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EFFECT OF INDUCING ORIGINAL AND ACCESSORY CORPORA LUTEA WITH GnRH OR hCG ON FERTILITY IN RECIPIENT DAIRY HEIFERS AND COWS

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People I can really count on. People who really believe in my potential, vibrate in my victories and support me in my defeats. And for which I will always be there.

To my family.

I DEDICATE

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“There is no real ending. It's just the place where you stop the story.”

(Frank Herbert)

RESUMO GERAL

Três estudos foram desenvovidos com o objetivo de caracterizar o efeito da gonadotrofina coriônica humana (hCG) e do hormônio liberador de gonadotrofina (GnRH) na atividade lútea e na taxa de prenhez por transferência de embriões (P/TE) em novilhas nulíparas e vacas leiteiras. O primeiro objetivo foi determinar o efeito do hCG ou GnRH administrado no último dia (d0) do programa de sincronização 5d-CIDR Synch com ou sem administração de hCG após 5 dias (d5), na área lútea e concentrações de P4 durante o diestro em novilhas. O segundo objetivo foi determinar o efeito de hCG ou GnRH sobre P/ET e perdas de prenhez em novilhas receptoras de embriões produzidos in vitro (PIV) ou in vivo. O terceiro objetivo foi determinar os efeitos da indução de um CL acessório usando GnRH ou hCG no dia da TE na fertilidade de nulíparas, primíparas e multíparas. Hipotetizamos que as novilhas que receberam hCG no d0 têm maior P4 durante o metestro e diestro em comparação com novilhas tratadas com GnRH, e as receptoras com CL acessório têm maior P4 e maior P/TE do que o controle. As novilhas que receberam hCG no d0 tiveram área lútea e P4 maiores no d5 do que d0-GnRH. As novilhas que receberam hCG no d0 e 5 apresentaram maior P4 no d7 do que demais tratamentos. Animais que receberam d0-GnRH e d5-hCG apresentaram maior P/TE aos 60d com embriões *in vivo* em comparação com d0-GnRH. Por outro lado, novilhas com tratamento apenas com d0-GnRH e aquelas que receberam hCG nos d0 e 5 tiveram maior P/TE aos 60d do que apenas d0-hCG transferidas com embriões in vivo e congelados. Novilhas tratadas com d0-hCG apresentaram menor P/ET aos 60d do que aquelas apenas com d0-GnRH e d0-GnRH seguidas de d5-hCG transferidas com embriões PIV. A perda de prenhez geral tendeu a ser maior em d0-hCG comparado com GnRH seguido de hCG. Quando o CL acessório foi induzido no dia da TE, paridade e características do embrião (blastocisto ou blastocisto expandido) impactaram fertilidade. Nulíparas com CL acessório (hCG+GnRH combinados), tiveram tendência a apresentar maior P/ET que controle. Além disso, nulíparas com GnRH transferidas com embriões PIV vitrificados tenderam e tiveram maior P/ET e parto/TE do que o controle, respectivamente. Primíparas que receberam embriões blastocisto expandido PIV, com CL acessório induzido por GnRH tiveram maior P/TE e parto/TE do que primíparas tratadas com hCG. Além disso, o hCG diminuiu o parto/TE em primíparas com blastocisto expandido comparados com o controle. No entanto, o parto/TE geral foi semelhante entre os todos grupos com CL induzido no dia da TE. Em conclusão, os efeitos da indução de CL acessório sobre P/TE, perda gestacional e parto/TE foram distintos de acordo com a interação, indutor ovulatório, tipo de embrião e estágio de desenvolvimento e paridade. Além disso, o hCG teve maior efeito luteotrópico do que o GnRH, mas em discordancia teve efeito prejudicial na P/TE quando usado para induções de CL original ou acessório de acordo com as características do embrião e paridade.

Palavras-chave: Progesterona. Prenhez. Transferência de embrião. hCG. GnRH

GENERAL ABSTRACT

We have developed a series of studies aimed to characterize the effect of human chorionic gonadotropin (hCG) and gonadotropin releasing-hormone (GnRH) on luteal activity and its effects on pregnancy outcomes in nulliparous dairy heifers and dairy cows submitted to embryo transfer (ET). The first objective was to determine the effect of hCG, or GnRH administered on the last day of a 5d-CIDR Synch program (d0) with or without an additional hCG 5 days later (d5) to create an accessory corpus luteum (CL) on the luteal area and P4 during the diestrus in dairy heifers. The second objective was to determine the effect of hCG or GnRH on P/ET and pregnancy losses in recipient dairy heifers receiving in vivo or in vitro produced (IVP) embryos. The third objective was to determine the effects of inducing an accessory CL on the day of ET fertility outcomes in nulliparous, primiparous, and multiparous. The main hypothesis was that heifers receiving hCG on the last day of a 5d-CIDR synch protocol would have greater P4 during metestrus and diestrus compared with GnRH-heifers, and the recipients with an accessory CL induced by hCG have greater P4, as well as greater P/ET than those receiving GnRH. Heifers receiving hCG on d0 had a larger luteal area and greater serum P4 on d5 than those receiving d0-GnRH. Heifers that received hCG on d 0 and 5 had greater P4 on d7 than the heifers in the other treatments. The recipient heifers receiving both d0-GnRH and d5-hCG had greater d60 P/ET with fresh embryos compared to heifers treated only with d0-GnRH. Conversely, heifers with only d0-GnRH treatment and those receiving hCG on d0 and 5 treatment had greater d60 P/ET than only d0-hCG transferred with frozen-fresh embryos. Heifers in d0-hCG also had lower d60 P/ET than those treated with only d0-GnRH and GnRH followed by injection of hCG using IVP embryos. The overall pregnancy loss tended to be greater on d0-hCG compared to GnRH followed by hCG. When accessory CL was induced on d of ET, parity and embryo stage (blastocyst or expanded blastocyst) were factors that impacted the fertility outcomes. Nulliparous with accessory CL, (hCG + GnRH combined), tended to have greater overall P/ET than controls. Additionally, GnRH-nulliparous receiving vitrified IVP embryos tended and had greater P/ET and calving/ET than controls, respectively. Primiparous receiving expanded blastocyst fresh IVP embryos with GnRH-accessory CL had greater P/ET and calving/ET than primiparous hCG-cows. Moreover, hCG decreased calving/ET in primiparous with expanded blastocyst fresh embryos than controls. Yet, the overall calving/ET was similar between treated groups. In conclusion, effects of accessory CL induction with hCG, or GnRH on P/ET, pregnancy loss, and calving/ET were distinct according to interaction of ovulatory inducer and embryo produced type, and stage of embryo development and recipient parity. Moreover, hCG had a greater luteotropic effect than GnRH, but had detrimental effects on P/ET when used for original or accessory CL inductions according to embryo characteristics and parity.

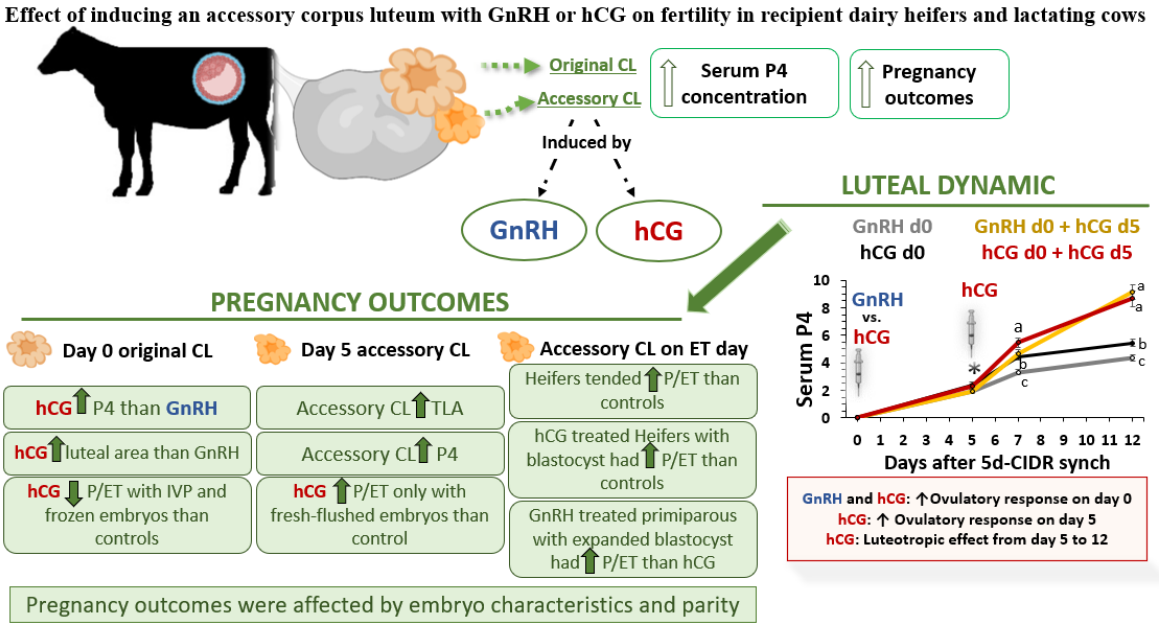
Keywords: Progesterone. Pregnancy. Embryo transfer. hCG. GnRH

INTERPRETATIVE SUMMARY AND GRAPHICAL ABSTRACT

Effect of inducing original and accessory corpora lutea with GnRH or hCG on fertility in recipient dairy heifers and cows

Performed by **Marcelo Siqueira El Azzi** and supervised by **José Camisão de Souza** and **João Paulo Nascimento Martins**

The objectives of this thesis were to determine the effect of replacing GnRH with hCG on the last day of the 5d-CIDR Synch (day 0) and inducing CL formation with hCG (day 5) on circulating progesterone concentrations, luteal dynamics. Also, determine effects on pregnancy outcomes with hCG or GnRH-induced accessory CLs on day 5 and immediately prior to ET (day of ET). Nulliparous heifers receiving hCG on day 0 had a larger luteal area and greater circulating progesterone concentration on day 5 than heifers receiving GnRH. Heifers receiving hCG on days 0 and 5 of the estrous cycle had greater progesterone concentrations on day 7 than the heifers in the remaining treatments. The hCG-accessory CL increased the luteal area and the progesterone concentration on day 12. Interestingly, hCG on day 0 appears to be detrimental to pregnancy maintenance with fresh-IVP and frozen embryos. The effects of hCG or GnRH accessory CLs induction on P/ET and calving/ET are distinct according to different development stages (blastocyst and expanded blastocyst), produced type embryos, and parity of recipients. Accessory CLs induced by hCG, or GnRH improve pregnancy with conventional *in vivo* and stage 6 (blastocyst) in vitro-produced embryos in recipient heifers. Similar positive hCG effects occur with vitrified *in vitro*-produced embryos in heifers. Moreover, primiparous with GnRH-induced accessory CL cows have higher P/ET and calving /ET than primiparous hCG-induced cows. Finally, pregnancy loss was not slightly affected by accessory CL induction with a tendency to decrease pregnancy losses when hCG-accessory CL is induced on day 5.



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KEY TO ABBRECIATIONS

PGFM	13,14-Dihydro-15-Keto-$PGF_{2\alpha}$
3ßHSD	3-ß hydroxysteroid dehydrogenase enzyme
AI	artificial insemination
CIDR	controlled internal drug release
CL	corpus luteum
ET	embryo transfer
PGDH	enzyme 15-hydroxyprostaglandin dehydrogenase
E2	estradiol
TAI	fixed time AI
BNCs	giant binucleate cells
GnRH	gonadotropin releasing-hormone
HDL	high-dense lipoprotein
hCG	human chorionic gonadorelin
ISG	IFN-stimulated genes
IVP	*in vitro* produced
IGF1	insulin-like growth factor 1
IFNT	Interferon-τ
CIDR or PRID	intravaginal dispensers
LH	luteinizing hormone
MRP	maternal recognition of pregnancy
OT	Oxytocin
OTR	oxytocin receptor

P450scc	P450 cholesterol side chain enzyme
PAGs	pregnancy associated glycoproteins
P/AI	pregnancy by artificial insemination
P/ET	pregnancy per ET
PSPB	pregnancy-specific protein B
P4	Progesterone
PG	prostaglandin
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
TMR	Total mixed ration

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1 INTRODUCTION

Embryo transfer has consistently increased in the last decade world widely (Viana, 2018a; b, 2020). According to the International Embryo Technology Society annual report, only in North America, the increase of in vitro embryo transfer was ~1,000%, from 28,100 transfers in 2010 to 339,716 in 2020 (Stroud, 2011; Viana, 2021). The enormous increase in the use of *in vitro*-produced embryos during the last decade was based on the improvement of two assisted reproduction technologies, the synchronization of ovulation (Wiltbank and Pursley, 2014), and the embryo *in vitro* production systems, with a greater number of commercial laboratories available, and on a steadily growing demand for herd genetic improvement (Viana, 2020). As a result of this growth, part of the research efforts has focused on the physiology related to early embryo development aimed at increasing survival and consequent fertility outcomes in dairy cattle (Wiltbank et al., 2016; Besbaci et al., 2020; Cunha and Martins, 2022).

One of the most critical periods of pregnancy in cattle is between days 3 and 7 post-ovulation when uterine changes influence the subsequent development of the embryo and its chances of survival (Carter et al., 2008, 2010; Forde et al., 2009; Clemente et al., 2011). During early embryo development, circulating progesterone concentrations have been positively associated with uterine hystotrophs synthesis, embryonic growth, and survival (Faulkner et al., 2013). Suboptimum circulating progesterone concentrations in heifers during this period are related to a delay in the endometrial changes, including the delay in the loss of progesterone receptors, which reduces uterus receptivity to embryo implantation (Forde et al., 2011). Conversely, progesterone supplementation, and consequent elevated circulating progesterone concentrations from day 5 to 9 of the estrous cycle, have been associated with conceptus development, related to pregnancy success in cattle after AI (Mann et al., 2006). Thus, optimizing circulating progesterone concentration during early embryo development is fundamental for the establishment of pregnancy.

Many studies have used different strategies of progesterone supplementation to increase its circulating concentrations during early embryo development in cattle; however, the use of these strategies has yielded inconsistent pregnancy results. Intravaginal controlled internal drug release dispensers have been used in embryo transfer programs to increase progesterone concentrations. The studies utilizing progesterone devices successfully increase progesterone concentrations and it seems that supplemented dairy cows are more likely to conceive, but

with no differences in pregnancy loss compared with unsupplemented animals (Garcia-Ispierto and López-Gatius, 2017; Steichen and Larson, 2019)

Other strategies to increase circulating progesterone concentrations have been tested, such as the induction of an accessory corpus luteum (**CL**) with gonadotropin releasing-hormone (**GnRH**) or human chorionic gonadorelin (**hCG**) during the first follicular wave (Santos et al., 2001; Nascimento et al., 2013). A recent study indicated that GnRH 5 days after synchronization of ovulation protocol induced an accessory CL in 84% of GnRH-treated heifers and increased serum progesterone concentrations on day 12 of the estrous cycle (García-Guerra et al., 2020). In that study, recipient dairy heifers of stage 7 embryos, expanded blastocyst, treated with GnRH on day 5 had lower pregnancy loss compared to untreated heifers (García-Guerra et al., 2020). Niles et al. (2019) treated heifers with 2,000 I.U. hCG on day 7 after artificial insemination (**AI**), reported an increase in serum progesterone concentration from day 11 to 67 compared with no treatment on day 7. In the same study, authors described a reduction of 12.5% in pregnancy loss between days 32 and 67, when hCG was used (Niles et al., 2019).

Human chorionic gonadotropin has also been used to optimize CL capacity to produce progesterone during the early luteal development. Administration of 3,000 I.U. of hCG 2 days after fixed time AI (**TAI**) increased circulating progesterone concentrations on days 7, and 14 in lactating dairy cows (Sánchez et al., 2018). Human chorionic gonadotropin has been used to increase circulating progesterone concentrations because it is a glycoprotein with alpha and beta-subunits that acts similarly to luteinizing hormone (**LH**). The beta-subunit is required to confer the specific biological activity of hCG, as it has high homology (~80%) with the LH beta-subunit (Humaidan et al., 2005; Stenman et al., 2006). Human chorionic gonadotropin binds directly to LH receptors in follicular or luteal cells, and it is more effective in inducing ovulation and increasing steroidogenesis and circulating progesterone concentrations in dairy cattle than GnRH (Binversie et al., 2012; Cabrera et al., 2021).

Although compelling evidence that the accessory CL induction by GnRH and hCG increases circulating progesterone concentrations, its effects on fertility are still undefined in recipient dairy heifers and lactating dairy cows. The overall objective of this doctorate thesis is to determine the effects of GnRH and hCG in the beginning of the estrous cycle, e.g., days 0, 5, and 7, on the fertility of recipient dairy heifers and lactating dairy cows. The specific objectives are: 1) determine the differences between GnRH and hCG in their efficiency of inducing an original CL, on luteal dynamics and on pregnancy per embryo transfer in

recipient dairy heifers receiving fresh embryos; 2) determine the day 5 accessory CL induction by hCG on luteal dynamics and pregnancy per embryo transfer in recipient dairy heifers receiving fresh embryos and; 3) determine the effects of accessory CL induction on day of ET by hCG or GnRH on pregnancy per embryo transfer in recipient dairy heifers and lactating dairy cows receiving *in vitro* produced embryos. The overall hypothesis of the present thesis is that recipient dairy cattle receiving hCG on the last day of a 5d-CIDR synch protocol have greater circulating progesterone concentration during metestrus and diestrus compared with GnRH-treated heifers, and the animals with an accessory CL induced by hCG have greater progesterone, as well as greater pregnancy per embryo transfer than those receiving GnRH.

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2 CHAPTER 1

LITERATURE REVIEW

Introduction

Progesterone (**P4**) is a steroid hormone derived from cholesterol, that has as its main source a high-dense lipoprotein (**HDL**) in ruminants. The P4 production process is driven by the luteinizing hormone (**LH**) surge and the increase of the P450 cholesterol side chain enzyme (**P450scc**) and 3-ß hydroxysteroid dehydrogenase enzyme (**3ßHSD**) in luteal cells. The P4 production is primarily modulated by P450scc, which catalyzes the conversion of cholesterol to pregnenolone in the inner of mitochondria (Diaz et al., 2002). Once pregnenolone diffuses from mitochondria to the smooth endoplasmic reticulum, it is converted to P4 by the 3ßHSD enzyme (Diaz et al., 2002; Wiltbank and Pursley, 2014). Circulating P4 concentrations in cattle are the result of the balance between its production and metabolization (Wiltbank et al., 2014). The P4 metabolism occurs in other tissues in ruminants, but it is mostly driven by the liver being dependent on the hepatic blood flow in heifers and cows. High feed intake, characteristic of high-producing dairy cows, has a positive correlation with post-prandial liver blood flow increases and consequent P4 metabolization and clearance occurring immediately after the high feed intake in cows (Sangsritavong et al., 2002; Doyle et al., 2019).

The circulating P4 concentration in cows begins to increase when follicular cells from ovulatory follicle luteinize into large luteal cells. The modification of granulosa cells into luteal cells occurs after the LH surge and the consequent development of the corpus luteum (**CL**; McNatty, 1979; Wiltbank et al., 2014). The increase in circulating P4 concentration occurs in luteinized follicles in cows, even when ovulation does not occur (Kesler, 1981; Brown et al., 1982; Hernandez-Ledezma et al., 1982). Despite the effect of low circulating pre-ovulatory LH concentrations in decreasing ovulatory follicle size and increasing the number of follicles undergoing ovulation (Gomez-Leon et al., 2022), pulsatile LH has no apparent effect on P4 production (Baird, 1992; McNeilly et al., 1992). After ovulation and CL formation, circulating P4 concentrations increase gradually and stay elevated despite the lower circulating LH concentrations (Ginther et al., 2001).

Circulating P4 concentrations increase gradually during the first 3 days post-ovulation (Lonergan and Sánchez, 2020). Between days 3 and 5 post-ovulation, the P4 concentrations in the oviduct ipsilateral to the CL can be greater than those contralateral (Cicinelli et al., 2009; Takahashi et al., 2016). Additionally, the oviduct contains nuclear P4 receptors in the ampulla and isthmus and P4 receptors components in the membrane. This suggests that P4 may

control the muscular contraction in the oviduct and the gamete transport during the first days after ovulation (Saint-Dizier et al., 2012; Bylander et al., 2015). Conversely, direct effects on gamete development were not yet determined (Wiltbank et al., 2016; Lonergan and Sánchez, 2020). Between days 3 and 6 after ovulation, circulating P4 concentrations increase approximately 3-fold in heifers and cows, from ~1 ng/mL to ~3 ng/mL. Circulating P4 concentrations increase steadily until approximately day 14 of the estrous cycle decreasing more abruptly after luteolysis (Sartori et al., 2004; Wiltbank et al., 2016). Luteal weight, volume, area, and histomorphology are highly correlated with circulating P4 concentrations (Maciel et al., 1992; Singh et al., 1997; Rocha et al., 2019).

Adequate circulating P4 concentrations on the first days of the estrous cycle are crucial to the development of the conceptus. Greater circulating P4 concentrations during the first days of pregnancy are associated with increased uterine hystotroph synthesis, conceptus length, and the chance of embryo survival (Forde et al., 2009a; Faulkner et al., 2013; Wiltbank et al., 2016). This review will highlight the importance of circulating P4 concentrations on conceptus development during the pivotal period of early pregnancy establishment in dairy cattle.

Early bovine conceptus development and progesterone

Pregnancy establishment in bovine only occurs after a series of extremely well-synchronized events. After ovulation and ensuing fertilization in the oviduct, the bovine embryo undergoes the first mitotic cleavage division. Approximately on day 4, a cleavage embryo with 16 cells, morula, is directed to the uterus. Afterward, on day 7 the embryo acquires an inner cell mass, a single layer of trophectoderm containing a fluid-filled blastocoel cavity in a spherical shape, forming a blastocyst (Guillomot, 1981). During the blastocyst stage, embryos are autonomously regulated and do not need a maternal environment to develop. This does not suppress the fact that the maternal environment positively influences the quality of embryos parameters (Rizos et al., 2002). Previously, authors have observed ~1% more in vivo-derived bovine embryos developing to blastocysts compared to in vitro-derived, demonstrating a positive effect of the maternal environment on early embryo development (Rizos et al., 2002). In fact, embryos produced in postpartum dairy cows had a compromised development compared to embryos produced in nulliparous heifers

(Rizos et al., 2010), and flushed nonlactating cows had a greater number of blastocyst recovered and higher metabolites associated with fertility, such as insulin-like growth factor 1 (**IGF1**), than in postpartum dairy cows (Maillo et al., 2012). Moreover, pre-hatching embryos induce changes in the uterine environment of lactating cows. The presence of embryos in the first week of development induced changes in the concentrations of lipoxygenase-derived metabolites, amino acids, biogenic amines, acylcarnitine, and phospholipids signaling. These changes are local and may play an important role in pregnancy success (Sponchiado et al., 2019).

On day 8 or 9 of the estrous cycle, after hatching from the zona pellucida, the blastocyst assumes an ovoid shape. Between days 12 and 14 embryos start to elongate, and the conceptus secretes Interferon-τ (**IFNT**), preventing the luteolysis triggered by Prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**) synthesis through the inhibition of the activation of oxytocin receptors (Guillomot, 1981; Guillomot and Guay, 1982; Robinson et al., 1999). The bovine embryo starts to change its morphometry from a day 7 spherical blastocyst to a usually thin-walled or expanded, and sometimes already hatched, structure on day 8. On day 9, the bovine embryo expands to a spherical shape with a well-defined inner cell mass. The flat inner mass inner is composed of hypoblast and epiblast cells (Brandão et al., 2004). After that, the conceptus changes from a blastocyst to an ovoid structure on days 12-13; then, to a tubular form between days 14 and 15, until it finally achieves a filamentous form on days 16-17 (Degrelle et al., 2005). The bovine conceptus grows exponentially from day 9 to 19. The process involves increases in the weight and length of the trophectoderm which doubles in length from day 9 to 16. The conceptus was described as having ~2 mm on day 12, ~6 mm on day 14, ~60 mm on day 16, and ~200 mm or more on day 19 of development (Betteridge et al., 1980; Berg et al., 2010). This elongation is important for the attachment process around days 18-19, maintenance of the CL, and the consequent successful establishment of gestation provided by the elongated conceptus signaling (Wiltbank et al., 2016).

