Mechanisms underlying reduced fertility in anovular dairy cows

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ABSTRACT

Resumption of ovulation after parturition is a coordinated process that involves recoupling of the growth hormone (GH)/insulin-like growth factor (IGF)-1 axis in the liver, increase in follicular development and steroidogenesis, and removal of negative feedback from estradiol in the hypothalamus. Infectious and metabolic disorders associated with extensive negative energy balance during early lactation disrupt this pathway and delay first ovulation postpartum. Anovulatory conditions exert long-lasting detrimental effects on fertility in dairy cows including the lack of spontaneous estrus, reduced pregnancy per artificial insemination (AI), and increased risk of pregnancy loss. Concentrations of progesterone in anovular cows subjected to synchronized programs for AI are insufficient to optimize follicular maturation, oocyte competence and subsequent fertility to an insemination. Ovulation of first wave follicles, which develop under low concentrations of progesterone, reduces embryo quality in the first week after fertilization and pregnancy per AI (P/AI) in dairy cows. Although the specific mechanisms by which anovulation and low concentrations of progesterone impair oocyte quality have not been defined, studies with persistent follicles support the involvement of premature resumption of meiosis and degradation of maternal RNA. Suboptimal concentrations of progesterone before ovulation also increase the synthesis of PGF_{2α} in response to oxytocin during the subsequent estrous cycle, which explains the greater incidence of short luteal phases after the first AI postpartum in anovular cows compared with estrous cyclic herd mates. It is suggested that increased spontaneous luteolysis early in the estrous cycle is one of the mechanisms that contributes to early embryonic losses in anovular cows. Anovulation also leads to major shifts in gene expression in elongated conceptuses during pre-implantation stages of pregnancy. Transcripts involved with control of energy metabolism and DNA repair were downregulated

whereas genes linked to apoptosis and autophagy were upregulated in Day 15 conceptuses collected from anovular cows compared with estrous cyclic counterparts. Similar changes in conceptus transcriptome were not observed in estrous cyclic cows induced to ovulate follicles that grew under low and high concentrations of progesterone, indicating an effect of anovulation on embryonic development that is not mediated solely by progesterone concentrations before ovulation. Finally, risk factors for anovulation have direct effects on embryo development and uterine receptivity to pregnancy that complement those determined by insufficient concentrations of progesterone during follicular growth. One approach to minimize the impact of anovulation on fertility is supplementation with progesterone during the antral stages of development of the ovulatory follicle. It is suggested that a minimum of 2.0 ng/mL of progesterone is needed during growth of the preovulatory follicle to achieve P/AI similar to that of cows growing the preovulatory follicle during diestrus.

Keywords: anovular; conceptus; corpus luteum; disease; progesterone

1. Introduction

Reproductive efficiency plays a key role in the economic success of dairy herds, as it influences milk production per day of calving interval and culling policies [1]. In spite of recent advances related to management of reproduction with experiments achieving pregnancy per artificial insemination (**P/AI**) in high-producing Holstein cows of 45 to 50% [2-3], still less than 35% of the lactating dairy cows subjected to artificial insemination (**AI**) carry gestation to term [4,5]. The establishment and maintenance of pregnancy relies on a delicate crosstalk among the maternal hypothalamic-pituitary-gonadal axis, the endometrium, and the developing embryo. As expected, physiological, metabolic, and pathological conditions that disrupt these pathways

largely impair fertility. Extensive research supports the concept that anovulation is a major impediment to reproductive performance in dairy cows. The proportion of cows that remain anovular by the end of the voluntary waiting period varies among herds, genetic groups, and management schemes, ranging between 5 and 40% [6-8]. In addition to the lack of estrous behavior that precludes insemination because of lack of estrous cyclicity, anovular cows subjected to synchronization protocols consistently have reduced **P/AI** and increased risk of pregnancy loss compared with estrous cyclic herd mates [5,7].

