Effect of follicular aspiration just before ovulation on corpus luteum characteristics, circulating progesterone concentrations and uterine receptivity in single-ovulating and superstimulated heifers

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Abstract

The aim of this study was to investigate, in unstimulated and superstimulated heifers, the effect of follicle aspiration just before ovulation on corpus luteum (CL) development, circulating progesterone (P₄) concentrations and the ability of the uterus to support embryo development. Following follicle aspiration or ovulation timed from GnRH administration, CL development was assessed by daily ultrasonography, and CL function was assessed in terms of the capacity to produce P₄ and the expression of genes involved in steroidogenesis in luteal tissue. The capacity of the uterine environment to support conceptus development was assessed following transfer and recovery of *in vitro*-produced embryos. Follicular aspiration just before the expected time of ovulation leads to a significant reduction in CL diameter, CL area and area of luteal tissue. This was associated with a decrease in circulating P₄ in both unstimulated and superstimulated heifers. Follicle aspiration leads to a reduction in conceptus length and area on day 14 in unstimulated heifers only. Follicle aspiration leads to a reduction in the expression of *LHCGR* in luteal tissue from unstimulated heifers compared with those in which the CL formed after ovulation. Superstimulation significantly reduced the expression of *STAR* in luteal tissue in both ovulated and follicle-aspirated heifers. In conclusion, in stimulated and unstimulated heifers, aspiration of the preovulatory dominant follicle(s) just before expected ovulation interferes with the subsequent formation and function of the CL, in terms of size and P₄ output and this, in turn, is associated with a reduced capacity of the uterus to support conceptus elongation in unstimulated heifers.

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Introduction

Controlled ovarian hyperstimulation (COH) is a routine procedure used in assisted reproduction to stimulate the growth of multiple follicles in naturally single-ovulating species such as cattle and humans. In cattle, this procedure is used to increase the number of offspring from genetically valuable females; such females are stimulated with gonadotropins to induce the ovulation of a variable number of follicles, artificially inseminated with semen from a high genetic merit sire and the resulting embryos are non-surgically flushed from the uterus 7 days later for transfer to surrogate recipients or cryopreservation and transfer at a later date. Similarly, many women undergoing *in vitro* fertilisation (IVF) treatment undergo COH to induce the development of multiple dominant follicles. In contrast to cattle, multiple mature oocytes are recovered by transvaginal follicle aspiration just before ovulation, inseminated *in vitro* with partner/donor sperm and then embryos are transferred back to the same women, often in the same

cycle. Because of this fact, the endocrinology associated with COH and the environment of the reproductive tract following such procedures are important.

In women, stimulated IVF cycles are associated with a defective luteal phase in almost all cases due to the supraphysiological concentrations of steroids associated with COH, which lead to suppression of pituitary LH secretion (reviewed by *Fatemi et al. (2007)* and *Messinis et al. (2009)*). Exogenous supplemental progesterone (P₄), often extending up to week 12, is a common practice in IVF-stimulated cycles because of the associated luteal phase defects (*Kolibianakis & Devroey 2002, Pritts & Atwood 2002, Kolibianakis et al. 2003*). Although the benefit of P₄ administration as luteal phase support has been well documented in IVF (*Fatemi et al. 2007*), a question surrounds the optimum time for its discontinuation (*Griesinger 2011*) based on the results of a retrospective uncontrolled study of 400 pregnancies (*Schmidt et al. 2001*) and two randomised trials (*Nyboe Andersen et al. 2002, Kyrrou et al. 2011*),

which suggest that supplementation beyond 4 weeks of gestation is unnecessary.

One other explanation put forward to explain luteal dysfunction is the removal of granulosa cells during follicular aspiration and the consequent effect on corpus luteum (CL) formation and function (P_4 secretion; Garcia *et al.* 1981, Vargyas *et al.* 1986). Similar findings were reported in non-human primates (Kreitmair *et al.* 1981). In contrast, Kerin *et al.* (1981) reported that aspiration of follicular fluid from the immediately preovulatory follicle did not impair the steroid secretion capacity of the subsequent CL compared with that in spontaneously ovulating women.