Studies have shown differences in conceptuses length during the first days of gestation. Berg et al. (2010) demonstrated that 14 days-old in vitro-produced (**IVP**)-embryos recovered from heifers were larger than embryos recovered from lactating dairy cows at the same gestational stage. The mechanisms for those differences in conceptus length between heifers and lactating cows was previously proposed by Sartori et al. (2002, 2004). The authors did not measure the size of the embryos, but lactating dairy cows that had an inferior

embryonic development measured by embryo quality, on the first days of gestation (day 5 of the estrous cycle) also had lower circulating P4 concentrations in comparison to dairy heifers (Sartori et al., 2002, 2004). Forde et al., (2011a) concluded that circulating P4 concentrations affect the endometrial transcriptome during the estrous cycle in lactating cows. The lower P4 concentrations were associated with reduced uterus capacity to support embryo development after embryo transfer (**ET**) on day 7 of the estrous cycle (Forde et al., 2011a). Moreover, greater circulating P4 concentrations increase the expression of genes associated with energy sources or contributors to embryo hystotrophs, indicating a P4-endometrial regulation on conceptus development (Forde et al., 2009b).

Additionally, lower circulating P4 concentration on the first week of gestation was associated with lower IFNT synthesis on day 14 in dairy cattle, due to delayed conceptus elongation. Otherwise, augmented circulating P4 concentrations may increase the synthesis of IFNT with an effect on conceptuses or because of the permissive effect on the expression of IFN-stimulated genes (**ISG**), which modulate the maternal immune system, and the maternal recognition of pregnancy or MRP (Bazer et al., 2011; Forde et al., 2011a). Interferon-τ is the major protein produced from the elongated embryo trophectoderm cells. IFNT production begins on days 10-12 of embryo development and peaks around days 15-17 of pregnancy, and it is essential for the MRP around day 16 (Hansen et al., 1985; Imakawa et al., 2017). Moreover, this pivotal period of pregnancy, between days ~7 and ~19, requires all those well-orchestrated steps described so far, which provides an adequate environment for pregnancy establishment (Brandão et al., 2004; Bazer et al., 2011; Forde et al., 2011a).

Later, approximately on days 20-22, the conceptus attachment to the endometrial epithelium is characterized by the development of a maternal-conceptus interface (caruncles). After that, focal villous aggregation (cotyledons) appears and attaches to the maternal uterine caruncles to form placentomes. Placentomes increase in size and become the structures responsible for supplying nutrients and metabolic fetal-maternal exchanges involving the conceptus and the dam throughout pregnancy (Atkison et al., 1984; Haeger et al., 2016). Before its development, the conceptus nutrition is supplied by the yolk sac, a structure that develops between days 18 and 23 of gestation (Assis Neto et al., 2010). At the attachment, the trophectoderm conceptus cells proliferate, and the adhesion between the trophectoderm and endothelium epithelium occurs. This adhesion is a marker for the differentiation of trophectoderm cells into giant binucleate cells (**BNCs**). The BNCs migrate and fuse with

endometrial epithelium forming a temporary syncytium. Those BNCs differentiate into trinucleate cells releasing their granulomas and cytoplasm into the maternal tissue and circulation (Guillomot, 1981, 1995; Guillomot and Guay, 1982). These secretions are called pregnancy associated glycoproteins (**PAGs**). Pregnancy associated glycoproteins are glycoproteins essential for the establishment and maintenance of pregnancy (Wallace et al., 2015).

Circulating PAG concentrations have been related to embryonic death. Pohler et al. (2013) investigated the relationship between circulating PAG concentrations and embryonic survival during early gestation. Authors demonstrated that pregnancy loss is highly correlated to PAG concentrations. The authors also demonstrated that there is a significant increase in PAG concentrations between days 22 and 24 of conceptus development and that increased PAG concentrations on day 28 lead to a greater chance for pregnancy maintenance through day 72 of gestation. Other authors have shown that PAG concentrations were reduced on day 28 in heifers that lost pregnancy on day 44 of gestation (Reese et al., 2019). None of these authors reported a decrease in circulating P4 concentrations at the same time as circulating PAG concentrations around day 28 of gestation (Pohler et al., 2013; Reese et al., 2019). In those studies, mean circulating P4 concentrations remained elevated and similar in animals that maintained pregnancy or those losing pregnancy after 6 weeks of gestation. Interestingly, some authors have suggested a luteotropic effect of PAGs in cattle. Those authors have tested the use of PAGs, specifically the pregnancy-specific protein B (**PSPB**), in vitro on bovine luteal cells. They have shown that PSPB increases progesterone in cultured luteal cells and also increases prostaglandin E_2 (**PGE_2**), a luteotropic and anti-luteolytic prostaglandin (Del Vecchio et al., 1996; Arosh et al., 2004).

Circulating PSPB concentrations have been recently described as a possible marker for embryo attachment. The PSPB concentration increases after day 23 of gestation in pregnant nulliparous heifers and pregnant cows, while non-pregnant animals maintain the average optical density of PSPB concentration from day 15 to 35 after artificial insemination (**AI**). This increase in PSPB may be a marker of embryo attachment in those animals (Middleton et al., 2022). Moreover, circulating PSPB concentration has already been proposed as a determinant for pregnancy in cattle between days 28-31. Recently, authors indicated that the circulating PSPB concentration can be used accurately to determine pregnancy in cattle (Martins et al., 2018; Middleton and Pursley, 2019). It was demonstrated that at day 28 post-

AI in lactating dairy cows pregnancy was positively diagnosed when circulating PSPB concentrations were above or equal to 60 ng/mL (Martins et al., 2018).

Following conceptus development, the active placentation period starts after day 28, with endometrium remodeling and binucleate cell migration. The link between maternal endometrium and the fetal trophoblastic-cell layer on day 30 of gestation is tenuous and easily discontinued (Melton et al., 1951; King et al., 1979). From day 30 of gestation, the placentome development is characterized by the growth in strengthening maternofetal connection and size up to day 70 post-AI and also by the remodeling of its structure. At about day 60 of gestation the yolk sac disappears, and the conceptus nutrition is assumed completely by the placentomes. On day 70 of gestation, the development of the placentomes guarantees a firm environment and connection between the fetus and maternal tissue (Assis Neto et al., 2010).

Luteolysis

Luteolysis is the physiological process of luteal regression mediated by $PGF_{2\alpha}$ that occurs in a non-pregnant uterus and culminates in a decrease of circulating P4 concentration. The uterus secrets $PGF_{2\alpha}$ pulses beginning on days 14-15 of pregnancy in cows, but luteolysis requires the occurrence of episodic surges of $PGF_{2\alpha}$ (Bazer et al., 1986; McCracken et al., 1999). Schramm et al., (1984) have demonstrated that low doses of $PGF_{2\alpha}$ (0.9mg/mL) infused in placental lactogen and administrated once daily for 9 hours on day 12 of the estrous cycle was not able to cause complete luteolysis in ruminants allowing for the recovery of circulating P4 mid-cycle luteal concentrations, while 5 administrations as a bolus of $PGF_{2\alpha}$ over 25 hours successfully induced luteolysis. Later, Ginther et al., (2007, 2010) measured the metabolite of $PGF_{2\alpha}$, 13,14-Dihydro-15-Keto-$PGF_{2\alpha}$ (**PGFM**), during the estrous cycle and demonstrated that $PGF_{2\alpha}$ pulses lead to an apparent and significant decrease on circulating P4 concentrations in cattle. These pulses occur in an interval of 7 to 12 hours. Indeed, multiple pulses preceding a higher $PGF_{2\alpha}$ pulse from the uterus are needed to occur natural luteolysis, and this luteolysis is followed by a rapid drop of circulating P4 (Mezera et al., 2019).

Oxytocin is widely accepted as one of the triggers in the luteolytic process. Moreover, the expression of OTRs is equally important in luteolysis. On the other hand, as mentioned before, TFNT is a protective component against luteolysis. Additionally, other mechanisms

were investigated as part of luteolysis activation in bovines, such as estradiol (**E2**). The onset and high circulating E2 concentration may up-regulate the OTR, and later expression of estradiol receptors were associated with delayed OTR responsiveness (Araujo et al., 2009; Domingues et al., 2020). That mechanism may play an orchestrated role in the onset of the luteolysis mechanism.

In ruminants, natural luteolysis happens locally, involving the uterine horn and its ipsilateral ovary by a vascular countercurrent exchange system. The $PGF_{2\alpha}$ secreted by endometrium flows to the uterine vein and lymph system. Sequentially, $PGF_{2\alpha}$ diffuses from the uterus into adjacent ovarian and CL via the apposition of the uterine and ovarian arteries facilitated by the prostaglandin (**PG**) transporter protein (Mapletoft et al., 1976b; a; Heap et al., 1985). Once $PGF_{2\alpha}$ enters the ovarian tissue it binds to specific receptors and goes to the plasma membrane of luteal cells via its Ca^{++} channels to induce apoptotic events (Wright et al., 2014). This local pathway allows $PGF_{2\alpha}$ to exert its luteolytic action directly in the luteal cells without entering the systemic and pulmonary circulations. The systemic pathway could lead to an excessive inactivation of $PGF_{2\alpha}$ by the enzyme 15-hydroxyprostaglandin dehydrogenase (**PGDH**) and its metabolization to PGFM by the lungs, disabling the luteolytic process (Piper et al., 1970; Heap et al., 1985).

MRP

The MRP is a critical moment involving two independent biological systems, the conceptus, and the uterus. Pregnancy requires cross-talk between those two systems, with embryo signaling promoting CL maintenance and essential uterine metabolism (Thatcher et al., 1984; Wiltbank et al., 2016). If the MRP does not occur, luteolysis triggers the decrease of circulating P4 and a new estrous cycle (Spencer and Hansen, 2015).

The IFNT is a type I interferon that belongs to the family of cytokines (González-Navajas et al., 2012) produced exclusively by the trophoblast cells of ruminants embryos (Leaman and Roberts, 1992). It is secreted by the conceptus during the beginning of its development and is considered the major factor responsible for the occurrence of MRP in ruminants. The conceptus begins the secretion of IFNT on day 4 when cultured in vitro (Yao et al., 2009). In ruminant early gestation, IFNT was detected in vivo in low concentrations on days 10-12 and peaking on days 15-17 (Hansen et al., 1985; Wiltbank et al., 2016). Maternal

recognition of pregnancy in cattle occurs when IFNT reaches its maximum concentration, on day 16 post-conception (Forde et al., 2011b).

The major effect of IFNT on MRP is its antiluteolytic effect on the CL. IFNT down-regulates the oxytocin receptor (**OXTR**) gene in the endometrial luminal and superficial glandular epithelium. The lack of OXTR expression inhibits the production and secretion of $PGF_{2\alpha}$ by the endometrium. Oxytocin (**OT**) is produced by the CL and the hypothalamus and is stored in the neurohypophysis. When OT binds to the OXTR, $PGF_{2\alpha}$ synthesis and consequently luteolysis are inhibited (Flint and Sheldrick, 1983; Robinson et al., 1999; Dorniak et al., 2013). Moreover, IFNT stimulates the expression of ISG induced in the uterine endometrium and many other sites, e.g., cervical, and vaginal tissues (Mansouri-Attia et al., 2009; Bazer et al., 2011; Kunii et al., 2018). Furthermore, IFNT is linked to a critical role in immunomodulation of the uterine environment with innate and adaptative responses (González-Navajas et al., 2012). Interferon-τ protects the uterine and consequently embryo against viral and other infections that may occur during pregnancy. Additionally, the combination of IFNT and P4 production leads to the prevention of luteolysis, allowing the establishment of MRP (Niswender et al., 2000). Carvalho et al., (2017) demonstrated that circulating P4 concentration during MRP may not be dependent of IFNT production, but high P4 concentration during this period is likewise important to the successful conceptus development. The authors performed one trial with lactating cows with low P4 concentrations and reported that conceptus in a low P4 environment had delayed development in pregnant cows compared to conceptus in a high P4 environment, even IFNT did not change (Carvalho et al., 2017).

Summarizing, the failure in the MRP by the inadequate communication between the conceptus and maternal tissue leads to failure of CL maintenance and consequent luteolysis and a decrease in circulating P4 concentrations (Spencer et al., 2016).

Strategies to increase progesterone in recipient dairy heifers and cows

The circulating P4 increment has been associated with advancement in conceptus development in ruminants. Additionally, increases in circulating P4 were associated with increases in INFT and conceptus survival (Lonergan and Sánchez, 2020). Many studies have used different strategies to improve circulating P4 concentrations in heifers and lactating dairy

cows across recent years, reporting positive, neutral, and negative results in the establishment of pregnancy in cattle (Lonergan, 2011; Wiltbank et al., 2016; Besbaci et al., 2020). In trials aiming to increase circulating P4, some strategies have been used, such as, supplementing exogenous P4 via intramuscular injection, via intravaginal dispensers (**CIDR** and **PRID**; Forde et al., 2009b), and endogenous P4 supplementation by ovulatory inducers, GnRH and hCG (Vasconcelos et al., 2011; Niles et al., 2019; El Azzi et al., 2022). These strategies were used in different dairy cattle categories, nulliparous heifers and lactating dairy cows and their subcategories, such as primiparous and multiparous (Garcia-Ispierto et al., 2016; García-Guerra et al., 2020; El Azzi et al., 2022). Moreover, P4 supplementation strategies were used during AI and ET reproductive programs in dairy cattle (Monteiro et al., 2014, 2015; García-Guerra et al., 2020).

There have been many studies in cattle using strategies to increase circulating P4 to increase pregnancy odds by artificial insemination (**P/AI**). Many of those studies reported that the increment of P4 concentration was successful in increasing fertility post-AI. For the assessment of fertility, a large subset of studies was analyzed in a meta-analysis (Besbaci et al., 2020). Besbaci et al., (2020) analyzed 52 articles (from 1982 to 2019) using GnRH (33 trials), hCG (29 trials), or both (GnRH or hCG, 10 trials) during the luteal phase (days 4 to 15 after AI) in heifers and cows' post-insemination. The aggregation of studies demonstrated that the relative risk for P/AI is 7% higher by P4 supplementation, as well as for the use of hCG or GnRH as options to increase circulating P4 in cows. Moreover, when herd fertility was included in the model, the chance of P/AI increased to 17% compared with no treatment. Thus, increasing P4 in the luteal phase after AI has different effects when herds are stratified by fertility history. Cows with good fertility had 22% less chance to impregnate than cows with very-low fertility. Moreover, the author also evaluated the effect of treatments by parity and observed that the effect of higher circulating P4 concentrations after AI in primiparous increased by 13% the risk for P/AI compared to no treatment. This higher risk of P/AI linked to P4 increment strategies seems to be clear in dairy cattle.

Taken together, the results of a large number of studies using AI followed by GnRH and hCG treatments during the luteal phase seems to be a reliable strategy in dairy cattle breeding programs (Besbaci et al., 2020). The explanation that corroborates these benefits of P4 use in AI should be related to adequate P4 concentrations during embryo development, leading to increasing embryo survival and development, especially elongation, and adequate

TNFT production which improves attachment and MRP (Mann et al., 2006; Lonergan, 2011; Lonergan et al., 2016).

Fewer studies, compared to AI, have tested the effect of increasing circulating P4 prior to ET in dairy cattle (Lonergan and Sánchez, 2020; Cunha and Martins, 2022). Even with the same physiological mechanism involving strategies to increase P4 in recipients in relation to post-insemination animals, P4 supplementation in recipients does not present comparable consistent results among the studies. Progesterone supplementation was reported in studies with positive (Garcia-Ispierto et al., 2021), negative (Monteiro et al., 2015), or no effects (Vasconcelos et al., 2011; Niles et al., 2019; Steichen and Larson, 2019) on P/ET in dairy cattle. The challenges involved in ET involve parity differences responses (García-Guerra et al., 2020), also reported in AI programs (Vasconcelos et al., 2011), in addition to synchrony between embryo and recipients. These inconsistent results are challenging and lead to the need for more research aimed at improving P/ET using P4 supplementation strategies in dairy cattle.

In order to characterize the data based regarding P4 supplementation in recipient dairy heifers and cows receiving ET a term co-occurrence map was performed (VOSviewer, version 1.6.10; Figure 1). Term co-occurrence is used in scientometrics to investigate conceptual structures in research fields. The term co-occurrence relatedness is based on the analysis of the frequency with which the term co-occurs in scientific publications. The co-occurrence map is built on cluster structures and the distance-based is shown according to their relatedness in a similarity matrix (van Eck and Waltman, 2010, 2018).

Data were obtained in 2022 from the Scopus database in bibliography research with scientific research articles ranging from 1974 to 2022. The following query was used: “Cattle” and “Recipients” and “Progesterone” and excluded Note, Review, and Conference document types. The query returned 139 articles. The author keywords from the returned articles were used to analyze occurrences to create the co-occurrence network map (Figure 1). This process included a total of 266 terms with 66 containing at minimum 2 occurrences to minimize the appearance of disconnect reproductive publications. All of 66 terms were selected on a relevance score by the software and were utilized in the co-occurrence network map. The labels and circle sizes are proportional to the number of occurrences, lines identify major links between terms, and line thickness represents association strength. The distance between terms also reflects association strength. The colors of clusters (Cluster 1: red; Cluster

2: green; Cluster 3: blue; Cluster 4: yellow) synthetases association strength between terms (Figure 1A) and identifies the normalized average year (from dark blue to yellow) of terms occurrence in articles (Figure 1B; van Eck and Waltman, 2018).

The co-occurrence map (Figure 1A) shows “embryo type”, “cattle”, “progesterone”, “pregnancy”, and “corpus luteum” as major terms occurring. Moreover, into clusters, the most apparent terms are “embryo type” (red), “progesterone” (green), “pregnancy” (blue), and “superovulation” (yellow). Cluster 1 (red) indicates a large-scale interest in the protocol using ovulatory inducers hCG and GnRH e.g., in dairy cows and its possible effects on corpus luteum, but this research field appears far from terms linked with embryo terms such as “embryo” (blue), “recipient” (blue), “uterus” (blue), “endometrium” (blue), and “IVP” (yellow). Additionally, “CIDR” (green) appears more closed to “beef cattle” (green) than “dairy cows” and differently, “dairy cows” (red) are more linked to “heat stress” (red) and “artificial insemination” (red) than with embryo produced terms (blue).

The fact that “dairy cows” or “lactating dairy cows” and hCG or GnRH are separate from embryos terms by different clusters highlights the need for more publications linking those in a same field of research. This could be inferred also by few publications reported in recent reviews (Lonergan, 2011; Cunha and Martins, 2022) as described previously. In the next section the use of CIDR, hCG and GnRH will be described to increase circulating P4 concentration prior to ET and its effects on P/ET.

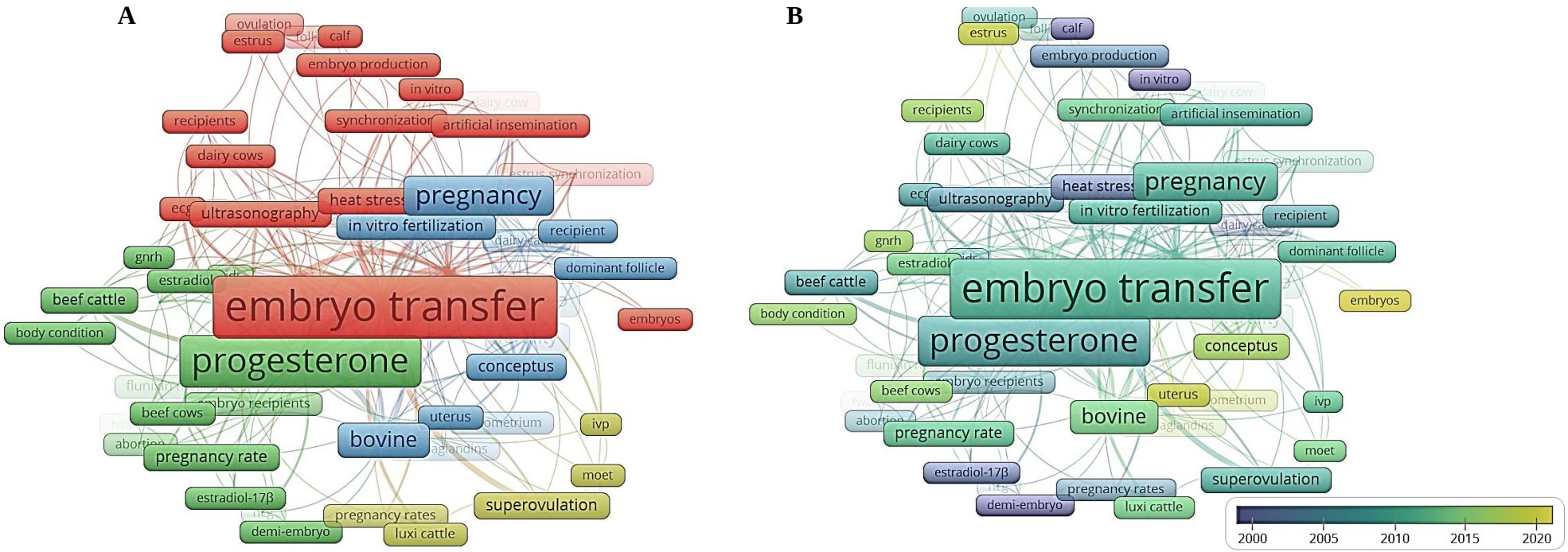

Figure 1. Term co-occurrence network maps (**A** and **B**) of the specific search term "cattle" and "recipients" and "progesterone" in the Scopus database in research articles. The labels and circle sizes are proportional to the number of occurrences, lines identify major links between terms, and line thickness represents association strength. The distance between terms also reflects association strength. Some term labels are not displayed because of scale e.g., **A:** Color identifies cluster of terms (Cluster 1: red; Cluster 2: green; Cluster 3: blue; Cluster 4: yellow). **B:** Colors identify the normalized average year of terms occurrence in articles. Earlier research terms are colored blue and more recent ones are in yellow. The normalization was performed by the software subtracting the term average from the overall mean and dividing it by the standard deviation.

Use of CIDR to increase circulating progesterone concentration and pregnancy in recipient dairy heifers and cows

Intravaginal devices containing P4 have been used prior to ET to increase circulating P4 concentrations in dairy recipient heifers and cows (Clemente et al., 2009; Monteiro et al., 2015; Steichen and Larson, 2019). Kenyon et al. (2013) studied the effect of inserting CIDRs during the beginning of conceptus development in different periods aiming to analyze the probability of pregnancy in lactating dairy cows in a constant circulating P4 releasing after ET. The authors utilized new CIDRs replaced every 7 days, in a total of 21 days with CIDR insertion after ET (day 7) in 28 cows previously receiving $PGF_{2\alpha}$ on days 4 and 5 after ovulation. Control (n= 153) animals did not receive any treatment. Authors reported that animals with circulating P4 concentration fold change between days 0 and 7 were more associated with chance of pregnancy on day 42, while cows with P4 <5ng/mL between days 0 and 14 were more likely to suffer pregnancy losses from day 28 to 63.

Indeed, embryo exposure to a uterine environment of cows previously treated with PRID, a similar CIDR dispositive (van Werven et al., 2013), to increase circulating P4 seems to increase the chance of conceptus survival and the establishment of pregnancy (Carter et al., 2008, 2010; Clemente et al., 2009). Clemente et al. (2009) started recipient cows on PRID treatment on day 3 of the estrous cycle. The IVP fresh embryos were transferred on day 7. The intravaginal device increased circulating P4 between days 3 and 6 of the estrous cycle but P4 concentration dropped after day 7. The authors observed a positive effect of P4 on conceptus length on day 14 of development. Day 14-embryos recovered from P4-treated cows were ~3-fold larger than those of untreated cows. Results from similar P4 treatments on embryo length between days 13 and 16 were demonstrated by other authors (Carter et al., 2008). Even though these authors had significant results regarding the survival characteristics of embryos, none of them were designed to achieve pregnancy.

