ABSTRACT

In late gestation and in the first weeks postpartum, lipid droplets accumulate in the hepatic tissue resulting in approximately 40 to 50% of the dairy cows developing hepatic lipidosis in the first weeks of lactation. Elevated concentrations of triacylglycerol in the hepatic tissue are associated with increased risk of peripartum diseases and impaired productive performance. Cows with hepatic lipidosis need to dispose the excess of hepatic triacylglycerol, but this is a slow process in the bovine liver and relies on primary mechanisms such as complete oxidation and ketogenesis because of the limited export of triacylglycerols as lipoproteins. Choline is a lipotropic compound because, among other functions, it facilitates the export of lipids from the liver. Supplementing choline as rumen-protected choline (RPC) to diets of feed-restricted dairy cows reduces the degree of triacylglycerol infiltration into the hepatic parenchyma in part by enhancing export of triacylglycerol as nascent lipoprotein. The reduced accumulation of triacylglycerol in hepatic tissue in feed-restricted cows fed RPC might affect secondary pathways involved in hepatic disposal of fatty acids such as increased cellular autophagy and lipophagy and minimize endoplasmic reticulum stress response and hepatocyte inflammation. Collectively, these effects on secondary pathways might further reduce the severity of hepatic lipidosis in cows. One of the benefits of supplementing RPC is improved fat digestibility, perhaps because choline, through phosphatidylcholines, facilitate lipid transport in the enterocyte by increasing the synthesis of chylomicrons. Finally, when supplemented during the transition period, RPC improves productive performance of cows, irrespective of their body condition, that extends well beyond the period of supplementation. This review summarizes the current understanding of hepatic lipidosis in early lactation, recapitulates the absorption, transport and metabolism of choline, and discusses its role on hepatic metabolism and gastrointestinal functions, which collectively results in improved performance in dairy cows.

Key words: choline, fatty liver, milk yield, transition cow

INTRODUCTION

Dairy cows experience negative nutrient balance that leads to extensive lipomobilization during the last week of gestation and the first 3 to 4 wk after calving to support lactation (Drackley, 1999). The resulting increased concentrations of fatty acids in blood increases the uptake of those lipids by hepatocytes, thus increasing re-esterification to triacylglycerol in hepatic tissue. Concentrations of hepatic triacylglycerol increase 3 to 6-fold from 21 d before to 21 d postpartum (Piepenbrink and Overton, 2003), and remains elevated at least for the first 3 wk of lactation in dairy cows (Zenobi et al., 2018b). Circulating fatty acids from mobilized fat depots are taken up by multiple tissues, including the liver where they are either completely oxidized in the mitochondria to generate ATP, undergo ketogenesis with export of water-soluble ketones to other tissues, used for synthesis of other lipid products, or re-esterified to triacylglycerols that can later be used as energy source or exported as lipoproteins (Emery et al., 1992; Bobe et al., 2004). Although mitochondrial oxidation and ketogenesis facilitate the disposal of fatty acids, the increased arterial supply and uptake of lipids by the liver (Reynolds et al., 2003) can lead to excessive re-esterification to triacylglycerol, which results in the development of lipidosis (Figure 1; Bobe et al., 2004). Extensive lipidosis leads to injury of hepatocytes resulting in release of proinflammatory cytokines (Li et al., 2015). Additionally, pro-inflammatory cytokines can further increase the accumulation of triacylglycerol in the bovine liver (Bradford et al., 2009).
The liver plays a central role in nutrient metabolism (Reynolds et al., 2003), and infiltration of triacylglycerols induces inflammation and impairs hepatocyte functions (Mashek et al., 2002; Li et al., 2015). Arshad and Santos (2022) showed that concentrations of triacylglycerol above 4 to 7% on wet-basis of the hepatic tissue were associated with reduced productive performance in dairy cows. Moreover, the concentration of hepatic triacylglycerol was linearly associated with increased morbidity in early lactation and reduced survival of cows. Therefore, management and dietary interventions are required to reduce the degree of hepatic lipidosis in dairy cows.

Generally, an essential nutrient is a substance that is required for normal cellular function but cannot be synthesized in an adequate amount in the body and must be obtained through diet. A required nutrient is a broader term that includes essential and non-essential nutrients, which are necessary for the body to function optimally. Choline, a quaternary (2-hydroxyethyl-trimethyl) amine, is acknowledged as an essential nutrient (Institute of Medicine, 1998) and dietary requirements have been established for several farm animal species (i.e., poultry, swine, and fish); nevertheless, the specific amounts of dietary choline required for dairy cows have not been established (NASEM, 2021). Choline is needed for the synthesis of phospholipids and assembly of lipoproteins such as very-low density lipoproteins (VLDL; Lombardi et al., 1968). The ruminant liver has inherently less capacity to secrete VLDL compared with other species (Emery et al., 1992), which limits the ability to export triacylglycerols and increases the risk of hepatic lipidosis in early lactation (Bobe et al., 2004). Jia et al. (2019) showed that cows affected by fatty...
liver have reduced transcript and protein abundance for apolipoprotein-B100 (APOB100), microsomal transfer triglyceride protein (MTTP), and apolipoprotein-E (APOE) in hepatic tissue, all required for the VLDL synthesis. Concentrations of numerous phosphatidyl- and lysophosphatidylcholines were lowest in the week of calving and then increased postpartum (Imhasly et al., 2015), and cows affected by fatty liver display smaller concentrations of phosphatidylcholine and sphingomyelins in plasma than those without fatty liver (Imhasly et al., 2014). Cows with inherently less capacity to synthesize phospholipids might be more susceptible to hepatic lipidosis. Conversely, choline is a component of phosphatidylcholine required for synthesis of lipoproteins and feeding choline as rumen-protected choline (RPC) reduces hepatic triacylglycerol content (Zenobi et al., 2018a; Arshad et al., 2023a) and benefits health (RPC) reduces hepatic triacylglycerol content (Zenobi et al., 2018a; Arshad et al., 2023a) and benefits health.

