Use of endometrial cytology and metabolic profiles for selection of embryo donor cows

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Abstract

The aim of this study was to evaluate the use of endometrial cytology and metabolic profiles for selection of donor cows in embryo transfer programmes. For this purpose, 69 clinically healthy Holstein cows were enrolled in the study. At the start of the superovulation procedure (Day 0), blood and endometrial samples were obtained to determine metabolic and uterine status, respectively. The cows were then subjected to porcine follicle stimulating hormone (pFSH) superovulation treatment, and embryos were recovered after 7 days. The mean number of embryos obtained per flush was 9.89 ± 8.21 (4.63 ± 5.34 viable embryos, 0.82 ± 2.01 degenerated embryos and 4.57 ± 6.44 unfertilized ova). The following statistically significant variables were entered in a regression model: beta-hydroxybutyrate, serum cholesterol, body condition, number of calvings and percentage of neutrophils. In almost all cases, the model explained some percentage of the variance: total number of embryos, 4.8% ($p < 0.05$); number of degenerate embryos, 4.2% ($p = 0.051$); and number of unfertilized ova, 14.2% ($p < 0.01$). Statistical models for the percentage of viable embryos and unfertilized ova accounted for 24.0% and 29.4% of the variance, respectively, and both were statistically significant ($p < 0.01$). The model for the percentage of degenerated embryos was statistically significant ($p < 0.05$) and explained 4.4% of the variance. In conclusion, we have demonstrated that positive energy balance and healthy uterus can improve ovarian response and the proportion of viable embryos in cows. Efficient tools for monitoring the metabolic and uterine status should therefore be used in bovine embryo transfer programmes.

Additional key words: dairy cattle; embryo transfer; serum chemistry parameters; endometritis; neutrophils.

Introduction

Variability in the response to superovulation (SOV) protocols and the time and effort required to administer treatments have affected the widespread application of embryo transfer (ET) in genetic improvement programmes for cattle (Bó et al., 2008). Differences in the superovulatory response have been associated with gonadotrophin preparation, batch and total dose, duration and timing of treatment, and the use of additional hormones in the superovulatory protocol (Mapletoft et al., 2002). Environmental and individual cow factors such as nutritional status, reproductive history, age, season, breed, ovarian status and repeated SOV treatments can also affect the superovulatory response (Mapletoft et al., 2002).

Early studies of the metabolic profile and blood biochemistry in cows clarified the relationship between some blood components and physiological events (Payne, 1970). Metabolic profiles have been used to identify metabolic problems that may directly or indirectly affect fertility and productivity in cattle.

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Abbreviations used: BHB (beta-hydroxybutyrate); ET (embryo transfer); LH (luteotrophic hormone); MOET (multiple ovulation and embryo transfer); NEB (negative energy balance); NEFA (non sterified fatty acid); pFSH (porcine follicle stimulating hormone); PMN (polymorphonuclear neutrophils); SOV (superovulation).
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(Ramos et al., 2007). Several studies have also demonstrated a relationship between metabolic disorders and reproductive efficiency and, subsequently, embryo production (Chorfi et al., 2007; Ramos et al., 2007; Pfeifer et al., 2009).

A relationship between uterine health and reproductive performance has also been established (Gilbert et al., 2005; Santos et al., 2009; Senosy et al., 2009). Subclinical endometritis is common in dairy cows, affecting as many as 50% of all cows from 7 weeks postpartum in high-producing dairy herds (Hammon et al., 2001; Gilbert et al., 2005; Sheldon et al., 2006). This can severely impair subsequent reproductive performance (LeBlanc et al., 2002; Kasimanickam et al., 2004) by exerting a detrimental impact on embryo quality due to inflammatory mediators (Hill & Gilbert, 2008).

Useful information for diagnosing reproductive disorders in cows can be obtained by standard techniques: transrectal palpation (Azawi, 2008), vaginoscopy (McDougall, 2001; LeBlanc et al., 2002), collection of vaginal mucus (McDougall et al., 2007) and ultrasonography (Quintela et al., 2012). However, these routine types of examination are not always very efficient. Cytological examination of endometrial samples from cows can be used to obtain an earlier and more precise diagnosis of subclinical endometritis (Gilbert et al., 1998; Kasimanickam et al., 2004; Azawi, 2008; Barlund et al., 2008).

The aim of this study was to evaluate the influence of subclinical endometritis and several biochemical parameters on an embryo collection resulting from a multiple ovulation and embryo transfer (MOET) programme for dairy cows.

