New Insights on the Role of Essential Fatty Acids on Reproduction in Dairy Cattle

Eduardo Ribeiro[1], José Sprícigo, Elvis Ticiani, Bruna Mion, and Murilo Carvalho
Department of Animal Biosciences, University of Guelph

Introduction

For the past few decades, dairy researchers and the allied industry allocated major efforts to develop strategies to improve estrous detection and insemination rate in lactating cows. As a consequence, reproductive performance of dairy herds, measured by 21-d pregnancy rate, has improved in recent years (Ribeiro, 2018). Even though there is still room for further improvement in insemination rate in the average herd, the next major challenge in reproductive management of lactating cows is to find ways to consistently reduce embryo and fetal losses, and consistently increase pregnancy and calving per AI. Approximately 60% of fertilized eggs in lactating cows fail to develop to term (Ribeiro, 2018). Accordingly, pregnancy per AI remains low and stagnant for many years, and the economic burden of abortions and culling related to reproductive failures continues to lessen production sustainability of dairy herds (Ribeiro, 2018).

Pregnancy failures are ultimately caused by impaired developmental competence of the embryo or by inadequate uterine environment, which in turn are influenced by the genetics of the cow and embryo, and by environmental factors affecting ovarian and uterine biology of the cow (Ribeiro et al., 2018). Therefore, solutions for pregnancy failures will likely come from the discovery of genetic markers specifically associated with pregnancy survival and from management strategies that improve the quality of gametes and uterine environment either directly or indirectly through reduction risk factors. Advancements in nutrition management of cows during the transition and breeding periods will likely be important for achieving these goals.

Although responses are highly variable, fat supplementation improves milk production and reproduction in dairy cows (Rabiee et al. 2012; Rodney et al., 2015), which suggest that most cows are actually underfed lipids. Limited feeding of lipids is especially critical for the supply of essential fatty acids, which cannot be synthesized by bovine cells and must be supplied by the diet. In fact, the observed benefits of lipid supplementation in reproduction are often attributed to non-caloric effects of essential fatty acids (Santos et al., 2008). Nonetheless, the mechanisms by which fatty acids affect reproduction are still not completely understood. A better understanding of their role(s) in regulation of reproduction will be an important step towards development of nutraceutical strategies that consistently improve fertility in lactating cows. This review will focus on recent discoveries related to the importance of essential fatty acids to dairy cow physiology and reproduction, and their relevance to nutritional management of dairy cows.

[1] Contact at: Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada; E-mail: eribeiro@uoguelph.ca.

Elongation of the Preimplantation Conceptus

A significant portion of pregnancy losses in dairy cows occurs during early stages of conceptus development, and include the phase of elongation. Elongation of the preimplantation conceptus is a prerequisite for maternal recognition, implantation and survival of pregnancy. It entails remarkable expansion of extraembryonic tissues along the uterine lumen in a short window of development. In cattle, elongation starts around Day 14 and, within 3 days, the conceptus grows from < 5 mm to approximately 250 mm and occupies almost the entire extension of the uterine horn (Ribeiro et al., 2018).

The exponential increase in tissue mass is explained mainly by rapid proliferation of trophectoderm cells (cells of the developing placenta), which is induced by driver signals and demands substantial supply of nutrients (e.g. lipids, amino acids, sugar, nucleotides) for energy expenditures and synthesis of biomass. The required signals and nutrients are provided by the uterine histotroph (also known as uterine milk), whose secretion from endometrium and its composition are modulated by the activity of ovarian steroids and conceptus-derived molecules in endometrial cells (Ribeiro et al., 2018), and are influenced by the physiological stage and nutrition of the cow (Ribeiro, 2018).

Role of Polyunsaturated Fatty Acids on Conceptus Elongation

The specific components of the uterine histotroph that drive conceptus elongation are not completely known. Nonetheless, the nature of these components were recently investigated by two independent studies (Barnwell et al., 2016; Ribeiro et al., 2016b) that evaluated the biology of conceptus cells at the onset of elongation, and how they sense and respond to the histotroph stimuli. Both studies revealed substantial and consistent changes in transcriptome of trophectoderm cells with the onset of elongation, which suggest the nature of the elongation drivers of the uterine histotroph.