Although the detrimental effect of anovulation on reproduction is conspicuous, defining the specific mechanisms contributing to the poor fertility observed in anovular dairy cows continues to challenge researchers. The hallmark of anovulatory conditions is the absence of corpus luteum (**CL**) and ensuing insufficient concentrations of progesterone during growth of the ovulatory follicle, which reduces embryo quality in the first week after fertilization [9,10], increases the release of prostaglandin- $F_{2\alpha}$ (**PGF_{2\alpha}**) in response to oxytocin during the subsequent estrous cycle [11,12], and decreases P/AI in lactating dairy cows [13]. On the other hand, some of the risk factors for anovulation also have direct effects on fertility responses. Delayed resumption of ovulation is associated with dystocia, extensive negative energy balance and loss of body condition postpartum, as well as the occurrence of diseases during early lactation [6-8, 14]. The hormonal and metabolic milieus imposed by such circumstances have been shown to decrease the frequency of luteinizing hormone (**LH**) pulses [15,16], impair follicular maturation and oocyte competence [17-19], upregulate the expression of inflammatory mediators in the endometrium [20], and impair conceptus elongation [21]. Therefore, strategies to improve reproduction in high-producing dairy cows must combine therapies to increase fertility of

anovular cows that is coupled with adequate management during the transition period to promote health and expedite resumption of ovulation postpartum.

2. Insufficient concentrations of progesterone during follicular growth

Decreased fertility in anovular cows subjected to timed AI programs is largely mediated by insufficient concentrations of progesterone during growth of the ovulatory follicle. For instance, P/AI did not differ between anovular cows and estrous cyclic herdmates without a functional CL at the first injection of GnRH of the synchronization protocol [13]. As opposed to cows in diestrus at the initiation of the synchronization protocol, anovular and estrous cyclic cows without CL are induced to ovulate a first wave follicle at insemination, which develops simultaneously with the CL under low concentrations of progesterone. The fact that the wave of the ovulatory follicle had a greater impact on P/AI (i.e., P/AI in cows ovulating the 2nd wave follicle was greater than cows ovulating the 1st wave follicle) than did estrous cyclicity (i.e. P/AI in anovulatory cow was similar to that of estrous cyclic cows ovulating 1st wave follicle), suggests that the hormonal milieu during the final stages of follicular maturation is a major determinant of poor fertility in anovular cows [13]. In fact, supplementation with progesterone during growth of the ovulatory follicle improved embryo quality in cows induced to ovulate first wave follicles [10] and restored P/AI in anovular cows at the initiation of the timed AI program [3,22]. Such findings support a causal role of progesterone during growth of the ovulatory follicle affecting P/AI. Moreover, cows that initiated a resynchronization protocol without a CL had reduced P/AI compared with cows with a CL and supplemental progesterone was effective at reestablishing fertility in cows without a CL similar to that of cows in diestrus [22].

It remains unknown the ideal concentrations of progesterone during follicle growth to optimize fertility in anovular cows or cows that lack a CL when the pre-ovulatory follicle is

recruited for subsequent ovulation. Wiltbank et al. [23] clearly indicated the importance of high concentrations of progesterone during the pre-ovulatory period coupled with very low concentrations during proestrus. Their group showed that not only P/AI increased, but pregnancy loss decreased (6.8 vs. 14.3%) when cows had high compared with low progesterone concentrations during the follicular wave that resulted in the ovulatory follicle [9]. Lactating dairy cows in mid diestrus have concentrations of progesterone that are highly variable and dynamic [23], but usually range from 3 to 6 ng/mL [3,11,22]. Evidence from experiments in which progesterone concentrations were manipulated pre-AI suggest that a minimum of 2.0 to 3.0 ng/mL are needed to achieve P/AI in anovular cows that resemble those of cows in diestrus [22].

