The role of proestrus on fertility and postovulatory uterine function in the cow

M. Binelli[1,4], G. Pugliesi[1], V.V. Hoeck[1], M. Sponchiado[1], R.S. Ramos[1], M.L. Oliveira[1], M.R. França[1],
F.L. D'Alexandri[1], F.S. Mesquita[2], C.M.B. Membrive[3]

[1]Departament of Animal Reproduction, São Paulo University, Pirassununga, SP, Brazil.
[2]Federal University of Pampa, UNIPAMPA, Uruguaiana, RS, Brazil.
[3]University of São Paulo State "Julio de Mesquita Filho", UNESP, Dracena, SP, Brazil.

Abstract

Modern protocols to synchronize ovulations for timed artificial insemination and timed embryo transfer that include manipulations in the proestrus period (i.e., between luteolysis and estrus) affect fertility in cattle. Specifically, stimulating pre-ovulatory follicle growth and exposure to estrogens after CL regression increase the proportion of cows pregnant and decrease late embryo mortality. Such effects may be due to both pre-ovulatory actions of estrogens and post-ovulatory actions of progesterone, as concentrations of the later hormone may be changed in response to manipulations conducted during proestrus. In the first portion of this paper we describe strategies used recently to manipulate the proestrus period in protocols for synchronization of ovulation, and to present evidence of their effects on fertility. Manipulations of timing and prominence of sex steroids during the proestrus and early diestrus that affect fertility may act on targets such as the endometrium. This tissue expresses receptors for both estrogens and progesterone and these hormones change endometrial function to support conceptus growth and pregnancy maintenance. However, specific cellular and molecular mechanisms through which fertility is affected via manipulations of the proestrus are poorly understood. In the second portion of this paper we describe a well-defined animal model to study changes in endometrial function induced by manipulations conducted during the proestrus. Such manipulations induced endometrial changes on sex steroid receptors expression, cell proliferation, oxidative metabolism and eicosanoid synthesis in the uterus, but not on glucose transport to uterine lumen. In summary, evidence is accumulating to support a positive role of increasing duration and estrogen availability during the proestrus on fertility to synchronization protocols. Such positive effects may be through changes in endometrial function to stimulate conceptus growth and survival.

Keywords: artificial insemination, cattle, endometrium, estradiol, progesterone.

Introduction

During the estrous cycle of cattle, the proestrus phase can be defined as the period between functional luteolysis and the beginning of standing estrus. Anatomically, during this period the CL reduces in size, and the dominant follicle of the ovulatory wave grows quickly to reach its pre-ovulatory size. Endocrinally, proestrus starts when circulating concentrations of progesterone (P4) reach basal levels, allowing LH pulse frequency and estradiol (E2) concentrations to increase and peak. Protocols for timed artificial insemination (TAI) aim to program follicle development for ovulation of a follicle carrying a healthy oocyte at a moment compatible with fertilization by the sperm. The final portion of most protocols includes manipulations to generate a proestrus-like phase, in which P4 concentrations are induced to decrease to allow final pre-ovulatory follicle growth and ovulation. Recent research has targeted variables associated with the proestrus period of protocols. This is because manipulations that affect the proestrus period change fertility of protocols (Perry *et al*., 2005; Meneghetti *et al*., 2009; Peres *et al*., 2009; Dadarwal *et al*., 2013). Manipulations include inducing earlier luteolysis and stimulating pre-ovulatory follicle growth and function. Consequences are longer periods in the absence of luteal P4, longer periods in the presence of follicular E2, higher pre-ovulatory E2 concentrations and/or higher proportion of cows displaying estrus. Secondary consequences are that ovulation of larger follicles lead to the formation of larger and more functional CLs, capable of producing larger amounts of P4 earlier during diestrus, and these effects are known to stimulate conceptus elongation (Forde *et al*., 2009). Most important, such consequences may be stimulatory to fertility, individually or combined. Collectively, specific causative effects of manipulating the proestrus on fertility are usually confounded and could be due to several of the above mentioned consequences working together. The first aim of this paper is to describe strategies used recently to manipulate the proestrus period in protocols for synchronization of ovulation and to present evidence of their effects on fertility.

Cellular and molecular mechanisms through which fertility is affected via manipulations of the proestrus are much less understood. Changes in proestrus may affect fertility through modifications in several processes as final oocyte maturation, preparation of follicular cells for luteinization, and the endocrine control of the oviductal and uterine environments for

---

gamete and embryo development (Pohler *et al*., 2012). In this paper we will focus on the endometrium. The endometrium expresses both P4 and E2 receptors and endometrial functions, such as histotroph secretion and composition, which are modulated by changes in P4 and E2 concentrations associated with proestrus (Robinson *et al*., 2001; Bazer and Slayden, 2008). Histotroph characteristics (i.e., volume and composition) are important for pre-implantation conceptus development. This is because, during pre-implantation, conceptus relies exclusively on histotrophic nutrition for provision of nutrients and paracrine factors for growth (Bazer *et al*., 2010). Thus, inappropriate timing or concentrations of sex steroids and tissue expression of the respective receptors around estrus and ovulation may affect endometrium function and negatively influence growth of conceptus and, consequently, pregnancy outcome. Despite their critical importance, specific biological processes and molecular pathways affected by peri-ovulatory variations in sex steroids are only now starting to be elucidated and have not been sufficiently studied and explored in the endometrium. We have recently reported an animal model to manipulate pre-ovulatory follicle growth and, consequently, proestrus E2 and early diestrus P4 concentrations (Mesquita *et al*., 2014). Based on this model, the second aim of this paper is to report our recent data on the influence of manipulating proestrus on a number of pathways associated with endometrial function and, potentially, conceptus survival.

