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The Effects of Supplemental Progesterone via a CIDR Insert on Pregnancy Rates in Dairy Heifer Recipients of IVP Embryos

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By

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In previous research, progesterone has been supplemented using various techniques, at differing time points during peri-insemination in cattle, with conflicting results. Little research has been conducted on the effects of intravaginal delivery of supplemental progesterone following transfer of embryos produced *in vitro*, and no research was found to investigate this method exclusively in the period post-transfer. Pregnancy rates following the transfer of embryos produced in vitro are considered less than ideal, especially if these embryos have been cryopreserved. Embryos produced *in vitro* are less equipped to handle the freezing process due to alternative ultrastructural differences, and display lesser developmental potential in the post-transfer environment compared to embryos derived *in vivo*. The objective of this study was to evaluate the effect of a CIDR insert following the transfer of IVP embryos on pregnancy rates and circulating concentrations of progesterone of dairy heifer recipients.

DEDICATION

This thesis is dedicated to my family and friends. I would have not made it this far without them, their unwavering support and motivation. My grandparents, Jim and Darlene Steichen, have instilled in me the value of determination and their phone calls, care packages, surprise visits, and continual reassurance were so appreciated during my time at Mississippi State. I am forever thankful to be their granddaughter. To my dad, Joe Steichen, thank you for always being a shoulder to lean on, an ear to listen and a voice of reason. As the hardest working person I know, I am so grateful that a sliver of that work ethic is reflected in me, and whether or not I appreciated it at the time, your trial by fire worked. To my sisters Amyjo and Jillian, thank you for your continual love and laughter at the littlest thing. Finally, thank you to my Mississippi family; you know who you are. Taking me in for the holidays, weekends, treating me as your own, and having the utmost confidence in me. It is immensely appreciated, more than you know. Thank you is not a strong enough word, for all of them, but these people have significantly contributed to the person I am today, and this degree would not have been attainable without them.

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CHAPTER I

LITERATURE REVIEW

Progesterone

In mammals, the ovary is the primary site of synthesis and secretion of progesterone and estrogen, which mediate several actions throughout the estrous cycle (Graham and Clark, 1997). A continuous supply of cholesterol is imminent for steroidogenesis. Three potential sources are de novo synthesis, hydrolysis of stored cholesterol esters, or exogenesis high- and low- density lipoproteins, with the latter being the primary source for steroid production in the corpus luteum (CL), a transient endocrine organ formed in the ovary following ovulation (Stocco et al., 2007). Progesterone is the pro-gestational hormone that is primarily synthesized by the CL, but can also be produced by the placenta and adrenal glands. Progesterone acts on various target tissues, but the principal tissues include: the hypothalamo-pituitary axis, reproductive tract, and mammary gland (Niswender, 2002). In the uterus, progesterone prepares the endometrium for the initiation and maintenance of pregnancy by inducing cell differentiation (Cummings and Yochim, 1984), glandular secretions and alterations in protein secretions of endometrial cells (Maslar et al., 1986) while suppressing contractility of the myometrium (Parkington, 1983). The initial, rate-limiting step of steroidogenesis is the transport of cholesterol from the cytoplasm into the inner mitochondrial membrane via the steroidogenic acute regulatory (StAR) protein (Stocco et

al., 2007). Here, cholesterol is converted to pregnenolone by mitochondrial cytochrome p450 side chain cleavage (P450scc; Jefcoate et al., 1992) then to progesterone via and 3-beta hydroxysteroid dehydrogenase (3β-HSDII) in the smooth endoplasmic reticulum (Labrie et al., 1992).

The CL is a heterogeneous gland comprised of large steroidogenic luteal cells and small steroidogenic luteal cells, with large luteal cells producing 2- to 40- fold more progesterone than small luteal cells in ruminants (Stocco et al., 2007). While both large and small luteal cells contain receptors for lutenizing hormone (LH), only small luteal cells respond to LH, or cyclic adenosine monophosphate (cAMP) with a 5- to 15-fold increase in secretion of progesterone (Niswender, 2002). When LH binds to its receptors on small luteal cells, protein kinase A (PKA) second messenger pathway is activated, stimulating secretions of progesterone (Niswender, 2002). In contrast, large luteal cells contain prostaglandin F2alpha (PGF2α) receptors; when PGF2α binds to activate protein kinase C (PKC) and inhibit secretions of progesterone, and also increase concentrations of intracellular free calcium (Wiltbank et al., 1991) which promotes apoptosis and cellular degeneration (Niswender, 2002). These small and large luteal cells are derivatives of theca and granulosal cells, respectively.

Chronic secretions of progesterone from the CL are mitigated via LH stimulation, and its control mRNA concentrations coding for enzymes and proteins required for steroidogenesis (Juengel et al., 1995). Every luteal cell has immediate contact with numerous capillaries, giving the CL one of the largest rates of blood flow of any tissue, and allowing for efficient outputs of progesterone (Stocco et al., 2007). In many species, progesterone is primarily transported in the blood via albumin (Pardridge and Mietus,

