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CRediT authorship contribution statement

Rob Simmons: Funding acquisition, Conceptualization, Project administration, Investigation, Writing - Review & Editing. **Des Tutt:** Investigation, Formal analysis, Writing - Review & Editing. **Wing Yee Kwong:** Methodology, Investigation. **Gizem Guven-Ates:** Investigation, Writing - Review & Editing. **Remi Labrecque:** Resources, Writing - Review & Editing. **Federico Randi:** Funding acquisition, Conceptualization, Writing - Review & Editing. **Kevin Sinclair:** Funding acquisition, Conceptualization, Resources, Project administration, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing.

Enhanced progesterone support during stimulated cycles of transvaginal follicular aspiration improves bovine *in* vitro embryo production

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Abstract

The *in vitro* production (IVP) of cattle embryos requires that germinal-vesicle stage oocytes undergo a period of maturation *in vitro* prior to fertilization and culture to the blastocyst stage. Success of IVP in taurine cattle is enhanced following ovarian stimulation prior to oocyte retrieval (OPU), particularly if preceded by a short period of FSH withdrawal ('coasting'). However, evidence regarding the importance of progesterone (P4) support during OPU-IVP is equivocal. The current study, therefore, determined the effects of increased peripheral P4 concentrations during FSH-stimulated ('coasted') cycles of OPU. Progesterone support was provided by either an active *corpus luteum* (CL) and/or one of two intravaginal P4 releasing devices (i.e., CIDR® [1.38g P4] or PRID® Delta [1.55g P4]). Expt. 1 established an initial estrus prior to OPU, allowing CL formation (single luteal phase) spanning the first two of five cycles of OPU; the remaining three cycles were supported by either a CIDR® or PRID® Delta. Expt. 2 commenced with two cycles of dominant follicle removal (including prostaglandin $F_{2\alpha}$) undertaken seven days apart prior to six cycles of OPU. The absence of a CL meant that these cycles were supported only by a CIDR® or PRID® Delta. As each experiment involved several sequential cycles of OPU, the cumulative effects of device use on vaginal discharges were also assessed. Each experiment involved 10 sexually mature Holstein heifers. In the absence of a CL, peak plasma P4 concentrations were greater (P=0.002) for the PRID® Delta (4.3±0.22) than for the CIDR® (2.9±0.22). In Expt. 1 there was an interaction (P<0.05) between CL presence at OPU and P4 device on Day 8 blastocyst yields, indicating an effect of P4 device only when the CL was absent. The percentage hatching/hatched blastocysts of matured oocytes for the CIDR® and PRID® Delta was 44.3±5.04 and 41.0±5.40 in the presence, and 17.1±3.48 and 42.2±3.76 in the absence, of a CL (P=0.018). Combined analyses of data from Expt. 1 and 2, when no CL was present, confirmed that Day 8 blastocyst yields were greater (P=0.022) for the PRID® Delta than the CIDR®. Vaginal discharge scores were higher (P<0.001) for the PRID® Delta than the CIDR® in Expt. 1 but not in Expt 2; however scores were low, did not increase with repeated use, and thus were deemed of no clinical or welfare concern. In conclusion, enhanced P4 support during FSH-stimulated cycles of OPU-IVP can improve *in vitro* embryo development.

Key words Cattle, Progesterone, Ovarian Stimulation, OPU, *In Vitro* Culture, Blastocyst

1. Introduction

Contemporary systems for *in vitro* production (IVP) of cattle embryos require that germinal-vesicle (GV) stage oocytes undergo a period of maturation *in vitro* (IVM) prior to fertilization (IVF) and culture (IVC) to the blastocyst stage, typically by Day 7 [1]. As *Bos indicus* cattle possess a larger population of antral follicles, most oocytes from this sub species are retrieved from non-stimulated cycles of transvaginal follicular aspiration (Ovum Pick-Up; OPU), whereas protocols embracing a period of controlled ovarian stimulation are generally favoured prior to oocyte retrieval in *Bos taurus* genotypes [1,2,3]. Over the years various protocols for ovarian stimulation and oocyte retrieval in taurine cattle have been developed [4,5], including that described in the current report which is based on a short period (~42 h) of gonadotrophin withdrawal ('coasting') prior to OPU [6,7]. Although such protocols lead to comparatively high yields of transferable embryos [8], they result in modest live-birth rates of around 50% following single-embryo transfer [9]. Additionally, a degree of between-donor variability has been observed in the production of IVP embryos using this system of OPU-IVP [8,10].

A contributing factor to both within and between donor variability in IVP success could be the degree of 'maturational' synchrony of oocytes at the point of collection arising from the extent to which individual donors respond to a given ovarian stimulation regime [11]. Whilst the level and timing of FSH provision prior to OPU could be adjusted to facilitate this, equally IVP success could be influenced by the level of progesterone (P4) support during controlled ovarian stimulation [12]. However, supporting data in this regard is sparse and conflicting, appearing to differ between FSH-stimulated and non-stimulated cycles [12,13]. Therefore, the primary aim of the current study was to determine the effects of increased peripheral P4 concentrations during FSH-stimulated cycles of OPU as described above [7,8]. Enhanced P4 support was provided by either undertaking stimulated cycles of OPU in the presence of an active *corpus luteum* (CL) or in the presence of one of two commercially available intravaginal P4 releasing devices (i.e., CIDR® [Zoetis] or PRID® Delta [CEVA]). Previous studies identified benefits of using either device for fertility management [14], but the PRID® Delta releases more P4 into peripheral circulation [15,16], and the current study sought to determine if this could influence IVP outcome following OPU. Given that the experiments described in this report involved several sequential cycles of OPU, and that intravaginal devices can increase the incidence of vaginal discharges [17,18], a secondary objective of this study was to assess the cumulative effects of extended device use on the extent of vaginal discharge in young sexually mature heifers.