Monteiro et al. (2015) performed a series of experiments using CIDR aimed to access the circulating P4 concentration effect on the fertility of lactating dairy cows. Cows were randomly assigned to 3 groups: control, CIDR between day 4 and 7 after estrus detection, and CIDR from day 4 after estrus detection until 10 days after ET. Embryos were transferred 7 days after estrus detection. Surprisingly, P/ET was negatively affected by higher circulating P4 concentrations induced by day 4-CIDR insertion in dairy cows. Animals receiving CIDR for 3 days prior to ET had ~45% less P/ET, and cows with CIDR for 14 days had ~60% less

P/ET than controls on day 32. These differences in P/ET were observed also on the last pregnancy diagnosis on day 88. The same experimental design in TAI increased P/AI in primiparous cows compared to multiparous cows, suggesting a positive interaction effect of P4 supplementation and parity on MRP. Recently, Steichen and Larson (2019) performed a similar ET trial with a total of 452 recipient Holstein heifers randomly assigned to 2 groups: control, and CIDR insertion for 12 days after IVP-frozen embryo transference on day 7 post-ovulation. Pregnancies per transfer were similar between groups, with no effect on additional parameters evaluated, such as embryo quality grade, embryonic development stages, or P4 concentration.

The lack of consistent positive results in the studies reported above may be related to the asynchrony between uterine changes induced by P4 and embryo developmental status (Lonergan and Sánchez, 2020). Moreover, the use of intravaginal progesterone devices in recipient dairy heifers and cows would be associated with increased chance of vaginitis in some animals and consequently risk of uterine infection at the time of ET (Ahmadi et al., 2007), and perhaps embryo losses further into gestation.

The use of human chorionic gonadotropin and GnRH to increase circulating progesterone concentrations in recipient dairy heifers and recipient lactating dairy cows

Gonadotropin-releasing hormone

Gonadotropin-release hormone is a decapeptide produced by the hypothalamus. From over 20 decapeptides' different forms, three were identified in mammals. The GnRH I form regulates gonadotropins, in the classical hypothalamic-pituitary-gonadal axis, and GnRH II regulates sexual behavior, acting in extra-pituitary sites in mammals. GnRHs also act in reproductive tissues, with direct effects on primary, secondary, and tertiary follicles, also granulosa cells, and on luteal tissue (Millar, 2005). A GnRH-like molecule was identified in the granulosa cells of bovine follicles (Aten et al., 1987; Ireland et al., 1988). In follicular granulosa cells, GnRH has a dose-dependent action in the conversion of androgen to estrogen by aromatase (Janssens et al., 2002).

GnRH is produced by enzymatic processing in the hypothalamus, in the preoptic area, packaged in cellular storage granules, and transported down the axons to medial basal

hypothalamus-median eminence, to be released on target tissue via portal vessels. The GnRH release occurs each 30-120 minutes stimulating LH and FSH secretions from the pituitary gonadotrophs. GnRH binds to the specific-gonadotroph cell receptors and starts a sequence of events culminating in the release of LH and FSH. The sequence of events includes micro-aggregation and internalization of GnRH receptors, activation of second messengers, transduction of signals, *de novo* synthesis, and release of LH and FSH (Gibson et al., 1984; D'Occhio et al., 2000; Millar, 2005). Elevated circulating LH concentrations induce ovarian follicle maturation, leading to ovulation. The regulatory mechanism of GnRH synthesis and surge involves the increase of circulating E2 released by the dominant follicle and the decrease in circulating P4 as a result of luteolysis. Moreover, during the luteal phase, high P4 circulating concentrations inhibit the pulsatile release of GnRH (Chenault et al., 1975; Stumpf et al., 1991). Additionally, elevated circulating GnRH when it is administrated leads to an ovulatory response even in a high P4 circulation environment in dairy cattle, suppressing its inhibitory P4 effect on ovulation (Kittok et al., 1973; El Azzi et al., 2022).

The use of exogenous GnRH has been increasingly adopted in TAI programs in dairy cattle. In addition, the use of GnRH in breeding programs has become usual due to its efficiency on increasing ovulatory responses in proestrus cows, induction of accessory CLs, and also due to improvements in anovulatory conditions (Wiltbank and Pursley, 2014).

Human Chorionic Gonadotropin

Human chorionic gonadotropin is part of the glycoprotein hormone family and comprises the α and ß-subunits. The α subunit is similar among glycoprotein-based hormones, while the ß-subunits confer biological activity to hCG. The similarity between hCG and LH subunits gives them high homology (~80%), hence the ability of hCG to induce ovulation (Humaidan et al., 2005; Stenman et al., 2006; Theofanakis et al., 2017). The hCG has a half-life, 36 hours longer than that of LH (10-12 hours), thus it has greater functional activity on luteal cells (Chenault et al., 1975; Niswender et al., 1985; Theofanakis et al., 2017), characteristic observed when hCG is used as accessory CL inducer and as well luteotropic hormone on original CL (El Azzi et al., 2022). Biological endogenous hCG, in women, has the luteotropic function of LH in maintaining adequate P4 concentrations during early pregnancy (Casarini et al., 2018). hCG binds directly to the LH receptors in the

follicular cells with a strong binding affinity to the LH granulosa cell receptors, or CL, which allows this hormone to be used as an ovulatory inducer in ruminants by inducing ovulation and increasing steroidogenesis and circulating P4 concentrations in lactating dairy cows and dairy heifers (Binversie et al., 2012; Theofanakis et al., 2017).

To demonstrate the effect of hCG on luteal cells of original CL, Helmer and Britt (1986, 1987) administrated hCG between days 2 and 4 of the estrous cycle of dairy heifers (n =48; n =9, respectively) and cows (n =110 Jerseys and 105 Holsteins). Animals receiving hCG had greater circulating P4 concentrations after day 7 of the estrous cycle, indicating an effect of hCG on the original CL. The effect of hCG on original CLs as an ovulatory inducer was investigated in cattle (Sianangama and Rajamahendran, 1996; Aslan et al., 2011). In these studies, Holstein heifers and cows were used and no differences in circulating P4 concentration, luteal size, or luteal blood flow were observed after treatments. Despite the lack of possible positive results probably due to the small number of animals, the hCG treatment was linked to a considerable ovulatory response, prompting further investigations of hCG on day 0 in dairy cattle. Indeed, the use of hCG in beef cattle, n =467, on day 0 of the estrous cycle promoted an ovulatory response of 97% (Geary et al., 2001), and an 83% ovulatory response in the early luteal phase (days 4-7) in beef cattle (n =12; Price and Webb, 1989).

The effect of hCG (3,300 I.U.) given on day 5 of the estrous cycle on luteal size was analyzed in a large group of dairy cows (n =406). Cows treated with hCG had greater luteal area and volume of the largest CL (original) than saline treated control cows (Santos et al., 2001). Additionally, hCG (2,000 I.U.) applied on day 5 of the estrous cycle increased the size of small and large bovine luteal cells in heifers (Schmitt et al., 1996a). The CL is mainly composed of two categories of cells: ~40% of steroidogenic large cells and ~30% of small cells of the total cellular tissue after completed CL maturation (O'shea et al.; Hansel et al., 1987, 1991). Small luteal cells are originated from a population of stem cells (Niswender et al., 1985). During the estrous cycle, the luteal cells turn into steroidogenic large cells, controlled by LH stimulation (Farin et al., 1988), and the large luteal cells are responsible for a great proportion of P4 production in the luteal tissue (Rodgers et al., 1985; Niswender et al., 2007). The proportion of steroidogenic large luteal cells increases after hCG treatment in comparison to small luteal cells; this explains the increase in luteal weight of hCG-treated animals (Farin et al., 1988). The increased luteal size cells in hCG treatments would be genotypically explained by the increased expression of genes such as periostin, a suggested

factor associated with ovulation, luteinization, and tissue remodeling (Lussier et al., 2017). Additionally, hCG increases, not only CL area or volume but also circulating P4 concentrations with a luteotropic effect on the original CL in nulliparous dairy heifers and lactating dairy cows (Cunha et al., 2022; El Azzi et al., 2022).

The use of hCG in dairy cattle has been mostly directed toward the induction of accessory CLs (Besbaci et al., 2020; Cunha and Martins, 2022). Induced ovulation with hCG after day 4 of the estrous cycle occurs only if the follicle is responsive (Price and Webb, 1989; Sartori et al., 2001). The follicle acquires detectable LH receptor mRNA on granulosa cells after day 4 of wave emergence when the follicle has an average diameter of 10.8 mm (Xu et al., 1995). Induction of ovulation by hCG is possible only after the ovulatory follicle acquires responsivity and it would delay the mechanisms of the luteolysis. Ovulation of the first-wave dominant follicle induces an advanced emergence of a second wave, hastening the onset of functional luteolysis (Marques et al., 2012; Cunha et al., 2022).

Price and Webb (1989) demonstrated that the administration of hCG (1,500 I.U.) during the early (day 4-7) or late (day 14-16) luteal phase was capable of successfully inducing an accessory CL in dairy heifers. Indeed, hCG-induced accessory CLs with 1,000 I.U. or higher doses (Cabrera et al., 2021). The induction of an accessory CL in response to 2,500 I.U. of hCG was also demonstrated by Cabrera et al. (2021), where doses of 2,500 or 3,300 I.U. had a greater ovulatory response on day 7 of the estrous cycle in dairy cows. The ovulatory response to hCG on days 5 or 7 of the estrous cycle leads to an increase in the CL area or volume, and circulating P4 concentrations on the second week of pregnancy in heifers and lactating dairy cows (Cabrera et al., 2021; El Azzi et al., 2022). The greater circulating P4 concentrations during the beginning of pregnancy are related to the increased viability of post-hatched embryos (Carter et al., 2008, 2010), higher pregnancy rates (Nascimento et al., 2013), and lower pregnancy losses (Niles et al., 2019).

GnRH and hCG as accessory CL inducers in recipients

GnRH and hCG treatments are used as ovulatory inducers with similar effects on ovaries when applied at the beginning of the estrous cycle in heifers and cows (Fricke et al., 1993; Stevenson et al., 2007; El Azzi et al., 2022). These hormones are capable of inducing ovulation, forming original and accessory CLs, and increasing circulating P4 concentrations in ET programs (Besbaci et al., 2020; Cunha and Martins, 2022). GnRH is well known as a

potent ovulatory inducer when applied in breeding programs, with a reported ovulatory response of 84.9% in primiparous and multiparous dairy cows (Fricke et al., 1998), and 98.2% in nulliparous heifers (El Azzi et al., 2022) on day 0 of the estrous cycle. The induction of an original CL was high using hCG on day 0 of the estrous cycle in a few studies (Aslan et al., 2011; Garcia-Ispierto et al., 2019; El Azzi et al., 2022). The ovulatory response on day 0-hCG treatment when animals had been previously synchronized was 98.1% (3,300 I.U.; El Azzi et al., 2022).

Cabrera et al. (2021) showed that the ovulatory response to hCG (92.9%; 2,500 I.U.) is greater than that of GnRH (79%; 100µg) on day 7 of the estrous cycle. The differences on ovulatory response using hCG or GnRH may be explained by the ovulation responsiveness to hCG even in individuals with high circulating P4 at the time of treatment (El Azzi et al., 2022). On the contrary, GnRH treatment in cows did not recover the LH surge magnitude in a high P4 environment in cows, independently of the dose (100 or 200µg), in comparison with a low P4 environment (Giordano et al., 2012). Regardless of the slight difference in ovulation rate at the beginning of diestrus, both treatments, GnRH and hCG, are considered efficient in inducing an accessory CL. (Niles et al., 2019; García-Guerra et al., 2020; El Azzi et al., 2022).

Both treatments, GnRH or hCG administration, seem to be efficient in inducing an accessory CL in nulliparous Holstein heifers (El Azzi et al., 2022). When GnRH was given to nulliparous heifers on day 5 after a 5d-CIDR synch program, the proportion of accessory CLs was 83.5% (García-Guerra et al., 2020). The proportion of accessory CL seems to be consistent with hCG as the ovulatory inducer on day 5 after a 5d-CIDR synch program. Recently, a positive hCG result of 98.7% ovulation in nulliparous heifers with at least one accessory CL was reported (El Azzi et al., 2022). Cunha et al. (2021) gave GnRH or hCG between days 5 and 7 of the estrous cycle in lactating dairy cows reporting lower accessory formation with GnRH (74%) than hCG (86%) as the ovulatory inducer.

The luteotropic effect of hCG was recently accessed by El Azzi et al. (2022). In that study, nulliparous heifers previously induced to ovulate an original CL with GnRH or hCG were submitted to receive hCG on day 5 after synchronization. The circulating P4 concentration at day 7 was significantly higher in heifers receiving hCG than controls, and this increase was assigned partially to the luteotropic effect on original CLs. Accordingly, hCG effectively induces accessory CL in heifers and lactating cows, increasing

steroidogenesis in the original CL (Helmer and Britt, 1987; Schmitt et al., 1996b; El Azzi et al., 2022). A similar circulating P4 increase on day 11 was observed when the accessory CL was induced on day 7 of the estrous cycle (Niles et al., 2019). The circulating P4 concentration increases by 20% when the accessory CL is induced on day 5 in nulliparous recipient heifers and lactating dairy cows compared to control groups (no accessory CL; García-Guerra et al., 2020; El Azzi et al., 2022). Moreover, P4 remains high between days 14 and 18 of the estrous cycle when the accessory CL is induced between days 5 and 7, (Torres et al., 2013; Niles et al., 2019; Cunha et al., 2022). The gains in the original and accessory CL areas were linked to higher serum P4 concentrations (Cabrera et al., 2021; El Azzi et al., 2022). These results, regardless of the luteotropic effect 2 days after hCG administration, are similar between GnRH and hCG in dairy cattle (El Azzi et al., 2022).

Effect of accessory CL induced with GnRH and hCG on recipient fertility

The effects of accessory CL induction on fertility are controversial, ranging from positive to none in dairy recipient cattle. The induction of an accessory CL was tested in a tropical environment, in Brazil, by Vasconcelos et al. (2011) and Pinto et al. (2015). In the first study, authors performed two trials to investigate the effect of GnRH (100µg) and hCG (2,500 I.U.) on day 7 after synchronization in TAI and on ET programs. The P/ET on days 28 and 60 were 45.2% and 37.4 %, respectively. The data were collected throughout the year with mean temperatures ranging from 11.7°C to 30.3°C in cows housed in ventilated freestall barns. The induction (at ET) of accessory CL improved P/ET compared with non-induced control animals, but the treatment did not affect cows with accessory CL in TAI programs. The authors concluded that a high environmental temperature would damage the embryo and be detrimental to embryo survival and ET programs. The utilization of an accessory CL induction may minimize those negative effects. In the second study, authors performed a trial during the spring (temperature ranging from 16°C to 35°C) inducing accessory CL on days 6-8 of the estrous cycle using GnRH and reported a low CL induction rate of 27.4%. Animals were randomly allocated to treatments without any synchronization protocol, receiving either 10µg of Buserelin acetate or 750µg of Deslorelin acetate on the day of ET. Even with the low number of animals (control n =92; Buserelin n =86; Deslorelin n =89) authors reported higher pregnancy rates on days 30 to 40 in dairy cows with Buserelin-induced accessory CLs in comparison with cows with no accessory CL (38.27% and 24.05%, respectively).

The induction of an accessory CL on day 7 with 1,500 I.U. of hCG utilizing demi-embryos was used to analyze its effect on embryos and pregnancy (Torres et al., 2013). The authors reported improvement on P/ET receiving hCG compared to non-treated animals (26% and 50%, respectively) but no differences on day 42 embryo size (overall mean size of 21.5 ± 2.3mm). García-Guerra et al. (2020) aimed to investigate accessory CL induction by GnRH (200µg of gonadorelin acetate) in dairy heifers after a 5d-CIDR synchronization program. The study indicated an 84% accessory CL induction in GnRH-treated heifers and increased serum P4 concentrations on day 12 of the estrous cycle. In the same study, recipient heifers receiving day 7 expanded blastocyst and treated with GnRH on day 5 had 42% lower pregnancy losses between day 33 and 60 of pregnancy compared to non-treated heifers (15.2% and 27.1%, respectively). Similar pregnancy losses (22.25%) were observed in day 6 blastocyst embryos (GnRH: 23.6%; Control: 20.6%).

Recently, Niles et al. (2019) treated heifers with 2,000 IU hCG on day 7 after AI, resulting in increased serum P4 concentrations from day 11 to day 67 compared with no treatment (day of ET). In the same study, authors described a reduction of 12.5% in pregnancy losses between days 32 and 67 when hCG was used after AI. Even though, P/AI was not affected by treatments. The effect of accessory CLs was reported in many studies, ranging from positive to none in dairy cattle (Table 1). Moreover, the administration of GnRH and hCG to induce an accessory CL seems to be affected by many factors, such as season, parity, embryo development stage, and cryopreservation processing as described previously.

Table 1. Descriptive P/ET in six studies using GnRH or human chorionic gonadotropin to induce accessory corpus luteum in recipient lactating dairy cows and recipient dairy heifers

Study	Parous	n	Embryo type	Pre-synch	Control	Control P/ET (%)	GnRH P/ET (%)	GnRH dose (µg)	hCG P/ET (%)	hCG dose (IU)	Administration day (d) relative to the estrous cycle	Difference of P/ET (%) (GnRH - hCG)	*P*-value (Control ^{x} treatment)	*P*-value (GnRH ^{x} hCG)
Vasconcelos et al. (2011)	Cows	372	Fresh and frozen flushed stages 4 to 7	Ovsynch EC + CIDR	Untreated	38.1	52.4	100	45.1	2,500	7	7.3	0.03	0.10
Pinto et al. (2015)	Cows	267	Frozen IVP	Estrus detection	Saline	24.1	38.3	10	-	-	6, 7 and 8	14.22	0.02	-
Torres et al. (2013)	Cows	61	Demi-embryos	Estrus detection	Untreated	26.0	-	-	50.0	1,500	7	-	<0.05	-
Niles et al. (2019)	Heifers	291	Fresh IVP	5d-CIDR synch	Untreated	48.0	-	-	43.0	2,000	7	-	0.47	-
García-Guerra et al. (2020)	Heifers	1,562	Fresh IVP stage 6 and 7	Modified 5d-CIDR synch	Untreated	34.6	35.3	200	-	-	5	-	0.78	-

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3 CHAPTER 2

STUDY 1

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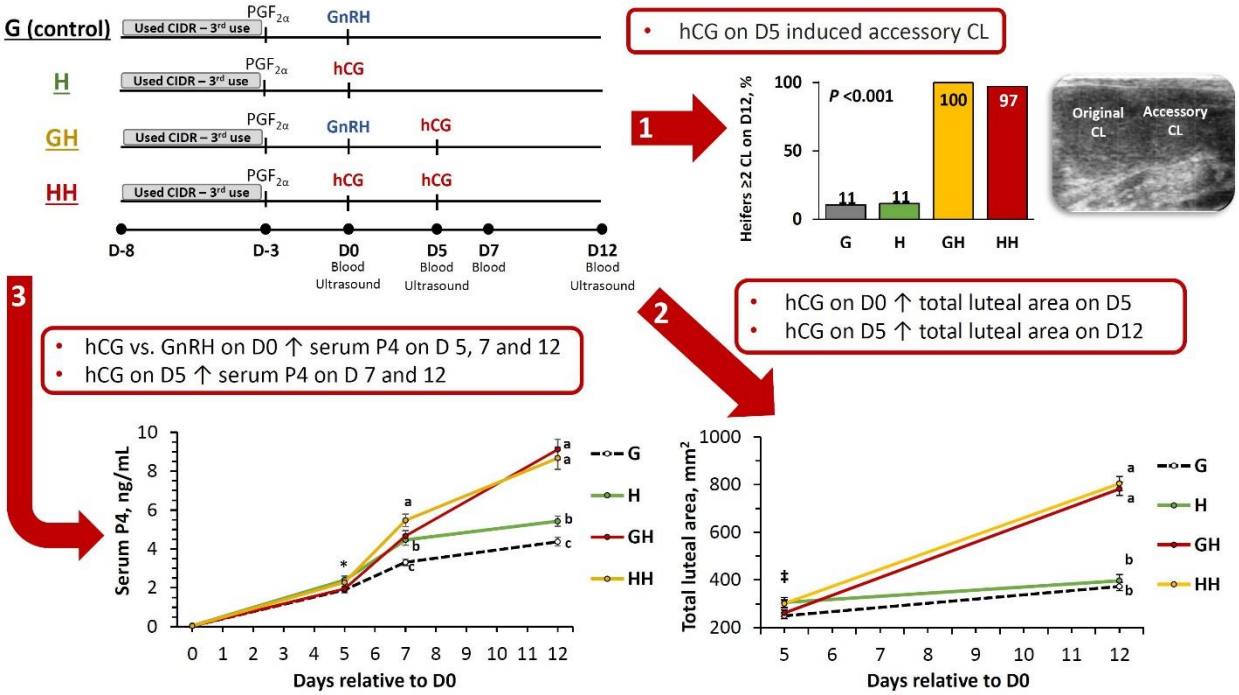
Graphical Abstract:



Highlights:

- hCG on the last d of the 5d-CIDR Synch (D0) enhanced original CL luteal area on D5
- hCG vs. GnRH on D0 increased serum P4 on D5, 7 and 12
- hCG on D5 induced accessory CL formation and increased serum P4 on D7 and 12
- hCG on D5 augmented original CL area on D12 compared with no hCG on D5
- hCG on D0 and 5 (HH) increased serum P4 on D7 compared with the other treatments

Summary: Our objective was to determine the effect of replacing GnRH with hCG on the last d of the 5d-CIDR Synch (D0) and inducing CL formation with hCG on D5 on serum progesterone concentration and luteal dynamics in dairy heifers. Heifers receiving hCG on D0 had a larger luteal area and greater serum progesterone concentration on D5 than heifers receiving GnRH on D0. Heifers that received hCG on D 0 and 5 had greater serum progesterone concentrations on D7 than the heifers in the other treatments. In addition, hCG on D5 promoted a greater proportion of heifers with ≥ 2 CL on D12 and a larger luteal area of the original CL, which resulted in a larger total luteal area on D12.

Running head: REPLACING GnRH WITH hCG IN THE 5-d CIDR-SYNCH

Effects of human chorionic gonadotropin on the last day of the 5-d CIDR Synch protocol and 5 d later on circulating progesterone concentrations and luteal area in Holstein heifers

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Abstract: The objective was to determine the effect of replacing GnRH with human chorionic gonadotropin (hCG) on the last day of the 5-d CIDR Synch protocol (d 0) and inducing accessory corpus luteum (CL) formation with hCG 5 d later (d 5) on serum progesterone (P4) concentrations and luteal dynamics in dairy heifers. Holstein heifers (n= 207) were synchronized with a 5-d CIDR Synch protocol (D-8: used CIDR inserted; D-3: CIDR removed and $PGF_{2\alpha}$). Heifers were randomly assigned to 1 of 4 treatments on d 0: control (G; n = 55), H (n = 50), GH (n = 53), and HH (n = 49). Heifers in G were treated with 100μg of GnRH on d 0, while H heifers received hCG (3,300 IU) on d 0. Heifers enrolled in GH were treated with GnRH on d 0 and hCG on d 5, while HH received hCG on D 0 and 5. Ovaries were scanned by ultrasound on D 0, 5, and 12, and blood was collected on D 0, 5, 7, and 12. Heifers that ovulated before or after the hCG or GnRH d 0 treatment and had P4 ≤ 0.50 ng/mL on d 0 were considered as synchronized. Overall protocol synchronization rate was 93.8.%, with no differences among treatments. Only synchronized heifers (n = 193) were included in the analyses of luteal dynamics after d 0. Serum P4 concentration and original CL luteal area on d 5 in heifers treated with hCG on d 0 (H+HH) were greater than in heifers treated with GnRH on d 0 (G+GH). Almost all heifers treated with hCG on d 5 had ≥ 2 CL on d 12 (98.6%). Ovulatory response for d 5 hCG treatment did not differ for GH vs. HH (97.2 vs. 94.7%). Heifers in HH had the highest serum P4 on d 7, and G had the lowest serum P4 on D 7 and 12. In contrast, serum P4 on d 7 did not differ for H vs. GH. On d 12, serum P4 and total luteal area were not different for GH vs. HH. In summary, heifers that received hCG on d 0 had a larger total luteal area and greater serum P4 concentration on d 5 than heifers treated with GnRH on d 0. Moreover, hCG on d 5 promoted a greater proportion of heifers with ≥ 2 CL on d 12 and a larger luteal area of the original CL, which resulted in a larger total luteal area on d 12. The HH treatment successfully increased serum P4 concentrations in heifers on d 7 compared with the other treatments.

