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Differences in the bovine milk whey proteome between early pregnancy and the estrous cycle

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ABSTRACT

Current bovine pregnancy detection methods are not reliable until at least day 28 post artificial insemination (AI). The bovine estrous cycle is approximately 21 days; consequently, producers miss an opportunity to rebreed at the next estrous event. Therefore, commercial interest exists for the discovery of novel biomarkers of pregnancy which could reliably detect pregnancy status at or before day 21 of pregnancy. The objective of the present study was to use liquid chromatography tandem mass spectrometry (LC-MS/MS) to perform a global, label-free, proteomics study on (i) milk whey and (ii) extracellular vesicle (EV) enriched milk whey samples, from day 21 of pregnancy, compared with day 21 of the estrous cycle, in order to identify potential protein biomarkers of early pregnancy. The estrous cycles of 10 dairy cows were synchronized, they went through one (control) estrous cycle and these cows were artificially inseminated during the following estrus. These cows were confirmed pregnant by ultrasound scanning. Milk whey samples were collected on day 21 of the estrous cycle and on day 21 post AI. Milk whey samples and EV enriched milk whey samples were analyzed by LC-MS/MS and subsequent analyzes of the label-free quantitative data was performed in MaxQuant and Perseus. Four proteins (APOB, SPADH1, PLIN2 and LPO) were differentially expressed between the proteomes of milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$). Ten proteins (PIGR, PGD, QSOX1, MUC1, SRPRA, MD2, GAPDH, FOLR1, GPRC5B and HHIPL2) were differentially expressed between the proteomes of EV enriched milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$). These proteins are potential milk whey biomarkers of early pregnancy.

1. Introduction

Dairy farming profitability in seasonal, pastoral based systems is dependent on compact calving and achieving a target of one calf per cow every 365 days [1]. This compact calving system is economically favorable as it enables producers to reduce feed costs by aligning calving and peak lactation with the maximum grass growth in spring. To achieve this target, high levels of reproductive efficiency are required, and in particular, the ability to determine the pregnancy status of cows as soon as possible after artificial insemination (**AI**) or mating.

One of the most common methods used for pregnancy detection is transrectal ultrasound scanning [2]. It is the gold standard method for pregnancy detection in dairy cows/heifers due to its superior sensitivity, ability to detect the presence of multiple fetuses or fetal death, and capability to assess uterine health and ovarian cyclicity if the animal is not pregnant [3,4]. However, this method cannot be performed until at least day 26–28 post AI and requires significant expertise and expensive equipment. An alternative pregnancy detection method is transrectal palpation [5], but similarly, this technique can only be used from 35 days after AI, and additionally, it poses the threat of induction of embryo loss [6]. Visual observation of estrus (often with the use of aids to detection; e.g. tail paint/heat pads) is commonly used for recognition of conception failure [7]. However, it is labor intensive and not very

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reliable due to the phenomenon of silent and/or missed heats [8].

Biological fluids such as blood or milk are ideal matrices for diagnostic assays as their collection is minimally invasive and they are protein abundant. Milk is the most desirable matrix as a sample can be easily obtained during routine milking of the cows and, therefore, will not impose any additional stress. There are bovine pregnancy detection methods currently in existence which utilize proteins in biological fluids for pregnancy detection. These include pregnancy associated glycoprotein (PAG) enzyme-linked immunosorbent assays (ELISA) [9], pregnancy specific protein B (PSPB) radioimmunoassays [10], early conception factor (ECF) lateral-flow assay [11,12], progesterone immunoassays [13], and in-line progesterone sensors [14]. However, while in-line progesterone testing can detect non-pregnancy, progesterone is not a specific biomarker of pregnancy, as elevated progesterone concentrations may be indicative of an extended estrous cycle or an ovarian pathological condition [15]. The PAG and PSPB ELISAs are only reliable indicators of pregnancy status after at least day 28 post AI [9,16]. The ECF test can be performed as early as day 6 post AI, however, the ECF test lacks specificity with reported values of detection of correctly identified non-pregnant cows of only 50% [12]. Therefore, these pregnancy diagnosis assays are of limited use for many producers. Commercial interest exists for the discovery of novel protein biomarkers of pregnancy which could reliably detect pregnancy status at or before day 21 post AI as this would allow producers the opportunity to rebreed at the next estrus event. As the majority of early embryonic death occurs before day 16 post insemination [17,18], a diagnostic test that provides valid results between days 17 and 21 post AI would be most desirable.