1979), while transcortin, a corticosteroid binding globulin that typically binds cortisol and corticosterone, accounts for 20% of transportation of progesterone in the bloodstream (Graham and Clarke, 1997). The physiological actions of progesterone on target cells are modulated through the binding to its nuclear receptors in one of two isoforms: A or B, or through non-genomic actions via progesterone receptor membrane component 1 (PGRMC1) and its homolog (PGRMC2). The nuclear receptor belongs to a family of ligand-activated nuclear transcription regulators, characterized as having a highly conserved, DNA-binding domain, and a carboxy-terminal ligand-binding domain (Graham and Clarke, 1997). The two isoforms differ only in length at the amino-terminus, with progesterone receptor-A having 164 additional amino acids (Kastner et al., 1990). Both isoforms of the nuclear progesterone receptor originate from differential promoter usage in a single gene, and bind progestins and modulate transcription of target genes, such as lipoprotein lipase (LPL) and connective tissue growth factor (CTGF) via progestin response elements located in promoter regions of these genes (Kastner et al., 1990; Graham and Clarke, 1997; Forde et al., 2010). Elevated progesterone was found to modify LPL expression in the bovine uterus (Forde et al., 2010). LPL codes for an enzyme involved in lipid metabolism and transport, specifically of triacylglyerols (TAGs) that are thought to function as an energy source for the conceptus prior to glucose availability at the blastocyst developmental stage (Fergusen and Leese, 2006). CTGF expression, involved in cell proliferation, migration and/or adhesion of conceptus trophectoderm during early pregnancy and likely associated with conceptus elongation, is also upregulated in the presence of progesterone (Forde et al., 2010). All steroid hormones exhibit acute effects, manifesting in seconds or minutes, on various secondary

messenger pathways and signal transducing pathways without transcriptional modulation of target genes, that can lead to extensive and sustained downstream consequences (Gellersen et al., 2009).

Finidori-Lepicard et al., (1981) reported progesterone is responsible for signaling the resumption of meiotic division of oocytes arrested in the G2 phase through means of adenylate cyclase inhibition and reduction of intracellular concentrations of cAMP. The expression of progesterone receptors is found in an abundance of tissue types, and is controlled by estrogen and progesterone, which increases and decreases subsequent progesterone receptor expression in target tissues, respectively (Graham and Clarke, 1997). The postovulatory rise in circulating concentrations of progesterone negates proliferative activity and contractility, and induces remodeling of endometrial tissue necessary for embryo growth and implantation (Dey et al., 2004; Gray et al., 2006). Myometrial contractions are reduced through increasing resting potentials and preventing electrical coupling between myometrial cells (Parkington, 1983), decreasing the uptake of extracellular calcium necessary for contraction of myometrial cells (Batra, 1986), and downregulating genes encoding voltage-dependent calcium channels (Tezuka et al., 1995). Rueda et al., (2000) reported a steroid- ligand relationship between lesser concentrations of progesterone and increased apoptosis in bovine luteal cells. Progesterone mitigates prostaglandin production in the uterine environment during gestation by inhibiting the expression of cyclooxygenase (COX-2) coupled with upregulating the prostaglandin catabolizing enzyme, 15-prostaglandin dehydrogenase (Graham and Clarke, 1997). Progesterone is an essential hormone in the establishment and maintenance of pregnancy, and inadequate concentrations (< 1 ng/mL) on d 6 post

estrus decrease early embryo survivability (Mann and Lamming, 2001). Concentrations of progesterone and their effects on embryonic development and pregnancy outcome have been thoroughly investigated (Mann and Lamming, 2001; Starbuck et al., 2006; Forde et al., 2011). Forde et al., (2011) reported that low concentrations of progesterone have deleterious effects on uterine histotroph and subsequent conceptus elongation, supporting previous research that indicates P4 regulates endometrial secretions through altering gene expression in the uterine endometrium (Bauersachs et al., 2006). Follicular development is also regulated by concentrations of progesterone and the negative – feedback control exerted on the frequency of pulsatile gonadotropin- releasing hormone (GnRH) and subsequent LH secretions (Kinder et al., 1996). In lactating dairy cows, the metabolism of estrogen and progesterone is 2.8 times higher compared with non-lactating animals, suggesting that milk production influences circulating progesterone concentrations (Sangsritavong et al., 2002) through increased liver blood flow and steroid metabolism (Wiltbank et al., 2006).

Supplemental progesterone

Exogenous progesterone has been supplemented in numerous studies through mechanisms such as: daily injections of progesterone (Butcher et al., 1992; Garrett et al., 1988), and vaginally via insertion of a controlled internal drug releasing (CIDR) device (Van Cleeff et al., 1996; Purcell et al., 2005; Larson et al., 2007; Chebel et al., 2010; van Werven et al., 2013) or progesterone-releasing device (PRID; Stevenson and Mee, 1991; Walsh et al., 2007; van Werven et al., 2013; O'Hara et al., 2014). Endogenous concentrations of progesterone can also be manipulated through exogenous

supplementation of human chorionic gonadotropin (hCG) to induce the formation of accessory CL (Schmidt et al., 1996; Kerbler et al., 1997; Santos et al., 2001; Lonergan, 2011; Torres et al., 2013), Yan et al., (2016) reviewed 37 studies and found that there was no difference in pregnancy rate odds ratio due to the source of exogenous progesterone, or the administration of progesterone versus progestogens. Supplementation of progesterone has produced varying effects on pregnancy rates (Mann and Lamming, 2001). The volume of literature reporting positive effects of supplemental progesterone on pregnancy (Larson et al., 2007; Stevenson et al., 2007; Forro et al., 2012; Colazo et al., 2013) is ambiguous with studies concluding no significant differences (Villarroel et al., 2004; Arndt et al., 2009; Scott et al., 2009; Monteiro et al., 2014; 2015). However, there are a handful of studies that found supplemental progesterone to have deleterious effects on pregnancy rates (VanCleeff et al., 1996; Parr et al., 2014; Monteiro et al., 2015).