This review provides insights on the current understanding of hepatic metabolism, recapitulates the transport and metabolism of choline, and discusses the role of choline to improve performance in dairy cows.

**HISTORY, TRANSPORT, AND METABOLISM OF CHOLINE**

A compound resembling phosphorus matter was isolated from fish eggs, egg yolk, and brain matter and named lecithin, from the Greek word “lekythos” for egg yolk (Gobley, 1850). This compound was later identified in pig and cattle bile, and named choline (Strecker, 1862). Thereafter, Liebreich (1865) identified a molecule in the human brain, which was named neurine; however, it was accepted that choline and neurine were the same molecule, and the name choline was adopted. Serendipitously, the discovery of insulin by future Nobel laureate Frederick Banting and his student, Charles Best was later linked with the discovery of the lipotropic actions of choline in mammals. Depancreatized dogs were the animal model to study insulin, and Best observed that those dogs developed hepatic lipidosis. Hershey and Soskin (1931) described the effects of feeding raw pancreas to panreatectomized dogs to supply lecithin, which extended their lifespan by minimizing liver failure. Subsequently, Best and colleagues (Best and Huntsman, 1932; 1935) showed that feeding choline to dogs and rats inhibited the deposition of fat in the liver, thus demonstrating the lipotropic effects on the mammalian liver. These early findings opened the doors for extensive research that followed on the role of choline in mammalian metabolism and health.

Choline is absorbed primarily in the small intestine using a protein-mediated transport system (Michel et al., 2006). The extent of radio-labeled choline absorbed by enteroctyes is high, approximately 80%, in intestinal slices of adult rats (Sheard and Zaeisel, 1986), and absorption takes place in all segments of the small intestine (Budowski et al., 1977; Sheard and Zaeisel, 1986). Kinetic studies with radio-labeled choline showed the existence of intermediate-affinity Na⁺-independent and high-affinity Na⁺+-dependent transport systems for choline. The intermediate-affinity Na⁺-independent choline transport system includes choline transporter-like (CTL) proteins, mainly CTL1 and CTL2, which are present in the intestinal epithelial cells and involved in the uptake of choline through the plasma membrane of enterocytes (Michel et al., 2006). Once choline is absorbed in the enterocytes, it is either readily metabolized to its water-soluble or lipid-soluble metabolites; nevertheless, the free choline or its associated metabolites are transported across different tissues of the body. The high-affinity Na⁺+-dependent choline transport system includes choline transporter 1 and is mainly expressed in the liver, kidney, and spinal cord; however, the extent of activity of this transport system is minimal in enterocytes (Michel et al., 2006). In addition to these 2 major transport systems, another one is comprised of organic cation transporters (OCT) and also known as low-affinity transporters of choline. These transporters include OCT1, OCT2, and OCT3, which are predominantly expressed in the liver and kidney, with limited expression in the small intestine (Nishimura and Naito, 2005). Although there is a lack of knowledge of the exact mechanisms for the intestinal absorption and transport of choline in the bovine gastrointestinal tract, it is possible that the mechanisms to transport choline are conserved across species.

In mammalian tissues, free choline participates in 4 enzyme-catalyzed pathways: acetylation, oxidation, phosphorylation, and base exchange and these pathways are illustrated in Figure 2. In dairy cows, lipid-soluble forms of choline (phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) are predominantly present in blood circulation and milk than water soluble compounds (choline, betaine, acetylcholine, cytidine diphosphate (CDP)-choline, phosphocholine, and glycerophosphocholine) (de Veth et al., 2016). It has been shown that concentrations of lipid soluble forms of choline moieties or total choline moieties in blood were the lowest, primarily in the week after calving compared with mid or late lactation period (Artegoitia et al., 2014; Imhasly et al., 2015).