2.1. Effects of LH on oocyte quality and early embryo development

Despite the fact that bovine cumulus-oocyte complexes are responsive to progesterone [24], intra-follicular concentrations are determined by steroidogenesis within the granulosa cells and are approximately 10-fold greater than those measured in plasma during the first follicular wave [25]. Therefore, the effects of low concentrations of progesterone in plasma on follicular maturation and subsequent embryo development are likely mediated by changes in LH pulse frequency rather than a direct impact on the follicle. Results from sheep and mice indicate that progesterone depresses the release of GnRH via the kisspeptin/GPR54 system at the hypothalamus [26,27]. In ewes, kisspeptin positive neurons also express progesterone receptor [28], and supplementation with progesterone to ovariectomized ewes depressed the number of neurons expressing mRNA for kisspeptin in the arcuate nucleus [28]. It is known that progesterone depress LH pulse frequency in cattle [29] and low concentrations of progesterone are associated with greater frequency of LH pulses during metestrus compared with diestrus [30].

Collectively, these data suggest that low concentrations of progesterone influence follicle and oocyte development through increased secretion of LH that accelerates follicle growth and can depress oocyte quality.

Throughout final stages of follicular development, oocytes remain arrested at the diplotene stage of prophase I by inhibiting activation of the maturation promoting factor (**MPF**; [31]). The pre-ovulatory surge of LH increases intracellular cyclic adenosine monophosphate (**cAMP**) in the cumulus cells, leading to activation of protein kinase (**PK**) A type II and PKC, expression of epidermal growth factor (**EGF**)-like factors, activation of mitogen-activated protein kinase (**MAPK**), and disruption of the gap junctions between cumulus cells and the oocyte [32,33]. The lack of communication between the oocyte and surrounding cells blocks cAMP transfer towards the oocyte, which in combination with its hydrolysis by phosphodiesterase 3A (**PDE3A**), reduces PKA activity and activates MPF. Resumption of meiosis is characterized by the breakdown of the germinal vesicle and extrusion of the first polar body [34]. Another important characteristic of oocyte maturation is the accumulation of maternal mRNA in order to provide templates for protein synthesis before embryonic genome activation. Among other mechanisms, maternal mRNA stored in the oocyte is protected from degradation through the binding of multifunctional Y-box protein 2 (**MSY2**; [35]). This protection begins to be removed following the germinal vesicle breakdown by MSY2 phosphorylation [36].

Changes in LH pulse frequency coupled with oocyte morphology and mRNA content have not been evaluated in anovular dairy cows; however, it is possible that excessive exposure to LH in cows with low concentrations of progesterone, via an extended period of increased LH pulse frequency, advances the events associated with resumption of meiosis, thereby reducing oocyte quality and subsequent embryo survival (**Fig. 1**). Oocytes from persistent follicles induced by

sub-luteal concentrations of progesterone presented morphological signs of germinal vesicle breakdown before ovulation [37], supposedly by long-term exposure to increased frequency of LH pulses. This advancement in oocyte maturation possibly hastens the depletion of maternal mRNA, increasing embryonic expression of cell death transcripts, and impairing development to the blastocyst stage [38]. Using a heifer model, Schmitt et al. [39] showed that those induced to have persistent follicles had reduced P/AI, which could be corrected with induction of a new follicular wave by administration of GnRH. Extending the period of follicular dominance reduced embryo quality on d 7 after AI [40] and decreased P/AI in cows inseminated following spontaneous estrus [41] and in those subjected to timed AI programs [42]. In a similar manner, ovulation of first wave follicles increased the proportion of degenerated embryos on Day 7 after AI [9,10], which was reversed by supplementation with progesterone during follicular growth [10].

