Animal Science Papers and Reports
vol. 38 (2020) no. 2, 135-144

Endocannabinoid system and early embryonic loss in Holstein dairy cows*

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(Accepted March 3, 2020)

The precise role of the endocannabinoid system (ECS) at the pregnancy of dairy cows remains elusive, therefore this study was designed to compare different components of the ECS between pregnant cows and those that had experienced early embryonic losses at the mRNA levels. Holstein cows (n=130, average parity 3.3, body condition score of 3.1 at calving) were selected for the study on 10th d postpartum. Cows were subjected to GnRH-based ovulation synchronization protocol for timed artificial insemination (TAI). Blood samples were collected on 16th d after calving to determine interferon-stimulated gene-15 (ISG-15) relative mRNA expression. Prediction of pregnancy status on d 16 after TAI was determined by determining interferon-stimulated gene-15 mRNA expression relative to standard genes (β actin and GAPDH). Results showed CNR1, N-acylphosphatidylethanolamine-specific phospholipase D (NAPEPLD), fatty acid amide hydrolase (FAAH) are differentially expressed in the uterus of cows with early embryonic loss (n=30) when compared to the expression in confirming pregnancies (n= 3). Expression of CNR1 and NAPEPLD were greater (3.56 and 4.72 fold respectively), while FAAH expression (6.75 fold) was lower in cows with early embryonic loss compared to healthy cows. Expression of mRNA for the other candidate genes, which included monoacylglyceride lipase (MGLL), N-acyl ethanolamine amino hydrolase (NAAA) and diacylglycerol lipase (DAGL) was not differentially expressed between compared groups ofcows. Cows with early embryonic loss had lower serum progesterone and greater prostaglandin F2α metabolite (PGFM) levels compared to healthy ones during first 20 days of pregnancy. Our data confirm for the first time that association between the components of the endocannabinoid system and early embryonic losses in dairy cows exists. We provide evidence that endocannabinoid system is altered in the endometrium of cows of early embryonic loss through

*This work was supported by funding from Sari Agricultural Sciences and Natural Resources University (SANRU) project No 03-1396-06.
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increased mRNA expression of *CNR1* and synthesis enzyme (*NAPEPLD*) and decreased mRNA expression of hydrolyzing enzyme (*FAAH*).

KEY WORDS: dairy cattle / embryo loss / endocannabinoids / ISG-15 / pregnancy

Thirty to 40% of pregnancies are lost during the first third of pregnancy, which has been hypothesized to be due to inadequate progesterone secretion by the corpus luteum [Weems *et al,* 2009]. Loss of luteal progesterone secretion during the oestrous cycle is mediated via uterine secretion of prostaglandin F2α [Dirandeh *et al.* 2015] and endocannabinoids (Dirandeh et al., 2019). Previous studies highlighted negative impact that an imbalance of the "endocannabinoid tone" exerts on the course of human pregnancy. Accordingly, it has been reported that women with non-viable first-trimester pregnancies have elevated serum levels of anandamide (AEA) [Taylor *et al.* 2011]. Similarly, women subjected to a successful *in vitro* fertilization of intracytoplasmic sperm injection had lower levels of AEA at the time of implantation when compared to those in which the procedure failed. Furthermore, increased levels of plasma levels of AEA and related endocannabinoids have been reported in women with ectopic pregnancies [Gebeh *et al.* 2013].