Supplemental progesterone has been administered pre- and post- timed artificial insemination (TAI) (Arndt et al., 2009; Colazo et al., 2013) and pre-transfer of *in vivo* derived embryos (Nishigai et al., 2002). One study has previously investigated the effects of a CIDR device on pregnancy rates of IVP embryos transferred to lactating dairy cows over a 14-day period (from 4 days prior to transfer through 10 days after transfer) (Monteiro et al., 2015), but literature is lacking in post- *in vitro* produced embryo transfer. Monteiro et al., (2015) concluded supplemental progesterone via CIDR insert from day -4 until day 10 post-transfer of IVP embryos decreased pregnancy rates compared to recipients receiving a CIDR for only 4 days (inserted 4 days prior to transfer and removed immediately before), or recipients that received no additional hormones

(15.2% vs. 21.3% vs. 39.7%, respectively). The timing of supplementation and the initial herd pregnancy rates are important factors affecting the efficacy of supplementing progesterone to cattle (Mann et al., 2006; Mann and Lamming, 1991). Supplemental progesterone during the peri-insemination period increased trophoblastic length and increased uterine concentrations of interferon-tau by 4- fold and 6- fold, respectively (Mann et al., 2006). Garrett et al. (1988a) recorded increased endometrial growth factors and protein secretions following supplementation of progesterone, which can be involved in early embryonic development and survival. Mann and Lamming (2001), noted an overall increase of pregnancy rates by 10.3% following review of several exogenous progesterone studies, indicating the early luteal phase to be the optimal time of supplementation. Supplemental progesterone accelerates normal, temporal modifications in gene expression of the endometrium, particularly those associated with uterine histotroph composition (Lonergan et al., 2016), which could facilitate higher rates of conceptus growth and development (Garrett et al., 1988a). Administration of progesterone enhances the secretion of interferon-tau by the conceptus (Mann et al., 2006), embryonic growth rate (Carter et al., 2010), and overall embryo survivability (Inskeep et al., 2004). Carter et al., (2010) recorded altered gene expression of 191 genes (114- down regulated and 71 up-regulated) exhibiting greater than 1.5-fold expression in embryos cultured from oviducts of heifers receiving supplemental progesterone via PRID compared to heifers without supplemental progesterone. Following intramuscular injections of progesterone during the first 4 days of pregnancy, Garrett et al., (1988b) observed shortened intervals of estrus and altered secretory properties of the uterus

consistent with earlier maturation of the uterine environment and advanced release of PGF2α from the endometrium (Garrett et al., 1987).

The administration of RU486, a progesterone receptor antagonist, negated the effect of supplemental progesterone on conceptus growth and elongation (Clemente et al., 2009). Insertion of a PRID into heifers from days 3 to 7 after embryo transfer increased circulating concentrations of progesterone 4- to 5- fold and increased conceptus elongation when recovered on day 14 of gestation (Clemente et al., 2009). However, Robinson et al., (1989) found that insertion of a PRID into dairy cattle from day 10 to day 17 following insemination increased pregnancy rates, but suppressed endogenous production of progesterone. Arndt et al., (2009) reported no difference in pregnancy rates of dairy cows that received a CIDR device from d 4 to 18 post-insemination compared to cows that received no exogenous progesterone. Carter et al., (2008) concluded that insertion of a PRID on day 3 of the estrous cycle increased concentrations of progesterone on subsequent days and was associated with greater embryonic size on days 13 and 16 of gestation, but did not alter stage of embryonic development on day 5 and 7 of pregnancy. Supplemental progesterone during culture of *in vitro* produced embryos resulted in no advantageous effects on embryo development (Clemente et al., 2009; Larson et al., 2011).

Intravaginal CIDR devices are primarily implemented to suppress ovulation and synchronize estrus, and the effects on estrus synchrony were similar to the PRID while providing easier application, better retention rates, and reduced incidence of vaginitis in virgin heifers (Broadbent et al., 1993). Intravaginal CIDR devices are comprised of a nylon spine and silicone skin. The wings are pliable, allowing for folding during the

insertion process, and expand to the original 'T' shape and apply pressure to the vaginal canal to remain in place. The first CIDR-B inserts contained 1.9 grams of micronized progesterone and were initially formulated to deliver progesterone over a 12-day period (Rathbone et al., 2002), and while efficient at synchronizing estrus and suppressing ovulation, the subsequent fertility was hindered due to an increase in the duration of dominance of follicles (Mihm et al., 1999). Currently, a commercially available CIDR device contains 1.38 grams of progesterone (Rathbone et al., 2002). When a CIDR is administered to ovariectomized Holstein cows, serum concentrations of progesterone begin increasing an hour after insertion, and decline just as rapidly once the device is removed (Rathbone et al., 2002).

In vitro embryo production

Assisted reproductive technologies such as *in vitro* production (IVP) of embryos have been associated with increased pregnancy failure when compared to artificial insemination and conventional embryo transfer techniques. This failure of pregnancy to establish may be due to a reduction in post-transfer, developmental competence of embryos produced *in vitro* compared to those produced *in vivo* (Hasler et al., 1995). Oocytes are obtained from donor females via transvaginal aspiration of follicular fluid through an ultrasound-guided needle (Galli et al., 2004). Ovum pickup (OPU) procedures can be performed every 12 to 18 days, and through the first 100 days of gestation (Phillips and Jahnke, 2016). Oocytes recovered on farm, or in a clinic are matured and fertilized in a laboratory before being frozen, or fresh-transferred into recipients. IVP embryos are typically 1 day older than *in vivo* derived counterparts, as