Lipid metabolism was one of the top molecular and cellular functions associated with the observed changes in gene expression in both studies. Among genes that had increased expression during elongation, some were involved with lipid uptake, lipid droplet formation, biogenesis of peroxisomes, activation, oxidation, desaturation and elongation of fatty acids, biosynthesis of phospholipids, mobilization of membrane phospholipids, biosynthesis of prostaglandins, and transport of prostaglandins and other lipids metabolites. In addition, peroxisome proliferator activated receptor gamma (**PPARG**) not only had transcript expression markedly increased during the onset of elongation but was also listed as an important upstream regulator of the transcriptome changes observed during elongation. In fact, PPARG is a nuclear receptor that functions as ligand-dependent transcription factor. Moreover, its transcript expression was highly correlated with the expression of other genes involved with lipid metabolism.

The PPARG have large binding pockets that interact promiscuously with multiple polyunsaturated fatty acids and their metabolites (Kliewer et al., 1997; Nagy et al., 1998). Itoh and coauthors (2008) examined crystal structures of PPARG bound to lipid ligands and concluded that the large binding pocket of the receptor confers remarkable versatility in ligand binding and could therefore act a cellular sensor of the varying composition of the cellular pool of fatty acid ligands. Moreover, multiple omega-3 and omega-6 fatty acids were able to bind and activate PPARG.

Binding of fatty acids into the ligand pocket of PPARG causes conformational changes in the receptor that facilitates the formation of heterodimers with retinoid X receptor (**RXR**). The dimer of nuclear receptors then binds to PPAR response elements (**PPRE**) in regulatory regions of target genes to regulate gene expression. Putative PPRE were identified in regulatory regions of several genes transcriptionally regulated during the onset of conceptus elongation, which suggests a direct effect of PPARG on the abundance of the respective transcripts (Ribeiro et al., 2016b).

The hypothesis that essential fatty acids and their nuclear receptor PPARG are important for elongation of the bovine conceptus is strongly supported by an elegant study performed in sheep. Brooks et al. (2015) performed a loss-of-function study by infusing morpholino antisense oligonucleotides in the uterus of pregnant ewes from Day 7 to Day 14 after breeding using osmotic pumps. In sheep, conceptus elongation starts around day 11. Morpholino antisense oligonucleotides for PPARG resulted in the recovery of growth-retarded conceptuses, while infusion of the designed control or PPAR-delta morpholinos resulted in normally elongated conceptus on Day 14.

Dynamics of Fatty Acids and Oxylipids in the Uterus

Ovarian steroid hormones influence lipid metabolism in endometrial cells. The amount of lipid droplets in the epithelium fluctuates according to the phase of estrous cycle in cows (Wordinger et al. 1977) and ewes (Brinsfield and Hawk 1973), being low during metaestrus and increasing during diestrus. Brinsfield and Hawk (1973) administered 25 mg of exogenous progesterone for 5 days in ovariectomized ewes and observed an accumulation of lipids in the endometrium that was comparable to the accumulation observed in a spontaneous estrous cycle, thus concluding that progesterone was the main factor inducing the accumulation of lipids during diestrus. The mechanism by which progesterone influences the formation of lipid droplets in epithelial cells is likely caused by modulation of transcript expression of diacylglycerol O-acyltransferase 2 (***DGAT2***) and lipoprotein lipase (***LPL***), which are increased and decreased, respectively (Forde et al., 2009; 2010). The former gene is involved in synthesis of triglycerides, whereas the latter in hydrolysis of triglycerides.

Recently, we investigated the lipid content of the uterus at early-, mid-, and late-diestrus in lactating cows (Ticiani et al., 2018). Cows (n = 30) had the estrous cycle and ovulation synchronized by administration of exogenous hormones; they were blocked by parity and assigned randomly to undergo transcervical uterine flushing and biopsy on estrous cycle Days 5 (early-diestrus), 10 (mid-diestrus) or 15 (late-diestrus). Flushing and biopsy were performed in the uterine horn ipsilateral to the corpus luteum. The recovered flushing was used for analyses of lipid composition by mass spectroscopy and the tissue collected for biopsy was used for investigation of lipid droplets abundance in the endometrium by immunohistochemistry. The abundance of lipid droplets in the endometrium increased 2-fold from Day 5 to Day 10, and another 2-fold from Day 10 to Day 15. Similarly, the concentration of fatty acids in the uterine fluid increased from Day 5 to Day 15 of the cycle. In addition, there was an enrichment of essential fatty acids and their metabolites on uterine fluid collected on Day 15 compared with Day 5 and 10. These results suggest that the uterus has a physiological mechanism to supply specific fatty acids to the uterine lumen at the time of the onset of conceptus elongation. In addition, accumulated lipids are also critical for synthesis of biomass and energy requirements of the fast growing conceptus.