Collectively, the present review will, first, provide evidence that manipulations of proestrus on protocols to synchronize ovulations affect fertility and, second, summarize our recent data on molecular pathways potentially affected by such manipulations.

Influence of proestrus on fertility

Important aspects of the preovulatory follicle growth phase are the decreasing concentrations of P4 and rising concentrations of E2. Factors determining timing and prominence of changes in P4 and E2 concentrations during proestrus include preovulatory follicle growth rate and final size, and onset of luteolysis. Other factors as physiological status of the cow (heifer, non-lactating and lactating), nutrition, breed and purpose (dairy *vs*. beef) may also influence the synthesis and clearance of sex steroid hormones during proestrus. All these factors ultimately determine the length of proestrus and are recognized as key factors determining fertility rates.

Endocrine profiles are key players impacting pregnancy outcome. For example, in dairy cows, the size of the ovulatory follicle has been positively correlated with the CL size (Vasconcelos *et al*., 2001; Sartori *et al*., 2002) and, consequently, higher circulating P4 concentrations (Moreira *et al*., 2000). In contrast, dairy cows with low preovulatory E2 concentrations and low postovulatory P4 concentrations

are more likely to have reduced fertility (Folman *et al*., 1973; Fonseca *et al*., 1983; Ahmad *et al*., 1995; Larson *et al*., 1997; Bisinotto *et al*., 2010). Also, in beef cattle, such a positive relationship between preovulatory follicle size and E2 concentrations has been reported and it is assumed that the size of the pre-ovulatory follicle can be an indicator of pregnancy probability (Busch *et al*., 2008; Vasconcelos *et al*., 2009; Jinks *et al*.; 2013). Indeed, ovulation of smaller follicles is associated with decreased circulating E2 concentrations at TAI and results in increased late embryonic/fetal mortality (Perry *et al*.; 2005). However, the effect of large follicles may not be consistent between dairy and beef cattle, as ovulation of large follicles (aged or persistent) result in lower fertility even though estradiol is higher in dairy cows (Fonseca *et al*., 1983; Bleach *et al*., 2004; Bisinotto *et al*., 2010).

Duration of proestrus is decisive for fertility outcome. The average duration of proestrus in cattle is 3 to 4 days. However, in most TAI protocols, proestrus is modified, for example, by the induction of ovulation with E2 esters, lutropin (pLH), or GnRH after progestin device withdrawal (Martinez *et al*., 2004). Therefore, a shortened or extended proestrus may affect fertility in synchronized estrous cycles by distinctive mechanisms. A longer proestrus, associated with a longer period of follicle dominance, can be detrimental to embryo quality in dairy cattle (Cerri *et al*., 2009), mainly due to the presence of a persistent dominant follicle and the negative effects on oocyte and embryo quality. In this context, premature oocyte maturation (Revah and Butler, 1996), altered pattern of oviductal proteins (Binelli *et al*., 1999), and low embryo quality (Ahmad *et al*., 1995) have been reported in cows with persistent dominant follicles. Consistently, Bleach *et al*. (2004) documented a negative correlation between the period of follicle dominance and pregnancy rate. On the other hand, a shortened proestrus resulting from an induced ovulation may decrease the fertility outcome by a distinctive mechanism. The importance of the P4 concentrations at time of AI might provide, at least to a certain extent, an explanation for the latter observations. More specifically, slight elevations in P4 concentrations near the time of AI, due to lack of complete CL regression, result in major reductions in fertility of dairy cattle (Souza *et al*., 2007; Brusveen *et al*., 2009; Santos *et al*., 2010; Martins *et al*., 2011; Pereira *et al*., 2013). Therefore, length of proestrus should be long enough to provide time for reduction of P4 concentrations, growth of dominant follicle and E2 priming after induced luteolysis, but not too extended to induce formation of a large follicle associated with persistency and low oocyte viability.

Preovulatory P4 and E2 concentrations are two critical aspects to be considered when evaluating proestrus manipulations and potential effects on fertility. The effects of circulating P4 concentrations during dominant follicle growth in TAI programs differ