The endocannabinoid system (ECS), comprises cannabinoid receptors (CNR1 and CNR2), endogenous cannabinoids (endocannabinoids, anandamide and 2-arachidonoyl glycerol), and the enzymes responsible for the synthesis and degradation of the endocannabinoids [Correa *et al.* 2016]. Anandamide and 2-arachidonoylglycerol (2-AG) are synthesized by a two-step enzymatic process from phospholipids, phosphoinositides and phosphatidylethanolamine. The endocannabinoids are converted to arachidonic acid by fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MGLL), respectively. Arachidonic acid is subsequently metabolized by prostaglandin-endoperoxide synthase-1 (PGH-1) and PGH-2 to produce prostaglandins (PGs). Cumulative evidence shows that the ECS plays a key role in reproduction, from egg fertilization to parturition [Correa *et al.* 2016]. In particular, the endometrium expresses greater levels of AEA than any other reproductive tissue. Interestingly, AEA levels and the expression of other components of the ECS, fluctuate with the menstrual cycle [Lazzarin *et al.* 2004] which indicates that this system is under hormonal control [El-Talatini *et al.* 2010]. Moreover, components of the arcs have been detected at all stages of oocyte maturation and fertilized eggs express high levels of CNR1. The precise role of the ECS in female reproductive physiology still remains elusive; however, several studies have demonstrated that an adequate "AEA tone" is required throughout gestation for a successful and healthy pregnancy [Paria *et al.* 1996, Guo *et al.* 2005]. Thus, during implantation (when the blastocyst adheres and invades the uterine wall), the uterine AEA level are tightly regulated by the uterine expression of the FAAH, since too high or too low levels of AEA are deleterious for gestation [Sun and Dey 2009]. Moreover, the blastocyst regulates its CNR1 expression in order to detect the appropriate level of AEA for a successful implantation [Wang *et al.* 2007].

The precise role of the ECS system in the course of pregnancy of dairy cows remains elusive and further research is warranted. Therefore, this study was designed to compare different components of the ECS between the full-gestation-cows and those experiencing early embryonic loss, at the mRNA levels.

Material and methods

Animals

One hundred thirty Holstein cows of average parity equalling to 3.3 and body condition score of 3.1 at calving entered the study on d 10 postpartum. Cows were housed in free stall barns equipped with fans. Cow diets were formulated to meet or exceed the nutrient requirements established by NRC (2001) for lactating Holstein cows weighing 650 kg, consuming 24 kg of DM, and producing 45 kg/d of milk.

Reproductive management

All cows were synchronized for the first AI after calving, with the modified G6G program (one injection of PGF2α, GnRH 4 days later, and a 7-day Ovsynch starting on 48±3 days in milk (DIM), as described by Heidari *et al.* [2017].

Progesterone and prostaglandin F2α metabolite (PGFM)

Blood samples were collected into vacutainer tubes containing EDTA (10.5 mg, Monoject; Sherwood Medical) on d 12, 14,16,18 and 20 after AI for PGFM measurement and from d 0 to 20 after AI (on alternate days) for progesterone measurement. The blood was centrifuged at 1500 × g for 20 minutes and was frozen at 20°C for subsequent analysis. Plasma progesterone (Diaplus, North York, Ontario, Canada) and PGFM were assayed using an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously [Dirandeh *et al*. 2013]. Inter- and intra-assay coefficients of variation for PGFM were 6.34 and 7.10%, respectively. Intra- and inter-assay coefficients of variation for progesterone were <5%.

Determination of ISG15 mRNA expression and early embryonic loss

Blood samples were collected oon d 16 after timed AI by Vacutainer tubes containing EDTA and stored at -80°C until analysis. Prediction of pregnancy status on d 16 after TAI was determined based on Mohtashamipour *et al*. [2020].

RNA isolation and cDNA synthesis

Total RNA was extracted from blood samples isolated with the RNAeasy Micro kit (Cat. No. 74004; Qiagen, GmbH, Germany) using established protocols in our laboratory [Dirandeh *et al.* 2019] and stored at -80°C pending cDNA synthesis. Any residual genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington,

DE, USA). The purity of RNA (A260/A280) for all samples was above 1.81. The RNA was reversely transcribed in the presence of 1 mmol/L oligo (dT) primer and 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions.

Real-time PCR

Real-time polymerase chain reaction (PCR) was implemented to determine the relative transcripts of ISG15 and endocannabinoid system. Sequence and primer information were published by Abolghasemi *et al.* [2017]. Expression of β-actin and GAPDH transcript were used as an internal housekeeping gene that was stable under our lab condition. Quantification of all transcripts was performed using QuantiFast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) in a 15 μL reaction volume containing 1 μL single-strand cDNA, 7.5 μL of master mix, 1 μL of each forward and reverse primers and 4.5 μL of distilled H2O in a Rotor-Gene 6000 Real-Time PCR software (Corbett Research, Sydney, Australia). The relative levels of mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen 2001].