It is clear that luteal tissue can be formed following follicular aspiration before ovulation; however, follicular diameter at aspiration can influence the subsequent CL formation and function (Hinrichs *et al.* 1991, Mozzaquatro *et al.* 2011). Follicular diameter at ovulation and the duration of proestrus have been identified as sources of variation in conception rate in cattle (Lamb *et al.* 2001, Peters & Pursley 2003, Perry *et al.* 2005, Mussard *et al.* 2007). Premature ovulation of a dominant follicle with GnRH can reduce the size of the ovulatory follicle, decrease subsequent luteal function and reduce fertility, perhaps associated with compromised uterine receptivity given the unequivocal role of P_4 in creating an optimal uterine environment for pregnancy establishment (Clemente *et al.* 2009, Forde *et al.* 2009, 2011a).

Here, in unstimulated and superstimulated heifers, we tested the hypothesis that follicular aspiration before ovulation interferes with the subsequent formation and function of the CL, leading in turn to impaired endometrial receptivity and conceptus development. Following aspiration or ovulation, CL development was assessed by daily ultrasonography, and CL function was assessed in terms of the capacity to produce P_4 and the expression of genes involved in steroidogenesis in luteal tissue. The capacity of the uterine environment to support conceptus development was assessed following transfer and recovery of *in vitro*-produced embryos.

Results

Follicle diameter at aspiration

In the unstimulated heifers, one follicle was aspirated per heifer. In the superstimulated group, a mean of 19.2 ± 1.8 follicles were aspirated per heifer (range per heifer, 14–24 follicles aspirated). Mean ($\pm$ S.E.M.) follicle diameter at aspiration was 14.56 ± 0.63 mm ($n=9$) in unstimulated and 8.21 ± 0.13 ($n=96$) in superstimulated heifers ($P<0.05$).

CL development

The dimensions of the CL measured using ultrasound scanning between days 3 and 13 in unstimulated heifers are shown in Fig. 1. Follicular aspiration just before

the expected time of ovulation leads to a significant reduction ($P<0.05$) in CL diameter (from day 6 onwards), total CL area (from day 6 onwards) and area of luteal tissue (i.e. CL area corrected for the presence of a cavity, if any, from day 5 onwards; Fig. 1). This difference was also apparent at slaughter on day 14 in terms of CL weight, diameter and area (Table 1; Fig. 2).

CL characteristics in the superstimulated heifers undergoing follicle aspiration before the expected time

Figure 1 Maximum corpus luteum (CL) diameter (A), total CL area (B) and total area of luteal tissue (i.e. minus any cavity area; C) in unstimulated (i.e. single-ovulating) heifers undergoing ovulation (filled square, $n=10$) or undergoing follicle aspiration just before expected time of ovulation (filled diamond, $n=10$). Significant difference ($P<0.05$) was present for diameter (A) and total CL area (B) from day 6 onwards and for total area of luteal tissue (C) from day 5 onwards.

Table 1 Corpus luteum (CL) characteristics at slaughter on day 14 in unstimulated (i.e. single-ovulating) heifers undergoing ovulation ($n=9$) or follicle aspiration just before expected time of ovulation ($n=9$).

Treatment	CL weight (g) ^d	CL diameter (cm) ^d	CL area (cm ²) ^d	Luteal tissue area (cm ²) ^{d,a}
Ovulation ^b	8.42 ± 1.24	2.73 ± 0.12	59.42 ± 4.69	47.20 ± 4.89
FA ^c	4.16 ± 1.24	1.99 ± 0.12	32.19 ± 4.69	27.75 ± 4.89
P value	0.02	0.006	0.0008	0.01

FA, Follicle aspiration.

^aCorrected for the presence of a cavity. ^bExcluding one heifer due to the presence of a double ovulation. ^cExcluding one heifer as ovary with CL not recovered at abattoir. ^dData represented as mean ± S.E.M.

of ovulation or undergoing natural ovulation were recorded after slaughter on day 14 and are presented in Table 2. The mean number of CL differed between groups (23.0 ± 8.13 vs 14.4 ± 2.84 for ovulated and aspirated respectively), a reflection of variation in animal response to superstimulatory treatments. Follicular aspiration just before the expected time of ovulation leads to a significant reduction ($P < 0.05$) in mean CL weight, diameter and area compared with controls.

Circulating P₄ concentrations

Circulating concentrations of P₄ for unstimulated and superstimulated animals are presented in Fig. 3. In both groups, follicle aspiration just before ovulation reduced serum P₄ concentration. Similarly, in both groups, circulating P₄ concentrations were lower at all time points from days 3 to 14 but were only statistically significant from day 7 onwards ($P < 0.05$).