between dairy and beef cattle. In dairy cows, recent studies indicated that oocyte/embryo quality (Rivera *et al*., 2011) and fertility (Bisinotto *et al*., 2010) are reduced when follicle development in TAI protocols is performed in cows with transitionally low circulating P4 concentrations. In contrast, reducing P4 concentrations during dominant follicle growth and the resulting larger preovulatory follicle have been associated with higher pregnancy rates in beef cattle (Menegethi *et al*., 2009; Peres *et al*., 2009; Dadarwal *et al*., 2013). Dadarwal *et al*. (2013) demonstrated that lower circulating P4 concentrations stimulate preovulatory follicle growth, subsequent CL size and function and, ultimately, pregnancy success. In contrast, shortened proestrus has a pregnancy-reducing effect. Furthermore, subluteal blood P4 concentrations during follicular growth in beef cows results in oocytes with improved *in vitro* fertilization capacity (Jaiswal, 2007). Consistently, a study from our group (Pugliesi *et al*., 2014) in *Bos indicus* beef cattle, documented that the presence of a functional CL during follicle growth is less favorable to pregnancy success due to a 30% reduction in the proportion of cows ovulating to the protocol and lesser incidence of estrous behavior. Such important differences between the dairy and beef cattle could be a consequence of the effects of lactation, genetic selection, size of animals and steroid metabolism that modify the availability of circulating E2 and P4. In this regard, circulating P4 concentrations during dominant follicle growth could be lower in dairy compared to beef females because of the higher blood flow and steroid metabolism in the liver of high producing dairy cows (Sangsritavong *et al*., 2002; Vasconcelos *et al*., 2003). Thus, several strategies have been proposed in order to trigger efficacious CL regression. One of them is to advance the PGF injection in relation to progestin withdrawal. This allows a uniform decline in P4 concentrations among cows, following progestin withdrawal. The result is a proestrus period with a longer duration that stimulates the pre-ovulatory follicle to grow and achieve its full estrogenic capacity (Mussard *et al*., 2003; Meneghetti *et al*., 2009; Peres *et al*., 2009; Dadarwal *et al*., 2013).

Benefits of estrogen priming during proestrus on pregnancy rates need to be considered in order to improve fertility success. Several strategies have been proposed in order to optimize pre-estrus E2 effects, such as: the addition of E2 at the end of the synchronization protocols, stimulation of ovulation using E2 injections, increasing the interval between the luteolytic injection and the time of expected ovulation (as mentioned above) and increasing gonadotrophic support through injections of equine chorionic gonadotropin (eCG). However, this later strategy will not be addressed in the present paper. Administration of estradiol esters, such as estradiolcypionate (ECP), at the moment of P4 device withdrawal increased pregnancy rates in beef cows submitted to TAI (Sá Filho *et al*., 2011). In addition,

Atkins *et al*. (2013) reported that E2 treatment previous to ovulation affected pregnancy rate of embryo recipient cows independent of P4 concentrations on day 7 post-estrus. In dairy cattle, similar strategies have included the addition of E2 to the end of the Ovsynch protocol (Souza *et al*., 2007), stimulation of ovulation using E2 injections (Pancarci *et al*., 2002; Cerri *et al*., 2004; Stevenson *et al*., 2004), and increasing the interval between the luteolytic injection (PGF) and the time of expected ovulation (Pereira *et al*., 2013).

Collectively, precise management of pre-ovulatory follicle growth and control of timing of ovulation, associated with earlier and complete luteolysis are associated with greater pregnancy success. However, positive fertility results depend on factors such as animal category (e.g., beef *vs*. dairy, heifers *vs*. cows, lactating *vs*. non-lactating, anestrous *vs*. cyclic), timing and types of hormones used in the synchronization protocol.

The influence of proestrus on fertility: mechanistic insights provided by an animal model

Preovulatory circulating E2 and P4 concentrations control several physiological processes that contribute to the establishment and maintenance of pregnancy, including effects on follicular cells, the oocyte, gamete transport and preparation of the uterine environment (Pohler *et al*., 2012). Thereby, the physiological reason(s) for reduced fertility of animals with reduced preovulatory E2 and postovulatory P4 concentrations could include compromised oocyte quality or deficient uterine support for the developing conceptus.

When focusing on the gamete effects, circulating E2 concentrations during proestrus are important regulators of sperm capacitation, fertilization, zygote transport and early embryonic development (Binelli *et al*., 1999; Inskeep, 2004). Furthermore, P4 seems to interfere with gamete development, as dairy cows with lower blood P4 concentrations during the pre-ovulatory follicle growth display a reduced fertility and supplementation of P4 at this development phase increased pregnancy rate (Folman *et al*., 1990; Wehrman *et al*., 1993; Bisinotto *et al*., 2013). In line with these observations, a decrease in oocyte quality, and consequently, low embryo quality (Ahmad *et al*., 1995), may also be caused by a premature maturation of the oocyte (Revah and Butler, 1996) in cows with increased pulse frequency of LH under low circulating P4 during dominant follicle development (Roberson *et al*., 1989; Adams *et al*., 1992). However, whether these observations are direct P4-induced effects on the embryo or indirect, through P4 influences in the reproductive micro-environment provided by the mother, remains to be elucidated.