2.2. *Effect of expression of estrus on subsequent fertility responses*

The growth of ovarian follicles and maturation of the enclosed oocyte are tightly controlled processes that must occur simultaneously. Estradiol produced by the dominant follicle stimulates estrous behavior and triggers a pre-ovulatory surge of LH to induce its own ovulation [27]. In view of that, expression of estrus at AI is an indicator of follicular health and adequate timing of the events involved with ovulation. It is interesting to note that anovulation [21] and insufficient concentrations of progesterone during growth of the ovulatory follicle [3,22] only reduced P/AI in cows not detected in estrus on the day of insemination (**Fig. 2**). Moreover, the reduction in P/AI observed in cows that did not express estrous behavior at insemination was reversed by progesterone supplementation [3,22]. A recent study in grazing dairy cows suggest that plasma concentrations of anti-Müllerian hormone (**AMH**) are related with the interaction between

progesterone concentration during follicular development and expression of estrus during timed AI programs [43]. The proportion of cows detected in estrus at timed AI was influenced by AMH concentrations (low AMH = 49.3%, intermediate AMH = 39.4%, and high AMH = 34.5%). For cows with progesterone concentrations in plasma < 1.0 ng/mL at the initial GnRH injection of the timed AI protocol, those with low concentrations of AMH had P/AI that was greater than cows with intermediate or high AMH concentrations (40 vs. 25%). No differences in P/AI were detected between AMH categories in the presence of a functional CL, and P/AI averaged 47%.

2.3. *Legacy effects on PGF_{2α} release and subsequent luteal lifespan*

During pre-implantation stages, bovine conceptuses must elongate rapidly and synthesize a plethora of bioactive products that are essential for maintenance of pregnancy. Among such molecules, the conceptus must secrete large amounts of interferon-tau (**IFN-τ**) to inhibit the pulsatile release of PGF_{2α} by the endometrium and prevent luteolysis [44,45]. Conceptuses grow from a third of a millimeter to 25 cm between Days 11 and 17 of gestation, and length of the conceptus is directly related to concentrations of IFN-τ in the uterine lumen [21]. Elongation and consequently survival of the conceptus is dependent on histotroph secretion by the glandular epithelium of the endometrium [44]. Adequate provision of nutrients, growth factors and hormones in the histotroph stimulates cellular remodeling, proliferation, and differentiation in the conceptus, which follows time-dependent events. It is well accepted that inability of conceptus to develop properly or asynchrony between conceptus development and endometrial maturation can potentially increase the risk of pregnancy loss because of the inability of a less advanced conceptus to block the luteolytic cascade or because of early spontaneous luteolysis. The incidence of short luteal phases (< 11 days) following timed AI is greater among anovular

cows compared with estrous cyclic counterparts [11,46]. Similar to the effects on oocyte quality and early embryo development, hastening of the luteolytic cascade is mediated by low concentrations of progesterone during preovulatory follicular growth. The incidence of short estrous cycles, defined as reinsemination on estrus between 5 and 17 days after a previous AI, was greater for anovular and estrous cyclic cows that ovulated first wave follicles compared with herdmates in diestrus at the first GnRH injection of the synchronization protocol [3,13]. Moreover, the incidence of short estrous cycles was reduced by supplementing progesterone to cows without CL at the initiation of the timed AI program [3].

The mechanism by which exposure to low progesterone concentrations during follicle growth affect luteal lifespan in the subsequent estrous cycle involves uterine responsiveness to oxytocin. Endometrial expression of oxytocin receptors in ruminants is suppressed until mid-diestrus [47,48], when progesterone downregulates its own receptors in the luminal epithelium and superficial glands allowing for oxytocin to trigger the luteolytic cascade [47]. Cows with low concentrations of progesterone during preovulatory follicle growth had an anticipated increase in protein expression for estrogen receptor- α in the endometrium [11] and greater concentrations of 13,14-dihydro-15-keto PGF_{2 α} (**PGFM**) in plasma following an oxytocin challenge in the subsequent diestrus [11,12]. On the other hand, supplementing postpartum beef cows with a progestin (i.e. norgestomet) during follicular growth increased the expression of progesterone receptors [49,50] and downregulated oxytocin receptors in the endometrium on Day 5 after ovulation compared with untreated controls [49]. In addition, some experiments have shown that low concentrations of progesterone during the preceding estrous cycle accelerates pre-ovulatory follicle growth [11,51], which can result in larger CL in the subsequent estrous cycle and greater concentrations of progesterone. Perhaps, the combined low and high concentrations of

progesterone during the preceding and subsequent estrous cycles, respectively, induce the premature luteolytic signaling that leads to short estrous cycles in some cows.