Material and methods

Animals and location

Forty-one high genetic merit Holstein donors at the Bovine Embryo Transfer Unit (FEFRIGA, A Coruña), in northwestern Spain, were used for this study. The cows were housed in individual stalls and were fed a total mixed ration (25.5 kg dry matter intake; 16.5 crude protein; 1.5 Mcal kg⁻¹ dry matter intake net energy). Dry cows were fed a grass-based diet supplemented with rye-grass hay, in accordance with National Research Council (NRC, 2001) guidelines. The cows were clinically healthy, with an average number of calvings of 2.28 and mean body condition score of 3 (Ferguson et al., 1994).

Sample collection and serum determination

Donor cows were selected for SOV treatment. Blood samples were obtained by puncture of the coccygeal vein by using vacuum tubes without anticoagulant. The blood samples were centrifuged at 1500 g for 20 min, and serum was aliquoted and stored at −20°C until analysis. A digital photometer (Selecta MD200, Barcelona) was used for all analyses, except protein analysis, for which a portable refractometer was used. Glucose, total cholesterol, triglyceride and albumin concentrations were determined by a colorimetric endpoint method, with Biosystems reagents (Biosystems S. A., Barcelona); urea was also assayed by a colorimetric enzymatic method with Spinreact reagents (Spinreact, S.A.U., Sant Esteve de Bas, Spain), and non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) were analyzed by use of kinetic enzymatic kits from Randox Laboratories (Antrim, UK).

Endometrial sampling was performed with the aid of a small cytobrush (length 20 mm and diameter 0.6 mm) twisted onto the plunger of insemination catheter (Quicklock 2000, Minitube Iberica, Barcelona). The catheter and cytobrush were protected by an insemination sheath and surrounded by a plastic sanitary sleeve (Minitube Iberica, Barcelona). The catheter was passed through the vagina to the external cervical opening. At the cervix, the tip of the sanitary sheath was punctured with the insemination catheter and the device comprising catheter, cytobrush and insemination sheath was passed into the uterine body. The plunger was depressed to extend the cytobrush, and a sample was obtained by rotating the cytobrush against the adjacent uterine wall. Before being completely removed, the cytobrush was retracted into the catheter to prevent cellular contamination from the cervix or vagina. The cytobrush was removed and the sample was rolled onto a glass slide and allowed to air-dry. Slides were stained with Quick Panoptic reagents (Quick Panoptic kit, Química Clínica Aplicada S.A., Tarragona, Spain). The slides were then examined under an optical microscope (CHT, Olympus Iberia S.A.U., Barcelona, Spain), at 100× magnification, by one examiner who counted at least 150 cells (nucleated cells: epithelial cells, large vacuolated epithelial cells, neutrophils and lymphocytes) (Ahmadi & Nazifi,
2006) for quantitative assessment of endometrial inflammation.

Superovulation and embryo collection

Sixty-nine SOV treatments were applied. The genital tract of each cow was thoroughly explored by rectal palpation, ultrasound and vaginoscopy, and any cows with signs of clinical endometritis or abnormalities were excluded from the study.

When a dominant follicle was present at the beginning of the SOV treatment (Fig. 1, Day 0 of the SOV protocol), transvaginal ablation was performed. A progesterone-releasing intravaginal device (PRID®, Ceva Salud Animal S.A., Barcelona) was then inserted. Treatment with pFSH (Folltropin®, Bioniche Animal Health Europe Ltd., Dublin, Ireland) was started on Day 2: a total dose of 360 mg was given over four days by administration every 12 h of a dose that was decreased daily (60, 60, 50, 40, 40, 30 and 30 mg). Luteolysis was induced by intra muscular (i.m.) injection of 150 µg D-Cloprostenol (Dalmazin®, Fatro Ibérica, Barcelona) at the same time as the fifth and sixth doses of pFSH were administered. PRID® was withdrawn after the eighth dose of pFSH. At the onset of oestrus or 24 h later, donors were injected i.m. with 25 µg Lecireline (Dalmarelin®, Fatro Ibérica, Barcelona, Spain) and were inseminated twice, 12 h and 24 h later.