Supplementation of Omega-3 Fatty Acids to Support Conceptus Development

Multiple studies have described benefits of supplemental omega-3 fatty acids on pregnancy success in lactating cows [reviewed by Santos et al. (2008) and Ambrose et al. (2016)]. A reduction in late pregnancy losses is the most common positive outcome, which might be associated with enhanced implantation and placental function. More recently, Sinedino et al. (2017) evaluated the effects of supplementing 100 g of an algae product rich in docosahexaenoic acid (**DHA**) in the diet of lactating cows, starting 30 days postpartum, on fatty acid composition of milk and blood phospholipids, milk production, and reproduction. Compared with non-supplemented controls, cows fed the algae product had greater incorporation of omega-3 fatty acids on milk and blood phospholipids, a 1.1 kg increase in daily milk production, greater pregnancy per artificial insemination (**AI**; 41.6 vs 30.7%) for all breedings, and fewer days open (102 vs. 124 days). Interestingly, pregnant cows in the algae group had greater gene expression of *RTP4* in peripheral blood leukocytes on Day 19 after AI, which is an indicative of enhanced elongation of the preimplantation conceptus. Altogether, these studies support a positive effect of omega-3 fatty acids on pregnancy success in lactating dairy cows, which could be mediated by better conceptus development.

Omega-6 Fatty Acids Are Also Important for Conceptus Development

Reducing the amount of omega-6 fatty acids, especially arachidonic acid, in the endometrium of dairy cows during the breeding period has been suggested as a strategy to minimize synthesis of luteolytic prostaglandins, to protect the corpus luteum at late diestrus, and to reduce embryonic mortality around the time of maternal recognition of pregnancy (Thatcher et al., 2006). For instance, feeding a diet rich in omega-3 fatty acids to cows increased the proportion of those fatty acids and reduced the proportion of arachidonic acid in the endometrium (Bilby et al., 2006), which resulted in a smaller prostaglandin response to an oxytocin challenge in late diestrus (Mattos et al., 2002). These associations have been suggested as a possible explanation for the improvements in reproduction observed in cows supplemented with omega-3 fatty acids (Thatcher et al, 2006). Nonetheless, the current knowledge of the biology of elongating conceptus and its high demand for arachidonic acid and prostaglandins suggest that positive effects of omega-3 supplementation on reproduction are likely caused by the higher concentration of those fatty acids per se in the endometrium and not by the reduction of omega-6 fatty acids.

Prostaglandins are produced and secreted abundantly by the developing placenta during elongation and have intracrine, autocrine and paracrine functions on conceptus and endometrium cells, which express both membrane and nuclear receptors for prostaglandins (Ribeiro et al., 2016c). Moreover, transport of prostaglandins across cell membranes is enhanced by increased expression of a prostaglandin transporter in both endometrium and conceptus cells (Ribeiro et al., 2016c). Prostaglandins are also natural ligands of PPARG and, therefore, might be important for coordination of gene expression and cell biology of elongating conceptuses. Moreover, intrauterine infusion of prostaglandins in ewes during diestrus induced expression of several genes in the endometrium that are known to stimulate trophectoderm cell proliferation and migration during the elongation phase (Dorniak et al., 2011). Intrauterine infusion of meloxicam, a specific inhibitor of cyclooxygenase-2 (**COX-2**) activity, reduced the amount of prostaglandins in the uterine fluid of pregnant ewes and precluded conceptus elongation (Dorniak et al., 2011).

The endometrium is likely the major source of omega-6 fatty acids for the developing conceptus. In fact, Meier et al. (2011) observed a negative association (R^2 = 0.55; P = 0.01) between size of the conceptus and concentration of arachidonic acid in the endometrium of the gravid horn (i.e. conceptuses longer in length were associated with less endometrial arachidonic acid on Day 17 of gestation). In addition, Ribeiro et al. (2016b) reported a negative association (R^2 = 0.28; P < 0.05) between the concentration of arachidonic acid and prostaglandins in the uterine fluid on Day 15 of the cycle or pregnancy (R^2 = 0.28; P < 0.05). Thus, the presence of a healthy elongating conceptus in the uterus in late diestrus should prevent luteolytic pulses prostaglandin $F_{2\alpha}$ by endometrial cells. Also, the focus of fatty acids supplementation should shift from manipulating the production of luteolytic prostaglandin pulses to the discovery of the best fatty acid profile that supports conceptus development, which likely include both omega-3 and omega-6 fatty acids.