Embryo recovery and development

Significantly fewer embryos were recovered from unstimulated heifers undergoing ovulation (39/72, 54.2%) than those undergoing follicle aspiration (56/88, 63.6%). Follicle aspiration before ovulation leads to a significant reduction in conceptus length ($P = 0.002$) and area ($P = 0.01$) on day 14 compared with controls (Table 3). In the superstimulated group, there was no difference in embryo recovery rate between those undergoing ovulation (15/20, 75%) or follicle aspiration (20/25, 80%). Furthermore, conceptus dimensions were not affected by treatment (Table 4).

CL gene expression analysis

Luteal expression of *CYP11A1*, *HSD3B1* and *STAR* was not affected by follicle aspiration before ovulation or by superstimulation treatment. Follicle aspiration leads to a reduction in expression of *LHCGR* in luteal tissue from unstimulated heifers compared with those in which the CL formed after ovulation ($P < 0.05$).

Superstimulation significantly reduced the expression of *STAR* in luteal tissue, irrespective of follicle aspiration or ovulation (Fig. 4).

Discussion

The main findings from this study are that follicle aspiration before ovulation results in a reduction in CL size and P₄ output and, in unstimulated animals, decreased expression of *LHCGR* in luteal tissue and a compromised uterine capacity to support conceptus elongation. These findings are relevant not only for cattle, where oestrous synchronisation programmes involving GNRH-induced ovulation may lead to the ovulation of a premature follicle, a smaller CL with a lower P₄ output and thereby a compromised uterine environment, but also for human infertility treatment given the high reported frequency of luteal dysfunction following ovarian stimulation and follicle aspiration as evidenced by the fact that P₄ supplementation post-embryo transfer is common.

Follicular diameter at ovulation and the duration of proestrus have been identified as sources of variation in conception rate in cattle (Lamb *et al.* 2001, Peters & Pursley 2003, Perry *et al.* 2005, Mussard *et al.* 2007). P₄ concentrations and CL cross-sectional area are reduced in animals induced to ovulate prematurely. For example, Mussard *et al.* (2007) demonstrated that premature ovulation of a dominant follicle with GNRH reduced the size of the ovulatory follicle, reduced fertility and decreased subsequent luteal function. Furthermore, granulosa cells make a substantial contribution to the P₄ output of the CL and aspiration of the preovulatory follicle inevitably involves removal of follicular fluid and a variable number of granulosa cells. This process has been implicated in the subsequent compromised formation and function of the CL leading to impaired endometrial receptivity (termed luteal insufficiency in the human literature). CLs formed after removal of a large number of granulosa cells from the preovulatory follicle in cattle have a limited capacity to produce P₄. Milvae *et al.* (1991) reported that aspiration of follicular fluid and granulosa cells from bovine preovulatory follicles resulted in a reduction in circulating P₄ in the subsequent luteal phase. Interestingly, removal and return of follicular fluid resulted in similar

Table 2 Corpus luteum (CL) characteristics at slaughter on day 14 in superstimulated heifers undergoing ovulation ($n=4$) or follicle aspiration just before expected time of ovulation ($n=5$).

Treatment	CL number per heifer ^a	CL weight (g) ^a	CL diameter (cm) ^a	CL area (cm ²) ^a
Ovulation	23.0 ± 8.13	1.95 ± 0.10	1.51 ± 0.041	18.73 ± 0.97
FA	14.4 ± 2.84	1.16 ± 0.12	1.26 ± 0.048	13.69 ± 1.13
P value	<0.05	<0.0001	0.0005	0.003

FA, Follicle aspiration.

^aData represented as mean ± S.E.M.

Figure 2 Representative images of dissected corpora lutea (CL) from unstimulated (i.e. single-ovulating) heifers undergoing ovulation (left) or undergoing follicle aspiration just before expected time of ovulation (right). Note the larger CL following ovulation and the presence of a blood clot in CLs formed after follicular aspiration.

P₄ concentrations to controls. In humans and non-human primates, luteal dysfunction after follicle aspiration of preovulatory follicles and failures in human IVF have been related to lower maternal P₄ (Garcia *et al.* 1981, Kreitmann *et al.* 1981, Vargyas *et al.* 1986).