day 0 in IVP embryos is the day of in vitro fertilization (IVF) while day 0 in embryos produced through superovulation and AI is deemed as the day of estrus of the donor (Hasler, 2014). Any recipients to be asynchronous with donor (6 days vs. 7 or 8 days post-estrus) at time of transfer of IVP embryos previously resulted in a reduction of pregnancy rates by 10 to 16% (Hasler, 1998; 2014). As of 2009, the vast majority of all beef and dairy embryos (99% and 94%, respectively) are frozen in a direct transfer cryopreservation system in 0.25 mL straws containing ethylene glycol as the cryoprotectant (Hasler, 2014). Pontes et al., (2010) reported a higher yield of oocytes from *Bos indicus* donors than *Bos taurus* breeds, and of all oocytes recovered on average (11.4 ± 3.9) from Holstein donors following OPU procedures, 69.9% (8.0 ± 2.7) were viable and 18.7% (2.1) yielded embryos without the use of exogenous hormones. However, despite breed effects on donor oocyte characteristics, pregnancy rates were similar between either source of oocytes (Pontes et a., 2010). On average, 9 days of *in vitro* culture are required from the time oocytes are aspirated from donors until resultant embryos may be vitrified or transferred into recipient females (Blondin, 2017). Sexed semen is often used in conjunction with IVP to predetermine embryo sex at conception, and optimize production and profitability (Morotti et al., 2014). In 2016, almost 5 million straws of sexed semen were processed in the United States, 90% of which were from dairy sires (Moore and Hasler, 2017). Xu et al., (2006) found that the *in vitro* fertility of bulls is affected by the sorting process and can have negative effects on blastocyst production.

The percentage of bovine oocytes that reach the blastocyst development stage following maturation, fertilization, and culture *in vitro* is about 30 to 40%, while oocytes

matured *in vivo* and subjected to IVF have a blastocyst rate of 60% (Rizos et al., 2002). Furthermore, Rizos et al., (2002) obtained in vivo matured oocytes via donor estrus synchronization, ablation of the dominant follicle on day 8 of the estrous cycle, twice daily injections of follicle stimulating hormone (FSH) from day 10 to 14, and follicular aspiration of follicles larger than 6 mm on day 14. The ablation of a dominant follicle can be achieved through various tactics such as: ultrasound-guided aspiration, estradiol-17β, and gonadotropin-releasing hormone (Bó and Mapletoft, 2014). Shaw and Good, (2000) compared recovery rates of oocytes and quality of resultant embryos produced from donors that underwent dominant follicle ablation to non-ablated donors. They reported more oocytes were recovered from donors that had the dominant follicle removed, but the amount of high quality embryos did not differ between groups, indicating the higher volume of oocytes resulted in primarily nontransferable embryos (Shaw and Good, 2000). Factors influencing the developmental potential, yield, and quality of IVP embryos can include: initial oocyte source, quality, and maturation and fertilization conditions, sperm preparation techniques, and embryo culture environments. Cumulus-oocyte complex morphology, the number of cumulus cell layers, oocyte coloration and diameter, the amount of periviteline space, and zona pellucida thickness and organization are all morphological factors that influence oocyte quality and future developmental capacity (Blondin and Sirand, 1995; Fair, 2003; Gabrielsen et al., 2001). The alteration of *in vitro* media culture from a one-step system utilizing serum supplementation with, or without cell co-culture to sequential defined, or semi- defined has markedly increased IVF embryo quality (Blondin, 2017). The variation in *in vitro*

media culture utilized in commercial IVF systems can account for differences in quantity, cryosensitivity, and pregnancy rates of high- quality embryos produced (Blondin, 2017).

A blastocyst consists of an inner cell mass (ICM) and blastocoele enclosed in a single layer of trophectoderm. While Hoelker et al., (2014) reviewed the relationship between insemination and time of first cleavage to affect the likelihood of embryos to reach the blastocyst developmental stage, Kubisch et al., (1998) concluded that the timing of cleavage does not accurately predict the timing of blastocyst development, or the incidence of hatching. Around day 8 of pregnancy, the blastocyst will hatch from the zona pellucida and proceed to form an ovoid, then tubular, and filamentous form that is known from then on as the conceptus (Spencer et al., 2016). In the ruminant conceptus, the primary secretory protein around the time of attachment is interferon-tau (Roberts et al., 1992); it can be detected in the trophoblastic cells from day 12 until day 25 (Farin et al., 1990). Kubisch et al., (1998) observed less production of interferon-tau by IVP embryos that achieved the blastocyst developmental stage earlier (day 7 or 8) than later (day 9 or 10), but previous research has indicated earlier developing blastocysts yield higher pregnancy rates post-transfer into recipients (Hasler et al., 1995). Kubisch et al., (1998) also found that the longer an embryo took to develop into a blastocyst, the more unlikely that embryo was to hatch.

Post-transfer, embryos produced *in vitro* can exhibit normal growth and development, end in abortion, or develop fetal and placental defects (Farin et al., 2006). Kruip and den Daas, (1997) reported higher rates of abortion, stillbirths (14.6% vs. 7.8%), perinatal calf mortality (26.8% vs. 9.5%), and incidence of hydroallantois (5.2% vs. 0.6%) in offspring originating from IVP embryos than artificial insemination. When

compared to AI, or *in vivo* embryo-derived calves, IVP calves were also heavier at birth; an observation that is only partially explained by a longer gestational length (Wagtendonk-de Leeuw et al., 2000). Embryos produced *in vitro* have yielded varying pregnancy rates, ranging from 27.7% (Block et al., 2010) to 39.7% (Monteiro et al., 2015) in recent studies, and as high as 45% (Thompson et al., 1989) previously.

Implications and conclusions

In vitro production of bovine embryos offers various advantages to milk and beef production compared to artificial insemination and *in vivo* embryo breeding schemes (Blondin, 2017). However, this process results in ultrastructural, metabolic, and genetic alterations not observed in embryos derived *in vivo* (Kruip and Daas, 1997; Khurana and Niemann, 2000: Lazzari et al., 2002). These alterations can lead to reduced abilities of embryos to establish and maintain a pregnancy.