**DEVELOPMENT OF RUMEN-PROTECTED CHOLINE PRODUCTS**

Dietary choline is transformed to trimethylamine and methane in the rumen of sheep (Neill et al., 1978). Lim-
Choline is absorbed in the gastrointestinal tract of ruminants because of extensive ruminal degradation (Sharma and Erdman, 1988), and most choline appearing in the phospholipids pool of sheep originates from de novo synthesis (Dawson et al., 1981). Hence, choline must be fed in a rumen-protected form to supply the nutrient post-rumen for absorption (Sharma and Erdman, 1988). Choline chloride is typically encapsulated with a lipid coating, using hydrogenated fatty acids and triacylglycerols, which reduces microbial degradation of the molecule. Such RPC products allow for delivery of choline for absorption in the small intestine of ruminants (de Veth et al., 2016). Several RPC products have been developed with variable degree of rumen protection and intestinal availability of choline for absorption.

Intestinal disappearance of choline in monogastrics is high. Budowski et al. (1977) fed a diet containing 0.1%
choline chloride to 30-d-old broiler chicks and reported 80.6% apparent digestibility of choline. This estimate, based on choline disappearance from the intestine, may overestimate choline digestibility because there is a possibility of microbial choline within the intestine and conversion to trimethylamine. Sheard and Zeisel (1986) used adult rat intestinal slices cultured in vitro and quantified the intestinal uptake of radio-labeled choline. The authors showed that uptake of choline takes place in all segments of the small intestine, which has also been shown in chicks (Budowski et al., 1977), and approximately 80% of the radio-labeled choline was recovered in the intestinal slices.

Because choline is typically fed as choline chloride in a rumen-protected source to ruminants, the product has to minimize microbial degradation in the rumen at the same time that it releases choline in the small intestine for absorption. The protection against ruminal degradability of choline in different RPC products ranged from 60% (Brüsemeister and Südekum, 2006) to 85% by Deuchler et al. (1998) using in vitro incubation methods. Measurements of intestinal disappearance of choline from RPC products is cattle is limited. Vazquez (1999) used 6 steers fitted with duodenal and ileal cannulas to estimate duodenal flow and intestinal disappearance of choline from a RPC product. Animals were fed 4.5 g/d of choline from the RPC product and the small intestinal disappearance of choline reaching the duodenum was calculated at 41.6%. de Veth et al. (2016) enrolled mid-lactation multi-catheterized dairy cows and evaluated the bioavailability of a RPC product containing molecules in plasma, lymph, and milk. The authors showed that uptake of choline chloride into the abomasum as choline chloride. On the other hand, the net portal flux of free choline was 61% relative to the amount infused into the abomasum as choline chloride. On the other hand, the net portal flux of free choline for the RPC product evaluated was 13% of that abomasally delivered choline showing that, the relative bioavailability of choline from the RPC product studied, compared with abomasal infusion of choline chloride was 13% (de Veth et al., 2016). The net portal flux of choline in cows not supplemented with choline chloride was approximately 4.3 g/d and it increased to 5.5 when fed 25 g/d of choline ion as RPC or to 17.8 g/d when abomasally infused with 25 g/d of choline ion. It is possible that either some of the RPC was destroyed by the rumen microbes and never reached the small intestine, or that the choline in the RPC product was overprotected and, although it reached the intestine, it was not available for absorption. Recent work by France et al. (2021) showed limited if any increase in plasma choline with different amounts of choline feeding from different RPC products, although trimethyl amine increased in milk thus suggesting microbial metabolism of choline either in the rumen or in the intestines of cows. Nevertheless, changes in choline or phosphatidylcholine concentrations in plasma and lymph have been observed in dairy cows fed RPC (Zenobi et al., 2018a; Arshad et al., 2023c; Arshad et al., 2023d). Furthermore, experiments with duodenal infusion of choline showed that the content of choline in milk is responsive to the post-ruminal supply of choline (Deuchler et al., 1998), and feeding RPC products to dairy cows increased the concentration of choline in milk (Elek et al., 2008; de Veth et al., 2016). The scarce data on intestinal digestibility and bioavailability of choline from RPC products fed to bovine indicate that a small fraction of choline is the RPC product is digested and absorbed in the intestinal tract; however, it can change the concentrations of choline containing molecules in plasma, lymph, and milk of dairy cows.

**ACTIONS OF CHOLINE: PRIMARY MECHANISMS**

Choline is a precursor for the synthesis of phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins which are essential constituents of cell membranes, with phosphatidylcholines being the main phospholipids in mammalian cells (Vance, 2008). Figure 3 illustrates the possible mechanistic effects of choline in hepatic and extrahepatic tissues.

**Trafficking and Oxidation of Fatty Acids**

Supplementing choline might alter the trafficking of fatty acids by changing the fluidity of cell membranes, the latter carry solute-carrier proteins or trans-membrane transporters required for uptake of fatty acids into the hepatic tissue (Figure 3A). Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are the predominant phospholipids in cellular membranes, and a reduced ratio of phosphatidylcholine to phosphatidylethanolamine leads to disruption in membrane integrity and leakage of cellular enzymes (Costa et al., 2004). During periods of negative nutrient balance in dairy cows, the uptake of fatty acids by hepatic tissue increases (Reynolds et al., 2003), which enhances mitochondrial β-oxidation and ketogenesis. Increased supply of choline might improve the integrity of outer and inner mitochondrial membranes and avoid leakage of reactive oxygen species typically generated during oxidative reactions in the mammalian cell (Chandler and White, 2017; Figure 3B).

