphocholine, PC, LPC, and sphingomyelin were extracted from all biological

samples and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Koc et al. (2002) with modifications described by Yan et al. (2014). Trimethylamine *N*-oxide and trimethylamine were measured using the methodology described by Wang et al. (2011) with modifications by Yan et al. (2012). Enrichment percentages of isotopically labeled choline metabolites in plasma, liver, milk, and urine were calculated in accordance with Chew et al. (2011) and Yan et al. (2014).

We confirm that the abomasal infusion of choline chloride at 13.5 g/d with 20% enrichment with methyl-d9-choline chloride was adequate to achieve a steady state plasma concentration of d9-choline by d 5 of infusion. Bacterial degradation of choline to trimethylamine and TMAO was evident with plasma enrichments of d9-trimethylamine (2.78 and 4.01% during pregnancy and lactation, respectively; $P = 0.21$) and d9-TMAO (13.2 and 4.87% during pregnancy and lactation, respectively; $P < 0.05$). We observed evidence for choline oxidation to betaine by detecting plasma enrichment of d9-betaine (9.83 and 12.1% during pregnancy and lactation, respectively; $P < 0.05$) and d6-dimethylglycine (33.3 and 27.2% during pregnancy and lactation, respectively, $P = 0.46$). We detected evidence for choline utilization by the CDP choline pathway including enrichment of plasma d9-PC (4.85 and 5.71% during pregnancy and lactation, respectively), liver d9-phosphocholine (5.43 and 6.38% during pregnancy and lactation, respectively) and d9-PC (5.40 and 6.28% during pregnancy and lactation, respectively), and milk d9-phosphocholine and d9-PC (4.82 and 4.18% during lactation, respectively). We also provide direct evidence for the use of choline methyl donors for hepatic methylation of PE including enrichment of plasma d3-PC (0.22 and 0.16% during pregnancy and lactation, respectively; $P = 0.33$) and d3-choline (0.07 and 0.14% during pregnancy and lactation, respectively; $P = 0.08$), and liver d3-PC (0.40 and 0.41% during pregnancy and lactation, respectively; $P = 0.96$) and d3-choline (0.24 and 0.25% during pregnancy and lactation, respectively; $P = 0.87$), and milk d3-phosphocholine and d3-choline (0.15 and 0.15% during lactation, respectively). Evidence for endogenous recycling of choline via bile was also detected (e.g., enrichment of plasma d3-trimethylamine at 6.23 and 9.28% during pregnancy and lactation, respectively; $P = 0.19$).

The presentation will provide a complete evaluation of plasma, liver, milk, and urinary isotope enrichments of choline and choline metabolites. We will highlight liver enrichment ratios to consider enzyme activation or deactivation. We will also provide estimates for the extent of post-ruminal choline degradation to trimethylamine, the amount of abomasal-infused choline chloride used for milk choline and choline metabolite production, and the amount of choline and choline metabolites excreted in urine or secreted in milk in response to abomasal choline chloride infusion.

Conclusion

The use if stable isotope methodology has provided us definitive evidence that choline is a methyl donor in the pregnant and lactating dairy cow. In addition, we provide evidence for choline utilization by the CDP choline and PEMT pathways, and endogenous recycling of absorbed choline via bile. Our findings also provide insight into the degree of post-ruminal choline degradation to trimethylamine, which will emphasize the importance of correcting estimates of metabolizable choline when in vitro systems are applied. We also demonstrate that the use of methyl-d9-choline chloride and mass spectrometry are means to assess choline bioavailability in the cow.

Acknowledgement

We acknowledge Andres F. Ortega (Cornell University), Andrew Richards (Cornell University), Miranda Farricker (Cornell University), and the Cornell University farm crew for their support. We also acknowledge the Cornell Institute of Biotechnology Centers for Advanced Technology Grant and Balchem Corporation for funding this project.

References

Arshad, U., M. G. Zenobi, C. R. Staples, and J. E. P. Santos. 2020. Meta-analysis of the effects of supplemental rumen-protected choline during the transition period on performance and health of parous dairy cows. J. Dairy Sci. 103:282-300.

Chew, T. W., X. Jiang, J. Yan, W. Wang, A. L. Lusa, B. J. Carrier, A. A. West, O. V. Malysheva, J. T. Brenna, J. F. Gregory III, and M. A Caudill. 2011. Folate intake, MTHFR genotype, and sex modulate choline metabolism in mice. J. Nutr. 141:1475–1481.