3. Effects of anovulation on conceptus biology and late embryonic/fetal mortality

In addition to the detrimental effect on P/AI, previous studies indicate that anovulatory conditions increase the risk of late embryonic and fetal mortality in lactating dairy cows [5,13,23]. As opposed to the effects on P/AI, however, pregnancy loss is mostly affected by estrous cyclic status and not by concentrations of progesterone during growth of the ovulatory follicle. In fact, the incidence of pregnancy loss between Days 30 and 53 of gestation was greatest for anovular cows (15.0%), intermediate for cyclic cows in diestrus at the initiation of the synchronization protocol (13.5%), and lowest for cyclic cows induced to ovulate first wave follicles that develop under low progesterone concentrations [10.0%; 13].

Recent work by our group with lactating dairy cows revealed major shifts in gene expression in conceptuses cells from anovular cows [21]. These changes in conceptus transcriptome were not observed in estrous cyclic cows with low concentrations of progesterone before AI [51]. Transcriptome analyses of Day 15 elongated conceptuses detected 500 transcripts affected by estrous cyclicity of which 262 were upregulated and 238 were downregulated in anovular compared with the referent estrous cyclic Holstein cows. Eighty-seven probesets were upregulated in conceptuses from anovular cows in association with progression of conceptuses from tubular to filamentous stages, reflecting the increase in the length of the trophectoderm in anovular cows compared with estrous cyclic counterparts. Nevertheless, most of the differentially expressed transcripts were not related to improved embryo development and may explain the greater occurrence of pregnancy loss in anovular cows. Transcripts involved with phosphatidylinositol/AKT signaling (*IMP1A1*, *IMP1A2*, *PIP5K1A*, and *AKT2*) were downregulated

in conceptuses from anovular cows. Because phosphatidylinositol/AKT is a key downstream mediator of insulin-like growth factor 1 (**IGF-1**) actions [52] and affects the mechanistic target of rapamycin (**mTOR**) signaling, its downregulation might affect energy metabolism, cell survival, proliferation and migration during establishment of gestation [53]. The transcripts associated with DNA repair were also downregulated in response to anovulation, including *TOP1* and *NBN* which are required for embryo survival in mouse models [54-56]. Moreover, the expression of transcripts involved with apoptosis (*CASP6* and *GSK3B*) and autophagy (*ATG4A*, *MAP1LC3B*, and *LAMP1*) were upregulated in conceptuses from anovular cows, which has been associated with embryo mortality and termination of pregnancy [57]. It is interesting to note that conceptus length and the concentrations of IFN- τ in the uterine flush fluid were greater for anovular than estrous cyclic cows, providing no morphological evidence of poor embryonic development in anovular cows for conceptuses that develop to Day 15 of gestation [21]. The collective results from the transcriptome analysis pointed to differences in canonical pathways related to apoptosis, 14-3-3 protein signaling, and autophagy, perhaps suggesting greater cellular stress and the need to eliminate aberrant protein aggregates and malfunctioning organelles. Thus, the aforementioned findings support the hypothesis that some of the detrimental effects of anovulation on embryo survival, although already imprinted during the pre-implantation stages, will be expressed later in gestation and provides biological support to explain the greater incidence of later embryonic/fetal mortality in anovular cows [3,13,21].