Cooke et al. (2007) subjected dry, late pregnant Holstein cows to a 10-d feed restriction such that cows consumed 30% of the NE₃ required concurrent with supplementation of either 0 or 12.9 g/d of choline ion as RPC. Cows fed RPC had reduced hepatic triacyl-
glycerol content, coinciding with reduced concentrations of fatty acids in plasma. In a second experiment, supplementing RPC after induction of fatty liver accelerated the rate of disposal of hepatic triacylglycerols (Cook et al., 2007). The data from Cooke et al. (2007) demonstrated that feeding RPC prevented and alleviated the risk of fatty liver in feed-restricted dry dairy cows. Additionally, the data from Cooke et al. (2007) implicated reduced lipolysis as a potential mechanism by which RPC attenuates the degree of hepatic lipodosis in cows. Nonetheless, in follow-up experiments with 187 dairy cows (Zenobi et al., 2018a; Arshad et al., 2023a), it was reported that feeding RPC does not affect concentrations of fatty acids in plasma in cows under negative nutrient balance. Cows fed RPC had reduced hepatic triacylglycerol independent of changes in plasma fatty acids or BHB (Zenobi et al., 2018a; Arshad et al., 2023a). Indeed, Goselink et al. (2013) showed that supplementing 12.9 g/d of choline ion as RPC to transition cows did not affect the abundance of transcripts involved in adipose tissue lipolysis. When cows are in negative nutrient balance, feeding RPC does not seem to affect adipose tissue mobilization or hepatic ketogenesis, thus suggesting that the reduction in hepatic triacylglycerol from supplementing choline might be mediated by mechanisms other than reduced lipolysis.

Figure 3. Schematic diagram of the possible effects of choline on hepatic and extrahepatic tissues. During periods of extensive lipomobilization, the hepatic uptake of fatty acids (FA) increases, and choline is involved in the synthesis of phosphatidylcholine that might alter the plasma membrane configuration and affect the uptake of FA in hepatic tissue (A). Feeding choline increases the supply of FA to mitochondria via increased synthesis of carnitine that facilitates the transport of activated FA across the mitochondrial membranes. Choline might affect the rate of supply of very-long chain fatty acids (VLCFA) to peroxisomes as acyl-CoA or acyl-CoA ester, which can increase peroxisomal oxidation of VLCFA. Also, the supply of choline increases the membrane stability of mitochondria and decreases the leakage of reactive oxygen species (ROS), coinciding with the greater synthesis of antioxidants (B). Feeding choline increases the synthesis and assembly of very-low density lipoprotein (VLDL) particles and increases the export of triacylglycerol (TAG) from hepatic tissue (C). Feeding choline might increase the synthesis of autophagosomes that carry-out the degradation of lipid droplets (LD) in autophagolysosomes. Choline feeding might provide substrates for the synthesis of organelles involved in protein folding with increased membrane integrity and increase their efficiency for the synthesis of proteins (D). Supplementing choline attenuates the inflammatory response either via the anti-inflammatory effects of phosphatidylcholine or reduced synthesis of proinflammatory cytokines thus attenuating the acute phase response [synthesis of haptoglobin (HP) or serum amyloid A (SAA)]; such effect might reduce the damage to hepatocytes (E). Feeding choline increases glycogenogenesis or reduces glycogenolysis to maintain the glycogen content in hepatic tissue (F). Feeding choline might increase the repair of enterocytes and facilitate the synthesis of chylomicrons for increased lipids absorption (G). Supplementing choline might increase the proliferation of mammary epithelial cells (MEC’s) that bear carry-over effects on the synthesis of milk yield throughout the lactation period of cows (H).
**Export of Hepatic Triacylglycerol**

Choline is a component of phosphatidylcholines and enhances the export of triacylglycerol as a component of hepatic VLDL particles in nonruminants (Lombardi et al., 1968), in hepatocytes from calves in vitro (Chandler and White, 2017), and in dairy cows in vivo (Arshad et al., 2023c). Supplementing choline to cows increases the plasma pool of lipid-soluble choline biomolecules (Zenobi et al., 2018a). Therefore, it is plausible to suggest that feeding RPC might facilitate the synthesis and assembly of VLDL particles and export of hepatic triacylglycerols as a component of VLDL particles is one of several potential mechanisms to decrease or prevent hepatic lipidosis as shown in feed-restricted late gestation dry dairy cows (Cooke et al., 2007; Arshad et al., 2023c). Indeed, Zenobi et al. (2018a) showed a linear decrease in hepatic triacylglycerol as the intake of RPC increased, concurrent with increased concentrations of phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins in plasma of cows.