The objectives of the present study were to use liquid chromatography tandem mass spectrometry (LC-MS/MS) to perform a global, label-free, proteomics study on (i) milk whey and (ii) extracellular vesicle (EV) enriched milk whey samples, from day 21 of pregnancy and day 21 of the estrous cycle, in order to identify potential protein biomarkers of early pregnancy.

2. Materials and methods

All animal procedures performed in this study were conducted by authorized individuals under experimental license from the Irish Medicines Board (HPRA Project Authorization No. AE18982/P047).

2.1. Animal model

This study is a component of a larger study examining possible reliable molecular biomarkers of early pregnancy in dairy cows (Malo Estepa et al. unpublished data). For clarity, the animal model is briefly summarized here. The estrous cycles of 81 multiparous Holstein-Friesian dairy cows on a commercial dairy farm in Co. Kildare, Ireland, were synchronized. An intra-vaginal progesterone releasing device (CIDR 1.38G vaginal delivery system for cattle, Zoetis Ireland limited, Dublin, Ireland), was inserted in the vagina of each cow. Each cow simultaneously received intramuscularly, 100 μg of Gonadotropin-releasing hormone (Acegon 50 μg/ml solution for injection for cattle, Laboratorios Syva, León, Spain; day −31; Fig. 1). Seven days later, the cows received an intramuscular injection of 25 mg of prostaglandin (Lutalyse 5 mg/ml, dinoprost, Zoetis Ireland Limited, Dublin, Ireland) and either heat patches (Estrotec Heat Detector, Rockway Inc., Wisconsin, USA) or tail paint, were applied on the tail head of the cows, as aids to detect estrus (day −24; Fig. 1). The CIDRs were removed the following day (day −23; Fig. 1). Commencing two days later, the cows were examined for visual signs of estrus four times per day (day −21; Fig. 1).

All cows went through one (control) estrous cycle. On day 21 of the control cycle (i.e., day 0 of the following cycle), milk samples for proteomic analyzes were collected. Seventy-four cows were artificially inseminated 12 h following observation of estrus (day 0; Fig. 1). Milk samples for proteomic analyzes were collected 21 days post AI (day 21; Fig. 1). Forty-five cows were confirmed pregnant by ultrasound scanning on day 35 post AI (day 35; Fig. 1). Pregnancy and estrus were further confirmed by examining progesterone levels in additional milk samples using a radioimmunoassay according to the manufacturer's instructions (DIAsource ImmunoAssays SA, Louvain-LaNeuve, Belgium). Ten of these cows were selected for use in the present study (Table 1).

2.2. Comparison of the milk whey proteome between day 21 of pregnancy and day 21 of the estrous cycle

2.2.1. Sample collection and processing

Milk was collected at the routine morning milking. Whole milk was centrifuged for 30 min (4000 × *g*) at 4 °C. Fat was removed with a spatula and subsequently 12 ml of whey was recovered (avoiding the cell pellet) and placed in a sterile falcon tube. All samples were subsequently stored at −80 °C.

2.2.2. Sample preparation for LC-MS/MS analysis (Fig. 2)

Chemical reagents were purchased from Sigma Aldrich, Wicklow, Ireland, unless stated otherwise.

2.2.3. Protein precipitation

Milk whey (200 μl) was precipitated using 50 μl trichloroacetic

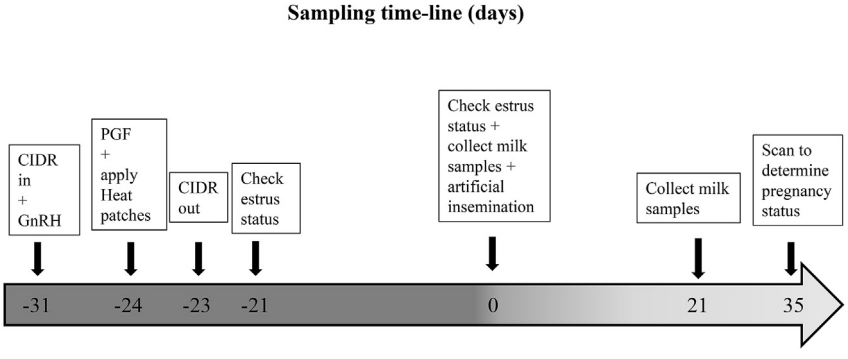

Fig. 1. Outline of the days that the cows were estrous cycle synchronized, artificially inseminated and milk sampled.
CIDR = Progesterone containing Controlled Internal Drug Release device.
GnRH = 100 μg of Gonadotropin-releasing hormone.
PGF = 25 mg of prostaglandin.