Using a different model with nonlactating Holstein cows to study the role of progesterone on conceptus biology, we showed that the transcriptome of Day 17 conceptus was not substantially affected by low concentrations of progesterone during the development of the preovulatory follicle [51]. Only nine probesets were affected by concentrations of progesterone during

follicular growth. Among the differently expressed genes, transcripts involved with cell adhesion (*CD97*), signal transduction and ion transport (*P2RY1*), cytoskeleton organization (*CNN2*), glucose metabolism (*PFKP*), and embryo survival (*CALCRL*) were upregulated in conceptuses collected from cows with low progesterone concentrations during follicular growth compared with cows with high progesterone concentrations. Although progesterone mediates a large portion of the effects of anovulation in reducing fertility in dairy cows [13], and sufficient supplemental progesterone can restore P/AI [3,22] and reduce pregnancy loss in anovular dairy cows [58], it is clear that the lesions caused by anovulation on conceptus cells are more complex and not explained simply by the endocrine milieu during the pre- and periovulatory periods. A major risk factor for anovulation is disease during the peripartum period [59], and cows that suffered disease in early lactation had conceptuses with inflammation-like changes in the transcriptome and up-regulation of major histocompatibility complex (**MHC**)-I heavy chain BOLA gene [60]. Such changes suggest that these conceptus cells are less likely to escape surveillance of the maternal immune system, thereby posing an increased risk for pregnancy loss [60].

4. Risk factors for anovulation also have impact on fertility responses

The transition from late gestation to early lactation is extremely challenging to high-producing dairy cows. A sharp increase in nutrient requirement occurs with the onset of lactation, which is not met by voluntary feed intake, leading to negative nutrient balance and extensive mobilization of body reserves [61]. In spite of homeostatic controls and homeorhetic adjustments that allow for partitioning of nutrients towards milk synthesis, 45 to 60% of dairy cows across different levels of milk production, breeds, and management systems develop health disorders postpartum [8,59,60]. Many of the events that affect cows in early lactation are known

risk factors for anovulatory conditions, but metabolic and infectious diseases that are common in the periparturient period have direct impacts on follicular maturation, embryo development, and uterine environment [60]. Thus, this complex array of events in early lactation contribute to the reduced fertility in dairy cows beyond simply delaying or preventing resumption of estrous cyclicity. This concept is supported by the observation that health disorders and anovulation have additive effects on depressing fertility in lactating dairy cows [60].

4.1. Negative nutrient balance and metabolic health during the transition period

Energy balance in early lactation has been associated positively with reproductive success, and cows that lose excessive body condition in the first weeks to two months postpartum have delayed resumption of estrous cyclicity [7,62]. In a similar manner, dairy cows with elevated concentrations of non-esterified fatty acids (NEFA) in serum (≥ 0.7 mM) are more likely to remain anovular by Day 49 postpartum [8]. The inclusion of saturated fatty acids into the culture media induced apoptosis and necrosis of cumulus cells, impairing fertilization and early embryonic development *in vitro* [63]. The concentrations of NEFA in the follicular fluid parallel those observed in serum in dairy cows in early lactation [63], and increased lipomobilization with the onset of lactation results not only in increased serum NEFA concentrations [64], but also NEFA with more saturated fatty acids [64]. Therefore, it is plausible to suggest that extensive lipolysis in early lactation not only influence the risk of anovulation, but it might also alter the lipid composition of the follicular fluid which might imprint negative characteristics on oocytes and granulosa cells from follicles that will ovulate during the breeding period.