Health Postpartum Is Critical for Future Fertility

Transition from the dry period to lactation is accompanied by major adjustments in the metabolism of the dairy cow to support milk synthesis. A steep increase in nutrient requirements occurs, and feed intake is inadequate to support the nutritional needs in the first weeks postpartum. Consequently, the caloric and nutrient requirements of the cow postpartum are only partially met by feed consumption, which causes extensive mobilization of nutrients from body tissues. Adipose tissue is particularly affected by reduced circulating concentrations of insulin, which up-regulate lipolytic signals for hydrolysis of stored triglycerides, increasing the availability of non-esterified fatty acids (**NEFA**) as energy substrate. As a consequence, lactating dairy cows usually lose large amounts of body mass postpartum, which varies according to the extent of the negative energy balance.

Extensive mobilization of body reserves during the peripartum period causes inflammation. The cellular environment of adipose tissue in cows with excessive mobilization of fatty acids stimulates infiltration of macrophages and differentiation into a pro-inflammatory phenotype (Contreras et al., 2017). Inflammatory mediators such as tumor necrosis factor-α (**TNFα**) and interleukin-6 (**IL6**) are secreted d from adipocytes, especially those in visceral fat, and they reduce the intracellular signaling of insulin. A large part of the NEFA mobilized from adipose tissue is composed of saturated fatty acids, mostly C16 and C18, which have the capacity to bind toll-like receptors and activate immune cells, thereby contributing to the metabolic stress postpartum (Contreras et al., 2010). The inflammation caused by the metabolic adaptation in the peripartum is further increased by microbial infections and incidence of clinical diseases commonly diagnosed in dairy cows postpartum such as metritis, mastitis, displaced abomasum, and laminitis.

Combined, excessive loss of body condition (**BCS**) and clinical diseases affect approximately 50% of dairy cows postpartum. Both conditions cause long-term negative effects on the reproductive biology cows, delaying the resumption of estrous cyclicity postpartum and impairing early embryonic development, including the elongation of preimplantation conceptuses (Ribeiro et al., 2016a; Ribeiro and Carvalho, 2017). The odds of pregnancy per AI is reduced approximately 30%, the odds of late pregnancy loss increases 2-fold, the odds of calving per AI is reduced 42%, the interval from calving to pregnancy is extended by weeks, and more cows are culled because of reproductive failure. Regarding conceptus elongation, cows with postpartum disorders have delayed elongation, reduced concentration of interferon-tau in the uterine fluid and expression of interferon-stimulated genes in peripheral leukocytes (Ribeiro et al., 2016a).

Essential Fatty Acids and the Paradox of Inflammation Postpartum

Because inflammation is a common feature of postpartum problems that result in compromised fertility in the breeding period, and multiple events have additive effects on the degree of inflammation and on the degree of reduction in fertility, the process of inflammation might have a central role in mediating the long-lasting effects of postpartum disorders on reproductive biology of dairy cows. Therefore, management solutions that reduce the incidence of clinical diseases or minimize the loss of BCS postpartum or mitigate inflammation are all likely to improve reproductive performance in dairy cows. Hence, feeding essential fatty acids early postpartum can be beneficial to the following breeding period for multiple reasons: 1) it increases the energy density of the diet and, if feed intake is maintained, improves the energy balance of the cow postpartum, minimizing loss of body BCS and metabolic stress; 2) it might improve immune cell function and reduce the incidence of clinical disease postpartum; and 3) it might be used to regulate the degree and enhance the resolution of inflammation postpartum. The outcomes, however, depends on the type of fatty acids supplemented.

Reduction in the incidence of disease postpartum has been described with supplementation of omega-6 fatty acids, which enhance the production of inflammatory mediators (i.e. series-2 prostaglandins) and might improve the ability of the immune system to fight infections postpartum (Silvestre et al., 2011). Nonetheless, clear evidence exists to support that inflammation is exacerbated in many dairy cows postpartum, especially in those that develop clinical diseases and have extensive loss of BCS.

Exacerbated inflammation leads to more intense sickness behavior, a more pronounced reduction in feed intake, increased partition of energy and nutrients to support the immune system, potentially longer interval for recovery, and larger consequences for milk production and reproduction performance. Control of inflammation, on the other hand, is generally obtained with supplementation of omega-3 fatty acids. Thus, the key question is whether inflammation postpartum should be enhanced by supplementation of omega-6 fatty acids or attenuated by supplementation of omega-3 fatty acids. Ideally, management strategies should strive to improve immune cell function without increasing the degree of inflammation postpartum, which might be seen as a paradox by many.