Follicle diameter at the time of aspiration was smaller in superstimulated heifers compared with unstimulated heifers. This is consistent with the fact that co-dominant follicles (or multiple dominant follicles) do not grow as large as singletons (Adams *et al.* 1993). In both stimulated and unstimulated heifers, CL weight, diameter and area were significantly lower following follicle aspiration compared with when ovulation took place. However, consistent with the follicle diameter data, CL weight, diameter and area were lower following superstimulation compared with unstimulated heifers. Early luteinisation of preovulatory follicles after follicular aspiration in mares has been described (Hinrichs *et al.* 1991). It was suggested that the diameter at aspiration may influence the subsequent CL formation. Mozzaquatro *et al.* (2011) reported that a functional CL can be induced in mares using follicular aspiration but that a follicular

diameter of at least 35 mm is necessary to reach P₄ serum concentration comparable with that of a CL produced by natural ovulation. Follicular aspiration resulted in a slower increase in serum P₄ concentrations compared with controls. In cattle, Hayashi *et al.* (2006) aspirated follicles before or after a GnRH-induced LH surge and observed that CL formation only occurred when aspiration took place after the LH surge indicating that the LH surge rather than follicle rupture is essential for CL formation. In the post-LH group, plasma P₄ concentration, CL volume and luteal blood flow were not different from the intact follicle group, which underwent ovulation.

Regardless of species, a dialogue between the developing conceptus and maternal uterus must be established during the peri-implantation period. The uterus must provide a microenvironment that supports growth and development of the conceptus and is receptive to implantation. During the same period, the conceptus must provide pregnancy recognition signalling to sustain the functional life of CL for production of P₄, which is essential for implantation and placentation. Indeed, in cattle, it has been shown that compromised

Figure 3 Serum progesterone concentrations in (A) unstimulated (i.e. single-ovulating, $n=20$) and (B) superstimulated ($n=9$) heifers undergoing ovulation (filled square) or undergoing follicle aspiration (filled diamond) just before the expected time of ovulation. In both groups, P₄ concentrations were lower at all time points from days 3–14 but were only statistically significant from day 7 onwards ($P<0.05$).

Table 3 Conceptus dimensions following transfer of day 7 bovine blastocysts to unstimulated (i.e. single-ovulating) heifers undergoing ovulation ($n=9$) or follicle aspiration just before expected time of ovulation ($n=10$) and recovery at slaughter on day 14.

Treatment	Number of embryos recovered/transferred (%)	Conceptus dimensions ^a		
		Length (mm)	Width (mm)	Area (mm ²)
Ovulation	39/72 (54.2)	4.06 ± 0.51	0.98 ± 0.08	4.71 ± 0.66
FA	56/88 (63.6)	1.99 ± 0.42	1.03 ± 0.07	2.41 ± 0.55
P value	<0.05	0.002	0.61	0.01

FA, Follicle aspiration.

^aData represented as mean ± S.E.M.

embryos that fail to elicit a normal response from the endometrium are less likely to survive (Heyman *et al.* 2002, Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009). Post-hatching conceptus elongation in ruminants is a maternally driven process. Evidence for this comes from the fact that post-hatching conceptus elongation does not occur *in vitro* (Brandao *et al.* 2004, Vejsted *et al.* 2006) and fails to occur *in vivo* in the absence of uterine glands (Gray *et al.* 2002, Spencer & Gray 2006). P₄ is central to this process. Elevated P₄ in the first week post-conception advances the normal temporal changes that occur in the uterine endometrial transcriptome (Forde *et al.* 2009) and this, in turn, leads to an advancement in conceptus elongation in cattle (Carter *et al.* 2008, Clemente *et al.* 2009). Furthermore, a delay in the post-ovulatory rise in P₄ is associated with retarded conceptus development (Forde *et al.* 2011a). Similarly, in women, following ovulation, P₄, produced by the CL, induces secretory transformations of the endometrium (Bourgain *et al.* 1990) and allows endometrial receptivity and embryo implantation (Kolibianakis & Devroey 2002). In the case of conception, trophoblast-derived human chorionic gonadotrophin (hCG) prevents CL regression and amplifies steroid secretion that consequently decidualises the endometrial stroma and supports early embryonic development.

The reasons for abnormal luteal function in women after ovarian stimulation for IVF are not clear but proposed mechanisms (Beckers *et al.* 2003) include the following: i) continued downregulation due to preceding follicular phase GnRH agonist coadministration may retard pituitary recovery, ii) induction of multiple follicle development could influence the duration of the luteal phase, iii) removal of large quantities of granulosa cells may diminish CL capacity to produce P₄ (Garcia *et al.* 1981, Vargyas *et al.* 1986) and iv) supraphysiological concentrations of steroids associated with multiple corpora lutea could inhibit LH release due to negative feedback (Fauser & Devroey 2003). Exogenous supplemental P₄ is a common practice in IVF-stimulated cycles because of associated luteal phase defects (Kolibianakis *et al.* 2003). Although the benefit of P₄ administration

as luteal phase support has been well documented in IVF (Fatemi *et al.* 2007), a question surrounds the optimum time for its discontinuation (Nyboe Andersen *et al.* 2002, Griesinger 2011, Kyrou *et al.* 2011).