Negative nutrient balance in early lactation influences dairy cows beyond the changes in blood NEFA that can affect reproductive tissues. Insufficient caloric intake reduces concentrations of insulin, which downregulates hepatic expression of GH receptor 1A and

uncouples the GH/IGF-1 axis in dairy cows [65]. It is known that insulin and IGF-1 in early lactation were linked with nutrient intake and energy balance, and cows that remain anovular are known to have reduced concentrations of these metabolic hormones in early lactation [21]. The low concentrations of IGF-1 in anovular cows might be the result of uncoupling of the GH-IGF axis, which compromises follicular steroidogenesis [66] and seems to diminish sensitivity to LH in ovarian follicles [67]. Furthermore, undernutrition and reductions in IGF-1 in early lactation not only compromises resumption of ovulation, but it can have direct effects on the embryo. It has been shown that increments in concentrations of IGF-1 with treatment with exogenous GH stimulate conceptus development and conceptus-maternal tissues cross-talk through IFN- τ , which ultimately resulted in improved maintenance of pregnancy in lactating dairy cows [68].

Resumption of ovulation postpartum is affected by calcium homeostasis and cows with subclinical hypocalcemia (≤ 2.14 mM) during the first week postpartum are more likely to be anovular at the end of the voluntary waiting period [8]. Hypocalcemia not only delayed estrous cyclicity [8], but it also depressed pregnancy rate in dairy cows [69]. To a great extent, the detrimental effects of hypocalcemia on fertility are likely mediated by the important role of calcium for proper function of immune cells and the ensuing prevention of uterine and other peripartum diseases. Induction of subclinical hypocalcemia reduced the percentage of neutrophils undergoing phagocytosis and impaired oxidative burst in dairy cows [70]. Cows with hypocalcemia in at least one of the first 3 days postpartum had greater risk of developing fever and metritis [69]. Hypocalcemia exacerbates lipomobilization [69,70] because of reduced release of pancreatic insulin [70] and reduced adipose tissue sensitivity to insulin induced by the high parathyroid hormone concentration [71]. The negative effects of hypocalcemia on insulin might further compromise the recoupling of the GH/IGF axis that is critical for follicular function and

steroidogenesis [65,66]. Furthermore, hypocalcemia directly depress dry matter intake [70], the main driving force influencing energy balance in dairy cows [59]. Collectively, the detrimental effects of hypocalcemia on energy metabolism and innate immune function likely explain the delay in estrous cyclicity postpartum and the increased risk of metabolic and inflammatory diseases known to depress P/AI in dairy cows [59,60].

4.2. Incidence of diseases postpartum

Inflammatory and metabolic diseases are major factors that underlie anovulatory conditions in dairy cows. For instance, metritis, respiratory problems and indigestion increased the odds of cows remaining anovular by Day 49 postpartum by 2.4, 4.1, and 4.2-fold, respectively [8]. Disease increased the proportions of anovular cows from 18.9 to 26.6%, and both disease and anovulation depressed P/AI in dairy cow (**Fig. 3**) [60]. Because diseases have such a profound impact on establishment and maintenance of pregnancy in dairy cattle [59,60], and cows with health problems are more likely to be anovular, it is often difficult to clearly separate the exact mechanisms linking those perturbations with depressed fertility in dairy cows. It is interesting to note that disease and anovulation had additive effects depressing P/AI in dairy cows (**Fig. 3**), thereby suggesting that some of the mechanisms might be independent and complementary relative to the lesions that impair establishment and maintenance of pregnancy in cattle. Fertilization, development to the morula stage, conceptus biology, conceptus-maternal cross-talk, and maintenance of pregnancy to term were all influenced by disease in dairy cows. It is noteworthy that P/AI was depressed and pregnancy loss was increased similarly by disease regardless if cows received AI or embryo transfer [60]. Collectively, these data suggest that transferring an embryo is not capable of correcting the problem caused by disease and imply that

the negative effects of disease, and potentially anovulation, are linked to the uterine environment in which the embryo develops.