Embryos were recovered with the aid of a Foley catheter (18 Gauge for bovine). The catheter was guided, by a sterile gloved hand, through the vagina and cervix until it reached the uterine horn; the catheter balloon was then inflated with 10 mL of air and each uterine horn was flushed repeatedly with 0.5 L of phosphate buffered saline solution (Vigro™ Complete Flush, Bioniche Animal Health Europe Ltd., Dublin, Ireland). This was carried out 7 days after the first insemination. The flushed medium was collected into sterile embryo collection filters (EZ Way filter, SPI™, PETS, Canton, TX, USA) and transported to the laboratory. The filters were searched for embryos under a stereomicroscope (Leica MZ 95, Leica Microsistemas S.L.U., Barcelona). Finally, ova/embryos were placed in a Petri dish containing holding medium (Vigro™ Holding Plus, Bioniche Animal Health Europe Ltd., Dublin, Ireland) and were classified as unfertilized ova, degenerated embryos or viable embryos (IETS Manual; Stringfellow & Givens, 2010).

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software package (SPSS, Chicago, IL, USA). Data were expressed as means ± standard deviation (SD). Differences in means were considered significant at $p \leq 0.05$. Relevant clinical parameters (number of calvings, body condition score, percentage of polymorphonuclear neutrophils, calving-SOV treatment interval, glucose, total cholesterol, triglycerides, albumin, total proteins, urea, β-hydroxybutyrate and non-esterified fatty acids) were included as independent variables in a multiple linear regression model to determine independent effects on the number of total embryos, unfertilized ova, viable and degenerated embryos, percentage of viable and degenerated embryos and percentage of unfertilized ova (dependent variables). Variables selected by a stepwise were entered in a final
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A multivariate linear regression model was used to describe the percentage of variance in the dependent variables that was explained by the multivariate model.

Results and discussion

The SOV procedure yielded a total of 9.89 ± 8.21 embryos or ova per flush. The mean number of viable and degenerated embryos was 4.63 ± 5.34 and 0.82 ± 2.01, respectively. We also collected per flush a mean of 4.57 ± 6.44 unfertilized ova.

Models obtained to evaluate factors (Tables 1, 2 and 3) related to SOV explained some of the variance for the total number of embryos (4.8%; \( p < 0.05 \)), number of degenerate embryos (4.2%; \( p = 0.051 \)), number of unfertilized ova (14.2%; \( p < 0.01 \)), and the percentage of viable embryos (24.0%; \( p < 0.01 \)), degenerated embryos and unfertilized ova (29.4; \( p < 0.01 \)).

| Independent variables | Mean±SE |
|-----------------------|---------|
| Number of calvings    | 2.28±0.13 |
| Body condition score  | 3.00±0.05 |
| Polymorphonuclear neutrophils (%) | 0.86±0.31 |
| Calving-superovulation treatment interval (mg dL\(^{-1}\)) | 354.78±48.89 |
| Glucose (mg dL\(^{-1}\)) | 73.62±1.41 |
| Total cholesterol (mg dL\(^{-1}\)) | 216.43±10.01 |
| Triglycerides (mg dL\(^{-1}\)) | 16.60±1.26 |
| Albumin (g L\(^{-1}\)) | 38.01±0.62 |
| Total proteins (g L\(^{-1}\)) | 64.99±0.70 |
| Urea (mg dL\(^{-1}\)) | 33.07±1.36 |
| Beta-hydroxybutyrate (mmol L\(^{-1}\)) | 0.53±0.03 |
| Non-esterified fatty acids (mmol L\(^{-1}\)) | 0.32±0.04 |

### Table 2. Results of multiple regression analyses with total and degenerated embryos and unfertilized ova as outcome parameters

| Dependent variable | Model variables | Non-standardized beta coefficient ± SE | Standardized beta coefficient | Significance level |
|--------------------|-----------------|----------------------------------------|-------------------------------|--------------------|
| Total embryos      | Constant         | 4.266±1.745                            | 0.017                         | 0.051              |
|                    | BHB              | -1.147±0.576                           | -0.236                        | 0.000              |
| Degenerated embryos| Constant         | 10.575±2.776                           | 0.000                         | 0.046              |
|                    | BCS              | -2.245±1.103                           | -0.241                        | 0.037              |
| Unfertilized ova   | Constant         | 6.373±2.101                            | 0.003                         | 0.003              |
|                    | BHB              | 8.170±2.690                            | 0.371                         | 0.003              |
|                    | Cholesterol      | -0.028±0.009                           | -0.370                        | 0.004              |

BHB: beta-hydroxybutyrate. BCS: body condition score. SE: standard error.