When focusing on reproductive tract effects, P4 plays a role in oviductal function. The oviduct epithelium undergoes dramatic morphological and

248

Anim. Reprod., v.11, n.3, p.246-253, Jul./Sept. 2014

functional changes throughout the estrous cycle (Abe and Oikawa, 1993). Changes include variations in the expression of oviductal mRNA coding for steroid receptors and epithelial markers and in the sperm-binding capacity of the epithelium in culture (Chen *et al*., 2013). Interestingly, several researchers currently focus on the importance of proestrus endocrine profiles on changes in uterine receptivity during diestrus. This is highly relevant, as previous studies reported that deficient uterine function is the primary factor responsible for infertility in beef (Mussard *et al*., 2003; Bridges *et al*., 2012) and lactating and non-lactating dairy (Sartori *et al*., 2006; Cerri *et al*., 2012) cows that ovulated smaller follicles. In this context, our group recently described a model to manipulate preovulatory follicle growth to produce groups of cyclic beef cows with distinctly different preovulatory E2 concentrations and early diestrus P4 concentrations (Mesquita *et al*., 2014). In this model, nonlactating, presynchronized Nelore cows received a P4-releasing device and E2 benzoate treatment, and were split to receive a PGF treatment (large follicle-large CL group; LF-LCL) or not (small follicle-small CL group; SF-SCL) at device insertion. The PGF treatment induced CL regression and resulted in lower plasma P4 concentrations during dominant follicle growth in the LF-LCL group (1.15 ± 0.14 ng/mL *vs*. 4.91 ± 0.16 ng/mL on the day of P4 device removal). After eight days, devices were withdrawn and PGF administered 42 to 60 h (LF-LCL) and 30 to 36 h (SF-SCL) before GnRH treatment (day 0) to control the length of proestrus. Reproductive tissues and uterine washings were collected at slaughter on day 7. The cows with larger dominant follicles and longer proestrus (LF-LCL group) had larger preovulatory follicle (13.24 ± 0.33 mm *vs*. 10.76 ± 0.29 mm), greater plasma E2 concentrations on day 0 (2.94 ± 0.28 pg/mL *vs*. 1.27 ± 0.20 pg/mL), and greater size and weight of CL (2.72 ± 0.32 cm^3 and 3.1 ± 0.37 g *vs*.1.89 ± 0.16 cm^3 and 2.08 ± 0.15 g) and plasma P4 concentrations (3.71 ± 0.25 ng/mL *vs*. 2.62 ± 0.26 ng/mL) on day 7 compared with the SF-SCL group. The phenotype of smaller follicles and short proestrus was associated with lower receptivity and capacity to support conceptus development, in comparison with the other group, manipulated to ovulate a larger follicle. Based on the contrasting ovarian and endocrine characteristics of these two groups of animals, we studied the endometrial transcriptomic profile by RNA sequencing and different candidate pathways involved in endometrial receptivity on day 7 after induction of ovulation. The results revealed differential enrichment of biological processes, as subsequently described for selected endometrial pathways.

Effects of estrogen and progesterone receptors

The temporal and spatial expression of E2 (ESR) and P4 receptors (PR) in the bovine uterus is critical for establishment of pregnancy (Bazer *et al*., 2010; Shimizu *et al*., 2010). Moreover, an inhibitory feedback mechanism caused by increased diestrus P4 concentrations on its own receptors has been proposed as a key event determining the receptive signature of the endometrium (Okumu *et al*., 2010). Our research group investigated to which extent endometrial mRNA and protein concentrations of ESR and PR can be temporally influenced in response to modulation of periovulatory endocrine milieu proposed by Mesquita *et al*. (2014). The data revealed that day 0 (day of GnRH treatment to induce ovulation) endometrial tissue obtained from cows that ovulated a larger preovulatory follicle and had longer proestrus displayed significantly increased abundance of transcripts coding for ESR1 (1.18 ± 0.16 *vs*. 0.87 ± 0.24), PR-A (1.43 ± 0.41 *vs*. 0.71 ± 0.27), PR-B (1.3 ± 0.21 *vs*. 0.77 ± 0.27) and PR-C (1.29 ± 0.29 *vs*. 0.81 ± 0.34) genes (P < 0.05). On day 4, endometrial tissue gene expression of PR-A (0.71 ± 0.27 *vs*. 1.02 ± 0.26), PR-C (0.83 ± 0.21 *vs*. 1.01 ± 0.15), PRMC (0.81 ± 0.14 vs. 1.01 ± 0.22) was significantly down-regulated in cows with larger preovulatory follicle and longer proestrus (P < 0.05). Similarly, day 7 endometrial tissue showed a down-regulated expression of PR-B (0.79 ± 0.08 *vs*. 1.03 ± 0.27), and PR-C (0.77 ± 0.12 *vs*. 1.04 ± 0.31) in cows with larger preovulatory follicle and longer proestrus (P < 0.05). However, in the latter cow group, expression of ESR2 (1.78 ± 0.65 *vs*. 1.02 ± 0.21) and PRMC (1.01 ± 0.18 *vs*. 0.82 ± 0.21) was significantly increased in day 7 endometrial tissue (P < 0.05). These results confirm that differential periovulatory endocrine profiles impact the transcription of sex steroid-receptor genes in a time-specific manner. Complex modulation of receptor expression by proestrus E2 and early diestrus P4 may fine-tune downstream expression of target genes in the endometrium to regulate uterine receptivity to the developing embryo.

Effects on the glucose transporter system

In mammals, glucose transport from the maternal circulation towards the uterine lumen is essential for conceptus development and pregnancy success (Schneider *et al*., 1981; Gao *et al*., 2009). Therefore, our research group explored whether glucose transport pathways can be influenced in response to modulation of periovulatory endocrine milieu proposed by Mesquita *et al*. (2014). Despite the reported (Forde *et al*., 2010) potential role of glucose transport pathways in the uterine environment during early pregnancy in cattle, the different endocrine patterns during the periovulatory period did not modulate the expression of glucose transporter transcripts, such as SLC2A1, SLC2A3, SLC2A4, SLC2A5, SLC5A1, SLC37A4, ATP1A2 and ATP1B2 on day 7 (França *et al*., 2014, Departament of Animal Reproduction, São Paulo University, Pirassununga, SP, Brazil, unpublished data).