Progesterone is the pro-gestational hormone responsible for optimizing the uterine environment for early embryonic development and attachment through reducing myometrial contractions and increasing glandular secretions from the endometrium (Forde et al., 2010). When progesterone has been supplemented exogenously in previous studies, conceptus length has increased to various degrees around the time of maternal recognition (between d 15 and 17; Carter et al., 2010; O'Hara et al., 2014). These larger concepti secrete larger amounts of the anti-luteolytic protein interferon-tau (IFN-τ), which suppresses pulsatile prostaglandin F2-alpha and disallows the CL to undergo luteolysis (Cheng et al., 2007). It is thought that supplemental progesterone in the form of a CIDR insert could negate developmental incompetence of bovine embryos produced *in vitro* and increase the establishment and maintenance of pregnancy.

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CHAPTER II

THE EFFECTS OF SUPPLEMENTAL PROGESTERONE VIA A CIDR INSERT ON PREGNANCY RATES IN DAIRY HEIFER RECIPIENTS OF IVP EMBRYOS

Introduction

The production of embryos *in vitro* has exhibited exponential growth since the 1990s, and since 2013 alone (Blondin, 2017). In 2014, 500,000 IVP embryos were produced and accounted for almost 50% of global embryo production (Blondin, 2017), but pregnancy rates following transfer of IVP embryos are still less than ideal (Block et al., 2010; de Souza et al., 2017). Early embryonic loss accounts for 75 to 80% of pregnancy loss in natural bovine pregnancies (Sreenan and Diskin, 1983), and approximately 40% of embryonic loss is estimated to occur between d 8 and 16 of pregnancy (Lonergan, 2011).

Factors contributing to such a loss may include poor embryo quality (Lussier et al., 1987), inefficient cross-talk between endometrial cells and the conceptus (Ulbrich et al., 2010), as well as asynchrony between donor and recipient females (Ferraz et al., 2016). In early gestation, endometrial secretions of growth factors, nutrients, steroids, ions, enzymes and immunosuppressive agents that are imperative for embryonic development are heavily influenced by progesterone (Graham and Clark, 1997). Manning and Lamming (2001) have established a link between lesser maternal

concentrations of progesterone and reduced embryo development coupled with a delayed post-ovulatory increase in plasma progesterone and reduced concentrations of progesterone. Lesser circulating concentrations of progesterone between days 3 to 8 post-ovulation have yielded smaller day 16 embryos, which can negatively affect production of interferon-tau and interfere with successful maternal recognition of pregnancy (Mann and Lamming, 2001). Conversely, increased concentrations of progesterone in the first week post-ovulation increases production of interferon-tau (Mann and Lamming, 1999), enhances endometrial gene expression that improve uterine histotroph (Forde et al., 2009), and accelerates conceptus growth parameters (Garrett et al., 1988). Progesterone has been exogenously supplemented in numerous studies through various methods: daily injections of progesterone (Garrett et al., 2008), and vaginally via insertion of a controlled internal drug releasing (CIDR) device (Van Cleeff et al., 1996) or progesterone-releasing device (PRID; O'Hara et al., 2014). Endogenous concentrations of progesterone can also be increased following supplementation with hCG to induce formations of accessory CL (Kerbler et al., 1997; Wallace et al., (2011).

Pregnancy rates after transfer of *in vivo* generated embryos (ET) have also increased in recipient females administered 1,000 IU human chorionic gonadotropin (hCG) at time of transfer compared to control animals (Wallace et al., 2011). Compared to their *in vivo* counterparts, IVP embryos are less cryotolerant and exhibit lower developmental competence post-transfer (Hasler et al., 1995). Monteiro et al., (2015) has previously studied the effect of supplemental progesterone, beginning before transfer and continuing through 10 days after, on IVP embryo transfer pregnancy rates via a CIDR insert and found progesterone to have a detrimental effect on pregnancy rates of

recipients. However, the administration of CIDR devices in recipient animals, after the transfer of IVP embryos, to rescue high-risk embryos is a field of reproductive physiology that warrants further investigation. The objective of this study was to determine whether supplemental progesterone via a CIDR immediately after embryo transfer (d 7) through d 19 would increase conception rates in dairy heifer recipients and influence circulating concentrations of progesterone. We hypothesized that supplemental progesterone after embryo transfer would increase pregnancy rates.

Materials and methods

Animals

Nulliparous Holstein heifers (n = 452; 479 $\pm$ 33 d of age) were used in this embryo transfer study. Only 175 of the 452 heifers in this study were assessed and scored for body condition by two trained individuals as previously described (1 to 5; Wildman et al., 1982; Edmonson et al., 1989). Heifers were housed on pasture and supplemented with a concentrate to meet or exceed nutritional requirements.

Embryo transfer

Heifers were grouped into 9 transfer groups based on age of heifer as well as lack of previous conception. Each group had at least 25 but no more than 84 heifers. The first transfer group received embryos in early December, 2016 and the last group in the study received embryos in April, 2017. For heifers in each group, ovulation was synchronized via a Co-Synch + CIDR protocol (Lamb et al., 2001). Estrotect patches (Western Point, Inc., Apple Valley, MN) and tailhead paint (Paintstik; LA-CO Industries, Inc., Elks