| Dependent variable | Model variables | Non-standardized beta coefficient ± SE | Standardized beta coefficient | Significance level |
|--------------------|-----------------|----------------------------------------|-------------------------------|--------------------|
| Viable embryos (%) | Constant         | 11.646±10.954                          | 0.292                         | 0.000              |
|                    | Cholesterol     | 0.197±0.048                            | 0.441                         | 0.000              |
|                    | PMN             | -4.615±1.556                           | -0.316                        | 0.004              |
| Degenerated embryos (%) | Constant     | 10.575±2.776                           | 0.000                         | 0.046              |
|                    | Nº of calvings  | -2.245±1.103                           | -0.241                        | 0.037              |
| Unfertilized ova (%) | Constant       | 80.382±11.744                          | 0.000                         | 0.000              |
|                    | Cholesterol     | -0.256±0.053                           | -0.541                        | 0.000              |
|                    | PMN             | 5.221±1.589                            | 0.337                         | 0.002              |
|                    | BHB             | 27.896±15.032                          | 0.206                         | 0.068              |

PMN: polymorphonuclear neutrophils. BHB: beta-hydroxybutirate. SE: standard error.
Variables entered in the models were related to energy balance (total cholesterol, BHB and body condition) as well as uterine health status (polymorphonuclear neutrophils [PMN]). The number of calvings affected the percentage of degenerated embryos, although the model only explained 4.4% of the variance.

In cows with negative energy balance (NEB) status, body fat is mobilized and the blood NEFAs increase and are metabolized in liver, to be transformed into ketone bodies (Wathes et al., 2003) or to be stored as triglycerides, which can cause fatty liver (Knegsel et al., 2007). BHB is the most stable ketone body, and serum values are used to diagnose subclinical ketosis (Oetzel, 2003). In the present study, we found a negative correlation between the level of BHB and the number of embryos collected. This suggests that low energy status induces a lower ovarian response during superovulatory treatment. NEB status in dairy cows is known to affect luteotrophic hormone (LH) secretion and, thus, ovulation (Jolly et al., 1995; Buttler, 2003). In this study, glucose had no effect on embryo production and was also not a good indicator of energy balance in cows, because of the homeostatic control exerted on this sugar (Reist et al., 2002). We also found positive correlations between BHB, the number of unfertilized ova, and the percentage of unfertilized ova in relation to the number of embryos collected (Tables 2 and 3). This suggests that the ability of oocytes to mature and be fertilized may be compromised by an increased concentration of BHB. Leroy et al. (2004) observed that changes in serum glucose concentration and BHB are reflected in the composition of dominant follicular fluid in bovine embryos cultured in vitro. These authors concluded that in cows experiencing clinical ketosis the biochemical environment prevents optimal oocyte maturation in follicular fluid and thus hampers developmental competence (Leroy et al., 2006). They also demonstrated that in cows with subclinical ketosis, in which the glucose concentration is moderately low, BHB aggravates the toxic effect of low glucose concentrations and induces a significant reduction in the ability of fertilized ova to develop into morulae or blastocysts. Gómez et al. (2002) demonstrated that early bovine embryos are capable of consuming BHB as an alternative energy source. However, BHB does not seem to be used as alternative source of energy in oocyte maturation when glucose concentration is low (Leroy et al., 2006). In cumulus cells, glucose is predominantly metabolized via the glycolytic pathway to produce pyruvate and lactate, the oocyte’s preferred substrates for ATP production (Cetica et al., 2002). Pyruvate and lactate molecules cannot be produced from BHB, because BHB is inserted as acetyl CoA in the Krebs cycle (Stryer, 1995). In the oocyte, glucose is predominantly metabolized in the pentose phosphate pathway (PPP) (reviewed by Sutton et al., 2003), and BHB cannot be utilized as an alternative substrate in this pathway (Nehlig, 2004). NEB cows may suffer long-term effects on fertility due to a deleterious effect on follicular development and on oocytes (Britt, 1994). Animals with high BHB levels during SOV treatment may have suffered NEB in early postpartum, and this may affect maturation of oocytes.