Aspiration of the preovulatory dominant follicle just before ovulation in unstimulated heifers was associated with a compromised CL in terms of size and P₄ output. Consistent with our previous observations, reduction in circulating P₄ leads to a reduction in conceptus length and area on day 14 in unstimulated (i.e. single-ovulating) heifers. This is important given the linear association between conceptus size and interferon- τ production (Rizos *et al.* 2012). Interestingly, while aspiration of multiple dominant follicles before the expected time of ovulation in superstimulated heifers was also associated with a compromised CL in terms of size and P₄ output compared with those that ovulated, there was no deleterious effect on conceptus size on day 14. The lack of an effect on conceptus growth is probably due to the fact that the reduced concentrations of P₄ in this group were still high, being greater than concentrations in unstimulated animals undergoing ovulation (see Fig. 3).

Alterations in differentiation of the CL were examined by measuring abundances of genes involved in the LH-induced signalling cascades and steroidogenesis in the CL (*LHCGR*, *STAR*, *CYP11A1* and *HSD3B1*). LH is the primary gonadotropin that regulates differentiation and function of the CL including STAR and P₄ production (Stocco *et al.* 2007). STAR is the mitochondrial enzyme involved in shuttling cholesterol into the inner mitochondrial membrane, which in turn is converted by the side-chain cleavage enzyme system into pregnenolone (Stocco & Clark 1996); as such, STAR is the rate-limiting step in steroidogenesis in many tissues including the CL (Stocco & Clark 1996). Luteal expression of *CYP11A1* and *HSD3B1* was not affected by follicle aspiration before ovulation or by superstimulation treatment. Follicle aspiration leads to a reduction in the expression of *LHCGR* in luteal tissue from unstimulated heifers compared with those in which the CL formed after ovulation; this effect was not observed after superstimulation. Superstimulation significantly reduced the

Table 4 Conceptus dimensions following transfer of day 7 bovine blastocysts to superstimulated heifers undergoing ovulation ($n=4$) or follicle aspiration just before expected time of ovulation ($n=5$) and recovery at slaughter on day 14.

Treatment	Number of embryos recovered/transferred (%)	Conceptus dimensions ^a		
		Length (mm)	Width (mm)	Area (mm ²)
Ovulation	15/20 (75.0)	3.51 ± 0.92	1.40 ± 0.13	4.84 ± 1.63
FA	20/25 (80.0)	3.44 ± 0.80	1.17 ± 0.11	5.41 ± 1.46
P value	0.73	0.96	0.21	0.89

FA, Follicle aspiration.

^aData represented as mean ± S.E.M.

Figure 4 QRT-PCR analysis of the expression of four candidate genes (mean CNRQ values $\pm$ S.E.M.) in the CL of unstimulated and superstimulated heifers formed following follicle aspiration just before ovulation or following ovulation. Black bars, unstimulated aspirated; white bars, unstimulated ovulated; light grey bars, superstimulated heifers aspirated; dark grey bars, superstimulated ovulated. An asterisk (*) indicates a significant difference in expression ($P < 0.05$). *CYP11A1*, cytochrome P450, family 11, subfamily A, polypeptide 1; *HSD3B1*, hydroxy- Δ^5 -steroid dehydrogenase, 3 β - and steroid Δ -isomerase 1; *LHCGR*, LH/choriogonadotropin receptor; *STAR*, steroidogenic acute regulatory protein.

expression of *STAR* mRNA in luteal tissue, irrespective of whether follicle aspiration or ovulation took place. High variation in ovarian reserves is associated with alterations in CL differentiation and function. Jimenez-Krassel *et al.* (2009) found that P_4 concentrations were lower during oestrous cycles for animals with lower vs higher antral follicle count. Animals with low antral follicle count also had a decreased capacity of luteal and granulosa cells to produce P_4 , reduced amount of *STAR* and mRNA for *STAR* and LH receptor in the CL.

