Oocyte maturation and quality in dairy heifers with diverse genetic merit for fertility

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Abstract

Oestrous behaviour, cumulus cell-oocyte complex (COC) quality, and steroid concentrations were characterised in dairy heifers of high and low values for fertility breeding value (FBV). The hypothesis was that the greater FBV is partly mediated through improved oocyte quality. Ovum pick-up (OPU) was used to collect COCs from the preovulatory follicle of 40 heifers observed in oestrus. Multiplex Taqman qPCR was used to investigate gene expression in cumulus cells (eight genes) and oocytes (three genes) of the recovered healthy COCs (n=9 per group). Plasma and follicular fluid steroid concentrations were measured using high-performance liquid chromatography. Low-FBV heifers were more active in the first eight hours of oestrus ($P<0.05$), leading to earlier detection and OPU ($P<0.05$). Consequently, COCs recovered from low-FBV heifers were morphologically less mature ($P<0.05$) and tended to have lower expression of genes regulating maturation compared with high-FBV heifers. Our findings suggest COC development is retarded in low-FBV heifers relative to the timing of visually observed oestrus, which may result in a poorly developed oocyte at ovulation.

Keywords: oocyte; fertility; oestrus; gene expression; genetic merit

Introduction

Conception rate, defined here as the proportion of viable pregnancies resulting from insemination events, is one of the key drivers of reproductive performance in seasonal dairy systems (Macmillan 1979). Previous studies indicate conception failure is largely a consequence of early embryonic death rather than fertilisation failure (Diskin & Morris 2008). Cows with a high fertility breeding value (FBV) achieve higher conception rates (Cummins et al. 2012). Better oocyte quality and lower embryonic loss may be, at least partially, responsible for these gains; however, this has never been directly examined.

The inherent capacity of the oocyte to support early embryonic cleavage events is essential for embryonic survival. This ability, termed 'developmental competency', is attained progressively throughout follicular development as the oocyte matures and accumulates maternal mRNA and protein stores (Hussein et al. 2006). These stores support and guide early embryonic development until the embryonic genome activates, which is at the eight-cell stage in cows (Alves et al. 2015). The oocyte must be sufficiently competent to support this development or the embryo will die.

The objective of this study was to investigate developmental competency, and thus quality, of oocytes in dairy heifers selected for high or low FBV. The hypothesis was that animals selected for high FBV would have greater expression of gene markers associated with oocyte quality than those with low FBV.

Materials and methods

Animals and synchronisation

This study was undertaken in Northland, New Zealand (36.76°S, 174.46°E) with approval of the Ruakura Animal Ethics Committee (#13934). Initially 60 Holstein-Friesian heifers were enrolled from the animal model herd described by Meier et al. (2017). Briefly, the animal model consists of dairy heifers selected for very high or very low FBV, while being comparable for other important traits. The genetic indices of the 40 animals which underwent trans-vaginal ovum pickup (OPU) are shown in Table 1.

All animals were fitted with a neck-collar device (Hi Tag, SCR Engineers Ltd, Netanya, Israel) that identified dates of activity-based oestrus, which were used to predict the animals that would spontaneously return to oestrus over a four-day period (Talukder et al. 2015). It was also used to identify heifers that would be receptive to a luteolytic dose of prostaglandin to synchronise oestrus during this same, four-day period. Of these, 29 high- and 31 low-FBV animals, balanced for live weight and number of previous cycles, were selected. All 60 animals received an intramuscular injection of 500 µg cloprostenol (2 mL Ovuprost, Bayer New Zealand Ltd) 48 hours before sampling began.

Beginning the afternoon prior to the four-day sampling period, oestrous behaviour was visually identified twice-daily. Heifers identified in oestrus underwent OPU (n=40) over a four-day period from August 27th-30th. Animals thought to be closest to ovulation, judged by current behaviour and time since first detection of behavioural oestrus, were prioritised to undergo OPU first each day.