Grove Village, IL) were used as estrus detection aids in addition to daily, visual inspection for estrous behavior. Heifers observed in estrus (designated as d 0) were evaluated on the day of transfer (6 to 8 d after estrus; 7.15 ± 0.48 d average) for suitability for embryo transfer. At the time of transfer, transrectal ultrasonography was used to examine ovarian structures by a single, experienced technician using a 7.5 MHz transducing probe (Ibex Pro; E.I. Medical Imaging, Loveland,CO). Animals with cystic structures, CL smaller than 10 mm in diameter, or anatomical tract abnormalities (n = 36) did not receive an embryo and were excluded from the study. Cavernous CL as well as location (right or left ovary) of CL were recorded. Before transfer, all heifers received epidural anesthesia (3 mL of 2% Lidocaine, MWI Animal Health; Boise, ID). Suitable recipients received frozen-thawed, IVF-produced (TransOva Genetics, Boonsboro, MD) embryos according to the standard embryo transfer technique from the International Embryo Transfer Society (IETS; Savoy, IL) that were transferred into the uterine horn ipsilateral to the CL by the same experienced technician. Embryos were products of 48 different matings of 35 Holstein donor females and sex-sorted semen from 12 Holstein bulls and allocated equally among treatments. Prior to freezing, all embryos were assigned a developmental stage and quality grade in accordance with the IETS standards. Developmental stage codes utilized in this study were: 4 = morula, 5 = early blastocyst, 6 = blastocyst, and 7 = expanded blastocyst. Quality grades were: 1 = (excellent/good) symmetrical and spherical embryo mass with individual blastomeres that are uniform in size, color, and density with at least 85% of the embryonic cells are an intact, viable, and cohesive mass, and 2 = (fair) moderate irregularities present in the shape of the embryonic mass, or in the size, color, and density of individual embryonic cells with at

least 50% of embryonic cells being an intact, viable, and cohesive mass. Heifers suitable to receive an embryo were randomly assigned to 1 of 2 treatments at the time of transfer: 1) control (n = 212) with no further treatment, or 2) to receive an intravaginal controlled internal drug release device containing 1.38 g of progesterone for 12 d (n = 228; Eazi Breed CIDR; Zoetis Animal Health, Parsippany, NJ).

Blood collection and analysis

Blood samples were collected from a subset of heifers (all heifers from 3 transfer groups; n = 186) via coccygeal venipuncture into 10 mL evacuated tubes (Vacutainer; Becton, Dickinson and Company; Franklin Lakes, NJ). Blood was collected at d 7 (ET) and d 19 (CIDR removal). After collection, blood was refrigerated at 4°C for 24 hr before being centrifuged at 4°C at 1,500 x g for 15 min. Serum was removed and stored at -20°C until analysis. Samples were later analyzed for concentrations of progesterone via immunoassay (Immulite 1000, Siemens, Malvern, PA) according to manufacturer's instructions.

Pregnancy diagnosis

Pregnancy was diagnosed via serum assay for pregnancy-specific protein B (PSPB; Bio Tracking, Inc. Moscow, ID) and confirmed with real-time ultrasonography or rectal palpation by an experienced technician. Pregnancy was diagnosed between d 36 and 57 of gestation (hereafter referred to as d 30) depending on transfer group and confirmed a month later (hereafter referred to as d 60).

Statistical analyses

Some independent variables were grouped for analysis and care was taken so they were evenly distributed among both treatment groups. Heifers were separated into 5 different age groups: 1 (403-449 days; *n* = 76), 2 (450-495 days; *n* = 248), 3 (496-541 days; *n* = 80), 4 (542-587 days; *n* = 16), and 5 (588-631; *n* = 5). Concentrations of progesterone on d 7 and d 19 were also separated into 11 different groups: 1 (0-0.5 ng/mL; n = 1); 2 (0.5-1.5 ng/mL; *n* = 3), 3 (1.5-2.5 ng/mL; *n* = 24), 4 (2.5-3.5 ng/mL; *n* = 47), 5 (3.5-4.5 ng/mL; *n* = 39), 6 (4.5-5.5 ng/mL; *n* = 33), 7 (5.5-6.5 ng/mL; *n* = 13), 8 (6.5-7.5 ng/mL; *n* = 5), 9 (7.5-8.5 ng/mL; *n* = 4), 10 (8.5-9.5; *n* = 2), and 11 (> 9.5 ng/mL; *n* = 2). Pregnancy rate was analyzed using the GLIMMIX procedure of SAS (9.4; SAS Institute, Inc., Cary, NC) and the initial model included treatment, age of heifer, BCS, donor-recipient synchrony, sire, date of transfer, embryo quality grade and developmental stage, ovulatory side, and serum concentrations of progesterone. Terms with the greatest P-value were removed individually from the model in a backwards, stepwise manner to derive the final model for each dependent variable. Two way interactions of all independent variables in the final models were included when $P < 0.10$. Observations from 440 heifers were included in the final model for pregnancy status at d 30 and d 60. The final model for pregnancy rate at d 30 included the effects of treatment, group, sire, and the interaction of treatment × sire. The final model for pregnancy rate at d 60 included the effects of treatment, sire, and the interaction of treatment × sire. The GLM procedure of SAS (9.4) was used to analyze concentrations of progesterone and the initial model included: treatment, age of heifer, BCS, donor- recipient synchrony, sire, date of transfer, embryo quality grade and stage of development, ovulatory side, and

pregnancy status. Observations from 175 heifers were included in the final model for concentrations of progesterone at d 7. Observations from 172 heifers were included in the final model for concentrations of progesterone at d 19. The final model for concentrations of progesterone at d 7 included date of transfer, donor-recipient synchrony, and the respective two-way interaction. The final model for concentrations of progesterone at d 19 included the effects of treatment, pregnancy status at d 30, date of transfer, and the interactions of d 30 pregnancy status × date of transfer and d 30 pregnancy status × treatment. Each mating(batch) was distributed equally amongst treatment groups, so donor and batch were excluded from final analyses. In all cases, statistical significance was declared at $P \leq 0.05$.