Interestingly, glucose transporter (GLUT1) protein concentration in the endometrium at day 7 was higher in cows with larger preovulatory follicle and longer proestrus compared to their small follicle counterparts (1.24 ± 0.52 ng/µl *vs.* 0.87 ± 0.19 ng/µl, respectively; P < 0.05), but glucose concentrations in the uterine washing did not differ between groups. Therefore, as also indicated by protein abundance and immuno-histochemical characterization of main glucose transporters, the modulation of endocrine profiles during the periovulatory period did not impact glucose availability in the uterine environment. We speculate that systems that regulate glucose homeostasis in the endometrium during early diestrus are not modulated by the proestrus manipulations reported by Mesquita *et al.* (2014).

Effects on the eicosanoid synthesis and signaling systems

Eicosanoids are known for their pivotal role in reproductive processes such as ovulation, implantation, maternal recognition of pregnancy, luteolysis and parturition in mammals (Narumiya *et al.*, 1999), and are involved in pregnancy disorders in humans (Liu *et al.*, 2010). Different endocrine profiles during proestrus and early diestrus using the model described by Mesquita *et al.* (2014) impacted on the endometrial transcript abundance of several genes involved in eicosanoid synthesis and signalling pathways. More specifically, when comparing the endometrial tissue from the LF-LCL cows with the SF-SCL cows, the expression of PTGES1 (fold change 1.32; P = 0.05), AKR1C4 (fold change 1.65; P = 0.04), AKR1C3 (fold change 1.84; P = 0.02), CBR1 (fold change 1.25; P = 0.07), PTGIS (fold change 1.22; P = 0.04), ALOX 12S (fold change 1.46; P = 0.04) were significantly increased, whereas the expression of the CYP2B gene was significantly decreased in the LF-LCL endometrium compared to the endometrium from the SF-SCL cows. Despite the difference in transcript abundance of the latter genes between the cows groups, concentrations of their respective eicosanoid products in the endometrium and in uterine flushings were similar. Interestingly, the transcript abundance of 9-HETE and 14,15-DIHETrE in the uterus was affected by the periovulatory endocrine profile: significantly lower concentrations of 9-HETE were observed in endometrial tissue from the LF-LCL group compared to the SF-SCL group (0.15 ± 0.05 pg/ml *vs.* 0.97 ± 0.28 pg/ml; P = 0.02), whereas the opposite was found for the 14,15-DIHETrE concentrations (121.4 ± 11.1 pg/ml *vs.* 72.5 ± 19.0 pg/ml; P = 0.04). However, the role of the latter metabolites in uterine function and receptivity needs further investigation. Similar to the glucose transport system, the uterine concentrations of eicosanoids were not affected by size of the preovulatory follicle and duration of the proestrus. This suggests a tight molecular control to maintain homeostasis of this class of molecules during early diestrus.

Effects on the oxidative stress defence mechanisms

The redox homeostasis is vital for several cellular processes, such as protein phosphorylation, phospholipid hydrolysis, activation of transcription factors, and inhibition of phosphatases (Thannickal and Fanburg, 2000). Therefore, our group hypothesized that the redox profile of the uterine milieu can be modulated by exposure to ovarian steroids during the periovulatory period.

The endometrial tissue of cows induced to ovulate smaller follicles displayed lower (P < 0.05) activity of catalase (0.79 ± 0.09 *vs.* 0.50 ± 0.07 U/mg protein, P < 0.001) and glutathione peroxidase (2.43 ± 0.39 *vs.* 2.00 ± 0.35 nmol NADPH/min/mg protein, P = 0.04), as well as higher lipid peroxidation (177.7 ± 46.5 *vs.* 255.7 ± 40.6 nmol/MDA/g tissue; P = 0.005) and superoxide dismutase activity (37.8 ± 3.9 *vs.* 44.8 ± 7.7 IU; P = 0.04) compared to the group of cows with larger follicles (P < 0.05; Ramos and Binelli, 2014, Departament of Animal Reproduction, São Paulo University, Pirassununga, SP, Brazil, unpublished data). Therefore, the periovulatory endocrine milieu of cows with smaller preovulatory follicle and shorter proestrus modulate the uterine redox machinery to inhibit response to oxidative stress. Such diminished response may be detrimental to pre-implantation conceptus development.