Trans-vaginal follicle aspiration and blood sampling

Prior to OPU, heifers were sedated and received an epidural anaesthesia, as previously described by Hudson et al. (2014). To perform OPU, a 7.5 mHz transvaginal sector probe (PieMed 200S; Pie Medical Imaging BV, The Netherlands) with a sampling wand using a 19G × 1.5″ BD

Table 1 Genetic indices of Holstein-Friesian heifers grouped by high or low fertility breeding value that underwent ovum pick-up. Estimates are presented as the mean ± SD for each fertility group.

Parameter[1]	High (n=20)	Low (n=20)
Fertility BV	5.61 ± 0.82	-4.53 ± 1.16
Breeding worth	153.54 ± 20.96	78.54 ± 17.71
Production worth	102.67 ± 23.02	120.49 ± 18.10
Volume BV	807.35 ± 132.81	757.15 ± 117.49
Fat BV	18.84 ± 4.77	22.05 ± 4.35
Protein BV	26.02 ± 2.63	26.45 ± 4.12
Live weight BV	37.57 ± 9.97	34.68 ± 9.17
Body condition score BV	0.066 ± .05	-0.096 ± 0.06
Gestation length BV	-3.43 ± 1.87	-2.04 ± 2.33
North American ancestry (%)	55.79 ± 5.42	61.79 ± 7.20
Residual survival BV	34.05 ± 44.68	50.50 ± 78.72
Total longevity BV	364.45 ± 38.57	147.75 ± 85.41
Somatic cell BV	-0.096 ± 0.12	0.153 ± 0.16
Live weight (kg)[2]	307.35 ± 16.55	305.35 ± 21.32
Age (days)[2]	386 ± 12.37	381 ± 14.19

[1]BV = breeding value; [2] as of 17/08/16

Precision-Glide needle (Becton Dickinson) attached to a 25 mmHg vacuum aspiration pump (Karl Storz, Tuttlingen, Germany) was used to aspirate the preovulatory follicle. A sample of the follicular fluid (FF) was collected into a capillary straw upon initial puncture. Recovery of the cumulus-oocyte complex (COC) was then attempted by repeatedly flushing media into the follicle. Following OPU, a blood sample was collected from a jugular vein for plasma.

The COC was identified in the flush media using the procedure described by Hudson et al. (2014) and morphologically categorised into 'compact', 'semi-expanded' or 'expanded' based on cumulus cell expansion. The oocyte, cumulus cell mass, and FF were individually snap-frozen using dry ice and stored at -80°C. Blood samples were centrifuged at 4°C at 1,120 x *g* for 12 minutes and the aspirated plasma was stored at -20°C. Steroid concentrations were determined in plasma and FF using mass spectrometry as previously described by Hudson et al. (2014).

Gene expression

Total RNA was extracted from each denuded oocyte and cumulus cell mass using the ArrayPure Nano-scale RNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) as described by Crawford & McNatty (2012). Extracted RNA was re-suspended in ultrapure dH_2O and reverse transcribed using the SuperScript VILO cDNA Synthesis kit (Invitrogen) to produce a final volume of 20 µL of cDNA.

Taqman qPCR was used to quantify gene expression as described by Crawford & McNatty (2012). Oocyte-derived genes included those involved in gap-junction communication between the oocyte and cumulus cells (connexin 37, *CX37*) and essential oocyte-specific growth factors (bone morphogenetic protein 15, *BMP15;* and growth differentiation factor 9, *GDF9*). Cumulus cell derived genes included those involved in maintaining meiotic arrest (natriuretic peptide receptor 2, *NPR2*; phosphodiesterase 8A, *PDE8A*), gap junction communication (connexin 43, *CX43*), cumulus expansion (hyaluronan synthase 2, *HAS2*; versican, *VCAN*), or receiving hormone or oocyte-secreted signals necessary for oocyte development (progesterone receptor, *PR*; follicle stimulating hormone receptor, *FSHR*; bone morphogenetic protein receptor 2, *BMPR2*).