BODY OF THE PAPER

Optimizing circulating progesterone (**P4**) concentration during early embryo development is fundamental for the establishment of pregnancy. Previous studies used different strategies to increase circulating P4 concentrations during early embryo development and improve fertility of recipient cows and heifers (Rizos et al., 2012; Monteiro et al., 2015; Steichen and Larson, 2019). However, the use of those strategies has yielded inconsistent pregnancy results after embryo transfer (**ET**). One of the strategies tested was to induce an accessory corpus luteum (**CL**) with gonadotropin releasing-hormone (**GnRH**) or human chorionic gonadotropin (**hCG)** during the first follicular wave (García-Guerra et al., 2020; Niles et al., 2019). The fertility results from those studies may not be consistent because of a delayed increase in circulating P4 concentrations. Recently, Garcia-Ispierto et al. (2021) tested the use of hCG (3,000 IU) replacing GnRH (100 µg) at the last treatment of a 5-d CIDR Synch protocol in recipient lactating Holstein cows. Although the study sample size was small (n=120), hCG increased the odds (3.3) for pregnancy at 28 d compared with GnRH. In that study, the effect of hCG on luteal development and circulating P4 concentrations during the estrous cycle was not determined. The use of hCG instead of GnRH at the last treatment of synchronization programs may induce the formation of a CL with greater P4 synthesis capability and greater circulating P4 concentrations earlier, improving fertility of recipient heifers or cows.

Therefore, our study aimed to determine the effect of 3,300 IU of hCG administered on the last day of the 5d-CIDR Synch program (d 0) with or without an extra administration of hCG 5 days later (d 5) on luteal development and circulating P4 concentrations in metestrus and diestrus of Holstein heifers. The primary hypothesis was that heifers treated with hCG on d 0 would have greater total luteal area and serum P4 concentration during metestrus and diestrus compared with heifers treated with GnRH on d 0. The second hypothesis was that heifers treated with hCG on d 0 and d 5 would have greater total luteal area and serum P4 concentrations during diestrus than those treated with hCG only on d 0 or d 5.

All animal handling and experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison (#A006300). This experiment was conducted in a commercial dairy farm in WI from September to November of 2019. A total of 207 recipient Holstein heifers between 11 and 22 mo old (mean ± SD = 15.0 ± 2.8; median = 14.5) with mean BCS ± SD of 3.3 ± 0.2 [1 = emaciated, 5 = obese (Ferguson et al., 1994)] were enrolled in the study. Heifers were housed in a free-stall barn, bedded with dried manure

solids, free access to water, and fed once daily with a total mixed ration formulated to meet or exceed the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg/d (NRC, 2001).

Weekly cohorts of heifers were synchronized with a 5-d CIDR Synch protocol using a controlled internal drug release (**CIDR**; 1.38 g P4, Eazi-Breed CIDR, Zoetis) previously used twice for 5 days each time. After each use, CIDR inserts were individually washed with water and soaked in a solution of 2% chlorhexidine diacetate (Novalsan Solution, Zoetis) for 15 min, as described by Sala et al. (2020). The 5-d CIDR Synch protocol consisted of intravaginal insertion of a CIDR on D-8 and withdrawal 5 days later, followed by the administration of $PGF_{2\alpha}$ (500 mg of cloprostenol i.m.; Estroplan, Parnell) on D-3. Three days later, on d 0, heifers were randomly assigned to receive 1 of 4 treatments: Control (**G**), **H**, **GH**, and **HH**. Heifers in treatment G (n = 53) were only treated with GnRH (100 µg i.m.; Gonabreed, Parnell) on d 0, while heifers in the H group (n = 49) were treated with hCG (3,300 IU i.m.; Chorulon, Merck Animal Health) on d 0. Heifers assigned to GH (n = 52) were treated with GnRH on d 0 and hCG 5 days later (d 5). In the HH treatment, heifers (n = 48) received hCG on d 0 and d 5.

A trained technician scanned ovaries by transrectal ultrasonography using a Easi-scan:Go ultrasound unit with a 7.5-MHz linear array probe (IMV Imaging) paired to an iPad mini 4 (Apple Inc). Ovarian ultrasonography examinations were performed on d 0 (n =202), d 5 (n =194), and d 12 (n =159). Ultrasound videos were recorded with the IMV Go Scan application (version 3.70; BCF Technology Ltd) using an iPad mini. Ovarian structures were measured using a video metrics analysis software (Kinovea 0.8.15; Kinovea.org), and calipers were calibrated with background gridlines (size=10 mm). Follicle ≥ 7 mm, CL, and CL cavity (if present) mean diameters were calculated by the average of the height and width. Total CL and CL cavity area were calculated by the equation 0.5 height × 0.5 width × π (Martins et al., 2011). The total luteal area of each CL was calculated by subtracting the CL cavity area from the total CL area.

Ovulation was defined upon detection of a new CL in the subsequent scan (d 5 or d 12) in the same location of a follicle with a diameter ≥ 9 mm from the previous scan (d 0 or d 5). Heifers missing scans on d 0 (n = 5) or d 5 (n = 13) were considered to ovulate in response to the d 0 GnRH or hCG treatments when P4 was ≤ 0.50 ng/mL on d 0 and ≥ 1 ng/mL on d 7. Heifers were considered to have ovulated before GnRH or hCG treatment on d 0 when they had P4 ≤ 0.50 ng/mL on d 0, no ≥ 9 mm antral-follicle present on d 0, and the presence of a

new CL ≥ 14 mm on d 5 that increased in size by d 12. Heifers that ovulated before or after the hCG or GnRH d 0 treatment and had P4 ≤ 0.50 ng/mL on d 0 were considered as synchronized.

Blood samples were collected by puncture of the coccygeal vein or artery into vacuum tubes (Vacuette Z serum clot activator, Greiner Bio-One International GmbH) on d 0 (n =195), d 5 (n =194), d 7 (n =193), and d 12 (n =164). Blood samples were stored in a cooler with ice, transported to the laboratory and processed within 8 hours after collection. Serum was separated by centrifugation at 2,000 × *g* for 10 min at 4°C and stored at -20°C for later hormonal assays. Serum P4 concentrations were measured using a solid-phase RIA kit (ImmuChem coated tube Progesterone; MP Biomedicals). Mean assay sensitivity was 0.02 ng/ml. Intra- and inter-assay CV were 6.0% and 1.75%, respectively.

This study used a complete randomized experimental design. A priori power analysis was performed in version 3.1.9.6 of the G*Power analysis software (Erdfelder et al., 2009). The required sample size was calculated using *t*-test, 2-tail, α = 0.05, power = 0.95, and 0.80 of effect size day. A total of 42 heifers per treatment was required to find a difference in serum P4 concentrations between two independent means of 2.00 vs. 2.40 ng/mL using SD within each group of 0.50.

All statistical analyses were performed using version 9.4 of SAS (SAS Institute Inc., Cary, NC). Binary outcomes such as ovulatory responses, ovulation before and after d 0-treatments, ovulation on day 5, the proportion of synchronized heifers, and CL presence were analyzed by generalized linear mixed models considering a binary distribution and a logit link function using the GLIMMIX procedure. Treatment was considered as a fixed effect in the model. Continuous outcomes such as follicle diameter, primary and accessory CL area, total luteal area, and circulating P4 concentrations were analyzed by ANOVA using the MIXED procedure. The model included treatment, days, and interaction as fixed effects. For the analysis of the effect of d 0 treatment (GnRH or hCG) and time of ovulation (before or after d 0) on mean luteal area of original CL and serum P4 concentrations, the model included the fixed effects of d 0 treatment, time of ovulation, and interaction d 0 treatment × time of ovulation. The LSMeans statement was used to detect differences in d 0 treatments (GnRH vs. hCG) within each time of ovulation (before or after d 0). Normality and homoscedasticity of residuals were evaluated by Studentized residual plots for each variable after fitting the model using the residual option of the MIXED procedure.

The effect of treatment on total luteal area and circulating P4 concentrations over time was analyzed using the MIXED procedure with the REPEATED statement with cow (treatment) specified in the SUBJECT option. The unstructured covariance structure was used for these analyses. Treatment, day, and treatment × day interaction were included as fixed effects in the model. For the analysis of circulating P4 concentrations over time, P4 concentrations were log-transformed to fulfill normality assumptions. Actual means ± SEM of the data were presented for clarity. Differences among treatments were considered significant when $P \leq 0.05$, whereas $P > 0.05$ and $P \leq 0.10$ were considered a tendency. Data were presented as means ± SEM for continuous outcomes and as proportions for binary outcomes.

Follicular and luteal parameters evaluated in response to the last two hormone treatments of the 5-d CIDR Synch protocol did not differ ($P > 0.52$) among treatments (Table 1). Overall proportion of heifers with P4 ≤ 0.50 ng/mL on d 0 was 95.2%. Overall mean ovulatory response before or after d 0 was 98.1% and did not differ among treatments (Table 1) or between hCG and GnRH (Table 2) on d 0. Approximately half (46.4%) of the heifers submitted to the 5d-CIDR Synch program in our study ovulated before the treatment with GnRH or hCG on d 0. These heifers were most likely on d 1 or 2 of metestrus, characterized by serum P4 concentrations < 0.50 ng/mL and absence of a CL > 14 mm in diameter on d 0. In a previous study using a similar 5d-CIDR Synch protocol with a 3rd or 4th use CIDR, time of ovulation averaged ~86h after CIDR removal (Sala et al., 2020). In that study, 33.9 % of the heifers ovulated before d 0 (Rodrigo Sala, ST Genetics, South Charleston, Ohio, USA, personal communication), which is lower than in the present study. Heifers with a dominant follicle in an advanced development stage at d-3 with complete luteolysis (P4 ≤ 0.50 ng/mL) before or after $PGF_{2\alpha}$ are more likely to ovulate before d 0. Using GnRH at D-8 might have decreased the proportion of heifers ovulating before d 0 because fewer heifers would be in later stages of follicular development at D-3 (day of $PGF_{2\alpha}$). However, in a previous study, GnRH at the initiation of a 5d-CIDR Synch protocol (D-8) with a new CIDR did not increase the proportion of heifers in estrus on d 0 (Lima et al., 2013).

Furthermore, hCG treatment on d 0 (H + HH) did not affect ($P = 0.90$) synchronization parameters in comparison to GnRH (G+GH; Table 2). The overall synchronization rate for the 5d-CIDR Synch program was 93.8%. The synchronization rate in the present experiment indicates the proportion of heifers that would be on d 0, 1, or 2 of the estrous cycle on study d 0 and most likely to be utilized as recipients for ET on study D6 and 7. Sala et al. (2020), using a similar protocol with 3rd use CIDR in Holstein heifers, also found a similar ovulatory

response (97%) and an ET utilization rate (transferred/treated) of 93.3%. In the same study, pregnancy per ET did not differ between heifers receiving ET 7 ± 1 d after detection of estrus and after a 5-d CIDR Synch protocol with a new or 2nd use CIDR (Sala et al., 2020).

Treatment on d 0 (hCG vs. GnRH) affected ($P < 0.01$) circulating P4 concentrations at d 5. Heifers treated with hCG on d 0 (H +HH) had greater ($P < 0.01$) serum P4 and original CL area on d 5 than heifers treated with GnRH on d 0 (G+GH; Table 2). Moreover, treatment with hCG on d 0 also tended ($P = 0.06$) to increase the proportion of heifers with ≥2 CL at d 5 (Table 2). The increase in original CL size supports the increase in serum P4 on d 5 for heifers treated with hCG on d 0.

Time of ovulation relative to d 0 treatment had an effect ($P < 0.001$) on circulating P4 concentrations and total luteal area on d 5. Heifers ovulating before d 0 had greater ($P < 0.001$) serum P4 concentration (3.60 ± 0.15 vs. 2.06 ± 0.13 ng/mL, respectively) and total luteal area (313 ± 10 vs. 246 ± 10 mm^2, respectively) on d 5 compared with heifers that ovulated after d 0. These results were expected because heifers with ovulation before d 0 would be in a later stage of the estrous cycle (1 or 2 days later) with a more mature CL on d 5, synthesizing more P4, compared to heifers with ovulation after d 0. No interaction between ovulation time relative to d 0 and d 0 treatment was found on d 5 total luteal area ($P = 0.50$) and serum P4 concentration ($P = 0.53$). These results suggest that exogenous administration of 3,300 IU of hCG had a greater steroidogenic effect than the GnRH-induced LH surge not only during early CL development (after ovulation) but also just before ovulation and formation of the CL. It is not clear whether this effect occurred in the pre-ovulatory follicle cells, luteal cells after ovulation, or both. We speculate that hCG may have induced faster or increased luteinization of granulosa cells and that hCG, due to its greater half-life than LH, acts for a longer period on progesterone synthesis after the transformation of follicular cells into luteal cells (Schmitt et al., 1996).

Ovulation to hCG on d 5 did not differ between GH and HH (Table 1). Overall mean ovulatory response to hCG treatment on d 5 was 96 % (71/74) which was similar to the overall ovulation to d 0 hCG (99.0 %) and GnRH (97.1%; Table 2). About 97% of heifers treated with hCG on d 5 had ≥2 CL on d 12, indicating that 3,300 IU of hCG on d 5 of the estrous cycle is highly effective in inducing ovulation and formation of an accessory CL in Holstein heifers. In contrast, fewer ($P<0.001$) heifers (11.8%) not treated with hCG on d 5 had ≥2 CL at d 12 due to double ovulation to d 0 treatment (GnRH or hCG). Besides the induction of an accessory CL, hCG treatment on d 5 also increased ($P<0.001$) the luteal area

of the original CL on d 12 for heifers with a single CL on d 5 (no hCG on d 5: 369 ±15 vs. hCG on d 5: 464 ±20). In a different study using 3,300 IU of hCG on d 7 of the estrous cycle, an increase in original CL volume during diestrus was observed (Cunha et al., 2022). This effect of hCG on original CL may have been due to the prolonged half-life of hCG (Yen et al., 1968) and high affinity for LH receptors (Schmitt et al., 1996). Treatments not including hCG on d 5 (G and H) had a smaller original CL area at d 12 than treatments with hCG on d 5 (GH and HH; Figure 1). In addition, the luteal area of the original CL at d 12 did not differ between GH and HH (Figure 1). These results suggest that the effect of hCG on original CL appears to be transient, and the difference in original CL area found between H and G was not maintained from d 5 to d 12. Furthermore, accessory CL area did not differ between GH and HH on d 12 ($P = 0.11$; Figure 1).

Treatment also affected circulating P4 concentrations on d 7 and d 12 (Figure 1). Heifers in G had the lowest mean serum P4 concentrations on d 7 and d 12 compared to the other treatments (Figure 1). Mean serum P4 at d 7 did not differ between H and GH ($P = 0.83$). Heifers treated with HH had the highest serum P4 at d 7 among treatments, but at d 12, serum P4 was similar between GH and HH. The greater mean serum P4 at d 7 and d 12 for H compared with G suggests that hCG has a prolonged effect on serum P4 compared to GnRH induced-LH surge. This effect of d 0 hCG on serum P4 does not appear to be only the effect of the increase in the total luteal area since the luteal area at d 12 did not differ between H and G. The effect of hCG at d 0 on serum P4 at d 12 was not evident when heifers received another hCG treatment at d 5, indicating that the major driver for the increase in serum P4 at d 12 was the formation of an accessory CL induced by hCG at d 5.

A recent study that used 1,500 IU of hCG on d 5 in cross-bred heifers found an effect of accessory CL side relative to original CL (ipsilateral vs. contralateral) on original CL area and circulating P4 concentrations (Hazano et al., 2020). In that study, contralaterally-induced CL had greater diameter and serum P4 concentration on d 7 and 14 ($P \leq 0.05$) in comparison with ipsilaterally-induced CL (Hazano et al., 2020). In contrast, in the present study, side of accessory CL relative to original CL did not affect original CL luteal area ($P = 0.20$; ipsilateral: 431 ± 28 vs. contralateral: 480 ± 25 mm^2), or accessory CL luteal area ($P = 0.64$; ipsilateral: 357 ± 24 vs. contralateral: 340 ± 27 mm^2) on d 12. Moreover, total luteal area ($P = 0.43$; ipsilateral: 787 ± 26 vs. contralateral: 820 ± 31 mm^2) and serum P4 concentrations ($P = 0.78$; ipsilateral: 11.74 ± 0.66 vs. contralateral: 11.47 ± 0.69 ng/mL) on d 12 did not differ for heifers with ipsilateral and contralateral accessory CL.

In summary, the administration of hCG on the last day of a 5-d CIDR Synch program increased circulating P4 concentrations and luteal area 5 days later compared to heifers treated only with GnRH independently of the ovulation time relative to d 0 treatment. The treatments with hCG on d 5 induced accessory CL formation and increased total luteal area on d 12 and serum P4 concentrations on d 7 and 12. The HH treatment successfully increased serum P4 concentrations in heifers on d 7 compared with the other treatments. If applied in ET programs in recipient heifers, this strategy may potentially increase pregnancy. Future research is warranted to determine the effect of the presented treatments on pregnancy per ET.

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Notes

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Table 1. Effect of treatment1 on follicular and luteal parameters from d 0 to d 12 in Holstein heifers that received the 5-d controlled internal drug release (CIDR) Synch protocol.

	G	H	GH	HH	*P*-value
Ovulation before d 0, % (n/n)	49.1 (26/53)	42.9 (21/49)	51.9 (27/52)	41.7 (20/48)	0.69
Ovulation after d 0, % (n/n)	49.1 (26/53)	53.1 (26/49)	42.3 (22/52)	56.3 (27/48)	0.54
Ovulation before and after d 0, % (n/n)	0 (0/53)	2.0 (1/49)	1.9 (1/52)	2.1 (1/48)	0.99
Total ovulation, % (n/n)	98.2 (52/53)	98.1 (48/49)	96.1 (50/52)	100 (48/48)	0.93
Largest ovulatory follicle diameter on d 0,[2] mm ± SEM	13.3 ± 0.4	12.5±0.3	12.6±0.5	13.1 ± 0.4	0.53
Heifers with P4 ≤ 0.50 ng/mL on d 0, % (n/n)	94.6 (52/55)	96.0 (48/50)	96.3 (52/53)	98.0 (48/49)	0.85
Heifers synchronized,[3] % (n/n)	92.7 (51/55)	94.0 (47/50)	94.5 (51/53)	93.9 (46/49)	0.99
Heifers with n ≥ 2 CL on d 5,[4] % (n/n)	3.9 (2/51)	8.5 (4/47)	2.0 (1/50)	10.9 (5/46)	0.31
Ovulation between d 5 and d 12,[5] % (n/n)	4.9[b] (2/41)	4.6[b] (2/44)	97.2[a] (35/36)	94.7[a] (36/38)	<0.001
Largest ovulatory follicle diameter on d 5,[6] mm ± SEM	9.3 ± 0.1[b]	10.9 ± 0.9[b]	11.5 ± 0.2[a]	11.8 ± 0.3[a]	<0.001
Double ovulation between d 5 and d 12,[6] % (n/n)	0 (0/2)	0 (0/2)	8.6 (3/35)	19.4 (7/36)	0.65
Heifers with n ≥ 2 CL on d 12,[5] % (n/n)	9.8[b] (4/41)	11.4[b] (5/44)	100[a] (36/36)	97.4[a] (37/38)	<0.001
Heifers with ipsilateral accessory CL on d 12,[6] % (n/n)	50 (1/2)	50 (1/2)	48.6 (17/35)	52.7 (19/36)	0.99

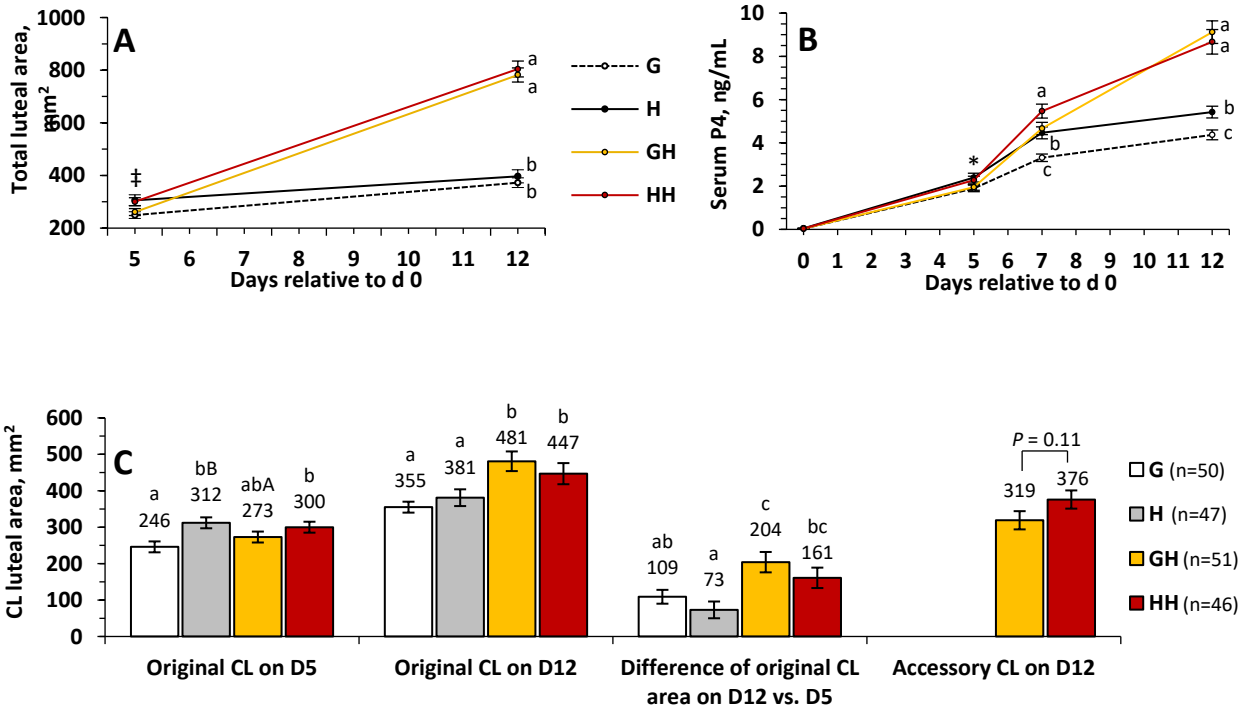
[1]Treatments: G= gonadotropin releasing-hormone (GnRH) on d 0; H= human chorionic gonadotropin (hCG) on d 0. GH= GnRH on d 0 and hCG on d 5; HH= hCG on d 0 and 5.
[2]Only heifers with ovulation after d 0 GnRH or hCG treatment.
[3]Heifers with P4 ≤ 0.50 ng/mL on d 0 and ovulation before or after the d 0 treatment (GnRH or hCG) were considered synchronized.
[4]Only synchronized heifers.
[5]Only synchronized heifers with scans on d 5 and d 12 (n=159).
[6]Only synchronized heifers with ovulation between d 5 and d 12 (n=75).
[ab]Means with distinct superscripts within row differ (P<0.05).

Table 2. Effect of d 0 treatment with GnRH vs. hCG (treatments[1] combined) on follicular and luteal parameters from d 0 to d 5 in Holstein heifers that received the 5-d controlled internal drug release (CIDR) Synch protocol.