In conclusion, aspiration of the preovulatory follicle resulted in a reduction in CL size and P_4 output in unstimulated and superstimulated cattle. In unstimulated animals, this was associated with a reduction in the capacity of the uterine environment to support conceptus elongation, while in superstimulated animals, the reduced P_4 concentrations were apparently still sufficient to drive this process. These data have relevance not only for oestrous synchronisation programs in cattle involving GNRH-induced ovulation but also for assisted human reproduction where oocytes derived from women undergoing COH are recovered by transvaginal preovulatory follicle aspiration and routinely replaced in the uterus of the same women during the same cycle, in contrast to the situation in cattle, where transfer to unstimulated surrogate recipients is the norm. The negative effect of ovarian stimulation on the uterine environment is highlighted by the fact that high-quality embryos transferred into unstimulated women involved as embryo recipients in a surrogacy procedure have

a higher likelihood of implanting than if they are transferred back into the donors (Check *et al.* 1992, Stafford-Bell & Copeland 2001).

Materials and Methods

Animals and treatments

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland. Protocols were in accord with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC and were sanctioned by the Institutional Animal Research Ethics Committee.

The experimental design is illustrated in Fig. 5. All animals were housed indoors on slats for the duration of the experiment and were fed a diet consisting of grass and maize silage supplemented with a standard beef ration. The oestrous cycles of crossbred beef heifers, predominantly Charolais and Limousin cross (mean age 23.7 ± 0.69 months, mean weight 584.98 ± 8.85 kg), were synchronised using an 8-day Controlled Internal Drug Release device (CIDR 1.9 g, Pfizer, Sandwich, Kent, UK) with administration of a prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) analogue (2 ml Estrumate; Schering-Plough Animal Health, Hertfordshire, UK, equivalent to 0.5 mg cloprostenol) given on the day before CIDR removal. Heifers were checked for signs of oestrus four times per day commencing 30 h after CIDR withdrawal and only those recorded in standing oestrus (day 0; $n = 29$) were used. Heifers were randomly assigned to be treated as unstimulated (i.e. single-ovulating, $n = 20$) or

Figure 5 Experimental design. (A) Unstimulated (i.e. single-ovulating) heifers ($n = 20$) were synchronised with an 8-day progesterone insert with prostaglandin (PG) administered the day before device removal. All heifers received GNRH 48 h after device removal and half underwent follicle aspiration 20 h later; the remainder underwent ovulation. Corpus luteum (CL) dimensions were assessed by ultrasound scanning from days 3 to 13. (B) Superstimulated heifers ($n = 9$) were administered eight injections of FSH starting on day 10 after a synchronised oestrus. Prostaglandin (PG $_2$) was administered with the sixth FSH injection. All heifers received GNRH 40 h after PG $_2$ and approximately half ($n = 5$) underwent follicle aspiration 20 h later; the remainder underwent ovulation. Blood samples were taken from days 0 to 14 to measure serum progesterone concentration.

superstimulated ($n=9$). As fewer animals were deemed necessary in the superstimulated group, once the desired number (originally 10, but one animal was lost) was reached, the remaining heifers were assigned to the unstimulated group. Within each group, approximately half of the animals underwent follicle aspiration just before the expected time of ovulation while the remainder were allowed to ovulate naturally. Follicle aspiration (removal of entire follicular contents) was achieved using an ultrasound scanner (SSD-500; Aloka, Tokyo, Japan) and a 5.0 MHz convex array transducer (UST-974-5; Aloka) attached to a 17 G $\times$ 540 mm stainless steel needle guide. The aspiration pressure was ~ 50 mm Hg.

Unstimulated heifers

Heifers received a GNRH agonist (2.5 ml Receptal, Intervet Ireland Ltd., Dublin, Ireland, equivalent to 0.01 mg buserelin) 48 h after CIDR removal to induce an LH surge. This treatment has been shown to induce a preovulatory LH surge 2 ± 3 h after injection (Bordignon *et al.* 1997, van de Leemput *et al.* 1999). Half of the animals underwent follicle aspiration 20 h later while the remainder underwent natural ovulation. The diameter of the dominant preovulatory follicle at puncture was measured using an optical calliper.