In the present study, cholesterol also strongly influenced the number of unfertilized embryos and the percentage of viable and unfertilized embryos (Tables 2 and 3). As a component of serum lipoproteins, serum cholesterol is a good indicator of total lipoproteins. The decrease in cholesterol levels observed in peripartum cows with elevated NEFA concentrations has been associated with altered liver metabolism (Vasquez-Anon et al., 1994) and with the occurrence of other diseases such as metritis (Lotthammer et al., 1971; Kweon et al., 1985, 1986). Although cholesterol did not affect the ovarian response—measured as the total number of embryos collected—it had a positive impact on the percentage of viable embryos. The number and percentage of oocytes were low in the cows under study, which suggests that the presence of cholesterol might improve the capacity of donor cows to produce oocytes that mature and become fertilized. In this respect, components of follicular fluid are known to affect granulose cells and oocyte, as they regulate follicle development and steroidogenesis (Brantmeier et al., 1987). Cholesterol, through HDL and LDL lipoproteins, is the main precursor of steroid hormones (Ramos et al., 2007). Herrera-Camacho et al. (2008) indicated that ovulation and fertilization are promoted when the oocyte develops and matures in a suitable endocrine environment. Staples et al. (1998) observed an increase in progesterone synthesis in follicular and luteal cells when the concentration of cholesterol increases as a result of dietary fat supplementation, producing a higher percentage of pregnancies in dairy cows. Although no increase in the number of viable embryos was observed in the present study (Table 2), other authors have observed that the number of transferable embryos was significantly higher in cows with ≥130 mg dL⁻¹ of serum cholesterol than in cows with lower concentrations of cholesterol (Kweon et al., 1985). In
a study carried out in crossbreed cows undergoing ovum pick-up, Pfeifer et al. (2009) obtained more follicles suitable for puncture in cows with cholesterol levels higher than 50 mg mL\(^{-1}\) than in cows with lower cholesterol levels, although the number and quality of recovered oocytes were not affected. Lucy et al. (1992) also found that the number and the size of follicles were higher in cows with higher blood cholesterol levels. In contrast, Chorfi et al. (2007) observed that serum cholesterol levels in superovulated cows did not significantly influence the number of transferable embryos.

Several authors (Green et al., 2009; Burke et al., 2010; Priest et al., 2013) have reported links between inflammation, liver function, subclinical endometritis and reproductive performance. Although these links are more likely to be associations that cause an effect, they constitute potential markers for subclinical endometritis in blood/serum. In a study of the association between liver proteins and subclinical endometritis diagnosed at day 21 and 42 postpartum, Green et al. (2009) found that cows with subclinical endometritis had lower plasma albumin and lower total protein concentration than control cows. However, we did not observe this relationship in the present study, and neither albumin or total protein index accounted for the variation in results (Tables 1 and 2), probably because the cows used in this study had calved for several months. Endometrial cytological sampling was used in this study to assess the presence of polymorphonuclear neutrophils, as previously described (Kasimanickam et al., 2004; Senosy et al., 2009). We observed that as the PMN increased, the percentage of viable embryos and fertilized ova decreased (Table 3). A lower number of fertilized ova and lower embryo quality associated with high uterine PMN may be affected by altered function of sperm and also by oocyte quality. It is well known that PMN can produce reactive oxygen species (Alőe et al., 2012) and that these may be damaging to sperm. Furthermore, Sheldon et al. (2009) concluded that uterine bacterial infections impair the function of the hypothalamus and pituitary and directly perturb steroidogenesis by ovarian granulose cells. Our results are consistent with those of a recent study by Carvalho et al. (2013), who reported a substantial reduction in embryo yield when the percentage of uterine PMN was increased before initiation of the superstimulation protocol. Moreover, an unfavourable uterine environment associated with inflammation might negatively affect embryo viability and development. Hill & Gilbert (2008) reported a reduction in blastocyst quality in bovine embryos cultured in vitro in media conditioned by fluid from an inflamed endometrium. These authors observed a lower number of total and trophectoderm cells, and a higher inner cell mass:trophectoderm ratio, although the number of degenerated or dead embryos did not increase, which suggests that aseptically-induced endometritis has a lower impact on embryo quality than expected for active bacterial endometritis. However, Drillich et al. (2012) did not find any difference in the quality of blastocysts produced referred to total cell number or apoptotic cell index, recovered from cows with or without high levels of PMN at insemination or flushing. They explained this on the basis of the relatively short time that embryos remained in the uterus cavity in comparison with the time that embryos remained in conditioned media. In this respect, Cerri et al. (2009) observed a lower fertilization rate in cows with subclinical endometritis, suggesting that decreased fertility is linked to decreased sperm transport or fertilizing capacity.

Our results show that the influence of parity was low. We found that as the number of calvings increased, the percentage of degenerated embryos decreased (Table 3).

In this study, we explored different factors (Table 1) that may influence the response to superovulatory treatments. These results can be improved by identifying negative energy balance (NEB) and by determining BHB and cholesterol levels. Endometrial cytological sampling can also be used to detect optimum uterine health status, as reflected by a low percentage of PMN, in order to select donor cows with a suitable uterine environment that allows the transport of spermatozoa, fertilization and the development of embryos.

In conclusion, we found that measurement of biochemical parameters (such as cholesterol and mainly BHB, which can be measured in situ) and the use of endometrial cytology, linked to previous exploration of the genital tract, can facilitate the selection of donor cows for in vivo embryo production.

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