During synthesis and assembly of VLDL particles in the Golgi cisternae, triacylglycerol is packaged in the core of lipoproteins which is encapsulated with the polar heads of phospholipids and structural proteins such as APOB100 and APOE. Both the CDP-choline and the phosphatidylethanolamine-N-methyltransferase (PEMT) pathways of phosphatidylcholine synthesis are required for VLDL formation and secretion (Vance, 2008). The core structural protein APOB100 is required for the synthesis of VLDL in bovine and hepatocytes from PEMT-deficient mice secrete approximately 70% less triacylglycerol and APOB100 (Noga et al., 2002). Transition cows fed RPC had reduced hepatic triacylglycerol content in early postpartum (Goselink et al., 2013), coinciding with an increase in the transcript abundance of APOB100 and MTTP in the hepatic tissue (Goselink et al., 2013). The increased expression of APOB100 and MTTP transcripts favors the assembly and secretion of VLDL to enhance hepatic disposal of triacylglycerol. Arshad et al. (2023b) corroborates the findings of Goselink et al. (2013) and showed that hepatic transcript abundance of APOB100 and MTTP increased in cows induced to develop hepatic lipidosis when fed RPC. Indeed, cows fed RPC had increased rate of secretion of hepatic triacylglycerol compared with cows fed no RPC (Arshad et al., 2023c). The increased secretion of hepatic triacylglycerol in cows fed RPC suggest increased hepatic secretion of nascent VLDL, thus corroborating the mechanisms already established in monogastric and supporting the concept that choline attenuates hepatic lipidosis in dairy cows with underlying mechanisms similar to those observed in other species (Figure 3C).

It is noteworthy to mention that most of experiments in which RPC was supplemented to transition cows did not show a reduction in hepatic triacylglycerol in the early postpartum period (Arshad et al., 2020), which differs from what is typically observed when feed-restricted late gestation cows receive supplemental RPC. The feed-restriction model used in several experiments (Cooke et al., 2007; Zenobi et al., 2018a; Arshad et al., 2023a; Arshad et al., 2023c) implemented a short-term abrupt reduction in DMI. Although the model resulted in a negative NE_L balance that resembles what is observed in early lactating cows, the abrupt change compared with a more gradual reduction in energy balance that typically happens with the onset of lactation might have implications to the lipotropic effects of choline in the liver. Also, the onset of lactation increases the needs for choline as milk of cows not supplemented with RPC contains approximately 100 to 120 mg of choline biomolecules per kg of milk (Artegoitia et al., 2014; de Veth et al., 2016). Perhaps, the increased needs for choline with the onset of lactation limits the ability of the supplemental choline from RPC to exert lipotropic effects in the liver. Perhaps, larger doses of choline might be needed in early lactation to understand if it can reduce hepatic triacylglycerol accumulation as typically observed in late gestation feed-restricted dry cows. Out of 9 experiments in which RPC was supplemented during the transition period and hepatic tissue collected in early lactation, only 2 showed a reduction in triacylglycerol content in the liver (Elek et al., 2013; Goselink et al., 2013). Additionally, in many of the experiments with supplemental choline as RPC to transition cows, yields of milk and milk components increased (Arshad et al., 2020), although a concurrent increase in DMI was not always observed during the postpartum period (Zenobi et al., 2018b; Bollatti et al., 2020a). It is possible that the lack of increase in DMI might limit the lipotropic effects of choline, thus not resulting in reduced hepatic triacylglycerol content. Thus, improvements in postpartum performance observed in cows supplemented with RPC occur, in many cases, irrespective of reduction in hepatic triacylglycerol content.

**ACTIONS OF CHOLINE: SECONDARY MECHANISMS**

Animal models employed to study non-alcoholic fatty liver disease (NAFLD) progression are based on the “multiple-hit” theory of its pathogenesis. In NAFLD, the initial trigger, known as the “first-hit,” is often associated with insulin resistance, obesity, and metabolic syndrome, which often ends up causing hepatic lipidosis (Petagine et al., 2023). Disease progression occurs when secondary mechanisms, referred to as “multi-hits,” come...
into play. These secondary mechanisms encompass impaired autophagy or lipophagy, dysregulated unfolded protein response, and inflammation, collectively contributing to liver damage (Mashek et al., 2015; Schulze et al., 2017; Petagine et al., 2023). In this review, we present preliminary evidence regarding the possible effects of choline on the regulation of these secondary mechanisms, which might play a role in reducing fatty liver in dairy cows.