Statistical analysis

Non-normally distributed data (Shapiro–Wilk test) were transformed to logarithms. Statistical analysis was performed with SAS (SAS Institute, Inc., Cary, NC, USA). The gene expression data were analyzed using t-test. The MIXED procedure of the SAS System (SAS version 9.2, SAS Institute, Cary, NC, USA) was used to perform repeated measures ANOVA (progesterone and prostaglandin $F_{2\alpha}$ metabolite (PGFM) concentrations). Cows were considered as a subject effect and time (weeks) as a repeated effect in the model. Means were compared by Duncan's multiple range test. Significance and tendencies were declared at $P \leq 0.05$.

Results and discussion

Cows with early embryonic loss had lower serum progesterone (P=0.01, Fig. 1) and greater PGFM (P=0.01, Fig. 2) levels compared to healthy cows during first 20 days of pregnancy.

Uterine endometrial CNR1, NAPEPLD and FAAH genes were found to be differentially ($P<0.05$) expressed in cows with early embryonic loss and healthy cows. The mRNA levels of CNR1 were 3.56 fold higher (P=0.02) in the cows with early embryonic loss than healthy cows (Fig. 3). In addition, mean gene expression for NAPEPLD increased 4.72 fold (P=0.04) in the cows with early embryonic loss than in healthy cows (Fig. 3), while expression of mRNA for FAAH was also nearly 6.75-fold lower (P=0.01) in the cows with early embryonic loss than healthy cows (P=0.03, Fig. 3).

Expression of mRNA for the other candidate genes, which included monoacylglyceride lipase (MGLL), N-acyl ethanolamine amino hydrolase (NAAA),

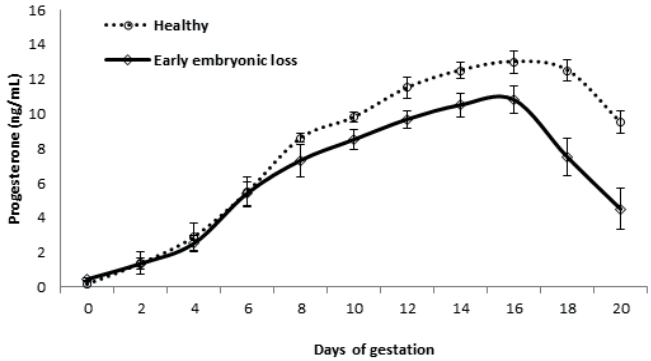

Fig. 1. Patterns of progesterone secretion in healthy cows and those with early embryonic loss. Prediction of pregnancy status on d 16 after AI by blood interferon-stimulated gene 15 (ISG15) mRNA was determined by ISG15 mRNA gene expression relative to β actin over the arbitrary level of -7.0 as previously described [17], the cows were divided into two groups, healthy (n=30), early embryonic loss (n=35).

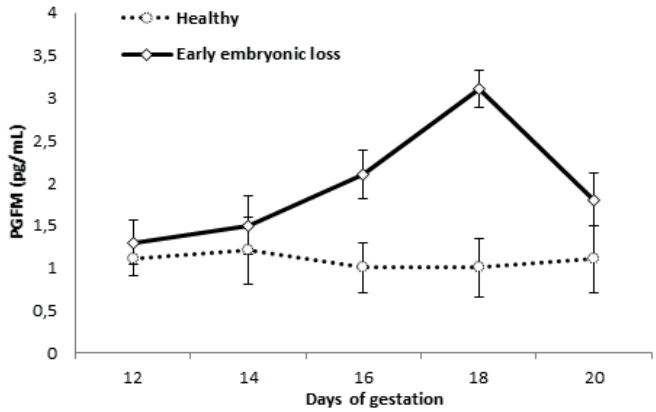

Fig. 2. Patterns of prostaglandin F2α metabolite (PGFM) secretion in healthy cows and those with early embryonic loss. Prediction of pregnancy status on d 16 after AI by blood ISG15 mRNA was determined by interferon-stimulated gene 15 (ISG15) mRNA gene expression relative to β actin over the arbitrary level of -7.0, the cows were divided into two groups, healthy (n=30), early embryonic loss (n=35).

and diacylglycerol lipase (DAGL) were not differentially expressed between cows with early embryonic loss and healthy cows (Fig. 3).