Cui, Z. and D. E. Vance. 1996. Expression of phosphatidylethanolamine N-methyltransferase-2 is markedly enhanced in long term choline-deficient rats. J. Biol. Chem. 271:2839-2843.

Davenport, C., J. Yan. S. Taesuwan, K. Shields, A. A. West, X.Jiang, C. A. Perry, O. V. Malysheva, S. P. Stabler, R. H. Allen, and M. A. Caudill. 2015. Choline intakes exceeding recommendations during human lactation improve breast milk choline content by increasing PEMT pathway metabolites. J. Nutr. Biochem. 26:903-911.

DeLong, C. J., Y. J. Shen, M. J. Thomas, and Z. Cui. 1999. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. J. Biol. Chem. 274:29683-29688.

de Veth, M., V. Artegoitia, S. Campagna, H. Lapierre, F. Harte, and C. Girard. 2016. Choline absorption and evaluation of bioavailability markers when supplementing choline to lactating dairy cows. J. Dairy Sci. 99:9732-9744.

France, T. L., W. A. Myers, A. Javaid, I. R. Frost, and J. W. McFadden. 2022. Changes in plasma and milk choline metabolite concentrations in repsonse to the provision of various rumen-protected choline prototypes in lactating dairy cows. J. Dairy Sci. 105:9509-9522.

Humer, E., G. Bruggeman, and Q. Zebeli. 2019. A meta-analysis on the impact of the supplementation of rumen-protected choline on the metabolic health and performance of dairy cows. Animals 9:566.

Javaid, A., F. Wang, E. A. Horst, M. E. Diaz-Rubio, L. F. Wang, L. H. Baumgard, and J. W. McFadden. 2002. Effects of acute intravenous lipopolysaccharide administration on the plasma lipidome and metabolome in lactating Holstein cows experiencing hyperlipidemia. Metabolomics 18:75.

Kabarowski, J. H. 2009. G2A and LPC: Regulatory functions in immunity. Prostaglandins Other Lipid Mediat. 89:73-81.

Koc, H., M. H. Mar, A. Ranasinghe, J. A. Swenberg, and S. H. Zeisel. 2002. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. Anal. Chem. 74:4734– 4740.

McFadden, J. W., C. L. Girard, S. Tao, Z. Zhou, J. K. Bernard, M. Duplessis, and H. M. White. 2020. Symposium review: One-carbon metabolism and methyl donor nutrition in the dairy cow. J. Dairy Sci. 103:5668-5683.

Neill, A. R., D. W. Grime, and R. M. C. Dawson. 1978. Conversion of choline methyl groups through trimethylamine into methane in the rumen. Biochem. J. 170:529-535.

Sharma, B. K. and R. A. Erdman. 1989. In vitro degradation of choline from selected foodstuffs and choline supplements. J. Dairy Sci. 72:2772-2776.

Wang, Z., E. Klipfell, B. J. Bennett, R. Koeth, B. S. Levison, B. DuGar, A. E. Feldstein, E. B. Britt, X. Fu, Y.-M. Chung, Y. Wu, P. Schauer, J. D. Smith, H. Allayee, W. H. Wilson Tang, J. A. DiDonato, A. J. Lusis, and S. L. Hazen. 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472:57–63.

Yan, J., X. Jiang, A. A. West, C. A. Perry, O. V. Malysheva, S. Devapatla, E. Pressman, F. Vermeylen, S. P. Stabler, and R. H. Allen. 2012. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am. J Clin. Nutr. 95:1060-1071.

Yan, J., X. Jiang, A. A. West, C. A. Perry, O. V. Malysheva, J. T. Brenna, S. P. Stabler, R. H. Allen, J. F. Gregory III, and M. A. Caudill. 2013. Pregnancy alters choline dynamics: results of a randomized trial using stable isotope methodology in pregnant and nonpregnant women. Am. J. Clin. Nutr. 98:1459-1467.

Yan, J., S. D. Ginsberg, B. Powers, M. J. Alldred, A. Saltzman, B. J. Strupp, and M. A. Caudill. 2014. Maternal choline supplementation programs greater activity of the phosphatidylethanolamine N-methyltransferase (PEMT) pathway in adult Ts65Dn trisomic mice. FASEB J. 28:4312-4323.

Zhou, Y. F., Z. Zhou, F. Batistel, I. Martinez-Cortes, R. T. Pate, D. L. Luchini, and J. J. Loor. 2018. Methionine and choline supply alter transmethylation, transsulfuration, and cytidine 5’-diphosphocholine pathways to different extents in isolated primary liver cells from dairy cows. J. Dairy Sci. 101:11384-11395.