**Autophagy, Lipophagy, Lipid Droplet Dynamics, and Endoplasmic Reticulum Stress Response**

Autophagy is a cellular process that involves the degradation and recycling of damaged or dysfunctional cellular components, and it plays a crucial role in maintaining cellular homeostasis. Autophagy has gained attention in the prevention or alleviation of NAFLD. Recent evidence suggests that fatty liver is associated with reduced autophagy in hepatic tissue in dairy cows. Du et al. (2018) showed that cows with severe fatty liver exhibited less abundance of autophagosome formation-associated transcripts compared with cows without fatty liver. The latter findings suggest that excessive hepatic lipid infiltration impairs autophagic activity that may exacerbate cellular damage and inflammation. Another interpretation is that reduced autophagy might be a mechanism for increased triacylglycerol accumulation into the hepatic parenchyma. Conversely, cows experiencing mild (1 to 5% triacylglycerol on wet-basis) to moderate (>5 to 10% triacylglycerol on wet-basis) degree fatty liver might not have impaired autophagy in hepatic tissue. Chen et al. (2020) showed that cows with mild to moderate degree of fatty liver had increased expression of transcripts related to autophagy compared with cows having no fatty liver. The findings of Du et al. (2018) and Chen et al. (2020) suggest that hepatocytes might adapt secondary pathways to degrade lipid droplets, especially in times of intensive lipomobilization and hepatic deposition of triacylglycerol; however, severe hepatic lipidosis, which affects 10 to 15% of the dairy cows (Bobe et al., 2004), might compromise the cellular machinery required for autophagy (Du et al., 2018). Andrejeva et al. (2020) showed that synthesis of phosphatidylcholine is required for the formation of autophagosomes using a human cell culture model. It has been shown that feeding RPC increases plasma concentrations of phosphatidylcholines and sphingolipids in feed-restricted dairy cows (Zenobi et al., 2018a). More recently, Arshad et al. (2023c) showed that feeding RPC to feed-restricted cows increased the transcript abundance of autophagy related gene 3 and reduced that of perilipin 2 in hepatic tissue coinciding with reduced hepatic concentrations of triacylglycerol compared with cows not supplemented with RPC. Therefore, authors proposed a model by which choline enhances the synthesis of autophagosomes, improves autophagy, and mobilizes lipid droplets for autophagolysosomal degradation in dairy cows (Arshad et al., 2023c).

Endoplasmic reticulum stress is an imbalance between the protein folding capacity of the ER and the protein load, resulting in the accumulation of unfolded or misfolded proteins in the ER lumen. An increase in damage to the unfolded protein response induces activation of apoptosis and cell death pathways, which are associated with the development of NAFLD in mice (Lee et al., 2012). Gessner et al. (2014) collected hepatic tissue from Holstein cows and showed increased abundance of transcripts involved in ER stress response from pre- to postpartum, concurrent with the elevation in hepatic triacylglycerol. The findings of Gessner et al. (2014) suggest that cellular ER stress-induced unfolded protein response in hepatic tissue with the onset of lactation might compromise the ability for proper folding of proteins such as APOB100 or APOE required for the synthesis of VLDL. Zhu et al. (2019) showed that abundance of transcripts and proteins involved in the ER stress response such as PERK, GRP78, IRE1α, ATF4, and ATF6 increased with the severity of fatty liver in dairy cows in the first week postpartum. Arshad et al. (2023a) showed that feeding choline ion as RPC reduced the transcript abundance of ERN1, a key sensor for the ER unfolded protein response. Also, increasing the intake of choline ion increased the expression of APOB100, suggesting that RPC might decrease tissue damage and improve proper folding of proteins in the ER compartments of cells to assemble VLDL particles, which should support hepatic triacylglycerol export (Arshad et al., 2023a; Arshad et al., 2023c). Identification of interventions that can alter the fate of cellular autophagy, lipophagy, or ER cellular stress response to promote removal of cellular lipids might prove beneficial to reduce the degree and accelerate the recovery from hepatic lipidosis in cows (Figure 3D). In fact, it has been shown that choline and methionine deficiency impaired autophagy and induced dysregulated unfolded protein response in the liver of mice, and such cellular alterations are associated with the development of NAFLD (Rinella et al., 2011). It is reasonable to suggest that supplementing choline might enhance the synthesis and proliferation of autophagosomes and reduce the ER stress response that helps explain the physiological pathways associated with the reduced risk of fatty liver in dairy cows. It is noteworthy to mention that the majority of data linking secondary mechanisms to hepatic lipidosis in dairy cows is of an associative nature, and the cause-and-effect relationships of these mechanisms have not been determined.
Inflammation and Hepatic Lipidosis

Lipid-soluble choline-derived biomolecules such as phosphatidylcholine or sphingomyelin act as second messengers by interacting with diacylglycerols, arachidonic acid, ceramides, and phosphatidic acid (Zeisel and Costa, 2009), and these signaling molecules can contribute to the progression of inflammation, arrest in cell cycle, and apoptosis. When LPS interacts with clusters of differentiation 14, the signal is transferred through the transmembrane toll-like receptor 4 leading to synthesis of cytokines including tumor necrosis factor α (TNFA) or interleukins. These cytokines bind to their respective receptors on hepatocytes and turn on transcripts for haptoglobin, serum amyloid A (SAA), C-reactive protein, and fibrinogen, which are released to sequester nutrients needed by pathogens, sequester bacteria, or reduce the inflammatory response. Bradford et al. (2009) showed that TNFA induces hepatic triacylglycerol accumulation in cows independent of the effects on DMI (Figure 3E). Supplementation of RPC has shown some beneficial effects to attenuate inflammation (Arshad et al., 2023a), and increasing intake of RPC linearly reduced the concentrations of haptoglobin in plasma of cows induced to develop fatty liver (Zenobi et al., 2018a). Arshad et al. (2023a) corroborated the findings of Zenobi et al. (2018a) and showed that feeding RPC reduced the concentrations of haptoglobin in serum in feed-restricted dairy cows, presumably because of the lipotropic effects of choline or the potential anti-inflammatory effects of phosphatidylcholine. The benefits of choline in reducing concentrations of triacylglycerol or in decreasing an acute phase response in feed-restricted dry cows indicated that hepatocytes might experience less damage and increase their metabolic efficiency (Mashek et al., 2002).