To our knowledge this is the first report showing that the endometrial genes, CNR1, NAPEPLD, and FAAH are differentially expressed in the uterus of cows with early embryonic loss when compared to the expression in confirmed pregnancies. Due to the key role of the ECS controlling embryo survival in human, identification of these changes in gene expression in the bovine uterus are critical to the improvement of our understanding of the biological mechanisms underpinning the regulation of uterine function and maintenance of pregnancy in cattle.

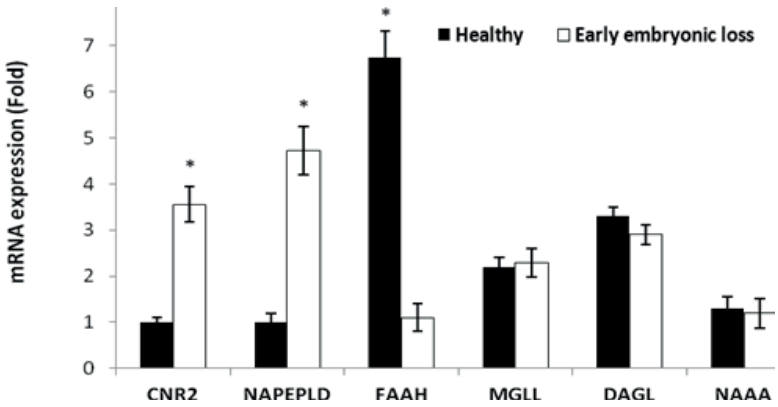

Fig. 3. Gene expression levels of the endocannabinoid system in peripheral blood leukocytes obtained from cows on 16 d after artificial insemination (AI). Prediction of pregnancy status on d 16 after AI by blood interferon-stimulated gene 15 (ISG15) mRNA was determined by ISG15 mRNA gene expression relative to β actin over the arbitrary level of -7.0 as previously described [17], the cows were divided into two groups, healthy (n=30), early embryonic loss (n=35). Data are expressed as relative ratios of the mRNAs to β actin and GAPDH. Values significantly different are marked with an asterisk (P≤0.05).

The impact of cannabis use in fertility and reproduction has been reported for years [Rapino *et al.* 2014, Metz *et al*. 2017, Szilagyi *et al*. 2019]. The use of cannabis during pregnancy is associated with intrauterine growth restriction, preterm labour and foetal neurodevelopment disturbances [Metz *et al*. 2017, Grant *et al*. 2018]. Cannabinoid signaling plays a key role during placental development [Costa *et al*. 2015, Maia *et al*. 2019]. Anandamide down-regulates human placental transporter expression and activity via CNR2-cAMP signaling [Szilagyi *et al*. 2019]. Moreover, the levels of the main endocannabinoid, AEA change across different stages of pregnancy, being proposed as a potential biomarker of pregnancy outcome [Rapino *et al*. 2014].

Endocannabinoids decrease fertility in mice [Maccarrone *et al.* 2002, Wang *et al.* 2006] and women [Habayeb *et al.* 2004] which is reported to be due to the interference with the implantation [Maccarrone *et al.* 2002. Wang *et al.* 2006]. Taylor *et al.* [2011] reported that AEA levels are increased in women with non-viable first trimester pregnancies when compared to the levels in confirmed viable pregnancies. Previous studies confirmed that for successful implantation, plasma AEA levels need to be maintained at a low level during both the implantation window and early pregnancy development [El-Talatini *et al.* 2009, Habayeb *et al.* 2004].