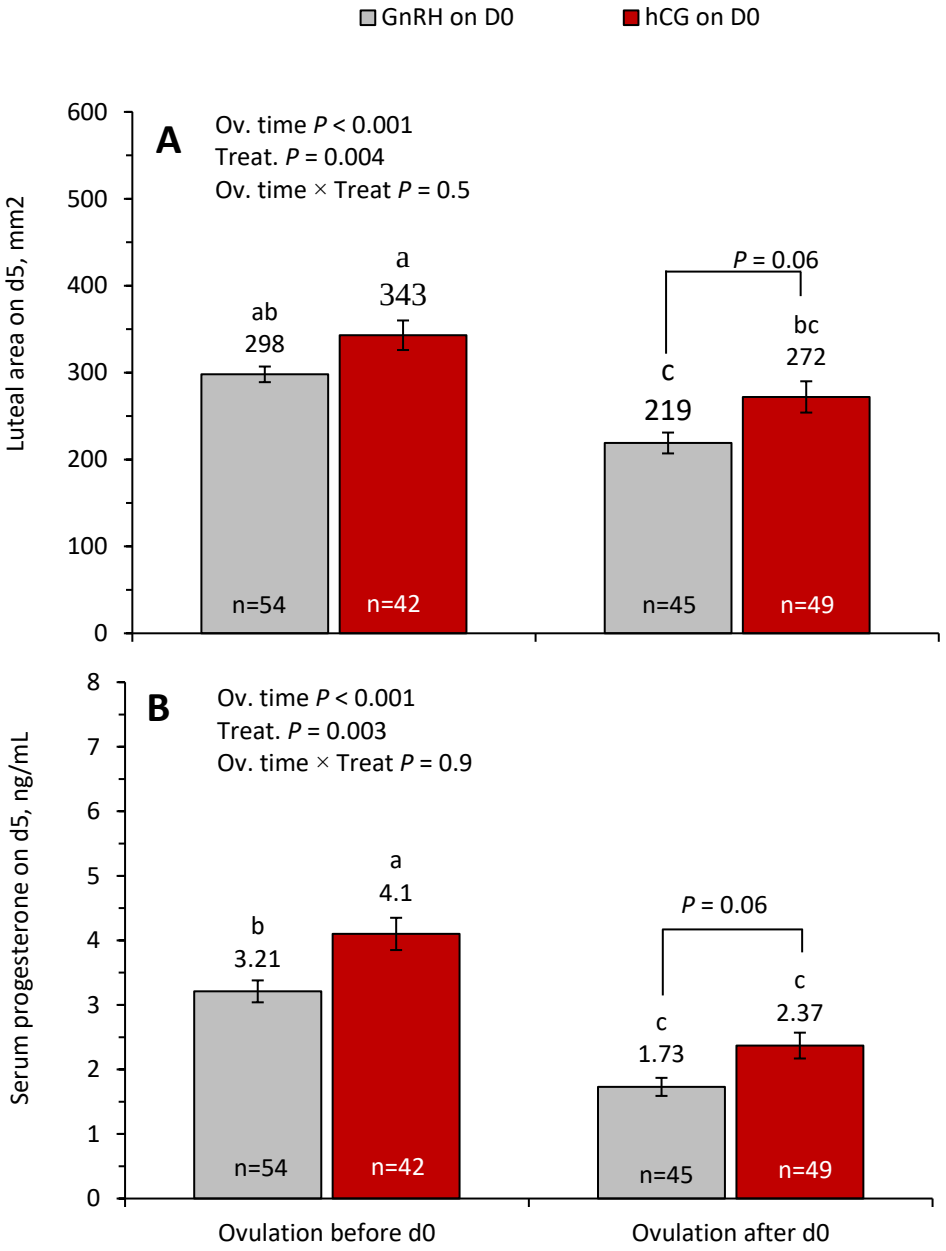
	GnRH on d 0 (G + GH)	hCG on d 0 (H + HH)	*P*-value
Ovulation before d 0, % (n/n)	50.5 (53/105)	42.3 (41/97)	0.25
Ovulation after d 0, % (n/n)	45.7 (48/105)	54.6 (53/97)	0.21
Ovulation before and after d 0, % (n/n)	0.9 (1/105)	2.1 (2/97)	0.50
Total ovulation, % (n/n)	97.1 (102/105)	99.0 (96/97)	0.37
Largest ovulatory follicle diameter on d 0,[2] mm ± SEM	12.9 ± 0.3	12.8 ± 0.3	0.73
Heifers with P4 ≤ 0.50 ng/mL on d 0, % (n/n)	95.4 (103/108)	97.0 (96/99)	0.55
Heifers synchronized,[3] % (n/n)	93.5 (101/108)	94.0 (93/99)	0.90
Heifers with n ≥ 2 CL on d 5,[4] % (n/n)	3.0 (3/101)	9.7 (9/93)	0.06
Serum P4 on d 5, ng/mL ± SEM	1.91 ± 0.10	2.37 ± 0.14	0.007
Luteal area of original CL on d 5,[5] mm^2 ± SEM	260 ± 9	306 ± 13	0.002

[1]Treatments: G= gonadotropin releasing-hormone (GnRH) on d 0; H= human chorionic gonadotropin (hCG) on d 0. GH= GnRH on d 0 and hCG on d 5; HH= hCG on D 0 and 5.
[2]Only heifers with ovulation after d 0 GnRH or hCG treatment.
[3]Heifers with P4 ≤ 0.50 ng/mL on d 0 and ovulation before or after the d 0 treatment (GnRH or hCG) were considered synchronized.
[4]Only synchronized heifers.
[5]Only heifers with only one CL on d 5 were used in this analysis.

Figure 1. Effect of treatment on (**A**) total luteal area and (**B**) serum progesterone (P4) concentrations over time after d 0. Also, effect of treatment on (**C**) original corpus luteum (CL) area on d 5 and d 12, the difference of original CL area on d 12 vs. d 5 and on accessory CL area on d 12. ‡Symbol indicates difference between G and HH (P = 0.01) on d 5. *Asterisks indicate differences between G and H on d 5 (P = 0.05). [abc] Means with different superscript letters differ (P < 0.05) within a day or CL type. [AB] Means with different superscript letters tend to differ (P < 0.10) within a CL type. Original CL analysis: treatment P < 0.002; day P < 0.001; and treatment × day P < 0.001. Treatments: G= gonadotropin releasing-hormoni (GnRH) on d 0; H= human chorionic gonadotropin (hCG) on d 0. GH= GnRH on d 0 and hCG on d 5; HH= hCG on d 0 and 5.



Supplemental content study 1



Supplemental figure 1. Effect of D0 treatment (GnRH or hCG) and time of ovulation (before or after d 0) on (**A**) mean luteal area of original CL (mm^2 ± SEM) and (**B**) serum progesterone concentrations (ng/mL ± SEM) on d 5. [abc] Columns with distinct superscript letters differ (P <0.05).

4 CHAPTER 3

STUDY 2

Manuscript formatted to be submitted to the Journal of Dairy Science

Effect of human chorionic gonadotropin on the last day of the 5d-CIDR-Synch protocol and 5 days later on pregnancy per embryo transfer in recipient dairy heifers receiving in vivo-derived and in vitro-produced embryos

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ABSTRACT

The objective of this study was to determine the effect of human chorionic gonadotropin (hCG; 3,300 IU) administered on d 0 and 5 of the estrous cycle on pregnancy per embryo transfer (P/ET) in recipient dairy heifers. Dairy heifers (n =1,297) were synchronized with a 5d-CIDR Synch program [d-8: CIDR inserted; d-3: CIDR removed + PGF2α; d0: GnRH or human chorionic gonadotropin (hCG)] and were randomly assigned to 1 of 4 treatments: G, H, GH, and HH. Control – G group – (n =325) was treated with 100μg GnRH on d 0 while heifers in H (n =322) group received hCG only on d 0. Heifers in GH (n =328) received GnRH on d 0 and hCG on d 5, and HH (n =322) heifers received hCG on d 0 and d 5 again. Ovaries were scanned by ultrasound on d 5 (n =1,285) and d 12 (n =882) to determine ovulatory response. Pregnancy diagnosis was performed on d 32, 46, and 60. Blood samples (n =218) were collected on d 5, 7, and 12 to measure circulating progesterone (P4) concentrations. Recipient heifers received embryos (n =1,152) once from d 6 to 9 [n=581 in vitro produced embryos (IVP); n=244 in vivo embryos (FRESH); and n=287 in vivo frozen embryos (FROZEN)]. Binary outcomes were analyzed by GLIMMIX and continuous outcomes by PROC MIXED of SAS. Fixed effects included treatment, embryo type, and two-way interaction. The proportion of heifers with CL on d 5 did not differ (P =0.81) from heifers treated with GnRH d 0 or hCG d 0. Ovulatory response to hCG d 5 did not differ (P =0.14) between GH (78.3%) and HH (72.2%). The hCG d 0 increased (P =0.005) the circulating P4 concentrations on d 5 in comparison to GnRH d 0 (2.97 ± 0.09; 4.34 ± 0.18, respectively). The hCG administration on d 5 successfully induced (P <0.001) an accessory CL (75.6%) in recipient heifers. Circulating P4 concentration on d 12 to hCG d 5 (12.74 ± 0.56) was greater (P =0.14) than no hCG d 5 (7.25 ± 0.29). Heifers GH had greater ($P \leq 0.05$) 60d P/ET than G when fresh IVD embryos were used (63.3% vs. 43.6%). The overall pregnancy loss (32 to 60 d) tended (P <0.10) to be lower on GH compared to H with IVD embryos (2.6 vs. 14.6%).In addition, recipients in d0-hCG with IVP had lower P/ET compared to others and greater pregnancy loss compared with controls. In conclusion, GH only improved P/ET in IVD embryos but did not affect P/ET in IVP embryos compared to G. Also, hCG on d 0 appears to be detrimental to pregnancy maintenance in FROZEN or IVP embryos.

Keywords: hCG, progesterone, pregnancy, embryo transfer, heifers

INTRODUCTION

Embryo transfer (**ET**) has consistently increased in the last decade in North America, which was driven by the use of in vitro-produced (**IVP**) embryos (Viana, 2018a; b, 2020). According to the International Embryo Technology Society annual report, ET using IVP embryos in North America increased from 28,100 transfers in 2010 to 339,716 in 2020 (Stroud, 2011; Viana, 2021). This tremendous increase in the use of IVP embryos during the last decade suggests an improvement in the system of the embryo in vitro production, more commercial laboratories available, and a greater demand for herd genetic improvement (Viana, 2020). Even though advances in the production of embryos in vitro have been made, pregnancy success after ET using IVP embryos is still similar to or lower than artificial insemination (AI; Sartori et al., 2016; Hansen, 2020). These results indicate that more pregnancy losses occur in heifers and cows receiving ET compared with AI (Sartori et al., 2016). One factor that has been associated with pregnancy loss following ET is the uterine environment receiving the embryo (Wiltbank et al., 2016). Thus, treatments that improve uterine quality and endometrial function may enhance pregnancy survival after ET.

Progesterone (P4) has been determined as a critical regulator of endometrial function for the establishment and maintenance of pregnancy. Recipient cows or heifers must have a functional corpus luteum (**CL**) on the ipsilateral side of the transfer to allow the ET to produce the most successful pregnancy results (Hansen, 2020). Although a CL is required to sustain a pregnancy, it is unclear whether different circulating P4 concentrations on days before or after the time of ET are positively associated with embryo survival after ET. Embryo physiological parameters, such as embryo size and interferon-tau production by elongating embryos, were positively related to circulating P4 concentrations (Carter et al., 2008; Clemente et al., 2009; Shorten et al., 2018). Moreover, expression of interferon-tau stimulated gene (**ISG**) in peripheral leukocytes on d 20 of pregnant heifers, fetus length on d 46 of pregnancy, and pregnancy-associated glycoprotein (**PAG**) concentrations were decreased in pregnant lactating Holstein cows that received a half dose of $PGF_{2\alpha}$ (12.5 mg of dinoprost) on d 4.5 after AI and had reduced circulating P4 concentrations. Nonetheless, different methods of P4 supplementation after AI and ET have produced inconsistent fertility outcomes.

One of these strategies is using CIDR post-AI or ET. Steichen and Larson used CIDRs from the day of ET or d 7 to d 19 in recipient heifers (Steichen and Larson, 2019). Heifers

treated with CIDR tended to have greater serum P4 concentration than controls, but pregnancy per embryo transfer (**P/ET**) was not different between treatments (Steichen and Larson, 2019). Garcia-Ispierto and López-Gatius compared P4 supplementation with CIDR during early embryo development (from d 3 to 5 post-AI) and maternal recognition of pregnancy (from d 15 to 17) with controls (no treatment) in high-producing lactating dairy cows (Garcia-Ispierto and López-Gatius, 2017). Cows receiving P4 supplementation were 1.7 times more likely to conceive with no differences in pregnancy loss compared with control (Garcia-Ispierto and López-Gatius, 2017).

Other strategies to increase circulating P4 concentrations have been tested, such as inducing an accessory CL with GnRH or hCG during the first follicular wave (Santos et al., 2001; Nascimento et al., 2013a). A recent study indicated that GnRH 5 d after a 5-day CIDR Synch protocol induced an accessory CL in 84% of GnRH-treated heifers and increased serum P4 concentration on d 12 of the estrous cycle (García-Guerra et al., 2020). In that study, recipient heifers of stage 7 embryos, expanded blastocyst, treated with GnRH on d 5 had less pregnancy loss compared to non-treated heifers (García-Guerra et al., 2020). Niles et al. (2019) treated heifers with 2,000 IU hCG on d 7 after AI, which resulted in an increase in serum P4 concentration from d 11 to 67 compared with no treatment on d 7. In the same study, authors described a reduction of 12.5% in pregnancy loss between d 32 and 67 when hCG was used after AI (Niles et al., 2019).

In a recent study, hCG (3,000 IU) was used to replace GnRH (100 µg) at the last treatment of a 5-d CIDR Synch protocol in recipient lactating Holstein cows (Garcia-Ispierto et al. 2021). The results from that study indicated that the odds (3.3) for pregnancy at d 28 increased when hCG was used in the pale of GnRH; however, the study used a limited number of subjects (n = 60/treatment; Garcia-Ispierto et al. 2021). Our laboratory recently tested the effect of replacing GnRH with hCG for the last treatment of a 5-d CIDR Synch protocol (d 0) on luteal and P4 dynamics during diestrus in recipient Holstein heifers (El Azzi et al., 2022). Heifers treated with hCG at d 0 had increased total luteal area on d 5 and serum P4 concentrations on d 5 and d 12 (El Azzi et al., 2022). These results indicate that hCG used as an ovulatory inducer at the end of the synchronization protocol may be a simple strategy to increase circulating P4 concentrations earlier during embryo growth and improve fertility outcomes after ET in recipient animals. Farms that use synchronization protocols to increase the availability of recipients could quickly implement this strategy.

Thus, the main objective of the current study was to determine the effect of replacing GnRH with hCG (3300 IU) on the last day of the 5-d CIDR Synch protocol and inducing accessory CL formation with hCG 5 d later on P/ET and pregnancy loss in recipient dairy heifers. Our secondary objective is to establish the effect of these treatments on the total luteal area (**TLA**) and circulating P4 concentrations on d 5 and d 12. We hypothesized that replacing GnRH with hCG for the last treatment of the 5d-CIDR Synch protocol would increase circulating P4 concentrations during early embryonic development (d 5 of estrus cycle) and P/ET and decrease pregnancy loss in recipient dairy heifers.

MATERIAL AND METHODS

All animal handling and experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison.

Animals, Housing, and Feeding

This study was conducted on a commercial farm in Ohio, USA, from May to October of 2020. A total of 1,297 recipient dairy heifers (80% Holstein, 8% Jersey and 12% other breeds) 16.2 ± 2.8 mo old (mean ± SD) and with 3.25 ± 0.5 BCS (mean ± SD; 1 – emaciated; 5 – obese; Ferguson et al., 1994) were enrolled in this study. Heifers were housed in freestall barns with water *ad libitum* and fed once daily with a formulated total mixed ration to meet or exceed the nutritional requirements of a 0.8 kg/d of gain in Holstein heifers weighing 360 kg (NRC, 2001).

Experimental Design

All treatments were performed weekly after synchronization (Figure 1) with a 5d-CIDR Synch protocol without a GnRH at the initiation of the program and only one $PGF_{2\alpha}$ (Lima et al., 2013). Recipients were synchronized using a new or 2^{nd}- or 3^{rd}-use CIDR (1.38 g P4, Eazi-Breed, Zoetis, Florham Park, NJ). The 2^{nd}-and 3^{rd}-use CIDR devices were previously used once or twice, respectively, for 5 d each time (Sala et al., 2020). All CIDR devices were individually washed and brushed with water and soaked in a solution of 2% chlorhexidine diacetate (Novalsan Solution, Zoetis Inc.) for 15 minutes (Sala et al., 2020). The synchronization protocol was initiated on d -8 with a CIDR device insertion, remaining for 5 d. The CIDR removal occurred on d -3 of protocol, followed on the same day by the

administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 500 mg of cloprostenol im; Estroplan, Parnell, Overland Park, KS).

Recipient heifers were randomly assigned to receive 1 of 4 treatments – **G**, **H**, **GH**, and **HH** on d 0 (Figure 1). Heifers (n =325) in the G (control) only received GnRH (100 mg of gonadorelin i.m., Parnell, Overland Park, KS) on d0, while heifers (n =322) in H received hCG (3,300 IU, i.m. of Chorulon: Merck Animal Health) on d0. Treatment GH (n =328) consisted of GnRH on d0 and hCG 5 days later on d 5. Lastly, HH heifers (n =322) were treated with hCG on d 0 and again on d 5.

Embryo Transfer and Ultrasonographic Examinations

To determine the presence, location, size, and number of CL, heifers (n =1,285) were scanned by transrectal ultrasonography using a 5-MHz linear probe (Ibex lite; EI Medical Imaging, USA) on d 5 after the 5d CIDR-Synch protocol. The same technician performed all transrectal ultrasonographic examinations. On the day of ultrasonography, BCS was determined by one technician. Recipient dairy heifers with the presence of CL received an embryo transference on d 7 or 8. Five experienced technicians performed the ET. Recipient heifers that received ET were scanned again on d 12 (n =882) by the same operator using the same ultrasound to evaluate CL characteristics. The largest cross-sectional diameters of CL and CL cavity, when present, were estimated using on-screen background with 10 mm side squares gridlines of the display. Total CL and CL cavity area were calculated by the equation $0.5\ H \times 0.5\ W \times \pi$ (Kastelic et al., 1990; Martins et al., 2011). The CL cavity area was subtracted from the total CL area to calculate the total luteal area.

Some heifers (n =147) did not receive ET for different reasons, including animals without the presence of a CL at the time of transfer (G, n =35; H, n =25; GH, n =27; HH, n =27), uterus infection (n= 1), lameness (n =1), cervix stenosis (n = 1), small rectum (n =1), adhesions (n = 1), and no embryo available at time of ET (n =23) and donor heifers (n = 3). Thus, a total of n = 1,152 ET was performed. Of the total embryos used, n =244 were conventional in vivo-derived not sexed embryos recovered by flushing (**IVD**); n =581 were sexed embryos produced in vitro (**IVP-FRESH**); n =287 were not sexed slow-frozen IVP embryos; and n =40 were not sexed vitrified IVP embryos. The stages of embryos transferred included morula (n = 19), early blastocyst (n = 496), blastocyst (n = 215), expanded blastocyst (n = 393), hatched blastocyst (n = 15), and non-classified (n = 14). Embryos were

classified as grade 1 (n = 927), 2 (n = 207), 3 (n = 4), and non-classified (n = 14). Pregnancy diagnoses were performed on d 32, 46, and 60 using transrectal ultrasonography examination of the uterus. A presence of an embryo or fetus with a heartbeat was required for a positive pregnancy diagnosis. Pregnancy loss was determined in non-pregnant heifers that were pregnant on a previous exam.

Blood Sample Collection and Progesterone Assay

Blood samples were collected from heifers in the last three weeks of enrollment (n =218; G, n =54; H, n =54; GH, n =55; and HH, n =55) to analyze serum P4 concentrations. Bloods were collected on d 5, 7, and 12 via coccygeal vein or artery using 20-gauge 3.8 cm needles and 9 mL vacuum tubes (Vacuette CAT Serum Clot Activator, Greiner Bio-One North America Inc.). Samples were stored in a cooler with ice, transported to the farm laboratory, and centrifuged within 6 hours after collection to separate serum. Serum aliquots were frozen at -20°C until the completion of on-farm data collection. All samples were shipped to Dr. Martins's laboratory at the Department of Medical Sciences, UW-Madison. Serum P4 concentrations were quantified using a solid-phase radioimmunoassay (RIA) kit (ImmuChem coated tube P4; MP Biomedicals, Costa Mesa, CA). The mean assay sensitivity, calculated as 2 SD less than the mean counts per minute of maximum binding, was 0.02 ng/ml. Intra- and inter-assay coefficients of variation were 1.75% and 6.00%, respectively.

Statistical Analysis

This was a completely randomized experimental design. The statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Binary outcomes, such as ovulation on d 5, number of CL on d 12, P/ET, and pregnancy loss, were analyzed by generalized linear mixed models with a binary distribution and a logit link function using the GLIMMIX procedure. The initial model included the effect of treatment, BCS, embryo quality (grades 1, 2, or 3), embryo stage, embryo type (IVD, IVP-FRESH, IVP slow-frozen, IVP vitrified), ET technician and service (ET) number (0 to 7) and the two-way interaction treatment × embryo type. No treatment × embryo type interaction was observed for analyses of pregnancy outcomes (P >0.20). Since we did not find treatment × embryo type interaction

and the number of transfers using IVP vitrified embryos was small (n = 40), we combined embryo type classification of in vitro-produced slow-frozen and vitrified embryos and named **IVP-FROZEN**. The final model for P/ET and pregnancy loss analysis included treatment, embryo type, and treatment × embryo type interaction as fixed effects. All other variables were non-significant (P >0.05) and removed from the model in a backward stepwise elimination fashion. The final model for other binary response variables (e.g., ovulatory response at d 5) only included treatment as a fixed effect.

Continuous response variables, such as total luteal area and circulating P4 concentration, were analyzed as repeated measurements by ANOVA using the MIXED procedure with the REPEATED statement with heifer nested in treatment specified in the SUBJECT option of SAS. The model consisted of treatment, day, and the interaction treatment × day as fixed effects. The unstructured covariance was used for the analyses. Normality and homoscedasticity of each variable residuals were evaluated using studentized residual plots after fitting the model using the residual option of the MIXED procedure. Variables that did not fulfill assumptions were transformed to the square root for the analyses. For clarity, actual means ± SEM obtained by the MEANS procedure are presented. Differences between treatments were considered significant when $P \leq 0.05$, whereas $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

RESULTS AND DISCUSSION

Ovulatory response

In the present study, 92.8% of recipient dairy heifers had at least one CL on d 5, and it was not different (P =0.55) between treatments. The proportion of heifers with CL on d 5 was close to this in our previous study using a similar synchronization protocol (99.2 %; El Azzi et al., 2022). In that study, similar treatments (G, H, GH, and HH) were used to determine luteal and progesterone dynamics after a 5d-CIDR Synch program, and a total of 93.8% of recipient Holstein heifers were considered as synchronized (P4 ≤0.50 ng/mL on d 0 and ≥1 ng/mL on d 7 of experimental protocol). In a recent study (Sala et al., 2020) using the same 5d-CIDR synch program in recipient heifers, authors reported a utilization rate (number of animals treated/transferred) of 91.3%. This indicates that most of the heifers were synchronized after the 5d-CIDR synch program.

The heifers treated with hCG on d 5 had 78.5% of overall ovulatory response after treatment (Table 3), with no differences (*P* =0.70) between GH (76.9%) and HH (73.3%) treatments (Table 1). The proportion of heifers with ≥ 2 CL on d 12 was the same (*P* =0.26) between hCG d5-treated groups (Table 1). The average proportion of heifers treated with d5-hCG with ≥2 CL on d12 was 75.6% (Table 3). These responses were lower than in previous studies when 3,300 IU of hCG were administered on d 5 [(93% (Galvão et al., 2006) or 7 (96% (Cabrera et al., 2021)] of the estrous cycle in lactating dairy cows. Studies that used lower doses of hCG (≤ 2,000 IU) in lactating dairy cows reported similar or slightly higher ovulatory responses [78% (Nascimento et al., 2013a) and 89% for 2,000 IU, and 77% for 1,000 IU of hCG (Cabrera et al., 2021] compared with those in the present study. Moreover, another study that used GnRH (100 µg) on d 5 in recipient Holstein heifers also reported a similar ovulatory response [84%; (García-Guerra et al., 2020)].