5. Conclusions

Anovulation is a complex condition that contributes to infertility in lactating dairy cattle by affecting several processes that are essential for the establishment and maintenance of pregnancy. The absence of a CL in anovular cows leads to insufficient concentrations of progesterone during growth of the ovulatory follicle, which has been shown to impair subsequent embryonic survival, shorten luteal lifespan around the time of maternal recognition of pregnancy, and reduce P/AI. Changes in the transcriptome of elongated conceptuses indicate that the detrimental effects of anovulation persist during the pre-implantation stages of gestation and are likely implicated in the increased embryonic/fetal mortality observed in anovular cows. Recent studies also indicate that anovulation and its predisposing factors have additive effects in reducing fertility in lactating dairy cows. For that reason, reproductive management in dairy herds should incorporate health and nutrition programs that minimize periparturient problems to reduce the prevalence of anovulation when breeding starts. Programs should also focus on methods to identify anovular cows that benefit from therapeutic interventions such as progesterone supplementation and require manipulation of the estrous cycle such as timed AI programs to ensure breeding at the proper time postpartum with adequate fertility.

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586 **Fig. 1.** Hypothetical model of the effects of elevated luteinizing hormone (LH) pulse frequency

587 on follicle and oocyte maturation and subsequent embryo development. Before the pre-

588 ovulatory surge of LH, the oocyte remains arrested at the diplotene stage of prophase I. Gap

589 junctions allow for the transport of cyclic adenosine monophosphate (cAMP) and cyclic

590 guanosine 3',5' monophosphate (cGMP) from cumulus cells to the oocyte, and cGMP

591 inhibits the hydrolysis of cAMP by phosphodiesterase 3A (PDE3A). Elevated

592 concentrations of cAMP within the oocyte activates protein kinase A (PKA), which

phosphorylates Wee1/Myt1, CDC25B, and cyclin-dependent kinase 1 (CDK1), thereby, inactivating the maturation promoting factor (MPF). Under low concentrations of progesterone, the frequency of LH pulses increases, which in turn increases cAMP production via adenylate cyclase in cumulus cells, thereby activating PKA type II and protein kinase C (PKC). The actions of PKA and PKC induce epidermal growth factor (EGF) like factors that activates mitogen-activated protein kinase (MAPK). This cascade of events culminates with the disruption of gap junctions between cumulus cells and the oocyte, blocking the transfer of cAMP and cGMP. The low cGMP favors hydrolysis of cAMP by PDE3A, therefore, further reducing cAMP, which causes activation of MPF. Hastening oocyte maturation results in germinal vesicle (GV) breakdown (GVBD), which might be accompanied by removal of multifunctional Y-box protein 2 (MSY2) from stored messenger ribonucleic acid (mRNA) and premature depletion of maternal RNA in the oocyte, all of which are associated with reduced embryo quality and embryonic mortality before the elongation of the blastocyst.

Fig. 2. Interaction among estrous cyclic status, concentration of progesterone during follicular growth, and expression of estrus behavior at insemination on pregnancy per AI (P/AI) in lactating dairy cows [3,21,22]. Cows were evaluated for presence of a corpus luteum (CL) and those without CL were randomly assigned to receive no additional treatment or two controlled-internal drug release (CIDR) containing progesterone. In Bisinotto et al [3,21] supplementing progesterone using two intravaginal inserts reestablished P/AI in anovular cows similar to that of cows in diestrus; however, the depression in P/AI caused by anovulation was observed only in cows that did not display estrus on the day of timed AI. Similarly, results from Ribeiro et al. [21] reiterate that the depression in fertility observed in anovular compared with estrous cyclic cows is observed primarily in those that do not show signs of estrus on the day of timed AI. ^{a,b,c} Within study, proportions with different superscripts differ ($P < 0.05$).

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624 **Fig. 3.** Proportion of dairy cows pregnant on Day 32 after AI according to incidence of diseases
 625 and estrous cyclicity before first insemination postpartum. Effect of disease ($P < 0.01$),
 626 anovulation ($P < 0.01$), interaction between disease and anovulation ($P = 0.70$), and the
 627 additive effect of disease and anovulation ($P < 0.01$). Disease and anovulation depressed
 628 pregnancy on Day 32 and their effects were additive. Data from Ribeiro et al. [60].

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