Each gene measured was incorporated into a quadriplex set of three candidate genes and one reference gene. Reaction mixes containing optimised concentrations of Taqman probes and primers, together with reagents from the Brilliant Multiplex QPCR Master Mix kit (Stratagene, La Jolla, CA, USA) and 1.04 µL of cDNA were prepared to a final volume of 52 µL for each sample. These were the aliquoted in duplicate (25 µL) into 0.1 mL strip tubes and amplified in a Rotor-Gene 6000 multiplexing system under the following conditions: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Controls were incorporated in every run and included reactions that omitted addition of template, or oocyte samples in which the reverse transcriptionase enzyme (Superscript_ III/RNaseout) was excluded to check for genomic DNA contamination. The amplification efficiencies of all genes measured in this study were >80%.

Comparative gene expression was quantified using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001). *RPL19*, previously shown to be a good reference gene for cumulus cells and oocytes (Ekart et al. 2013), was used for normalisation of target gene expression. Gene expression was calculated relative to the expression of a calibrator sample (100 bovine COC cDNA) that was present at the beginning and end of each reaction run.

Oestrus - OPU interval

The activity-monitoring system was used to retrospectively determine the time from onset of oestrus to OPU in every heifer. As the monitors report activity in two-hour blocks, the average activity for each block was calculated using activity data collected from August 24th to September 14th. The onset of oestrus was defined as the time when activity increased 25% above average for that time point and remained 25% above average for five of six consecutive two-hour blocks.

To compare the level of intensity between the groups, the fold change in activity relative to the average for that time point was averaged over the first eight hours of oestrus. For this comparison, oestrous activity from the wider selection group (n=52) was analysed. Animals which underwent OPU before, or within, 10 hours of the onset of oestrus were excluded for this purpose, as this procedure would have disturbed oestrous behaviour.

Statistical analysis

Statistical analysis was undertaken using SPSS statistical software version 23 (IBM Corp., Armonk, NY,

USA). The effect of FBV on COC morphology was assessed using a Pearson chi-squared test. Gene expression data were transformed using the natural log or square root to achieve a normal distribution. These data were then analysed using a one-way ANOVA with FBV group as a fixed factor. This analysis was repeated using the interval from oestrus onset to OPU as a covariate. The correlation between cumulus *NPR2* expression and FF oestradiol was determined using a Pearson product-moment correlation. The interval between onset of oestrus, as determined from the activity data, and OPU was compared between groups using a Student's *t*-test. Intensity of oestrus activity during the first eight hours was compared using this same procedure. Steroid concentrations in the FF and plasma were transformed using the natural log to achieve normal distribution then analysed using an ANCOVA with fertility group as the grouping variable and the interval between onset of oestrus and OPU as a covariate. The time of day the sample was collected was included as covariate for steroids that varied with time of day.

Three heifers were excluded from the analysis of COC data, seven were excluded from FF analysis, and five excluded from plasma analysis due to sample contamination, collection from atretic follicles or missing samples.

Table 2 Steroid concentrations (ng/mL) in the follicular fluid and plasma of heifers with high and low fertility breeding value at the time of ovum pick-up. Adjusted geometric means and SED are presented.