Effects on the cell proliferation mechanisms

Growth-promoting effects of E2 *in vivo* have been well documented in mammalian endometrium (Quarmby and Korach, 1984; Dallenbach-Hellweg, 1987), whereas P4 is mainly known for its differentiating effect. Hence, we investigated whether the endometrial cell proliferating activities are dependent/responsive to modifications in the periovulatory endocrine milieu. RNAseq data revealed that the global gene expression profile was different when comparing endometrium from cows with differential periovulatory endocrine profiles at early diestrus in beef cattle (Mesquita and Binelli, 2014, Federal University of Pampa, UNIPAMPA, Uruguaiana, RS, Brazil, unpublished data). When focusing on genes related to cell proliferation, a decreased expression (P < 0.0001) was observed, when comparing endometrial tissue from the LF-LCL group to the SF-SCL group, with particular focus on DKK3 (fold change 0.53) and WNT5A (fold change 0.46). The latter genes involved in WNT signaling pathways have been proposed as potential keys for endometrial tissue proliferation, differentiation and embryo growth (Wang *et al.*, 2009; Denicol *et al.*, 2014). Furthermore, a significant decreased expression was observed for genes involved in cell division and proliferation, such as PLK4 (fold change 0.38; P < 0.0001), ESPL1 (fold change 0.25; P < 0.0001), Cdk1 (fold change 0.35; P < 0.0001), KIF15

(fold change 0.18; P < 0.0001), KIFC1 (fold change 0.33; P < 0.0001) when comparing LF-LCL with SF-SCL endometrial tissue. Several of the latter genes have been proposed as potential markers for cancer cell phenotypes (Cheng, 2014). In this context, also genes specifically related to cancer cell signaling pathways, such as MYC (fold change 0.45; P < 0.0001) and NOV (fold change 0.16; P < 0.0001) were significantly down-regulated in LF-LCL *vs*. SF-SCL endometrial tissue. Considering the importance of the differently expressed genes (Cerri *et al*., 2012; Denicol *et al*., 2014), the preliminary transcriptome information, originating from RNA sequencing of the SF-SCL *vs*. the LF-LCL endometrial tissue, might indicate that cows synchronized to ovulate smaller follicles displays a proliferative transcriptome and phenotypic signature at day 7; whereas the endometrium of cows with larger ovulatory follicles reveals characteristics of a tissue that surpassed the proliferative status and is ready to proceed with other tasks. Such tasks may include the provision of molecules associated with optimal conceptus development. Using qRT-PCR, current research will quantify and re-evaluate the expression of the genes of interest more specifically. Furthermore, using immunohistochemical visualization of cellular proliferation markers, ongoing research will investigate to which extent the latter gene expression differences result in phenotypically relevant differences.

Concluding remarks

Strategic selection of drugs and changes in timing of drug administration in protocols of synchronizing ovulations for TAI or TET can change the length of proestrus in the synchronized estrous cycles. Most importantly, changes that affect the proestrus concentrations of, and time of exposure to E2 increase overall fertility to protocols. Mechanisms through which manipulations of the proestrus increase fertility are still being elucidated, but may include modulation of endometrial cell proliferation and regulation of the intrauterine redox environment.

References

Abe H, Oikawa T. 1993. Observations by scanning electron microscopy of oviductal epithelial cells from cows at follicular and luteal phases. *Anat Rec*, 235:399-410.
Adams GP, Matteri RL, Ginther OJ. 1992. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *J Reprod Fertil*, 96:627-640.
Ahmad N, Schrick FN, Butcher RL, Inskeep EK. 1995. Effect of persistent follicles on early embryonic losses in beef cows. *Biol Reprod*, 52:1129-1135.
Atkins JA, Smith MF, MacNeil MD, Jinks EM, Abreu FM, Alexander LJ, Geary TW. 2013. Pregnancy establishment and maintenance in cattle. *J*
Anim Sci, 91:722-733.
Bazer FW, Slayden OD. 2008. Progesterone-induced gene expression in uterine epithelia: a myth perpetuated by conventional wisdom. *Biol Reprod*, 79:1008-1009.
Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod*, 16:135-152.
Binelli M, Hampton J, Buhi WC, Thatcher WW. 1999. Persistent dominant follicle alters pattern of oviductal secretory proteins from cows at estrus. *Biol Reprod*, 61:127-134.
Bisinotto RS, Chebel RC, Santos JE. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. *J Dairy Sci*, 93:3578-3587.
Bisinotto RS, Ribeiro ES, Lima FS, Martinez N, Greco LF, Barbosa LF, Bueno PP, Scagion LF, Thatcher WW, Santos JE. 2013. Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol. *J Dairy Sci*, 96:2214-2225.
Bleach EC, Glencross RG, Knight PG. 2004. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. *Reproduction*, 127:621-629.
Bridges GA, Mussard ML, Pate JL, Ott TL, Hansen TR, Day ML. 2012. Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression. *Anim Reprod Sci*, 133:16-26.
Brusveen DJ, Souza AH, Wiltbank MC. 2009. Effects of additional prostaglandin F2alpha and estradiol-17beta during Ovsynch in lactating dairy cows. *J Dairy Sci*, 92:1412-1422.
Busch DC, Atkins JA, Bader JF, Schafer DJ, Patterson DJ, Geary TW, Smith MF. 2008. Effect of ovulatory follicle size and expression of estrus on progesterone secretion in beef cows. *J Anim Sci*, 86:553-563.
Cerri RL, Santos JE, Juchem SO, Galvão KN, Chebel RC. 2004. Timed artificial insemination with estradiol cypionate or insemination at estrus in high-producing dairy cows. *J Dairy Sci*, 87:3704-3715.
Cerri RL, Rutigliano HM, Chebel RC, Santos JE. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. *Reproduction* 137:813-823.
Cerri RL, Thompson IM, Kim IH, Ealy AD, Hansen PJ, Staples CR, Li JL, Santos JE, Thatcher WW. 2012. Effects of lactation and pregnancy on gene expression of endometrium of Holstein cows at day 17 of the estrous cycle or pregnancy. *J Dairy Sci*. 95:5657-5675.
Chen S, Einspanier R, Schoen J. 2013. In vitro mimicking of estrous cycle stages in porcine oviduct epithelium cells: estradiol and progesterone regulate differentiation, gene expression, and cellular function.