We expected a slightly higher ovulatory response to the d5-hCG treatment in the present study because of the hCG properties. The hCG is a glycoprotein that acts similarly to LH, with α and ß-subunits. The ß-subunits confer the biological activity of hCG, characteristic that gives it a high homology (~80%) with LH (Humaidan et al., 2005; Stenman et al., 2006). In addition, hCG binds directly to LH receptors in follicular or luteal cells, making it more effective in inducing ovulation and increasing steroidogenesis and circulating P4 concentrations in dairy cattle compared with GnRH (Binversie et al., 2012; Cabrera et al., 2021). One plausible explanation for the slightly lower d5-hCG ovulatory response observed on d 12 in the present study (Tables 1 and 3) is due to the possible proximity of the corpora lutea (original and accessory CL) on d 12, which may induce an incorrect counting from the technician that was scanning the recipient heifers. This type of error has been reported as a cause of a lower number of CL by other authors previously (Kastelic et al., 1990; Bicalho et al., 2017).

Total Luteal Area and circulating P4 concentration

In the present study, the effect of treatment (*P* <0.001), day (*P* <0.001), and interaction (*P* <0.001) between treatments and days (Table 1) were observed in serum P4 outcomes. The circulating P4 concentration on d 5 (Tables 2 and 3) was greater in the d0-hCG heifers compared to d0-GnRH heifers. Additionally, the same d0-hCG treated heifers had greater P4 concentration on d 7 compared with d0-GnRH treated heifers (Tables 1 and 3). The luteotropic effect of hCG is the primary reason for the increase of P4 in groups receiving hCG

on d 0 compared with GnRH. The hCG increases steroidogenesis by increasing the luteal volume of the CL (Binversie et al., 2012; Cabrera et al., 2021).

Group H had greater circulating P4 concentration than G and GH on d 5 and remained greater on d 7 than heifers only receiving GnRH on d0 (Tables 1 and 2). Mean circulating P4 concentration on d 7 was greater in HH than in G or H but similar to GH (Table 1). As expected, heifers receiving hCG on d 5 had greater circulating P4 on d 12 than heifers that had ovulation induced only on d 0, regardless of ovulatory inducer used, hCG or GnRH (Tables 1 and 3). In addition, d0-treatment [GnRH d0 (G + GH) or hCG d0 (H + HH)] did not affect circulating P4 concentrations on d 12 (Table 2). The TLA (Tables 1, 2, and 3) followed the same pattern as circulating P4 concentrations on d 5 and 12. The proportion of heifers treated with hCG on d 5 with CL number ≥ 2 on d 12 was 75.6%. All these results support the effectiveness of 3,300 IU of hCG in inducing accessory CL in dairy heifers on d5, in addition to the luteotropic effect of hCG.

Circulating P4 concentration in early embryo development is positively associated with the synthesis of uterine hystotroph and embryonic growth and survival (Faulkner et al., 2013). Heifers with lower circulating P4 concentrations during metestrus and early diestrus had delays in the initiation of proper endometrial function, including the loss of prostaglandin receptors, which may reduce uterus receptivity to embryo implantation (Forde et al., 2011). Conversely, P4 supplementation and consequently elevated circulating P4 concentrations from d 5 to 9 of the estrous cycle have been positively associated with pregnancy success in cattle (Mann et al., 2006). Many authors have shown that the hCG on d 5 is a potential strategy to increase P4 in dairy cattle at breeding programs (Nascimento et al., 2013a; Cunha et al., 2021). Nascimento et al. (2013a; b) delineated two studies to investigate the effect of hCG on d 5 of the estrous cycle. In those studies, the authors reported greater P4 concentrations in groups receiving hCG than controls from d 8 to 15 (Nascimento et al., 2013b) and on d 12 (Nascimento et al., 2013a). Cunha et al. (2021) determined that lactating dairy cows treated with GnRH and hCG on d 5 to 7 of the estrous cycle had greater P4 concentration on d14 of the estrous cycle compared with control cows (no hCG).

In contrast, to our knowledge, no study has evaluated the effects of using hCG on the last day of the 5-d CIDR Synch program and 5 d later in recipient dairy heifers on circulating P4 concentrations and fertility outcomes. Aslan et al. (2011) compared the effect of inducing ovulation 48 h after $PGF_{2\alpha}$ with GnRH or hCG on luteal dynamics (luteal diameter, blood

flow, and serum P4 concentration) at d 9 and 12 in twelve Holstein-Friesian heifers, and they did not find differences between treatments. In the present study, treatments with hCG on d 0 successfully increased the circulating P4 concentrations until d 7 of the estrous cycle. Still, this increase was insufficient to overcome the effect of hCG administrated on d 5 on circulating P4 concentrations at day 12. Our results with a larger number of heifers corroborated our previous study that evaluated the effect of the same treatments on luteal dynamics in Holstein heifers (El Azzi et al., 2022). In that trial, we reported greater circulating P4 concentration in heifers with hCG on d 0 (2.37 ± 0.14 ng/mL) compared with GnRH (1.91 ± 0.10 ng/mL). In addition, heifers treated with hCG on d 5 had greater circulating P4 concentration compared with heifers that did not receive hCG on d5 (El Azzi et al., 2022). This repeatability observed between these studies supports hCG treatments on d 0 and 5 of the estrous cycle as an efficient model to increase circulating P4 concentrations during late metestrus and early diestrus in dairy heifers.

Pregnancy outcomes

The overall P/ET on d 32 (G, H, GH, and HH treatments combined) in this study was 44.9%. Table 4 shows the effect of treatment and embryo type (IVD, IVP-FRESH, and IVP-FROZEN) on pregnancy outcomes. The overall d 32 P/ET by treatment did not differ (*P* =0.21) between G (47.6%), H (39.7%), GH (47.3%), and HH (45.3%) treatments. On d 46, P/ET tended to be lower in H compared with G (*P* =0.06) and GH (P =0.07). On d 60, heifers in H continued to have a tendency (*P* =0.06) for a decreased P/ET than G. However, P/ET was significantly (*P* =0.04) reduced for H than GH (Table 4). Heifers in H and HH tended (*P* =0.08) to have an increased total pregnancy loss (from d 32 to 60) compared with heifers in GH.

Embryo type had an effect (*P* < 0.001) on P/ET on d 32, 46, and 60. Heifers receiving IVD, and IVP-FROZEN embryos had greater P/ET than heifers receiving IVP-FRESH embryos on d 32, 46, and 60 (Table 4). Pregnancy loss between d 32 and 46 was greater for heifers receiving IVP-FROZEN or IVP-FRESH embryos compared with IVD embryos (Table 4). This increase in earlier loss contributed to a greater total pregnancy loss (from d 32 to 60) for IVP-FROZEN or IVP-FRESH embryos than IVD embryos (Table 4). In a trial performed to observe the factors that affect P/ET in two large dairy farms in the US, recipient heifers

received either in vivo-derived fresh or frozen embryos transferred 6, 7, or 8 days after spontaneous estrus (Chebel et al., 2008). In that study, frozen/thawed embryos had decreased d 35 P/ET than fresh embryos (44.2% vs. 56.9, respectively). Some authors may explain the pregnancy differences using fresh versus frozen embryos are due to different embryo stages and grades (quality) used (Hasler, 2001). However, in our study, the majority of embryos used were grade 1 (n=927; 88.1%) or 2 (n= 95; 19.7%), and grade did not affect (P >0.80) pregnancy outcomes.

Significant interactions between treatment and embryo type were observed for P/ET outcomes (Table 4; Table 5; Supplementary Table S1). Overall P/ET were greater for in vivo-derived embryos than for in vitro-produced embryos (Table 5). Recipients GH receiving IVD embryos had and tended to have greater P/ET than controls on d 46 and 60, respectively (Table 5). Treatment did not affect P/ET in the other treatments with IVD embryos. Moreover, treatment GH tended to have lower total pregnancy losses (d 32 to 60) compared to other treatments (Table 5). Curiously, the use of hCG only on d 0 of the experimental protocol hurt d 32 P/ET in heifers receiving IVP embryos (Table 5; Figure 2). Considering in vitro-produced embryos, heifers in H had lower overall d 32 and 46 P/ET compared with other treatments, while HH tended to be lower than controls only on d 46 (Table 5). Additionally, regardless treatment on d 5, recipients with IVP embryos ovulating an original CL by hCG had lower P/ET compared with those ovulating by GnRH independent of embryo type. In addition, d 0-hCG with IVP embryo increase total pregnancy loss (from d 32 to 60) compared with d 0-GnRH with IVD embryos but did not differ to others (Figure 2).

Based on our overall results and contradicting our central hypothesis, replacing GnRH with hCG to induce ovulation on the last treatment of the synchronization program was detrimental to fertility in recipient dairy heifers. The replacement of GnRH by hCG on d 0 increased circulating P4 concentrations on d 5 and 7 after the 5-d CIDR Synch program (Tables 1 and 3), which was expected to increase pregnancy outcomes in heifers receiving ET. However, in transfers using fresh or frozen in vitro-produced embryos, heifers treated with hCG only on d0 (H) resulted in lower d 60 P/ET than control heifers (G). In addition, although initial P/ET (d 32) in H heifers receiving IVD embryos tended to be greater than control heifers (G), total pregnancy loss tended to be increased. These results suggest that embryo type (in vitro-produced or in vivo-derived) and time of hCG treatment with subsequent increase in circulating P4 levels are essential factors that impact fertility

outcomes. Our results lead us to conjecture that an earlier increase in circulating P4 concentrations may have resulted in a premature luteolysis onset, which potentially caused early embryonic loss before pregnancy diagnosis. Previous studies supplementing P4 earlier during the metestrus reported an increase in premature luteolysis (Woody et al., 1967; Van Cleeff et al., 1996; Monteiro et al., 2014). Monteiro et al. (2014) determined the effect of supplementing P4 with one or two CIDR implants from d 4 to 18 post-AI on fertility of dairy cows. Cows treated with P4 supplementation had greater circulating P4 concentrations starting on d 5 post-AI, and d 34 and 62 P/AI were not different between treatments. Nevertheless, when only non-pregnant cows were analyzed, P4 supplementation increased the proportion of premature luteolysis (< d 19 post-AI) compared with control cows (51% vs. 24%, respectively).

Although hCG damaged fertility of recipient heifers when used on the last d of the synchronization program, an additional hCG 5 d later seems to have antagonized this early increase in circulating P4 concentration, as demonstrated in the HH treatment (Table 5). Two recent studies from our laboratory demonstrated that accessory CL formation induced by hCG from d 5 to 7 of the estrous cycle resulted in reduced expression of estrus, delayed onset of luteolysis and prolonged estrous cycle length in lactating dairy cows (Cunha et al., 2021, 2022). Therefore, we inferred that this hCG on d 5 and the subsequent formation of an accessory CL may have inhibited the premature luteolysis onset stimulated by hCG on d 0.

The successful use of hCG on d 5 to induce an accessory CL was confirmed in our study (Tables 1 and 3). Many studies have shown the use of hCG on d5 of the estrous cycle to increase circulating P4 concentration by inducing an accessory CL in dairy heifers (Helmer and Britt, 1986; Price and Webb, 1989; Rizos et al., 2012; A.M.Niles et al., 2019) or to stimulate the luteotropic effect on the original CL (Veenhuizen et al., 1972; Rizos et al., 2012; Maillo et al., 2014). The supplementation of circulating P4 concentration between d 3 and 7 was related to gene expression in the uterus after d 13 and an increase of conceptus length in heifers (Forde et al., 2009). Our hypothesis was that heifers treated with hCG only on d 5 (GH) would have an increase in P/ET. This hypothesis was due to the potential effects of greater circulating P4 concentrations in increasing conceptus size and interferon-tau production (Rizos et al., 2012). However, in our study, hCG use on d 5 (GH) only increased fertility outcomes compared to the GnRH on d0 (G) when fresh in vivo-derived embryos were used. This result indicates that the effect of accessory CL on fertility of recipient heifers may

be dependent on embryo type or stage. This interaction between accessory CL and embryo type or stage has also been demonstrated in a recent study that investigated the effect of GnRH on d 5 to induce accessory CL on fertility of recipient heifers (García-Guerra et al., 2020). In that study, GnRH on d 5 only reduced pregnancy loss in recipient heifers receiving expanded blastocyst, but it did not affect overall P/ET or pregnancy loss in heifers receiving blastocyst (García-Guerra et al., 2020).

CONCLUSIONS

In summary, hCG on d 0 increased the circulating P4 concentrations and TLA on d 5, indicating an evident luteotropic effect. However, it decreased P/ET of heifers receiving fresh or frozen in vitro-produced embryos, which may result from the earlier increase in circulating P4 concentrations. In fresh in vivo-derived embryos, the detrimental effect of hCG on d0 on fertility outcomes was not observed. Recipient heifers treated with hCG on d 0 tended to a greater d 32 P/ET but also greater pregnancy loss from d 32 to 60 of gestation compared to GnRH on day 0 and hCG again on d 5. Moreover, hCG on d 5 successfully induced accessory CL formation and increased circulating P4 concentration in recipient heifers during the diestrus. Unfortunately, the increased circulating P4 concentrations were not positively reflected in overall P/ET but improved P/ET only in fresh in vivo-derived embryos. Taken together, the effect of hCG treatments was dependent on embryo type, and this parameter should be considered in future studies.

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Table 1. Effect of treatment, day, and interaction treatment by day on luteal dynamic after treatment on d 5 in recipient dairy heifers

	Treatments[1]				*P*-value		
	G	H	GH	HH	Trt	Day	Trt * Day
Heifers with n ≥ 2 CL, % (no./no.)							
d5[2]	2.2 (7/324)	3.1 (10/318)	2.2 (7/325)	1.6 (5/319)	0.56	-	-
d12[3]	4.3 (9/220) [a]	7.3 (17/223) [a]	77.9 (173/222) [b]	73.3 (159/217) [b]	<0.001	-	-
Ovulatory response d5, % (no./no.) [3]	2.9 (9/220) [a]	6.3 (14/222) [a]	78.3 (173/221) [b]	72.2 (156/216) [b]	<0.001	-	-
TLA, mm ± SEM[4]							
d 5	270 ± 6 [a]	312 ± 8 [b]	275 ± 6 [a]	324 ± 8 [b]	<0.001	<0.001	<0.001
d 12	392 ± 10 [a]	419 ± 12 [a]	758 ± 18 [b]	767 ± 19 [b]	<0.001	-	-
Serum P4, ng/mL ± SEM[5]							
d 5[2]	3.01 ± 0.12 [a]	4.08 ± 0.26 [b]	2.93 ± 0.13 [a]	4.59 ± 0.26 [b]	<0.001	<0.001	<0.001
d 7[2]	5.38 ± 0.17 [a]	6.91 ± 0.33 [b]	7.97 ± 0.26 [bc]	10.96 ± 0.50 [c]	<0.001	-	-
d 12[3]	7.12 ± 0.20 [a]	7.38 ± 0.26 [a]	12.50 ± 0.31 [b]	13.00 ± 0.57 [b]	<0.001	-	-

[ab] Heifers with different superscript letters differ between treatments within rows (P<0.05).

[1] Heifers in group G received GnRH on d 0; group H received hCG on d 0; group GH received GnRH on d 0 and hCG on d 5; and group HH received hCG on d 0 and 5.

[2] Only heifers that were scanned on d 5.

[3] Only heifers that had scans on d 5 and 12; healthy animals with at least one visible CL ≥11mm by ultrasonography.

[4] Heifers with at least one visible CL ≥11mm on d 5 by ultrasonography; n heifers =1,193 (G, n =295; H, n =298; GH, n =304; HH, n =296).

[5] Subset of heifers; n = 218. G, n =54; H, n =54; GH, n =55; HH, n =55.

Table 2. Effect of treatment on d 0 (GnRH or hCG – treatments combined) day, and interaction treatments combined by day on luteal dynamic after treatment on d 0 in all dairy recipient heifers used in this study[1]

	Combined treatments[1]		*P*-value		
	GnRH d 0 (G + GH)	hCG d 0 (H + HH)	d0 Trt	Day	d 0 Trt*Day
Heifers with n ≥ 2 CL, % (no./no.)					
d 5[2]	2.0 (14/649)	2.4 (15/637)	0.81	-	-
d 12[3]	41.2 (182/442)	40.0 (176/440)	0.72	-	-
Ovulatory response d5, % (no./no.)	41.4 (182/441)	40.6 (170/438)	0.82	-	-
TLA, mm^2 ± SEM[4]					
d 5[2]	272 ± 4.1	318 ± 6	<0.001	<0.001	0.002
d 12[3]	578 ± 14	591 ± 14	0.99	-	-
Serum P4, ng/mL ± SEM[5]					
d 5[2]	2.97 ± 0.09	4.34 ± 0.18	0.005	<0.001	<0.001
d 7[2]	6.69 ± 0.17	8.92 ± 0.32	0.02	-	-
d 12[3]	9.80 ± 0.24	10.16 ± 0.35	0.99	-	-

[1] Heifers in group G received GnRH on d 0; group H received hCG on d 0; group GH received GnRH on d 0 and hCG on d 5; and group HH received hCG on d 0 and 5.
[2] Only heifers that were scanned on d 5
[3] Only heifers that were scanned on d 12; healthy animals with at least one visible CL ≥11mm by ultrasonography.
[4] Heifers with at least one visible CL ≥11mm on d5 by ultrasonography; n heifers =1,193 (G, n =295; H, n =298; GH, n =304; HH, n =296).
[5] Subset of heifers; n = 218. G, n =54; H, n =54; GH, n =55; HH, n =55.

Table 3. Effect of treatment on d 5 (hCG or not – treatments combined) day, and interaction treatments combined by day on luteal dynamic after treatment on d5 in all dairy recipient heifers used in this study[1]

	Combined treatments[1]		*P*-value		
	No hCG d 5 (G + H)	hCG d 5 (GH + HH)	Trt	Day	Trt*Day
Heifers with n ≥ 2 CL, % (no./no.)					
d 5[2]	2.7 (17/642)	1.9 (12/644)	0.44	-	-
d 12[3]	5.8 (26/443)	75.6 (332/439)	<0.001	-	-
Ovulatory response d5, % (no./no.)	3.9 (23/442)	78.5 (329/437)	<0.001	-	-
TLA, mm[2] ± SEM[4]					
d 5[2]	291 ± 4.9	299 ± 5.0	0.97	<0.001	<0.001
d 12[3]	406 ± 7.8	762 ± 12.7	<0.001	-	-
Serum P4, ng/mL ± SEM[5]					
d 5[2]	3.55 ± 0.25	3.76 ± 0.26	0.99	<0.001	<0.001
d 7[3]	6.15 ± 0.33	9.45 ± 0.51	<0.001	-	-
d 12[3]	7.25 ± 0.29	12.74 ± 0.56	<0.001	-	-

[1] Heifers in group G received GnRH on d 0; group H received hCG on d 0; group GH received GnRH on d 0 and hCG on d 5; and group HH received hCG on d 0 and 5.
[2] Only heifers that were scanned on d 5
[3] Only heifers that were scanned on d 12; healthy animals with at least one visible CL ≥11mm by ultrasonography.
[4] Heifers with at least one visible CL ≥11mm on d 5 by ultrasonography; n heifers =1,193 (G, n =295; H, n =298; GH, n =304; HH, n =296).
[5] Subset of heifers; n = 218. G, n =54; H, n =54; GH, n =55; HH, n =55.

Table 4. Effect of treatment and embryo type on pregnancies [% (no./no.)] per embryo transfer (P/ET) and pregnancy loss in dairy recipient heifers

Day	Treatments[1]				Embryo Type[2]			*P*-value		
	G	H	GH	HH	IVD	IVP-FRESH	IVP-FROZEN*	Trt	Embryo	Trt*Embryo
32	47.7 $^{A\,B}$ (135/283)	39.4 ^{A} (113/287)	47.6 ^{B} (138/290)	44.8 $^{A\,B}$ (128/286)	56.4 ^{x} (137/243)	37.8 ^{y} (218/577)	48.8 ^{x} (159/326)	0.32	<0.001	0.04
46	44.2 ^{B} (125/283)	34.2 $^{a\,A}$ (98/287)	43.5 $^{b\,A\,B}$ (126/290)	39.2 $^{A\,B}$ (112/286)	53.1 ^{x} (129/243)	33.3 ^{y} (192/577)	42.9 ^{z} (140/326)	0.14	<0.001	0.04
60	44.0 $^{a\,b\,B}$ (120/273)	34.2 $^{a\,A}$ (93/272)	44.2 $^{b\,A\,B}$ (123/278)	39.3 $^{a\,b\,A\,B}$ (106/270)	52.8 ^{x} (124/235)	33.2 ^{y} (183/551)	44.0 ^{z} (135/307)	0.09	<0.001	0.10
Loss 46	7.4 (10/135)	13.3 (15/113)	8.7 (12/138)	12.5 (16/128)	5.1 ^{X} (8/137)	11.8 ^{Y} (26/218)	11.9 $^{X\,Y}$ (19/159)	0.45	0.15	0.90
Loss 60	4.0 (5/120)	5.1 (5/93)	2.4 (3/123)	5.4 (6/106)	3.9 (5/129)	4.7 (9/192)	3.6 (5/140)	0.99	0. 99	0. 99
Loss rate	11.1 $^{A\,B}$ (15/135)	17.7 ^{B} (20/113)	10.9 ^{A} (15/138)	17.2 ^{B} (22/128)	9.5 ^{xX} (13/137)	16.1 ^{yY} (35/218)	15.1 ^{Y} (24/159)	0.14	0.13	0.86

^{abc} Heifers with different superscript letters differ within treatments (P <0.05)

^{AB} Heifers with different superscript letters tend to differ between treatments within treatments (P <0.10)

^{xyz} Heifers with different superscript letters differ within embryo types (P <0.05)

^{XY} Heifers with different superscript letters tend to differ within embryo types (P <0.10)

[1] Heifers in group G received GnRH on d 0; group H received hCG on d 0; group GH received GnRH on d 0 and hCG on d 5; and group HH received hCG on d 0 and 5.

[2] FRESH = recipient heifers receiving in vivo derived not sexed embryos; IVP = recipient heifers receiving fresh *in vitro* produced embryo with sexed semen; FROZEN = recipient heifers receiving frozen *in vitro* produced embryo with sexed semen

*Vitrified and slow-freezing embryos were combined from pregnancy analysis due to the reduced number of IVP-VIT transfers (n = 40).

Table 5. Effect of treatment and embryo type [*in vivo*-derived (FRESH) and *in vitro*-produced (IVP + FROZEN combined)] on pregnancies [% (no./no.)] per embryo transfer (P/ET) and pregnancy loss in dairy recipient heifers

Day	In vivo-derived				In vitro-produced				Embryo type		*P*-value		
	G	H	GH	HH	G	H	GH	HH	In vivo-derived	In vitro-produced	Trt	Embryo	Trt*Embryo
32	45.6 ^{A} (27/58)	61.2 ^{B} (41/67)	63.9 ^{B} (39/61)	52.6 $^{A B}$ (30/57)	48.0 ^{a} (108/225)	32.7 ^{b} (72/220)	43.2 ^{a} (99/229)	42.8 ^{a} (98/229)	56.4 (137/243)	41.8 (377/903)	0.50	<0.001	0.02
46	43.1 ^{a} (25/58)	56.7 $^{a b}$ (38/67)	62.3 ^{b} (38/61)	49.1 $^{a b}$ (28/57)	44.4 $^{a A}$ (100/225)	27.3 ^{b} (60/220)	38.4 ^{a} (88/229)	36.7 $^{a B}$ (84/229)	53.1 (129/243)	36.8 (332/903)	0.30	<0.001	0.01
60	43.6 ^{a} (25/56)	54.7 $^{a b}$ (35/64)	63.3 $^{b A}$ (38/60)	47.3 $^{a b B}$ (26/55)	43.8 ^{a} (120/217)	27.9 ^{b} (93/272)	39.0 ^{a} (85/218)	37.2 ^{a} (80/215)	52.7 (124/235)	37.1 (318/858)	0.19	<0.001	0.03
Pregnancy loss 32-46	7.4 (2/27)	7.3 (3/41)	2.6 (1/39)	6.7 (2/30)	7.4 ^{A} (8/108)	16.7 ^{B} (12/72)	11.1 $^{A B}$ (11/99)	14.3 $^{A B}$ (14/98)	5.8 (8/137)	11.9 (45/377)	0.60	0.05	0.68
Pregnancy loss 46-60	0.0 (0/25)	7.9 (3/38)	0.0 (0/38)	7.1 (2/28)	5.0 (5/100)	3.3 (2/60)	3.4 (3/88)	4.8 (4/84)	3.9 (5/129)	4.2 (14/332)	0.99	0.98	0.99
Total loss 32-60	7.4 $^{A B}$ (2/27)	14.6 ^{A} (6/41)	2.6 ^{B} (1/39)	13.3 $^{A B}$ (4/30)	12.0 (13/108)	19.4 (14/72)	14.1 (14/99)	18.4 (18/98)	9.5 (13/137)	15.7 (59/377)	0.17	0.05	0.63

^{abc} Heifers with different superscript letters differ within treatments (P <0.05)

^{AB} Heifers with different superscript letters tend to differ between treatments within treatments (P <0.10)

[1] Heifers in group G received GnRH on d 0; group H received hCG on d 0; group GH received GnRH on d 0 and hCG on d 5; and group HH received hCG on d 0 and 5.