Superstimulated heifers

Procedures for superstimulation and follicle aspiration were as described by Rizos *et al.* (2002). Beginning on day 10 of a synchronised oestrous cycle, heifers were superstimulated with a total of 455 IU FSH (13 ml Folltropin; Bioniche, Inverin, Galway, Ireland) given as twice daily i.m. injections over 4 days on a decreasing dose schedule. Luteolysis was induced with 2 ml Estrumate (PGF $_{2\alpha}$) given on day 12 with the sixth injection of FSH. All heifers received 2.5 ml Receptal (GNRH) at 40 h after PG, the expected time of the LH surge (Callesen *et al.* 1986) and follicle aspiration was performed ~ 20 h later (i.e. 60 h after PG). As above, the diameter of all punctured preovulatory follicles was measured using an optical calliper.

In vitro embryo production

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company. The techniques for producing embryos *in vitro* have been described in detail previously (Rizos *et al.* 2002). Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows collected at killing. COCs were matured for 24 h in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml epidermal growth factor at 39 °C under an atmosphere of 5% CO $_2$ in air with maximum humidity. For IVF, matured COCs were inseminated with frozen-thawed Percoll-separated bull sperm at a concentration of 1×10^6 spermatozoa/ml. Gametes were co-incubated at 39 °C under an atmosphere of 5% CO $_2$ in air with maximum humidity. Semen from the same bull was used for all experiments. At ~ 20 h post-insemination (hpi), presumptive zygotes were denuded and cultured in groups of 50 in 500 μ l synthetic oviduct fluid supplemented with 5% FCS. Cleavage rate was recorded at 48 hpi and blastocyst development recorded at day 7 pi.

CL ultrasonography

In order to assess the effect of follicle puncture on CL development, daily transrectal ultrasonography commenced on day 3 (oestrus=day 0) in the unstimulated heifers. This procedure was not carried out in the superstimulated groups because of the difficulty of correctly scanning the multiple CLs present. Ultrasound examination of the ovaries was performed using a Voluson portable ultrasound system (GE Healthcare, Vienna, Austria) equipped with an RNA 12L linear probe emitting a frequency of 12 MHz. All ultrasound examinations were carried out by the same operator. Upon visualisation of the ovary, three consecutive images of the CL were taken. Maximum CL diameter and diameter of a CL cavity, where present, were recorded. Images were saved and stored on the ultrasound system for later analysis. The area of the CL was calculated using the formula: $\text{area} = \pi \times (\text{radius})^2$. For unstimulated heifers, the area of a cavity, if present, was subtracted from the total CL area to get the total luteal tissue area.

P $_4$ measurement

Blood samples were taken daily by jugular venipuncture. Following collection, blood samples were refrigerated (4 °C) for 12–24 h before being centrifuged at 1500 g at 4 °C for 20 min. Serum was separated and stored at -20 °C until analysis to determine P $_4$ concentration by solid-phase RIA using a Coat-A-Count P $_4$ kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as described previously (Forde *et al.* 2011b). The sensitivity of the assay was 0.03 ng/ml. The inter- and intra-assay coefficients of variation for the were 13.9 and 14.6%, 8.3 and 6.9% and 9.4 and 4.5% for low-, medium- and high-quality control serum pools respectively.

Embryo transfer and recovery

In order to test the ability of the uterus to support embryo development and conceptus elongation, day 7 *in vitro*-produced blastocysts were transferred to the uteri of synchronised recipients (five to ten blastocysts per recipient) as described previously (Clemente *et al.* 2009, Forde *et al.* 2011b). All recipients were killed on day 14 to assess conceptus development. Following killing, the reproductive tract was removed, sealed in a plastic bag and placed in a sealed polystyrene box for transportation to the laboratory (within 60 min). After removal of the ovaries and the oviducts, the uterine horns were trimmed free of excess tissue before being flushed with 40 ml PBS. On recovery, the dimensions (length, width and area) of each conceptus were recorded.

CL processing

In the unstimulated group, at killing, the CL was dissected free from the ovary, weighed and the diameter was recorded. The CL was then cut into half and the diameter of any cavity present was also recorded. The area of the CL was calculated

using the formula: $\text{area} = \pi \times (\text{radius})^2$. For unstimulated heifers, the area of a cavity, if present, was subtracted from the total CL area to get the total luteal tissue area. A portion of the CL was snap frozen for subsequent gene expression analysis. In the case of the superstimulated heifers, the total number of CLs present on each ovary was recorded. The CLs were then dissected free from the ovaries, weighed and the diameter was recorded. Owing to the large number of CLs present in the superstimulated heifers, which increased dissection and processing time, cavities were not recorded to avoid tissue degradation before samples were snap frozen for further analysis. Thus, only CL diameter and CL area are presented for this group. A portion of up to five randomly selected CLs per ovary per heifer was snap frozen for gene expression analysis.