Hepatic Gluconeogenesis and Glycogenesis

Supplementation of choline increases the concentrations of glycogen in hepatic tissue of lactating or dry dairy cows. Piepenbrink and Overton (2003) reported a linear increase in hepatic glycogen content in postpartum dairy cows as intake of choline ion increased from 0 to 16.1 g/d. Zenobi et al. (2018a) and Arshad et al. (2023a) reported that feeding RPC increased the concentrations of hepatic glycogen in feed-restricted cows. Hepatic lipids can compromise hepatic gluconeogenesis (Mashek et al., 2002), which contributes carbon needed for glycogen synthesis. Choline is a lipotropic agent that reduces hepatic triacylglycerol accumulation, which might favor other cellular functions such as gluconeogenesis and glycogenesis. Arshad et al. (2023c) proposed a model for increased concentration of hepatic glycogen linked to either reduced glycolysis or increased glycogenesis, thus maintaining or more quickly replenishing hepatic glycogen when cows are supplemented with choline (Figure 3F). Baquet et al. (1990) showed that increasing weight of hepatocytes was positively associated with the synthesis of glycogen, and choline induces cell swelling by hypo-osmolarity of hepatocytes, coinciding with an increased synthesis of glycogen. Choline can be phosphorylated to synthesize phosphatidylcholine, and the latter can affect the fluidity of plasma membranes. Also, oxidation of choline to betaine, or hydrolysis of phosphatidylcholine to glycero-phosphocholine, which act as an osmoregulatory compounds, might increase the swelling of hepatocytes to stimulate glycogen synthesis (Baquet et al., 1990). Chandler and White (2019) cultured bovine hepatocytes in vitro in the presence of increasing concentrations of choline chloride and proposed a direct connection between methyl groups originating from choline, which, after undergoing oxidation to form betaine, play a significant role in the metabolic processes of glycine and serine. The authors suggested that methyl groups derived from choline, after oxidation to betaine, might increase the synthesis of glucogenic AA to be used for gluconeogenesis (Chandler and White, 2019); nevertheless this hypothesis needs to be validated.

ROLE OF CHOLINE ON GASTROINTESTINAL FUNCTIONS

Takahashi et al. (1982a) administered corn oil by gavage to rats fed either a choline-deficient or choline-adequate diet. Rats fed a choline-deficient diet had accumulated lipid droplets within the enterocytes and reduced chylomicrons in intestinal lymph compared with rats fed a choline-adequate diet (Takahashi et al. 1982a). The impairment in fat absorption was reversed when rats received phosphatidylcholine by gavage. The accumulation of lipid droplets in the enterocytes might be related to the impaired intracellular trafficking of lipids and secretion of chylomicrons. A diet deficient in choline not only disrupted the packaging of triacylglycerols in chylomicrons (Takahashi et al., 1982a), but also decreased the activity of calcium-ATPase in the enterocytes, which is required for intracellular transport and discharge of chylomicrons into lymph (Takahashi et al., 1982b). In another experiment, lactating rats fed a choline-deficient diet had reduced jejunal villus length, accumulated greater concentrations of triacylglycerol, and reduced concentrations of phosphatidylcholine and lysophosphatidylcholine in the intestinal tissue compared with rats fed a diet supplemented with choline (Silva et al., 2015). It is clear that choline and its lipid-soluble metabolites play important roles in gastrointes-
tinal functions, and their deficiency impairs intestinal lipid metabolism. Thus, it is plausible to suggest that adequate choline intake should facilitate the transport of lipids and fat-soluble vitamins from the small intestine to mesenteric lymphatic vessels in dairy cows. Phospholipids are essentially required to maintain the intact barrier of the gastrointestinal tract (Takahashi et al. 1982a; Silva et al., 2015). Imposing feed restriction to as low as 20% of the ad libitum intake for 5 d increased the blood concentrations of haptoglobin, SAA, and LPS-binding protein in dairy cows (Kvidera et al., 2017). Feed restriction induced alteration in the intestinal architecture that presumably increased intestinal permeability (Kvidera et al., 2017). Dairy cows have reduced DMI in the last days of gestation, concurrent with the smallest concentrations of numerous phosphatidylcholines and lysophosphatidylcholines in plasma around calving (Artegoitia et al., 2014; Imhasly et al., 2015). Therefore, it is possible that the integrity of the small intestine might be compromised in dairy cows during the transition period. We hypothesize that dietary supplementation of choline might promote enterocyte regeneration, enhance intestinal cell integrity, and stimulate chylomicron synthesis in dairy cows. These potential effects of choline could contribute to increased nutrient absorption in the digestive system of dairy cows. Zenobi et al. (2018a) suggested that choline might increase the integrity of intestinal cells or enhance the synthesis of chylomicrons during feed restriction. Cows fed RPC had increased concentrations of triacylglycerol in plasma after fed a dose of long-chain fatty acids (Zenobi et al., 2018a). Corroborating those findings, Arshad et al. (2023d) showed that supplementing RPC to feed-restricted cows increased digestibility of fat by 5.6-percentage units (control = 80.4 vs. RPC = 86.0%), serum concentrations of triacylglycerol, and lymphatic concentrations of choline and triacylglycerol metabolites. Choline is substrate for synthesis of lysophosphatidylcholine, which acts as an emulsifying agent in the intestine. In lactating cows, abomasal infusions of synthetic emulsifiers increased fatty acid digestion and tended to increase FCM yield (Prom et al., 2022). Thus, there is some indication that supplementation of choline enhances absorption of dietary fatty acids, although the exact mechanisms remain to be elucidated and might involve increased emulsification of fatty acids, improved enterocyte integrity or synthesis and secretion of chylomicrons (Figure 3G).