In the present study CNR1 and NAPEPLD relative mRNA expression were lower in cows with normal pregnancy compared with those subsequently losing their embryos. NAPEPLD and CNR1 and CNR2 receptors can be highly detected in the luminal edge of the fallopian tube epithelium [Gebeh *et al.* 2012]. The canonical biosynthetic pathway for AEA is the sequential action of *N*-acyltransferase (NAT) and *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPEPLD) [Di

Marzo *et al.* 1994]. Guo *et al.* [2005] reported a decrease in the activation pathways for anandamide mainly via its G-protein coupled receptor CNR1 which is essential for early pregnancy success. Weems *et al.* [2009] reported that CNR1 or CNR2 receptor agonists negatively affect cow luteal function *in vitro* and decreased progesterone concentrations. Activation of CNR1 results in *de novo* synthesis of ceramide and signaling via p38 MAPK phosphorylation, which leads to mitochondrial stress and ROS production and subsequently, apoptosis [Fonseca *et al.* 2013]. Wolfson *et al.* [2015] reported that increased levels of AEA exert toxic effects on decidua via activation of CNR1 receptors. The expression of the anandamide-generating enzyme NAPEPLD may be an indicator of anandamide concentrations and pregnancy outcomes; expression of NAPEPLD mRNA was greater in the first-trimester placenta from elective surgical termination than in those from spontaneous miscarriage [Trabucco *et al.* 2009]. Dirandeh *et al*. [2019] reported that CNR2 and DAGL mRNA showed 2.01 and 2.57-fold increase in oestrous cows compared to those in the luteal phase.

In the present study the relative mRNA expression of FAAH which catabolizes endocannabinoids was greater in cows with normal pregnancy compared with those which subsequently lost their embryo. Fatty acid amide hydrolase deficiencies in the oviduct and mouse blastocysts and high anandamide concentrations and low FAAH decrease pregnancy rates after *in vitro* fertilization (IVF) and embryo transfer [Wang *et al.* 2003]. Indeed, it has been suggested that the decreased activity and expression of FAAH in peripheral lymphocytes could be used as an early marker for first trimester miscarriage [Maccarrone *et al.* 2000]. During the implantation window in women with a successful pregnancy after IVF treatment and in women with normal menstrual cycles FAAH has been shown to have the greatest activity, whereas there is no change in CNR1 binding or NAPEPLD activity [Lazzarin *et al.* 2004]. Interestingly, FAAH and progesterone, show the same fluctuations during the menstrual cycle [Lazzarin *et al.* 2004]. Low progesterone levels were associated with greater plasma anandamide levels, through a mechanism that involves the direct stimulation of FAAH (key enzyme that degrades anandamide) in peripheral mononuclear cells [Maccarrone *et al.* 2002, 2003]. Dirandeh *et al*. [2019] reported that *FAAH* and *MGLL* were significantly down-regulated in oestrous cows compared with those in luteal phase, with a 5.01- and 2.44-fold difference in mRNA expression.

In the present study, prostaglandin $F_{2\alpha}$ metabolite concentrations were coincident with the decline in progesterone concentrations, as reported in the previous studies [Mann and Lamming 2006, Martin *et al.* 2015], moreover the mean concentrations of PGFM observed in the pregnant cows were similar to those reported before [Kotwica *et al.* 1998] for both pregnant and non-pregnant cows. Indeed, cows with early embryonic loss had lower serum progesterone and greater PGFM levels compared to healthy cows and similar to that described in the literature [Ginther *et al.* 2007, Martin *et al.* 2015]. It has been observed that the occurrence of five to eight pulses of PGF2α with a duration of 1 to 5 hours in a period of two to three days triggers luteolysis [Mann and Lamming 2006, Ginther *et al.* 2007].

Conclusion

Our results confirm that association between the components of the endocannabinoid system and the early embryonic loss exists. We provide evidence that ECS is altered in the endometrium of cows with early embryonic loss through increased mRNA expression of *CNR1* and synthesis enzyme (*NAPEPLD*) and decreased mRNA expression of hydrolyzing enzyme (*FAAH*). We believe that further studies on this association may lead to the identification of strategies to reduce the incidence of early embryonic loss or new therapeutics for pregnancy termination.

Acknowledgments. *Our gratitude is also extended to the owners and personnel of Mahdasht Meat & Milk Company (Northern Iran) for allowing us access to their cows and facilities to conduct this research.*

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