mRNA analysis

Total RNA was isolated from ~30 mg CL tissue in the single-ovulating group and from a random selection of five CLs from each ovary of the superstimulated group, using Trizol reagent as per manufacturer's instructions (Invitrogen). An on-column DNase treatment and clean up was performed and quality and quantity of RNA were determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the NanoDrop 1000 (Thermo Fischer Scientific, Inc., Wilmington, DE, USA) respectively. Following cDNA synthesis of 500 ng total RNA (high-capacity reverse transcriptase kit; Applied Biosystems, Carlsbad, CA, USA), the expression of selected genes in the CL tissue was analysed by quantitative real-time PCR using the primer concentrations listed in Table 5. Primers were designed using primer-BLAST Software (www.ncbi.nlm.nih.gov/tools/primer-blast/). Each QRT-PCR reaction was carried out on the 7300 Real-Time PCR System (Applied Biosystems) in a final reaction volume of 20 µl. Cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 1 min and were carried out with the inclusion of a dissociation curve to ensure specificity of amplification. A standard curve was included for each gene of interest including the normaliser genes to obtain primer efficiencies. All raw cycle threshold values were then imported into qbase^{plus} Software (Biogazelle, Zwijnaarde,

Belgium) where data were calibrated, normalised and expression values for each gene were determined in arbitrary units (calibrated normalized relative quantities values).

Statistical analysis

Data were checked for normality and homogeneity of variance using histograms, qplots and formal statistical tests in the UNIVARIATE procedure (version 9.1.3; SAS Institute, Cary, NC, USA, 2006). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate *P* values. The corresponding least squares means and S.E.M. of the non-transformed data are presented in the results for clarity. Variables having more than one observation such as the *P*₄ concentrations and ovarian ultrasonic measures were analysed for the unstimulated and superstimulated groups using a repeated measures analysis with the MIXED procedure of SAS. Fixed effects included experimental treatment (ovulate naturally or follicle aspiration before ovulation), day and their interaction. The interaction term if not statistically significant (*P* > 0.10) was subsequently excluded from the final model. Animal within treatment was included as a random effect in the model with the most appropriate covariance structure between records within animal determined by minimising the Akaike Information Criterion (AIC). Models were run under compound symmetry, unstructured, autoregressive or Toeplitz variance-covariance structures. Embryo-related data for the unstimulated and superstimulated groups were analysed using the PROC MIXED procedure of SAS. The model had experimental treatment (ovulate naturally or follicle aspiration before ovulation) as a fixed effect and 'animal within treatment' was included as a random effect. Similarly, gene expression data for the unstimulated and superstimulated groups were analysed using the PROC MIXED procedure of SAS. The model had experimental treatment (ovulate naturally or follicle aspiration before ovulation) as a fixed effect. Differences between treatments were determined

Table 5 Gene symbol, name and accession number used to design forward and reverse primers used for qRT-PCR. All primers were used at a final concentration of 300 nM.

Accession number	Entrez gene symbol	Gene name	Primer sequence
NP_776614.1	<i>STAR</i>	Steroidogenic acute regulatory protein	F: CCTCAAGGACCAAACTCACC R: GTGATTGGCAAATCCACCT
NM_174343	<i>HSD3B1</i>	Hydroxy-Δ-5-steroid dehydrogenase, 3β- and steroid Δ-isomerase 1	F: CGCGAGAGACCATCATGAAC R: CTATGGTGCTGGTGTGGATAAAG
NM_174381.1	<i>LHCGR</i>	LH/choriogonadotropin receptor	F: GAAAGCACAGCAAGGAGACC R: TGGAGTGTCTTGGGTAAGCA
NM_176644	<i>CYP11A1</i>	Cytochrome P450, family 11, subfamily A, polypeptide 1	F: AGGGGGTGTGAACACGACATCCA R: GCCTGGCGTCGGGCATTGAG
NM_174152	<i>PPIB</i>	Peptidylprolyl isomerase B	F: GGCCCAAAGTCACTGTCAAGGTGT R: AAAGAGACCGATGACCAACCCGG
NM_174760	<i>RPL10</i>	Ribosomal protein L10	F: TTCCTGGAGGGCGTCCGCAAT R: AAGCCAGGACGCGCTTGATCC

F, forward; R, reverse.

by *F*-tests using type III sums of squares. The PDIF command incorporating the Tukey test was applied to evaluate pairwise comparisons between treatment means.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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