	High	Low	SED	P-value
In follicular fluid:	n=18	n=15		
Cortisone	0.77	0.83	0.14	0.737
Cortisol	7.55	6.08	3.88	0.382
Corticosterone	0.10	0.19	0.03	0.016
11-Deoxycortisol[1]	1.83 (6.22)	1.48 (4.39)	0.17	0.047
Oestradiol	598.81	267.83	111.94	0.006
Androstenedione[1]	1.75 (5.77)	1.14 (3.11)	0.61	0.318
Testosterone[1]	-0.03 (0.97)	-0.72 (0.49)	0.62	0.278
Progesterone	77.42	77.55	12.60	0.992
In plasma[2]:	n=19	n=16		
Cortisone	2.31	2.65	0.31	0.280
Cortisol	16.47	21.46	0.15	0.145
Corticosterone[1]	-1.32 (0.27)	-0.74 (0.48)	0.36	0.076
11-Deoxycortisol[1]	-2.75 (0.06)	-2.04 (0.13)	0.33	0.042
Progesterone[1]	-2.67 (0.07)	-2.39 (0.09)	0.23	0.735

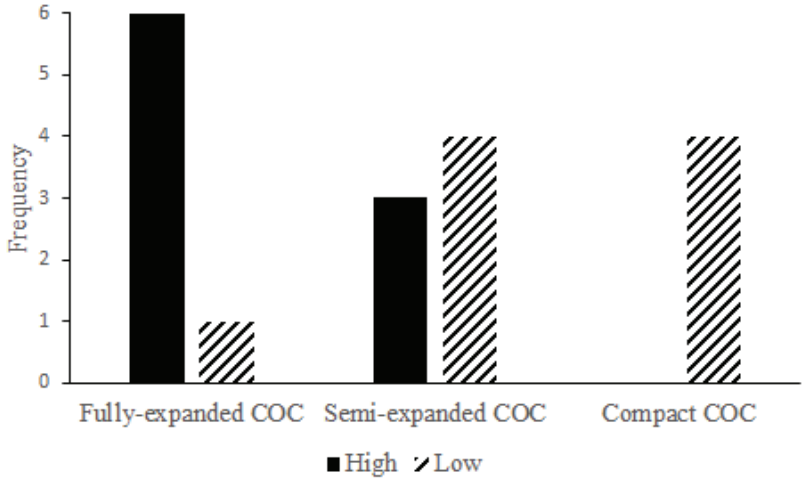
[1]Data were transformed to achieve normal distribution; the back-transformed means shown in brackets [2]Plasma androstenedione, oestradiol and testosterone were not detected in the majority of samples so these steroids were excluded from analysis

Results

Interval from onset of oestrus to ovum pick up

In total, 40 animals underwent OPU (Table 1) and 18 healthy COCs from presumptive preovulatory follicles were recovered. The interval from the onset of oestrus to COC collection was shorter in the low-FBV than high-FBV heifers for the animals with recovered COCs (n=18, High, 25.5 ± 1.81 h, Low 16.9 ± 2.75 h, P<0.05) and all animals that underwent the OPU procedure (n=36, High 24.6 ± 1.30 h, Low 19.8 ± 1.91 h, P<0.05). In addition, the low-FBV heifers were more active than high-FBV heifers in the first eight hours of oestrus (P<0.05).

Figure 1 Extent of cumulus expansion of cumulus-oocyte complexes (COC) recovered from Holstein-Friesian heifers with high fertility breeding value (n=9), or low fertility breeding value (n=9).



Morphology

The degree of cumulus cell expansion among the COC collected was different between FBV groups (P<0.05; Fig. 1). A greater proportion of fully-expanded COCs were recovered from high -FBV heifers (6/9), compared with low-FBV animals (1/9), and a greater proportion of COC recovered from low-FBV than high-FBV heifers had compact, nonexpanded cumulus cells (4/9).

Gene expression

There were no differences (P>0.05) between the FBV groups in the gene expression data, but the expression of two genes (*VCAN* and *PDE8A*) tended (P<0.1) to be influenced by FBV status. Abundance of *VCAN* mRNA and *PDE8A* mRNA tended to be greater in high-FBV heifers (P=0.08 and *P*=0.1, respectively). However, inclusion of the interval from oestrus onset to OPU as a covariate removed any tendency towards significance (P>0.1). A correlation was found between FF concentrations of oestradiol and the abundance of cumulus *NPR2* mRNA (P<0.01).