[2] In vivo-derived embryos (FRESH) = recipient heifers receiving in vivo-derived not sexed embryos; In vitro-produced embryos (IVP + FROZEN combined) = recipient heifers receiving fresh (IVP) and frozen (FROZEN) in vitro-produced embryos with sexed semen.

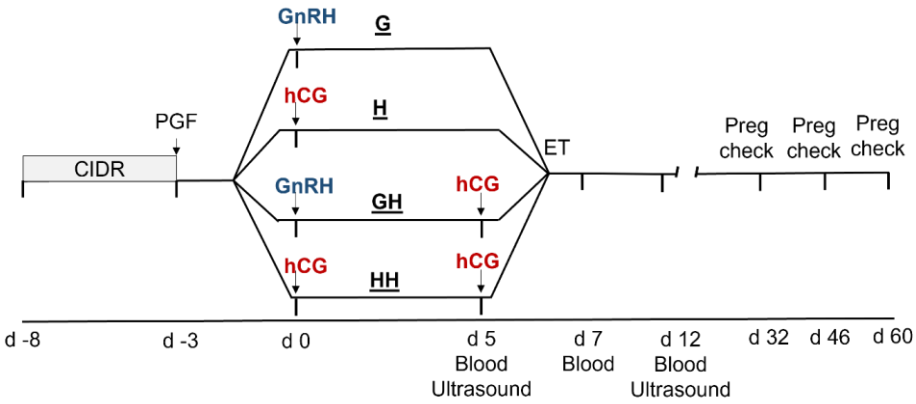


Figure 1. Schematic illustration of the experimental design. Controlled internal drug release (CIDR) device was inserted intravaginally on d -8 and removed d -3, followed by the administration of prostaglandin $F_{2\alpha}$ 500mg (PGF). Heifers on d 0 were randomly assigned to receive 1 of 4 treatments: G– GnRH 100mg on d 0; H– hCG 3,300UI on d 0; GH– GnRH on d 0, and hCG on d 5; and HH – hCG on d 0, and again on d 5. Ultrasound exams were performed on d 5 and 12. Blood samples were collected on d 5, 7, and 12 of the experiment. Pregnancy diagnoses (Preg check) were performed on d 32 by ultrasound.

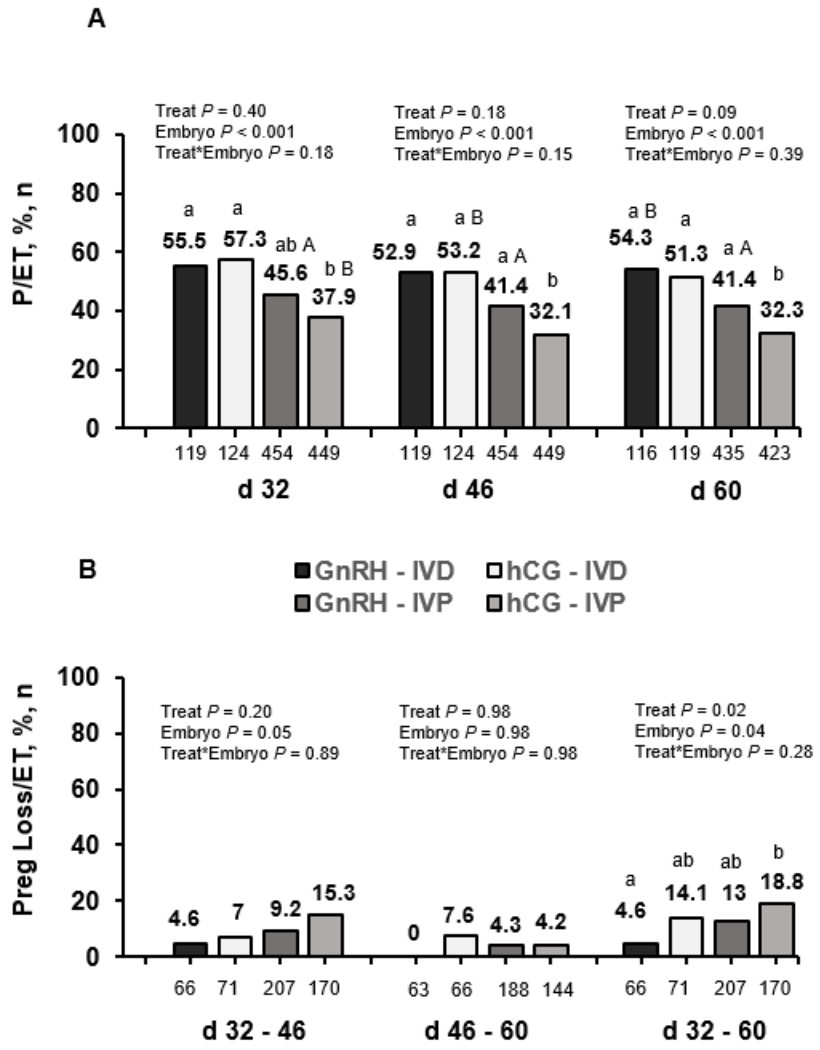


Figure 2. Effects of treatment (GnRH d0 and hCG d0), embryo type (IVD and IVP) and interaction treatment × embryo type on (**A)** P/ET d 32, 46, and 60 [(%), n], **(B)** pregnancy loss d 32-46, d 46-60, and d 32-60 [(%), n] in dairy recipient heifers receiving in vivo-derived [IVD (not sexed embryos)] or in vitro-produced [IVP (fresh, slow-freezing, and vitrified with sexed semen)]D embryos. GnRH [G (GnRH on d 0) and GH (GnRH on d 0 and hCG on d 5) combined]; hCG [H (hCG on d 0) and HH (hCG on d 0 and 5) combined]. $P \leq 0.05$ were considered a statistically significant difference. ^{abc} Columns with different superscript letters differ ($P<0.05$). ^{AB} Columns with different superscript letters tend to differ ($P<0.10$). Data is presented with adjusted *P* thresholds.

S1. Effect of treatment, embryo type and interaction on P/ET [% (no./no.)] and pregnancy loss in dairy recipient heifers

Day	G IVD	H IVD	GH IVD	HH IVD	G IVP-FRESH	H IVP-FRESH	GH IVP-FRESH	HH IVP-FRESH	G IVP-FROZEN	H IVP-FROZEN	GH IVP-FROZEN	HH IVP-FROZEN	*P*-value Trt	Embryo	Trt*Embryo
32	45.6 $^{a\,A}$ (27/58)	61.2 ^{a} (41/67)	63.9 $^{a\,B}$ (39/61)	52.6 ^{a} (30/57)	43.5 ^{a} (64/147)	29.8 ^{b} (42/140)	41.9 ^{a} (62/148)	35.2 $^{a\,b}$ (50/142)	57.6 ^{b} (38/66)	38.2 ^{a} (29/75)	44.1 $^{a\,b\,A}$ (30/68)	58.4 ^{B} (45/77)	0.39	<0.001	0.02
46	42.1 ^{a} (25/58)	56.7 $^{a\,b}$ (38/67)	62.3 ^{b} (38/61)	49.1 $^{a\,b}$ (28/57)	40.8 $^{b\,B}$ (60/147)	24.1 ^{a} (34/140)	37.2 ^{b} (55/148)	30.3 $^{a\,b\,A}$ (43/142)	51.5 ^{b} (34/66)	32.9 ^{a} (25/75)	38.2 $^{a\,b}$ (26/68)	49.4 ^{b} (38/77)	0.23	<0.001	0.02
60	43.6 ^{a} (25/56)	54.7 ^{a} (35/64)	63.3 $^{b\,B}$ (38/60)	47.3 $^{a\,b\,A}$ (26/55)	39.9 $^{b\,B}$ (57/143)	24.1 ^{a} (33/132)	36.9 ^{b} (52/141)	30.4 $^{a\,b\,A}$ (41/135)	53.2 ^{b} (33/62)	33.3 ^{a} (24/71)	40.6 $^{a\,b}$ (26/64)	51.4 ^{b} (36/70)	0.15	<0.001	0.05
Pregnancy loss 32-46	7.7 (2/27)	7.3 (3/41)	2.6 (1/39)	6.7 (2/30)	6.3 ^{a} (4/64)	19.0 ^{b} (8/42)	11.3 $^{a\,b}$ (7/62)	14.0 $^{a\,b}$ (7/50)	10.5 (4/38)	13.8 (4/29)	13.3 (4/30)	15.5 (7/45)	0.54	0.11	0.88
Pregnancy loss 46-60	0.0 (0/25)	7.9 (3/38)	0.0 (0/38)	7.1 (2/28)	5.0 (3/60)	2.9 (1/34)	5.4 (3/55)	4.6 (2/43)	2.9 (1/34)	4.0 (1/25)	0.0 (0/26)	5.3 (2/38)	0.99	0.99	0.99
Total loss 32-60	7.7 (2/27)	14.6 ^{B} (6/41)	2.6 ^{A} (1/39)	13.3 (4/30)	11.0 (7/64)	21.4 (9/42)	16.2 (10/62)	18.0 (9/50)	13.2 (5/38)	17.3 (5/29)	13.3 (4/30)	20.0 (9/45)	0.16	0.12	0.89

^{abc} Treatments groups with different superscript letters differ within embryo type (P <0.05)

^{AB} Treatments groups with different superscript letters tend to differ within embryo type (P <0.10)

[1] FRESH = recipient heifers receiving in vivo derived not sexed embryos; IVP= recipient heifers receiving fresh *in vitro* produced embryo with sexed semen; FROZEN = recipient heifers receiving frozen *in vitro* produced embryo with sexed semen

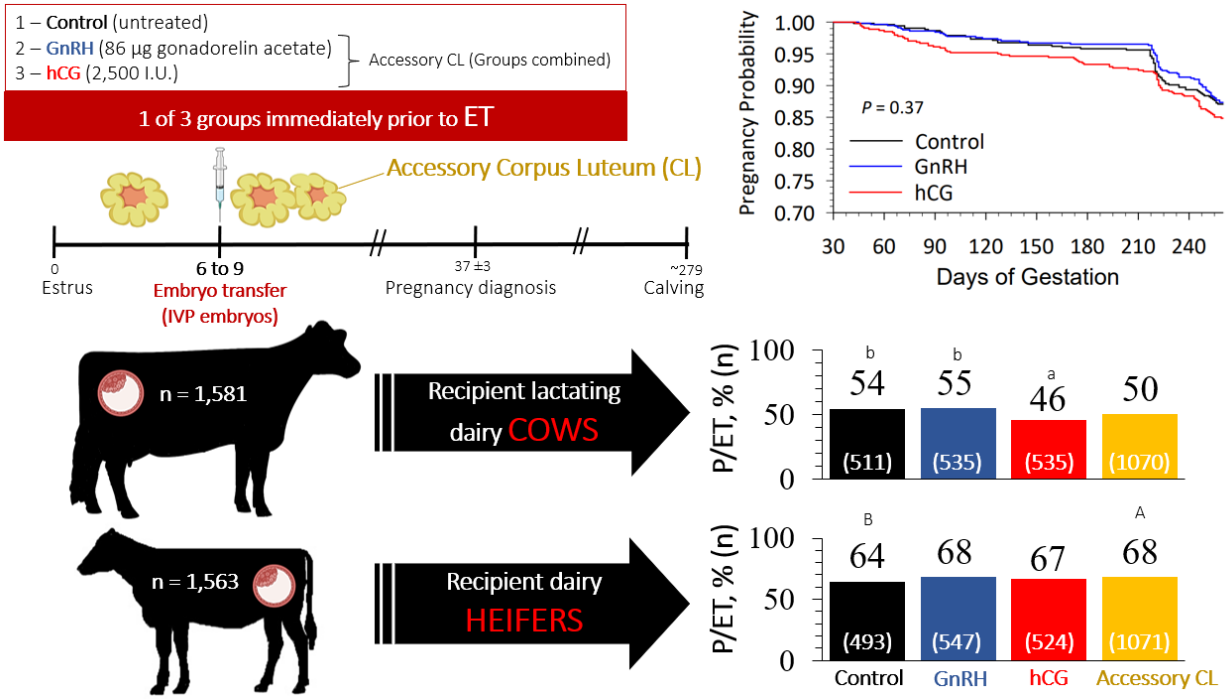
*Vitrified embryos were excluded from pregnancy analysis due to the reduced number of transfers (n = 40)

5 CHAPTER 4

STUDY 3

Manuscript accepted to be published in the JDS Communications, November 2022

Graphical Abstract:



Highlights:

- Accessory CL induced on d of ET tended to improve overall P/ET in heifers
- GnRH improved P/ET in primiparous cows receiving expanded blastocyst fresh IVP embryos vs. hCG
- hCG tended to decrease P/ET in primiparous cows receiving expanded blastocyst fresh IVP embryos
- hCG reduced calving/ET in primiparous cows receiving expanded blastocyst fresh IVP embryos
- GnRH tended to improve P/ET and calving/ET in animals receiving vitrified IVP embryos vs. controls

Summary: Our objective was to determine the effect of inducing an accessory CL using GnRH or hCG at the d of ET on P/ET, calving/ET, and pregnancy loss in recipient dairy heifers and lactating cows receiving IVP embryos. Recipients were randomly assigned to receive GnRH, or hCG, or no treatment (control) immediately before ET on d 6 to 9 of the estrous cycle. Parity and embryo characteristics were factors that impacted the fertility outcomes of GnRH and hCG treatments on d of ET. Overall, treatments did not have a major impact on P/ET or calving/ET and had no effect on pregnancy loss; however, the high P/ET results in the present study may have reduced any beneficial impact of accessory CL formation.

Running head: GnRH OR hCG ON THE DAY OF EMBRYO TRANSFER

Effect of inducing accessory corpus luteum formation with GnRH or human chorionic gonadotropin on the day of embryo transfer on fertility of recipient dairy heifers and lactating cows

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Abstract: The objective was to determine the effect of inducing an accessory corpus luteum (CL) using GnRH or human chorionic gonadotropin (hCG) on the day of in vitro produced (IVP) embryo transfer (ET) on pregnancy per ET (P/ET) and calving/ET in dairy heifers and lactating cows. Dairy heifers (11-15 mo of age; n =1,547) and lactating cows (n =1,480) detected in estrus by tail chalk (d0) were used as recipients. Before ET, the presence of a CL was evaluated by transrectal palpation from d 6 to 9 of the estrous cycle. Animals with a CL were randomly assigned to receive one of three treatments immediately before ET: control (no treatment; n = 1,009), GnRH (86 µg of GnRH; n = 1,085) and hCG (2,500 IU; n = 1,069). Embryos were implanted in the uterine horn ipsilateral to the ovary with a CL (fresh IVP embryos, n= 2,544; vitrified IVP embryos n= 545; slow-freezing IVP embryos, n= 74). Pregnancy diagnosis was performed on d 37±3 of gestation by transrectal palpation. Pregnancy loss data and calving records were collected from the dairy farm management software. Treatment did not affect P/ET, calving/ET and pregnancy loss either overall or within parity. When treatments inducing CL formation were combined (GnRH + hCG), heifers tended to have greater P/ET than controls (67.7 vs. 63.5 %, respectively). Yet, calving/ET were similar. Response variables were also analyzed within embryo type and parity. For heifers receiving stage 6 (blastocyst) fresh IVP-embryos, hCG had greater P/ET than controls (74.5 vs. 51.1%, respectively). In addition, GnRH tended to have greater P/ET than controls (67.8 vs. 51.2 %, respectively). However, calving/ET in heifers receiving blastocyst fresh IVP-embryos was similar among treatments. When only stage 7 (expanded blastocyst) fresh IVP embryos were considered, primiparous GnRH cows had greater P/ET (59.3 vs. 47.1%) and calving/ET (48.6 vs. 38.1%) than hCG. Moreover, hCG decreased calving/ET compared with controls in primiparous transferred with expanded blastocyst fresh IVP embryos. In summary, the effects of hCG or GnRH at ET on P/ET and calving/ET were inconsistent according to different embryo characteristics (e.g., embryo stage) and parity of recipients. Furthermore, treatment did not improve the overall fertility outcomes for recipient animals receiving IVP embryos.

BODY OF THE PAPER

The timing of progesterone (**P4**) increase after ovulation is crucial to the establishment and maintenance of pregnancy (Forde et al., 2011; Lonergan and Sánchez, 2020), promoting a uterine environment appropriate for embryo elongation (Clemente et al., 2009). Progesterone supplementation during early embryo development increases the expression of triglyceride synthesis and glucose transport-associated genes used as embryo energy sources (Forde et al., 2009). Moreover, circulating P4 concentrations > 1 ng/mL after the first 5 d of pregnancy in cows submitted to AI and an increase in P4 after d 7 in recipients have been correlated with an increased probability of pregnancy (Starbuck et al., 2001; Kenyon et al., 2013). Additionally, low circulating P4 concentrations are associated with decreased fertility (Lonergan, 2011). Yet, supplementation of circulating P4 concentrations during embryo development has not resulted in a consistent increase in fertility across studies (Monteiro et al., 2015; Steichen and Larson, 2019; García-Guerra et al., 2020).

The induction of an accessory corpus luteum (**CL**) has been used in the last two decades to increase circulating P4 concentrations in the early pregnancy or pre-implantation period (Besbaci et al., 2020). The ovulation inducers used for accessory CL formation are GnRH and its analogues, or human chorionic gonadotropin (**hCG**; Vasconcelos et al., 2011; Nascimento et al., 2013). The GnRH acts on specific sites located in the anterior pituitary, causing a release of an LH surge (Estes et al., 1977), while hCG has high homology with LH (Stenman et al., 2006), which makes it a more efficient ovulation inducer in cattle compared with GnRH (Cabrera et al., 2021b). A recent study indicated that cows on d 7 of the estrus cycle had a lower ovulatory response when treated with 100 µg of GnRH (gonadorelin acetate) compared with 2,000, 2,500 and 3,300 IU of hCG (79.0, 88.9, 92.9 and 95.6 %, respectively; Cabrera et al., 2021a). Besides the induction of an accessory CL, heifers and cows receiving hCG still have an increase in the original CL area induced by the luteotropic effect of the hCG (El Azzi et al., 2022). Moreover, the induction of an accessory CL by hCG during the early luteal-phase increases serum P4 concentrations (Cunha et al., 2022).

To the best of our knowledge, no studies have determined the effect of hCG and GnRH on fertility of nulliparous, primiparous and multiparous recipients receiving *in vitro*-produced (**IVP**) embryos using a large sample size. The studies found in the literature determined the effect of using either GnRH or hCG on the day of embryo transfer (**ET**) or using small sample sizes and the results are controversial, ranging from no difference to positive effects on P/ET (Vasconcelos et al., 2011; Niles et al., 2019; García-Guerra et al., 2020). In addition, Niles et al. (2019) reported that the administration of hCG on d of ET

seems to reduce pregnancy loss in dairy cattle ET programs. These results indicate a need for additional studies with large sample sizes on using GnRH and hCG to improve fertility after ET. Hence, our objective was to determine the effects of using GnRH or hCG immediately before ET on P/ET, pregnancy loss, and calving/ET in a large sample size of recipient dairy heifers and cows receiving IVP embryos. We hypothesized that GnRH or hCG immediately before ET would improve P/ET and Calving/ET and decrease pregnancy loss.

All animal handling and experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison. Lactating dairy cows were allocated in freestall barns and dairy heifers in drylots with shade and access to water *ad libitum*. Cows were fed twice and heifers once daily with a TMR formulated to meet or exceed the nutritional requirements of lactating dairy cows or dairy heifers (Nutrient Requirements of Dairy Cattle, 2001). Treatments and embryo transfers were conducted from July to August 2020 on a large commercial dairy herd in Oregon, USA. Recipient cows and heifers ET selection was based on daily detection of estrus by tail chalk. Cows ≥ 45 DIM and heifers at least 330 days old (~11 months of age) in estrus were eligible to receive ET. Animals not observed in estrus after two weeks of daily observation by tail chalk received $PGF_{2\alpha}$ weekly until estrus detection. Animals were eligible for receiving ET for the two first services.

Animals were randomly assigned to 1 of 3 groups (Control, GnRH, and hCG). Control did not receive any treatment before ET [d 6 (n = 2), d 7 (n = 824), d 8 (n = 176) or d 9 (n =7) of the estrous cycle]. Animals in GnRH received 86 µg of gonadorelin acetate (Fertagyl, Merk Animal Health, NJ) i.m. immediately before ET [d 6 (n = 2), d 7 (n = 882), d 8 (n = 193) or d 9 (n =8) of the estrous cycle]. Animals in hCG received 2,500 IU of hCG (Chorulon, Merk Animal Health, NJ) i.m. immediately before ET [d 6 (n = 1), d 7 (n = 886), d 8 (n = 177) or d 9 (n =5) of the estrous cycle]. Embryos were transferred to the ipsilateral uterine horn of the ovary with a CL detected by transrectal palpation performed by experienced veterinarians (n = 4). A total of 3.163 ET were performed on 3.027 animals (68% Holstein × Jersey crossbred, 29% Jersey, and 3% Holstein). Of the 3.027 animals, 136 (heifer, n = 18; primiparous, n = 86; multiparous, n = 32) received a second ET during the study after being detected in estrus following the first ET. At transfer, cows (n=1,480) averaged 89.5 DIM (53 to 196 DIM), and heifers (n=1.547) average age was 357.4 d (331 to 441 d).

Animals received fresh IVP embryos (**IVP-F**; n= 2,544), vitrified IVP embryos (**VIT**; n= 545) and slow-freezing IVP embryos (n= 74). All embryos were fertilized with

frozen-thawed sex-sorted semen, except for 14 embryos, in which conventional semen-fertilized fresh blastocysts were used (Control n=5; GnRH n=7; hCG n=2). The fresh embryos consisted of 29 early blastocysts, 335 blastocysts, 2,105 expanded blastocysts, 56 hatched blastocysts, and 19 expanded hatched blastocysts. The frozen and the VIT embryos consisted of blastocyst stage embryos.

Eighteen animals were culled before pregnancy diagnosis and were excluded from the analysis. Pregnancy was diagnosed on d 37 ±3 of gestation by transrectal palpation. Animals observed in estrus after ET were also considered not pregnant and received insemination or a subsequent ET (n = 136). Animals previously diagnosed as pregnant at d 37 ± 3 of gestation with signs of abortion or detected in estrus by tail chalk during gestation were considered to undergo pregnancy loss after confirmation by transrectal palpation. Pregnancy was reconfirmed before dry-off (~60 d before the due date) by transrectal palpation. Pregnancy loss was only considered in gestations with < 260 d. Data related to recipients (e.g., breed, age, parity), embryo transfers (e.g., estrus date, transfer date, technician, embryo used), pregnancy diagnoses, cullings, abortions, and calvings were retrieved from the farm management software (BovSync, Fond du Lac, WI).