Anim. Reprod., v.11, n.3, p.246-253, Jul./Sept. 2014

251

Biol Reprod, 89(3):54.

Cheng W-Y. 2014. *Attractor molecular signatures and their applications for prognostic biomarkers*. New York, NY: Columbia University .Thesis PhD.

Dadarwal D, Mapletoft RJ, Adams GP, Pfeifer LFM, Creelman C, Singh J. 2013. Effect of progesterone concentration and duration of proestrus on fertility in beef cattle after fixed-time artificial insemination. *Theriogenology*, 79:859-866

Dallenbach-Hellweg G. 1987. *Histopathology of the Endometrium*. New York: Springer-Verlag. pp. 55-86.

Denicol AC, Block J, Kelley DE, Pohler KG, Dobbs KB, Mortensen CJ, Ortega MS, Hansen PJ. 2014. The WNT signaling antagonist Dickkopf-1 directs lineage commitment and promotes survival of the preimplantation embryo. *FASEB J*. doi: 10.1096/fj.14-253112.

Folman Y, Kaim M, Herz Z, Rosenberg M. 1990. Comparison of methods for the synchronization of estrous cycles in dairy cows. 2. Effects of progesterone and parity on conception. *J Dairy Sci*, 73:2817-2825.

Folman Y, Rosenberg M, Herz Z, Davidson M. 1973. The relationship between plasma progesterone concentration and conception in post-partum dairy cows maintained on two levels of nutrition. *J Reprod Fertil*, 34:267-278.

Fonseca FA, Britt JH, McDaniel BT, Wilk JC, Rakes AH. 1983. Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J Dairy Sci*, 66:1128-1147.

Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P, Lonergan P, Roch JF. 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol Reprod*, 81:784-794.

Forde N, Spencer TE, Bazer FW, Song G, Roche JF, Lonergan P. 2010. Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. *Physiol Genomics*, 41:53-62.

Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. 2009. Select nutrients in the ovine uterine lumen. II. Glucose transporters in the uterus and periimplantation conceptuses. *Biol Reprod*, 80:94-104.

Inskeep EK. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. *J Anim Sci*, 82(E-suppl): E24-E39.

Jaiswal RS. 2007. *Regulation of follicular wave patterns in cattle*. Saskatoon: University of Saskatchewan. PhD dissertation.

Jinks EM, Smith MF, Atkins JA, Pohler KG, Perry GA, MacNeil MD, Roberts AJ, Waterman RC, Alexander LJ, Geary TW. 2013. Preovulatory estradiol and the establishment and maintenance of

pregnancy in suckled beef cows. *J Anim Sci*, 91:1176-1185.

Larson SF, Butler WR, Currie WB. 1997. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *J Dairy Sci*, 80:1288-1295.

Liu P, Lu Y, Recker RR, Deng HW, Dvornyk V. 2010. ALOX12 gene is associated with the onset of natural menopause in white women. *Menopause*, 17:152-156.

Martinez MF, Kastelic JP, Mapletoft RJ. 2004. The use of estradiol and/or GnRH in a two-dose PGF protocol for breeding management of beef heifers. *Theriogenology*, 62:363-372.

Martins JP, Policelli RK, Neuder LM, Raphael W, Pursley JR. 2011. Effects of cloprostenol sodium at final prostaglandin F2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. *J Dairy Sci*, 94:2815-2824.

Meneghetti M, Sá Filho OG, Peres RF, Lamb GC, Vasconcelos JL. 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows I: basis for development of protocols. *Theriogenology*, 72:179-189.

Mesquita FS, Pugliesi G, Scolari SC, França MR, Ramos RS, Oliveira M, Papa PC, Bressan, FF, Meirelles FV, Silva LA, Nogueira GP, Membrive CM, Binelli M. 2014. Manipulation of the periovulatory sex steroidal milieu affects endometrial but not luteal gene expression in early diestrus Nelore cows. *Theriogenology*, 81:861-869

Moreira F, de la Sota RL, Diaz T, Thatcher WW. 2000. Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. *J Anim Sci*, 78:1568-1576.

Mussard ML, Burke CR, Gasser CL, Behlke EJ, Colliflower KA, Grum DE, Day ML. 2003. Ovulatory response, luteal function and fertility in cattle induced to ovulate dominant follicles of early or late maturity. *Biol Reprod*, 68:332. (abstract).

Narumiya S, Sugimoto Y, Ushikubi F. 1999. Prostanoid receptors: structures, properties, and functions. *Physiol Rev*, 79:1193-1226.

Okumu L A, Forde N, Fahey A G, Fitzpatrick E, Roche JF, Crowe M A, Lonergan P. 2010. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. *Reproduction*, 140:143-153.