Results

Pregnancy rates

Of all 452 dairy heifer recipients, overall pregnancy rates obtained were 27.5 and 23.3% at d 30 and 60 of gestation, respectively (figure 1). Pregnancy rates were similar between treatments at either pregnancy diagnosis (d 30), or confirmation (d 60). For initial pregnancy diagnosis, there was a significant effect of sire, group (Table 1) and treatment×sire on pregnancy rates, while sire and the interaction of treatment×sire affected pregnancy rates at time of pregnancy confirmation. The number of heifers that experienced pregnancy loss (n = 41) was similar between treatments (15.5 vs. 25%) for CON and CIDR, respectively. Pregnancy rates were similar in heifers receiving quality grade 1 embryos compared to quality grade 2 embryos (d 30: 30.5 vs. 22.2%; d 60: 23.6 vs. 16.5%). Of the four embryonic developmental stages (4 = morula, 5 = early

blastocyst, 6 = blastocyst, and 7 = late blastocyst) of transferred embryos, regardless of quality score, there were no differences in pregnancy rates (d 30: 7.1 (*n* = 14), 31.8 (*n* = 192), 22.3 (*n* = 121), and 29.5% (*n* = 112); d 60: 8.3 (*n* = 12), 21.9 (*n* = 169), 17.9 (*n* = 106), and 25% (*n* = 104), respectively). Similarly, heifer BCS, age, and concentrations of progesterone at either sampling had no significant effect on pregnancy outcome at either diagnosis, or confirmation.

Concentrations of progesterone

Concentrations of progesterone in serum were similar between treatment groups on d 7 ($P = 0.8690$). As expected, heifers receiving the control treatment had lesser ($P = 0.0545$) circulating concentrations of progesterone at d 19 compared to heifers in the CIDR group (3.64 ± 0.27 vs. 4.38 ± 0.27 ng/mL). As expected, heifers that were pregnant on d 30 had increased ($P < 0.0001$) concentrations of progesterone on d 19 independent of treatment group when compared to non-pregnant animals (5.21 ± 0.32 vs. 2.81 ± 0.20 ng/mL). Although there was a significant effect of days post-estrus (DPE) and transfer group, there was also a significant interaction of DPE ×group on concentrations of progesterone at the time of embryo transfer (d 7; Table 4). There were significant interactions for treatment × d 30 pregnancy rates (Table 2) and group × d 30 pregnancy rates (Table 3) on concentrations of progesterone on d 19. Concentrations of progesterone at either d 7 and d 19 were unaffected by heifer age, BCS, embryo stage of development or quality grade.

Discussion

The overall pregnancy rates observed in this study are similar to some previously reported (28.3%; Vieira et al., 2014; 27.7%; Block et al., 2010; 26.7%; de Sousa et al., 2017), but less than others using beef or dairy cow recipients (45%; Thompson et al., 1998; 45%; Kruip et al., 1997; 36%; Pontes et al., 2010), and even with IVP embryos fertilized with sex-sorted sperm (40.9%; Xu et al., 2006). Although this current project was not designed to assess pregnancy loss, others have found pregnancy loss in IVP embryo recipients to be caused by failure of placental membrane development and a reduced placental blood vessel development (Farin et al., 2006). Despite the pregnancy rates observed, Hasler, (2014) found heifer recipients to have 10 to 23% greater pregnancy rates compared to cows, in general. Similar pregnancy rates were observed for all stages of embryonic development of embryos transferred in this study. However, Hasler (2012) found that embryos of a developmental stage 7 had decreased survival post-transfer when compared to both stage 5 and 6 embryos post-transfer (43.8 vs. 55.3 vs. 52.1%, respectively). In the current study, there was an observed effect of transfer group on pregnancy rates at d 30, where embryos transferred into recipients in March had the lowest pregnancy rates compared to transfer groups in December, January, February, and April. Lussier et al., (1987) observed that an interval of 40 to 50 days, equivocal to 2 estrous cycles, is required to grow follicles through the antral stage (0.13 mm) to the preovulatory size, indicating the oocyte and follicular cells in the Graafian follicle may be affected by adverse effects for an extended period of time prior to ovulation. Heat stress negatively impacted oocyte quality and increased the abundance of degenerate theca and granulosal cells (Roth et al., 2001), which could affect embryonic development and

quality. Pregnancy rates for both quality grades of embryos in this study did not differ, but previous studies have found that embryos assigned a quality grade 1 had 5- 10% higher pregnancy rates compared with those assigned to a quality grade 2 (Vieira et al., 2014; Ferraz et al., 2016). BCS of heifers did not alter pregnancy outcomes in this study. However, Ambrose et al., (1999) projected pregnancy rates of lactating Holstein cow ET recipients to increase by 37% for each whole unit increase in BCS.

Concentrations of progesterone at cessation of treatment for all pregnant animals, and pregnant animals that received a CIDR device, were similar to those found previously (Pandey et al., 2016). Previously, BCS has been positively associated with plasma concentrations of progesterone: with every whole unit increase of BCS in lactating Holstein ET recipients, Ambrose et al., (1999) projected an increase in circulating progesterone of 2.9 ng/mL. In vitro fertilization rates and blastocyst development (Palma et al., 2008; Mikkola et al., 2017), and even pregnancy rates (Tonello et al., 2005) can vary between bulls. However, due to the number of bulls used in this study, the treatment × sire interaction on pregnancy rates observed in this study may be of limited biological value. Palma et al., (2008) found IVP embryos fertilized with sex-sorted sperm have ultrastructural differences compared to those fertilized with non-sexed sperm. Despite these differences, Rasmussen et al., (2012) did not report a difference in pregnancy rates at either d 32 or d 60 of gestation between lactating cow recipients receiving IVP embryos produced from sex-sorted sperm compared to IVP embryos fertilized from non-sorted sperm cells (d 32: 28 vs. 24%; d 60: 18 vs. 21%, respectively). Though this study explicitly dealt with frozen-thawed IVP embryos, the survivability of IVP embryos following cryopreservation are lesser than cryopreserved *in*

vivo derived embryos (Ambrose et al., 1999; Block et al., 2010; Do et al., 2016). Pregnancy rates are influenced by donor-recipient synchrony, and embryo developmental stage and quality grade (Hasler et al., 2001), but no such effects were found in the present study. Heifer pregnancy rates were unaffected by days post-estrus of the recipient at embryo transfer, however it has been reported previously that animals that are only 6 days post-estrus at time of transfer are less likely to establish a pregnancy than those 7 and 8 days post-estrus (38.4 vs. 44.3 vs. 43%; Ferraz et al., 2016).