Steroids

The concentration of four steroid hormones in FF and plasma differed between the FBV groups (Table 2). Mean concentrations of corticosterone were higher, and 11-deoxycortisol and oestradiol were lower in the FF of the low-FBV group compared with the high-FBV group

($P<0.05$). The concentration of 11-deoxycortisol was also higher ($P<0.05$), and corticosterone tended ($P=0.06$) to be higher, in the plasma of low-FBV heifers compared with that of high-FBV heifers.

Discussion

The developmental processes of cumulus-cell expansion in low-FBV heifers was retarded, when compared with that of high-FBV heifers. However, the cause of these differences is more complex than merely variation in the rate of COC maturation following the luteinising hormone (LH) surge. The presented data indicate that there are also temporal differences in this ovulatory stimulus, which are not obviously reflected in the timing of visually detected oestrus.

It is possible that the retarded COC development observed in low-FBV heifers was due to a delayed, weakened LH surge stimulus. Previous studies report that a delayed LH surge relative to the onset of visually detected oestrus is characteristic of sub-fertile dairy cows (Bloch et al. 2006). This fits with our observations that low-FBV heifers had less-mature COCs than did high-FBV heifers, relative to visually detected oestrus. Also reported to be associated with sub-fertility and perturbed LH release, is an activated stress hormone axis (Dobson & Smith 2000). Corticosterone and 11-deoxycortisol, intermediates of steroidogenesis pathways in the adrenal cortex, were higher in low-FBV heifers compared with high-FBV heifers in the present study. It is possible, therefore, that increased activity of the adrenal cortex is delaying the LH surge in low-FBV heifers.

Increased adrenal activity may also impair follicular steroidogenesis in low-FBV heifers. Overall, low-FBV heifers had lower FF oestradiol concentrations than did high-FBV heifers, regardless of time of collection. This may indicate that the pre-ovulatory follicles in low-FBV heifers were less developed and the granulosa cells may have been fewer in number per follicle or have acquired fewer gonadotrophin receptors (Zimmermann et al. 1990). Elevated adrenal glucocorticoids can retard follicular development and diminish the preovulatory oestradiol rise and this may be occurring in the low-FBV heifers (Daley et al. 1999).

Low follicular oestradiol concentration may impair oocyte nuclear maturation as follicular oestradiol increases cumulus expression of *NPPC* and its receptor *NPR2*, which maintain intraoocyte conditions necessary for meiotic arrest (Zhang et al. 2011). In this study, cumulus *NPR2* expression was highly correlated with follicular oestradiol concentration. The lack of significant difference in *NPR2* mRNA expression between FBV groups may be due to a lack of statistical power. Low FF oestradiol concentrations and, thus, less *NPPC/NPR2* mRNA expression increases the likelihood that the oocyte will resume meiosis early, causing asynchrony in maturation and lowering oocyte competence.

Another possible cause of retarded COC development in low-FBV heifers is that these observations were made sooner after the stimulatory LH surge in low-FBV heifers than in high-FBV heifers. Although selection of heifers for OPU was based on visually detected oestrus with no intentional bias between fertility groups, retrospective analysis of activity data indicated the interval between oestrus onset and COC collection was shorter in low-FBV than in high-FBV heifers. Low-FBV heifers were more active than high-FBV heifers in the first eight hours of oestrus, possibly leading to earlier visual detection and earlier COC recovery. Earlier recovery of COCs from low-FBV heifers may explain the COCs immaturity relative to those from high-FBV heifers.

It was hypothesised that high-FBV heifers would have greater expression of gene markers associated with oocyte quality than low-FBV heifers. However, differences in the timing of COC collection meant not enough comparable COCs were available to test our hypothesis. Nevertheless, we can conclude from this study that COC development in low-FBV heifers is retarded relative to the timing of visually observed oestrus, and that this is underpinned by oestrus-related activity differences between these fertility strains.

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