Pancarci SM, Jordan ER, Risco CA, Schouten MJ, Lopes FL, Moreira F, Thatcher WW. 2002. Use of estradiol cypionate in a presynchronized timed artificial insemination program for lactating dairy cattle. *J Dairy Sci*, 85:122-131.

Pereira MH, Sanches CP, Guida TG, Rodrigues AD, Aragon FL, Veras MB, Borges PT, Wiltbank MC, Vasconcelos JL. 2013. Timing of prostaglandin F2α

treatment in an estrogen-based protocol for timed artificial insemination or timed embryo transfer in lactating dairy cows. *J Dairy Sci*, 96:2837-2846.

Peres RF, Claro I, Sá Filho OG, Nogueira GP, Vasconcelos JL. 2009. Strategies to improve fertility in *Bos indicus* postpubertal heifers and nonlactating cows submitted to fixed-time artificial insemination. *Theriogenology*, 72:681-689.

Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, Roberts AJ, Geary TW. 2005. Relationship between follicle size at insemination and pregnancy success. *Proc Natl Acad Sci USA*, 102:5268-5273.

Pohler KG, Geary TW, Atkins JA, Perry GA, Jinks EM, Smith MF. 2012. Follicular determinants of pregnancy establishment and maintenance. *Cell Tissue Res*, 349:649-664.

Pugliesi G, Lopes E, Diaza AMG, França MR, Ramos RS, Araujo ER, Sponchiado M, Scolari SC, Oliveira ML, Maio JRG, Binelli M. 2014. Strategies to modulate the peri-ovulatory endocrine milieu by controlling progesterone concentrations pre- and post-insemination in beef cows. *Reprod Fertil Dev*, 26:120. (abstract).

Quarmby VE, Korach KS. 1984. Influence of 17/S-estradiol on patterns of cell division in the uterus. *Endocrinology*, 114: 694-702.

Revah I, Butler WR. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. *J Reprod Fertil*, 106:39-47.

Rivera FA, Mendonça LG, Lopes G, Santos JE, Perez RV, Amstalden M, Correa-Calderón A, Chebel RC. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. *Reproduction*, 141:333-342.

Roberson MS, Wolfe MW, Stumpf TT, Kittok RJ, Kinder JE. 1989. Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. *Biol Reprod*, 41:997-1003.

Robinson RS, Mann GE, Lamming GE, Wathes DC. 2001. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction*, 122:965-979.

Sá Filho MF, Santos JE, Ferreira RM, Sales JN, Baruselli PS. 2011. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. *Theriogenology*, 76:455-463.

Sangsritavong S, Combs DK, Sartori R, Armentano LE,Wiltbank MC. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. *J Dairy Sci*, 85 2831-2842.

Santos JE, Narciso CD, Rivera F, Thatcher WW, Chebel RC. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination

protocol on reproduction of dairy cows. *J Dairy Sci*, 93:2976-2988.

Sartori R, Rosa GJ, Wiltbank MC. 2002. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci*, 85:2813-2822.

Sartori R, Gumen A, Guenther JN, Souza AH, Caraviello DX, Wiltbank, MC. 2006. Comparison of artificial insemination versus embryo transfer in lactating dairy cows. *Theriogenology*, 65:1311-1321.

Schneider NO, Calderon RO, de Fabro SP. 1981. Isolation and characterization of cell membranes from human placenta. *Acta Physiol Latin Am*, 31:283-289.

Shimizu T, Krebs S, Bauersachs S, Blum H, Wolf E, Miyamoto A. 2010. Actions and interactions of progesterone and estrogen on transcriptome profiles of the bovine endometrium. *Physiol Genomics*, 42A:290-300.

Souza AH, Gümen A, Silva EP, Cunha AP, Guenther JN, Peto CM, Caraviello DZ, Wiltbank MC. 2007. Supplementation with estradiol-17beta before the last gonadotropin releasing hormone injection of the Ovsynch protocol in lactating dairy cows. *J Dairy Sci*, 90:4623-4634.

Stevenson JS, Tiffany SM, Lucy MC. 2004. Use of estradiol cypionate as a substitute for GnRH in protocols for synchronizing ovulation in dairy cattle. *J Dairy Sci*, 87:3298-3305.

Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol*, 279:L1005-L1028.

Vasconcelos JL, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. 2001. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. *Theriogenology*, 56:307-314.

Vasconcelos JL, Sangsritavong S, Tsai SJ, Wiltbank MC. 2003. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. *Theriogenology*, 60:795-807.

Vasconcelos JL, Sá Filho OG, Perez GC, Silva AT. 2009. Intravaginal progesterone device and/or temporary weaning on reproductive performance of anestrous crossbred Angus x Nelore cows. *Anim Reprod Sci*, 111:302-311.

Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, Veldscholte J, van Doorn HC, Ewing PC, Kim JJ, Grootegoed JA, Burger CW, Fodde R, Blok LJ. 2009. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res*, 15:5784-5793.

Wehrman ME, Roberson MS, Cupp AS, Kojima FN, Stumpf TT, Werth LA, Wolfe MW, Kittok RJ, Kinder JE. 1993. Increasing exogenous progesterone during synchronization of estrus decreases endogenous 17 beta-estradiol and increases conception in cows. *Biol Reprod*, 49:214-220.