In conclusion, supplemental progesterone via a CIDR device did increase circulating progesterone concentrations, but had no effect on pregnancy rates of dairy heifers that received an IVP embryo. Further research is vital in this field of study to improve IVP embryonic survivability and pregnancy outcomes.

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Table 1 Pregnancy rates among transfer groups at pregnancy diagnosis

	Transfer Group								
	1	2	3	4	5	6	7	8	9
D 30 PR (%)	33.96^{bc} (18/53)	25.5^{abc} (13/51)	31.0^{abc} (26/84)	28.6^{abc} (12/42)	44^{c} (11/25)	25.8^{abc} (17/66)	16.7^{ab} (7/42)	12.5^{a} (5/40)	35.1^{bc} (13/37)
SEM	0.48	0.44	0.47	0.46	0.51	0.44	0.38	0.33	0.48

Pregnancy rates for each transfer group at pregnancy diagnosis (d 30) recorded as a percentage of heifers pregnant from the total number of recipients used within each group. Standard errors for each mean are presented. Lack of a common superscript indicates a statistical difference ($P \leq 0.05$).

Table 2 Concentrations of progesterone on d 19 between treatments and pregnancy status

TRT	D 19 P4 Concentrations (ng/mL)	
	Pregnant	Open
CIDR	5.19 ± 0.45^{c}	3.57 ± 0.29^{b}
CON	5.23 ± 0.46^{c}	2.04 ± 0.28^{a}

Concentrations of serum progesterone, in ng per mL of blood according to treatment group and pregnancy status on d 19. Standard errors for each mean are presented. Lack of a common superscript indicates a statistical difference ($P \leq 0.05$).

Table 3 Concentrations of progesterone on d 19 for each group based on pregnancy status at pregnancy diagnosis

Group	D 19 P4 Concentrations (ng/mL)	
	Pregnant	Open
1	4.09 ± 0.54^{b}	1.87 ± 0.39^{a}
2	5.5 ± 0.56^{c}	4.02 ± 0.35^{b}
3	6.03 ± 0.62^{c}	2.46 ± 0.33^{a}

Concentrations of progesterone, in ng per mL of blood at cessation of treatment (d 19) from the subset of heifers subjected to blood sampling in transfer group 1 (12/03/16), group 2 (1/08/2017) and group 3 (2/18/2017). Standard errors for each mean are presented. Lack of a common superscript indicates a statistical difference ($P \leq 0.05$).

Table 4 Table 4 Concentrations of progesterone on d 7 among heifers differing in days post-estrus among groups

Group	D 7 P4 Concentrations (ng/mL)	
	Days Post Estrus (DPE)	
	7	8
1	4.17 ± 0.26^{b}	5.84 ± 0.42^{c}
2	4.28 ± 0.25^{b}	4.38 ± 0.33^{b}
3	2.68 ± 0.19^{a}	3.44 ± 1.51^{abc}

Concentrations of progesterone, in ng per mL of blood at time of embryo transfer (d 7) between varying levels of donor-recipient synchrony from the subset of heifers subjected to blood sampling in transfer group 1 (12/03/16), group 2 (1/08/2017) and group 3 (2/18/17). Standard errors for each mean are presented. Lack of a common superscript indicates a statistical difference ($P \leq 0.05$).

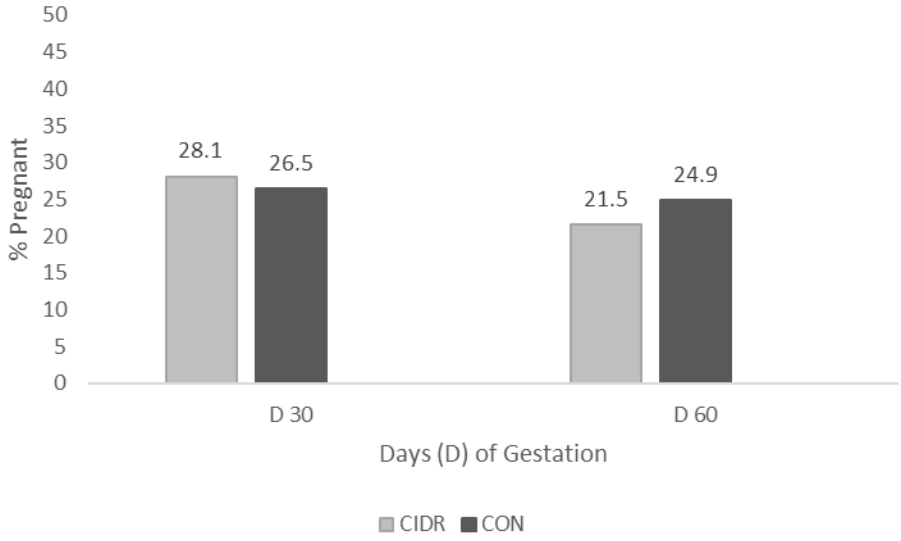

Figure 1 Pregnancy rates between treatment groups at pregnancy diagnosis (d 30) and confirmation (d 60)

Pregnancy rate of recipients in either treatment group at pregnancy diagnosis (d 30) and pregnancy confirmation (d 60) reported as the percentage of animals pregnant at each examination point amongst the total number of recipients used. Lack of a common superscript indicates a statistical difference ($P \leq 0.05$).

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