**ROLE OF CHOLINE ON PRODUCTIVE PERFORMANCE AND HEALTH**

Dairy cows experience health disorders, primarily within the first 3 wk of lactation, and disease results in inflammation that promotes accumulation of triacylglycerol in liver (Bradford et al., 2009). It is widely believed that the success of the transition period influences the remainder of the lactation. Consequently, nutritional interventions that limit inflammation, improve metabolism, or minimize the risk of disease are expected to benefit productive performance (Drackley, 1999). Supplementing choline to dairy cows as RPC during the transition period improves production performance and health. Arshad et al. (2020) conducted a meta-analysis and showed that feeding choline ion up to 25.2 g/d as RPC during the transition period increased yield of ECM by 2.18 kg/d and tended to reduce the risk of retained placenta and mastitis compared with non-supplemented cows. The meta-analysis showed no effect of feeding RPC during the transition period on postpartum concentrations of hepatic triacylglycerol (Arshad et al., 2020). Zenobi et al. (2018b) and Bollatti et al. (2020a) fed RPC from 3 wk before to 21 d after calving and observed that cows fed RPC had increased yield of ECM without a concurrent increase in DMI or decrease in concentrations of hepatic triacylglycerol postpartum. The improvements in efficiency of nutrient use in dairy cows supplemented with RPC with increased ECM per DMI might have masked the lipotropic effects of choline in early lactating cows as observed consistently in dry prepartum cows (Arshad et al., 2020).

An important and consistent finding with supplementing RPC to transition cows is that the increments in milk yield extend beyond the period of supplementation (Zenobi et al., 2018b; Bollatti et al., 2020a; Swartz et al., 2023). Zenobi et al. (2018b) and Bollatti et al. (2020a) showed that cows fed RPC during the transition period produced 2.1 and 2.0 kg/d more milk up to 40 and 25 weeks in lactation, respectively. Recently, Swartz et al. (2023) corroborated those findings and showed that cows supplemented with RPC until 21 DIM produced an additional 3.3 kg/d after supplementation ceased, from 22 to 84 DIM. Such carry-over effects of RPC might be related to potential benefits to health, intermediary metabolism, enhanced nutrient utilization, or proliferation of mammary epithelial cells (Figure 3H). Choline kinase, one of the rate-limiting enzymes in phosphatidylcholine synthesis, is required for mammary cell proliferation (Ramírez de Molina et al., 2004). It is possible that supplying additional choline during late gestation and early lactation, when concentrations of many choline-derived molecules are lowest (Artegoitia et al., 2014; Imhasly et al., 2015), might influence proliferation of mammary epithelial cells and influence subsequent milk yield; however, this hypothesis needs to be investigated in dairy cows.
Overconditioned cows are known to have increased risk of hepatic lipodosis (Bobe et al., 2004) and data from Zahra et al. (2006) suggested that response to RPC might be affected by the BCS of cows. To address this question, Bollatti et al. (2020b) studied 215 cows assigned to receive either 0 or 12.9 g/d of choline ion as RPC from 21 d pre- to 21 d postpartum. The authors showed that feeding RPC increased yield of ECM by 1.9 kg/d and improved the feed efficiency irrespective of body condition of cows when they entered the prepartum period. Their data supports the concept that responses to RPC are not dependent on the body condition score of cows prepartum.

CONCLUSIONS

Dairy cows during periods of extensive lipomobilization have increased accumulation of triacylglycerol into the hepatic tissue, which is associated with impaired lactational performance and increased risk of morbidity. Supplementing choline ion up to 25.8 g/d as RPC reduces the concentrations of hepatic triacylglycerol in feed-restricted late gestation cows; however, this effect has not been shown in postpartum cows. The reductions in hepatic triacylglycerol are mediated partly by an increase in hepatic secretion of triacylglycerol-rich lipoprotein, whereas increments in hepatic glycogen content prepartum or postpartum result either from increased glycosynthesis or reduced glycogenolysis. Supplementing RPC seems to influence secondary mechanisms involved in handling fatty acids by the liver such as increased autophagy and lipophagy concurrent with reduced ER stress response and inflammation, which altogether help hepatocytes to dispose of triacylglycerol. Feeding RPC increases the concentrations of phosphatidylcholine in plasma that might reduce inflammation, support the normal architecture of the intestine, and increase synthesis of chylomicrons that facilitates fat transport.

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