All statistical analyses were performed using the SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Binomial outcomes such as P/ET, calving/ET, and pregnancy loss were analyzed by logistic regression using the GLIMMIX procedure. Nulliparous (n= 1), primiparous (n= 6), and multiparous (n= 11) animals without pregnancy diagnoses were excluded from all analyses. The initial statistical model included the effect of treatment, parity (nulliparous, primiparous, and multiparous), technician, embryo type (IVP-F, VIT, and slow-freezing), or embryo developmental stage (early blastocyst, hatched blastocyst, blastocyst, expanded blastocyst, and expanded hatched blastocyst), day of ET (6 and 7 or 8 and 9), and two-way interactions with treatment. Non-significant variables ($P > 0.20$) and interactions ($P > 0.20$), besides parity × treatment, were removed from the model using backward stepwise elimination. The final model consisted of treatment, parity, and parity × treatment. The final model was also used to analyze the effect of treatment within three different embryo categories separately (IVP-F blastocyst, IVP-F expanded blastocyst embryos, and VIT).

The time to pregnancy loss detection was analyzed by Cox's proportional hazards regression using PHREG procedure. The model included treatment and parity (nulliparous, primiparous, and multiparous). The HAZARDRATIO statement with confidence interval

(**CI**) option was used to calculate the hazard ratio (HR) differences among parities, treatments, and treatments within parities and the 95 % CI to the pregnancy loss. Graphs of the probability of pregnancy survival after the first pregnancy diagnosis were generated by the LogRank Survival Analysis in the Kaplan-Meier option of Sigma-Plot (version 13.0, Systat Software Inc., San Jose, CA). Differences among treatments were considered significant when $P \leq 0.05$, whereas $P > 0.05$ and $P \leq 0.10$ were considered a tendency.

The overall P/ET at 37 ±3 d of gestation in our study was 56.5%. Treatment did not affect overall P/ET and calving/ET (Table 1). The overall P/ET in the present study was considered high compared to previous studies that have induced accessory CL in recipient dairy heifers or cows using GnRH or hCG (Vasconcelos et al., 2011; Niles et al., 2019). Vasconcelos et al. (2011) investigated the effect of GnRH or hCG 7 d after timed-AI or on the day of ET (d7) in lactating Holstein cows synchronized. In that study, overall P/ET on d 28 and 60 were 45.2% and 37.4 %, respectively. The authors performed AI and ET throughout the year on a Brazilian dairy farm with mean temperatures ranging from 11.7°C to 30.3°C with cows housed in ventilated freestall barns. In their study, induction of accessory CL with GnRH or hCG improved d 28 and 60 P/ET compared with controls (no treatment), but treatment did not affect cows receiving timed-AI. In contrast, our study was conducted only during the warm season (July and August) in Oregon, USA, and animals were housed in non-ventilated barns. However, our overall 37 d P/ET in cows was slightly higher (49.8 %) compared with their 28 d P/ET.

In the present study, we did not evaluate ovulatory response to GnRH or hCG, nor number of CL at time of transfer and later. Thus, we did not assess the effect of the interaction treatment by accessory CL formation on P/ET. Still, we speculate that most heifers and cows likely ovulated to either treatment and formed an accessory CL since most recipients were on d 7 (82 %) or 8 (17 %) of the estrous cycle at d of ET (Vasconcelos et al., 1999). Therefore, we combined both GnRH and hCG treatments to evaluate the effect of ovulation inducers on the outcomes. When treatments were combined, ovulation inducers tended ($P = 0.10$) to affect P/ET compared with Control only in heifers. This difference was due to a greater ($P = 0.02$) and a tendency ($P = 0.08$) for greater P/ET in heifers treated (GnRH + hCG) transferred with IVP-F (Table 2) and VIT embryos (Table 2), respectively. Nevertheless, P/ET was not different between treatments in heifers receiving expanded blastocyst IVP-F embryos, which accounted for most transfers in the study (Table 2).

Garcia-Guerra et al. (2020) reported no effect of GnRH administered on d 5 of the estrous cycle on P/ET transferred fresh IVP blastocyst and expanded blastocyst in heifers. Still, GnRH on d5 decreased pregnancy losses between days 33 and 60 of gestation compared with untreated heifers (15.2 vs. 27.1 %, respectively). Niles et al. (2019) also reported a reduction in pregnancy losses of recipient heifers treated with hCG compared with controls (10 vs. 22 %, respectively). However, the limited number of pregnant heifers in that study (n=131) did not provide enough statistical power for a sound evaluation of the effects of hCG on pregnancy losses. In our study, treatment did not affect overall heifers' calving/ET and pregnancy losses (Table 1). Still, in heifers receiving VIT embryos, GnRH tended to increase calving/ET compared with controls (Table 2).

On the contrary, in primiparous cows receiving expanded blastocyst IVP-F embryos, hCG decreased ($P = 0.01$) and tended to decrease ($P = 0.10$) P/ET compared with GnRH and Control, respectively (Table 2). This result is opposed to our hypothesis that hCG would increase P/ET. In addition, previous studies using 2,000 or 3,300 IU of hCG on d 5 after timed-AI increased or tended to increase P/AI only for primiparous cows (Nascimento et al., 2013a; Zolini et al., 2019). A recent meta-analysis, including 107 trials from 52 publications, evaluated the association of GnRH or hCG between d 4 and 15 after AI with P/AI in dairy cattle (nulliparous, primiparous, and multiparous; Besbaci et al., 2020). Their results suggested that GnRH and hCG only provided P/AI improvements in primiparous cows. Moreover, the benefits of GnRH or hCG treatments were only seen in studies with very poor (<30%) or poor (30.1 to 45%) P/AI, whereas ovulation inducers did not benefit cows with good (>60.1%) P/AI. The high P/ET for heifers and cows in the present study may have contributed to not finding major differences among treatments.

Parity had an effect ($P < 0.001$) on overall pregnancy outcomes with greater P/ET and calving/ET in nulliparous (66.4 % and 55.0 %, respectively) than in primiparous (52.0 % and 42.4 %, respectively) and multiparous cows (45.6 % and 35.7 %, respectively). Primiparous cows had greater ($P = 0.02$) P/ET and calving/ET than multiparous. A similar negative direct effect of parity number on P/ET using IVP embryos has been reported by Ferraz et al. (2016). This decrease in P/ET as recipient parity number increases appears to be related to the uterine environment or a systemic effect rather than an oocyte or embryo effect. Calving problems and metritis has been associated with a decrease in P/ET in recipient cows (Ferraz et al., 2016), which appears to contribute to the reduction in P/ET for cows vs. heifers recipients. As cows experience more parturitions, they may be more likely to have experienced calving problems and uterine diseases throughout their lives (Dohoo and Martin, 1984). However, we

did not find studies indicating if calving problems or uterine diseases occurring in previous parities would affect fertility in subsequent parities. Although parity affected P/ET and calving/ET, the proportion of pregnancy loss did not differ among parities (Table 1). These results indicate that parity effects on fertility after ET are most likely related to the pregnancy development during the first 37 d of gestation.

Time to pregnancy loss identification tended to differ ($P = 0.10$) among parities. The hazard to pregnancy loss detection was greater ($P = 0.05$) for multiparous compared with nulliparous (HR = 1.43; CI= 1.00 – 2.10), indicating that multiparous had earlier pregnancy loss compared to nulliparous (Figure 1). Hazard to pregnancy loss did not differ for nulliparous vs. primiparous ($P = 0.16$) or multiparous vs. primiparous ($P = 0.43$). A limitation of our study was that time of pregnancy loss was determined by signs of abortion or identification of estrus after the first pregnancy diagnosis at 37 ± 3 d of gestation or only at a second pregnancy diagnosis before dry-off (~60 d before the due date). Most animals in the study (34.5 %) were identified with pregnancy loss at the second pregnancy diagnosis from 210 and 230 of gestation, which may not be precise. Thus, time to pregnancy loss results in our study should be interpreted with caution because these animals may have failed to show signs of estrus or were not detected in estrus after an earlier pregnancy loss. Future research with more frequent pregnancy confirmations throughout gestation after ET using IVP embryos is required to identify a more accurate time of pregnancy loss.

In conclusion, parity and embryo characteristics were factors that impacted the fertility outcomes of GnRH and hCG treatments on d of ET. The interaction of these factors should be determined in future research to allow a clear interpretation of the effect of treatments on the fertility of recipient dairy heifers and cows. Overall, treatments did not have a major impact on P/ET or calving/ET and had no effect on pregnancy loss; however, the high P/ET results in the present study may have reduced any beneficial effect of GnRH or hCG on d of ET. Using different herds with a variation in fertility may elucidate this premise.

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Notes

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Table 1. Effect of treatment (Control = untreated, GnRH, or hCG immediately before ET) or ovulation inducers (treatments combined; GnRH+hCG) on P/ET, calving/ET, and pregnancy loss in recipient dairy heifers and lactating cows receiving fresh, frozen, or vitrified IVP embryos.

	Treatments			Ovulation inducers (GnRH+hCG)	*P*-value[2]				
	Control	GnRH	hCG		Treatment	Ovulation inducers	Parity	Treatment × Parity	Ovulation inducers × Parity
P/ET 37d, % (n)									
Overall	57.4 (1004)	59.6 (1082)	57.1 (1059)	58.4 (2141)	0.68	0.98	<0.001	0.39	0.23
Nulliparous	63.5 (493)	68.2 (547)	67.2 (524)	67.7 (1071)	0.25	0.10	-	-	-
Primiparous	53.0(362)	54.2 (349)	48.5 (342)	51.4 (691)	0.29	0.61	-	-	-
Multiparous	47.7 (149)	44.6 (186)	45.1 (193)	44.9 (379)	0.84	0.56	-	-	-
Calving/ET,[1] % (n)									
Overall	47.4 (966)	49.2 (1022)	45.7 (1004)	47.5 (2026)	0.38	0.70	<0.001	0.64	0.32
Nulliparous	52.7 (476)	57.6 (514)	54.5 (492)	56.1 (1006)	0.33	0.23	-	-	-
Primiparous	44.0 (348)	43.5 (333)	39.4 (325)	41.5 (658)	0.41	0.45	-	-	-
Multiparous	38.0 (142)	36.0 (175)	33.7 (187)	34.1 (362)	0.70	0.50	-	-	-
Pregnancy loss,[1] % (n)									
Overall	14.8 (538)	13.9 (585)	16.6 (550)	15.2 (1135)	0.38	0.82	0.82	0.55	0.88
Nulliparous	15.2 (296)	12.5 (340)	16.3(320)	14.6 (660)	0.47	0.77	-	-	-
Primiparous	15.6 (178)	16.2 (173)	14.1 (149)	15.2 (322)	0.82	0.99	-	-	-
Multiparous	14.1 (64)	12.5 (72)	22.2 (81)	17.7 (153)	0.27	0.67	-	-	-

[1]Pregnant culled animals (n=153 - sold for dairy n=15; sold for beef n=70; died n=68).

Table 2. Effect of treatment (Control = untreated, GnRH, or hCG immediately before ET) or ovulation inducers (treatments combined; GnRH+hCG) on P/ET, calving/ET, and pregnancy loss in recipient dairy heifers and lactating cows receiving stage 6 (blastocyst) and stage 7 (expanded blastocyst) fresh IVP embryos or vitrified IVP embryos.

	Treatments			Ovulation inducers (GnRH+hCG)	*P*-value				
	Control	GnRH	hCG		Treatment	Ovulation inducers	Parity	Treatment × Parity	Ovulation inducers × Parity
Fresh IVP Blastocyst embryos									
P/ET 37d, % (n)									
Overall	46.4 (112)	54.1 (114)	60.2 (108)	58.1 (222)	0.31	016	0.002	0.61	0.30
Nulliparous	51.2 (43) ^{aA}	67.8 (59) ^{abB}	74.5 (51) ^{b}	70.9 (110)	0.06	0.02	-	-	-
Primiparous	44.7 (47)	43.3 (30)	48.7 (37)	46.3 (67)	0.90	0.87	-	-	-
Multiparous	40.9 (22)	44.0 (25)	45.0 (20)	44.4 (45)	0.96	0.78	-	-	-
Calving/ET,[1] % (n)									
Overall	37.4 (107)	42.1 (107)	48.1 (102)	45.0 (209)	0.37	0.30	0.13	0.90	0.95
Nulliparous	42.5 (40)	51.0 (53)	52.2 (46)	51.2 (99)	0.63	0.34	-	-	-
Primiparous	35.6 (45)	31.0 (29)	47.2 (36)	40.0 (65)	0.37	0.64	-	-	-
Multiparous	31.8 (22)	36.0 (25)	40.0 (20)	37.8 (45)	0.86	0.63	-	-	-
Pregnancy loss,[1] % (n)									
Overall	14.9 (47)	21.0 (57)	17.0 (59)	19.0 (116)	0.80	0.66	0.98	0.80	0.21
Nulliparous	10.5 (19)	21.0 (34)	27.3 (33)	23.9 (67)	0.38	0.11	-	-	-
Primiparous	15.8 (19)	25.0 (12)	0.0 (17)	10.3 (29)	0.82	0.58	-	-	-
Multiparous	22.2 (9)	18.2 (11)	11.1 (9)	15.0 (20)	0.82	0.64	-	-	-
Fresh IVP Expanded blastocyst embryos									
P/ET 37d, % (n)									
Overall	60.7 (631) ^{B}	60.9 (723) ^{B}	56.2 (726) ^{A}	58.2 (1449)	0.14	0.28	<0.001	0.39	0.72
Nulliparous	65.9 (355)	65.8 (404)	64.8 (400)	65.3 (804)	0.93	0.84	-	-	-
Primiparous	55.2 (203) ^{abB}	59.3 (216) ^{b}	47.1 (208) ^{aA}	53.3 (424)	0.04	0.66	-	-	-
Multiparous	50.7 (73)	44.7 (103)	43.2 (118)	43.9 (221)	0.59	0.31	-	-	-
Calving/ET,[1] % (n)									
Overall	50.0 (608) ^{b}	50.3 (684) ^{abB}	45.0 (689) ^{aA}	47.6 (1373)	0.08	0.16	<0.001	0.42	0.41
Nulliparous	54.2 (343)	55.4 (381)	53.3 (377)	54.4 (758)	0.88	0.97	-	-	-
Primiparous	45.6 (195) ^{ab}	48.6 (208) ^{b}	37.6 (197) ^{a}	43.2 (405)	0.07	0.57	-	-	-
Multiparous	41.4 (70)	33.7 (95)	30.4 (115)	31.9 (210)	0.23	0.15	-	-	-
Pregnancy loss,[1] % (n)									
Overall	15.6 (360)	14.2 (401)	16.4(371)	15.3 (772)	0.50	0.55	0.56	0.60	0.49
Nulliparous	16.2 (222)	13.2 (243)	14.8 (236)	14.0 (479)	0.65	0.44	-	-	-

Primiparous	14.4 (104)	15.8 (120)	14.9 (87)	15.5 (207)	0.96	0.81	-	-	-
Multiparous	14.7 (34)	15.8 (38)	27.1 (48)	22.1 (86)	0.29	0.37	-	-	-
Vitrified IVP									
P/ET 37d, % (n)									
Overall	51.8 (197) [A]	59.9 (177) [B]	57.8 (161)	58.9 (338)	0.20	0.08	<0.001	0.74	0.47
Nulliparous	62.3 (69) [A]	76.9 (65) [B]	71.4 (56)	74.4 (121)	0.18	0.08	-	-	-
Primiparous	48.3 (87)	48.6 (72)	50.8 (65)	49.6 (137)	0.95	0.84	-	-	-
Multiparous	41.5 (41)	52.5 (40)	50.0 (40)	51.3 (80)	0.5	0.31	-	-	-
Calving/ET, [1] % (n)									
Overall	43.2 (190) [A]	53.0 (166) [B]	45.7 (151)	49.5 (317)	0.15	0.13	<0.001	0.42	0.27
Nulliparous	52.9 (68) [a]	73.0 (63) [b]	59.6 (52)	67.0 (115)	0.06	0.06	-	-	-
Primiparous	39.3 (84)	36.9 (65)	37.7 (61)	37.3 (126)	0.95	0.77	-	-	-
Multiparous	34.2 (38)	47.4 (38)	39.5 (38)	43.4 (76)	0.50	0.35	-	-	-
Pregnancy loss,[1] % (n)									
Overall	13.7 (95)	7.4 (95)	16.9 (83)	11.8 (178)	0.18	0.99	0.24	0.78	0.56
Nulliparous	14.3 (42)	4.2 (48)	13.9 (36)	8.3 (84)	0.25	0.31	-	-	-
Primiparous	15.4 (39)	14.3 (48)	20.7 (29)	17.5 (57)	0.78	0.78	-	-	-
Multiparous	7.1 (14)	5.3 (19)	16.7 (18)	10.8 (37)	0.50	0.70	-	-	-

[AB] Means within a row with different superscripts tend to differ ($P \leq 0.10$).
[1]Pregnant culled animals (n=153 - sold for dairy n=15; sold for beef n=70; died n=68).

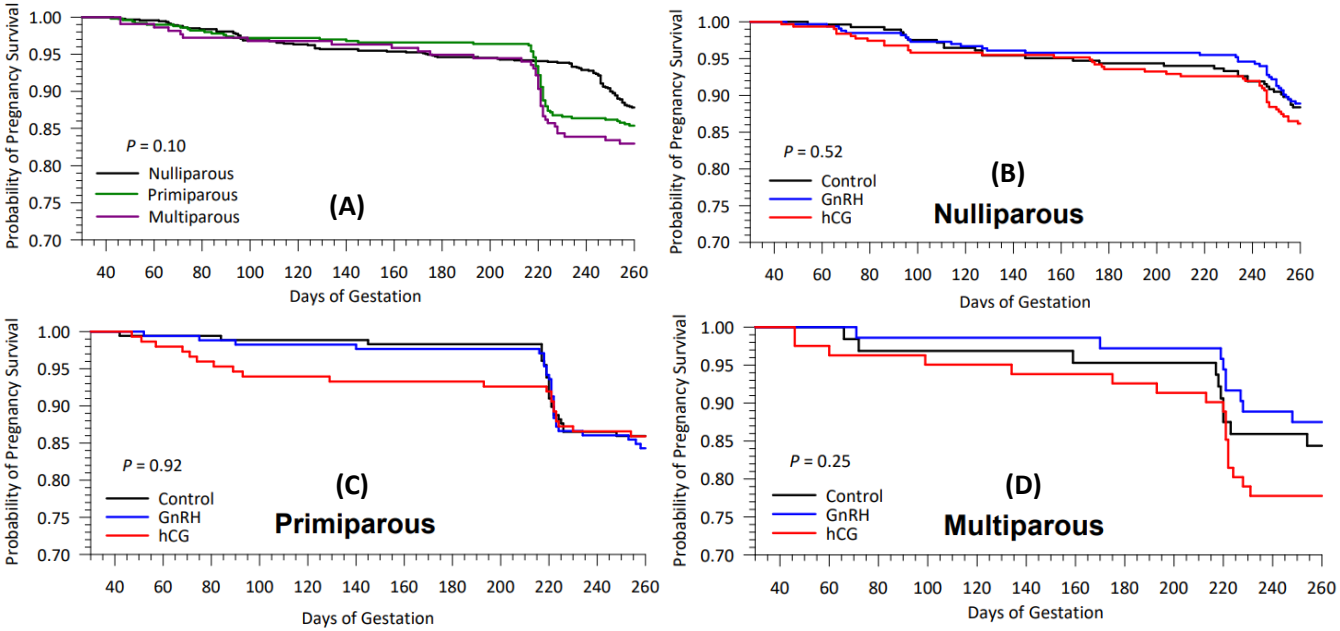

Figure 1. Kaplan-Meier survival curves for the probability of pregnancy survival after pregnancy diagnosis at 37 ± 3 d of gestation in different parities (A; n = 1,644) or only in heifers (B; n = 928), primiparous (C; n = 499), or multiparous (D; n = 217) not treated (Control) or treated with GnRH or hCG immediately before embryo transfer.

6 CONCLUSIONS

The present doctorate thesis was separated into four chapters. In the first chapter the importance of the circulating P4 concentration on conceptus development during the pivotal period of early pregnancy in dairy cattle is discussed with a focus on conceptus development, maternal recognition of pregnancy, luteolysis, and the use of strategies to increase circulating P4 concentration to improve fertility in recipient heifers and lactating dairy cows.

In chapters 2 to 4, the subjects are: Chapter 2, to evaluate in heifers the effect of 3,300 I.U. of hCG or GnRH administered on the last day of a 5d-CIDR Synch program with or without an additional hCG 5 days after the protocol of synchronization on the total luteal area and circulating P4 concentrations during the diestrus; Chapter 3, determine the effect of the administration of hCG or GnRH on the last day of a 5d-CIDR synch protocol and the accessory CL induced on day 5 on pregnancy per embryo transfer at days 32, 46 and 60 and pregnancy losses in recipient dairy heifers based on a previous study, and; Chapter 4, determine the effects of inducing an accessory CL using GnRH or hCG on the day of ET on pregnancy outcomes − P/ET, pregnancy losses, and Calving/ET in recipient nulliparous dairy heifers, primiparous, and multiparous dairy cows receiving IVP embryos. The first hypothesis was that recipient dairy heifers receiving hCG on day 0 of the estrus cycle increases circulating P4 concentrations during metestrus and diestrus compared to GnRH and consequently pregnancy outcomes, the second hypothesis was that recipients with accessory CL induced by hCG have higher circulating P4 concentrations and P/ET, as well fewer pregnancy losses than those induced by GnRH or those without an accessory CL, and the third hypothesis was that higher circulating P4 concentrations during first 12 days of early pregnancy improve P/ET rates.

The first and second thesis hypotheses were partially confirmed in chapters 2 and 3. In the second chapter, we demonstrated that administration of hCG on day 0 of the estrous cycle resulted in a larger CL and higher circulating P4 concentrations after 5 days of the estrus cycle in animals with induced original CL. We have also demonstrated that inducing an accessory CL promotes greater serum P4 concentrations in heifers from days 7 to 12, compared with accessory CL absence. Moreover, the circulating P4 concentration increased faster in heifers with hCG-induced accessory CL and an hCG-induced original CL compared to the other treatments in our study. In the third chapter, the luteal dynamics reported in chapter 2 were confirmed with higher P4 concentrations on those animals with hCG d0-induced original and accessory CLs. Moreover, we have reported the pregnancy outcomes

with the same strategy to increase circulating P4 concentration used in the second chapter. In that trial we demonstrated an interaction between treatment and embryo type on P/ET, demonstrating that different strategies would be required for different embryo types. Indeed, recipients with a successful increase of circulating P4 concentrations tended to have lower pregnancy losses (between days 32 and 60 of pregnancy). Additionally, accessory CL induction (GH) improved P/ET in recipients receiving fresh IVP embryos than in GnRH-induced original CL and in frozen IVP embryos (HH) than hCG-induced original CL. Contrarily, recipients with original CLs induced by hCG had lower pregnancies from frozen embryos, having also lower overall P/ET compared to other treatments, indicating its detrimental effects on pregnancy maintenance.

In the last chapter, an accessory CL was induced either with hCG or GnRH on the day of IVP-ET without previous treatment. Nulliparous heifers, primiparous, and multiparous cows receiving different embryo types, fresh blastocyst, fresh expanded blastocyst, and vitrified IVP-embryos were used. Induced accessory CL formation tended to improve overall P/ET in heifers. hCG-induced original CL increased and GnRH-induced original CL tended to increase P/ET in fresh-IVP blastocyst recipient heifers. While GnRH-induced original CL improved P/ET and calving/ET in primiparous compared to hCG-induced original CL. Conversely, hCG-induced original CL was detrimental to P/ET in primiparous.

In summary, we have concluded that the effects of inducing an accessory CL with hCG, or GnRH on P/ET, pregnancy loss, and calving/ET were distinct according to the hCG and GnRH treatment used to induce original CLs, different embryo characteristics, and recipient parity. The results from this doctorate thesis trial provided compelling evidence that the enhancement of P4 concentrations by an accessory CL increases fertility depending on the protocol, the type of embryo, and the animal category. Moreover, the hCG at day 0 detrimental effect on P/ET precludes its recommendation in IVP-ET dairy protocols. Studies investigating alternative patterns of hCG administration on day 0 of the estrous cycle to induce original CL in recipient dairy cattle could revert the negative effect on pregnancy reported in this thesis. Studies investigating the effect of accessory CL induction on different embryo stages of development in a better-controlled experimental environment would be necessary to understand the differences in P/ET found in the present work.