Studies evaluating the effects of injectable anti-inflammatory treatments might provide insights on whether controlling inflammation postpartum is good or bad for the dairy cow. Administration of flunixin meglumine, a nonsteroidal anti-inflammatory drug, within 24 h of parturition increased the odds of fever (odds ratio, **OR** = 1.7), retained placenta (OR = 2.6), and metritis (OR = 1.5) postpartum, and reduced milk production (1.6 kg/d) in the first 2 weeks postpartum (Newby et al., 2009). A similar study with meloxicam, another nonsteroidal anti-inflammatory drug more selective to COX-2, failed to show differences in health and milk production postpartum (Newby et al. 2013). Nonetheless, three consecutive daily drenches of sodium salicylate (125 g/cow/day) or one bolus of meloxicam (675 mg/cow), starting 12 to 36 h after parturition, resulted in greater milk production in the entire lactation (Carpenter et al., 2016). In cows diagnosed with puerperal metritis, applying a single dose of flunixin meglumine concurrent with the antimicrobial treatment protocol did not improve clinical cure, milk production in the week after diagnosis, and posterior reproductive performance (Drillich et al., 2007). However, adding 6 doses of flunixin meglumine into the treatment protocol of puerperal metritis (2.2 mg/kg twice daily on the first 2 days and once a day in the following 2 days) reduced the incidence of fever, shortened the interval from calving to resumption of estrous cyclicity, and seemed to improve uterine involution (Amiridis et al., 2001). Moreover, adding meloxicam to the antimicrobial treatment of clinical mastitis resulted in greater pregnancy at the first AI postpartum and increased proportion of cows pregnant by Day 120 after calving compared with the control group (McDougall et al., 2016). Altogether, these studies suggest that inflammation is critical on the day of parturition and likely involved with release of fetal membranes, but the effective control of inflammation a few days after calving, especially in situations in which exacerbated inflammation is expected, might contribute to health and subsequent performance of dairy cows. Thus, a balance between inflammatory and anti-inflammatory mediators postpartum would be ideal.

A study by Greco et al. (2015) evaluated the effects of altering the ratio between omega-6 and omega-3 fatty acids in the diet of lactating cows, starting on Day 14 postpartum. Dietary treatments were isocaloric and used multiple sources of supplemental fatty acids (Ca salts of fish, safflower, and palm oils) to establish the three distinct ratios in the diet, 6:1; 5:1, and 4:1 parts of omega-6 to omega-3 fatty acids. The total amount of fatty acids supplemented was identical among treatments. Reducing the ratio from 6:1 to 4:1 - thus increasing the proportion of supplemental omega-3 fatty acids - reduced the inflammatory response to an intramammary challenge with lipopolysaccharides and increased milk production. The approach used by Greco et al.

(2015), in which the ratio of omega-6 and omega-3 fatty acids is to prioritize and not to supply only one or the other, might be a sound alternative to balance the degree of inflammation postpartum and to minimize its long-term effects on milk production and reproduction.

Conclusions

Pregnancy losses are substantial in dairy cattle and threaten reproductive efficiency and sustainability of dairy herds. A substantial proportion of these losses occur during early stages of conceptus development, including the elongation phase. Elongation of the preimplantation conceptus is a prerequisite for maternal recognition, implantation, and survival of pregnancy. Lipids, especially essential fatty acids, in addition to be required for synthesis of biomass of the rapid growing conceptus, seem to be critical for coordination of cell biology during elongation. Thus, supplementation of essential fatty acids in the diets of lactating cows at the time of breeding has the potential to improve elongation of the conceptus and, consequently, pregnancy survival. In fact, multiple studies that evaluated the impact of supplementation of essential fatty acids, especially omega-3 fatty acids, have reported better pregnancy per AI and reduced pregnancy losses. The outcomes, however, remain variable, and a better understanding of the role of essential fatty acids on uterine physiology will contribute to the development of better and more consistent strategies to minimize early and late pregnancy losses.

Clinical diseases and excessive loss of body reserves are prevalent problems in postpartum dairy cows and represent important risk factors for pregnancy losses in the subsequent breeding period. Exacerbated inflammation postpartum seems to be a common feature of postpartum conditions that affect fertility of cows. Thus, supplementation of essential fatty acids early postpartum might also contribute to reproduction of dairy cows by increasing energy intake and minimizing loss of BCS, by improving immune cell function and minimizing the incidence of clinical disease, or by controlling the degree of inflammation postpartum and enhancing the recovery from clinical and metabolic problems postpartum. Finding a balance between pro-inflammatory omega-6 and anti-inflammatory omega-3 fatty acids seems to be a sound strategy to optimize the transition from the dry period to lactation, which substantially impacts performance in the entire lactation. A major challenge in nutrition management of transition cows is to find strategies that improve immune cell function without causing exacerbated inflammation postpartum, which continues to be seen as a contradiction by many dairy researchers.

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SESSION NOTES