2. Materials and Methods

2.1. Generic considerations

All procedures were performed under the auspices of the Animal Scientific Procedures Act (1986). Associated protocols complied with the ARRIVE guidelines and were approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Nottingham. All chemicals and reagents were sourced through Sigma-Aldrich Company Ltd (Dorset, UK).

2.2. *Experimental design, animals and treatments*

Two related experiments are reported that each involved stimulated ('coasted') cycles of OPU followed by IVP as described previously [6,7,8]. Post-pubertal Holstein-Friesian heifers, bred and accommodated in open cubicles at the University of Nottingham experimental dairy farm, were used. Heifers were fed a standard grass/maize silage-based diet formulated to meet the nutrient requirements of young females growing at around 0.8 kg/d [19]. In each experiment, one of two commercially available intravaginal progesterone (P4) releasing devices (i.e., either a CIDR® [Zoetis UK Ltd, Leatherhead, UK; impregnated with 1.38g P4] or a PRID® Delta [CEVA Santé Animale, Libourne, France; impregnated with 1.55g P4]) were inserted and subsequently replaced following each episode of dominant follicle removal (DFR) or OPU. In Experiment 1, ten 15- to 19-month-old virgin Holstein-Friesian heifers underwent five successive stimulated cycles of OPU-IVP, whereas in Experiment 2, ten 12- to 16-month-old virgin Holstein-Friesian heifers underwent six successive stimulated cycles of OPU-IVP. In each case, heifers were allocated at random to receive either a CIDR® or PRID® Delta in matched pairs based on antral-follicle count [20] established by transvaginal ultrasonography during the first session of DFR. Briefly, based on total antral follicle numbers, heifers were ascribed to one of three groups (low, intermediate or high), pair matched within group and then allocated at random to either the CIDR® or PRID® Delta treatment groups. Allocation to each of these two treatments was for the duration of each experiment. We opted for continuous exposure to either a CIDR® or PRID® Delta, as opposed to switching treatments between donors at the end of each cycle, to assess the cumulative effects of each of these devices on (i) embryo production over several cycles of OPU-IVP and (ii) vaginal health [17]. This better represents commercial practice (i.e., opting for continuous use of one device over the other) and is therefore of greater clinical interest.

The two experiments differed in that Experiment 1 involved establishing an initial reference estrus prior to commencement of procedures (Fig. 1A). This allowed the formation of a CL (i.e., a luteal phase) which spanned the first two cycles of OPU but regressed thereafter. In contrast, Experiment 2 commenced with two cycles of DFR undertaken seven days apart prior to the first cycle of ovarian stimulation and OPU (Fig. 1B). The regime involved administrating prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Enzaprost®, 25mg dinoprost trometamine, CEVA, Santé Animale, Libourne, France) at the initial cycle of DFR to ensure that no CL was present during subsequent stimulated cycles of OPU.

2.3. Ovarian stimulation and follicular aspiration

These procedures were identical for the two experiments and matched those described previously [8]. Briefly, sessions of OPU were undertaken every 14 days. Each cycle commenced with the ablation of all follicles ≥ 5 mm in diameter (DFR) at which point the intravaginal P4 device (CIDR® or PRID® Delta) was replaced. Prior to undertaking DFR, a vaginal discharge score (scale from 0 [no discharge] to 3 [significant discharge] units, subdivided to the nearest 0.25 unit [21]) was ascribed to each heifer. On this scale a score of 1 represents "mucus containing flecks of white or off-white pus", a score of 2 represents "< 50 mL exudate containing ≤ 50% white or off-white mucopurulent material", whereas a score of 3 represents ">50 mL exudate containing purulent material, usually white or yellow".

Each donor then underwent vaginal washing using a weak solution of Virkon™S (potassium peroxy-monosulfate and sodium chloride; LanXESS, Cologne, Germany) followed by rinsing with physiological saline. Ovarian stimulation (six injections [i.m.] of FSH [Folltropin, 70 IU dose per injection, Vetoquinol UK Ltd, Towcester, UK] given at 12 h intervals) commenced 48 h later (Fig. 1). Each session of OPU was undertaken 38-42 h following the last FSH injection in a dedicated, environmentally controlled theatre where the ambient temperature was maintained between 31 and 33 ºC. Aspiration was preceded by vaginal cleansing, as described above, and used a Cook Medical vacuum pump with a 7.5 MHz ultrasound scanner (Exapad, IMV Imaging, Glasgow, UK) with aspiration pressure set at -70 mm Hg. COCs from follicles ≥ 5 mm in diameter were aspirated through an 18G needle and 1.4 m of 1.4 mm (I.D.) silicone tubing into 5 mL Tyrodes lactate-based aspiration media contained in a 50 mL conical tube, hand-held to maintain temperature. Aspirants were then passed through a heated (~37°C) filter and rinsed repeatedly with pre-warmed media (~50 mL) to remove excess cell debris and blood. The filtrate was then rinsed from the filter into a 100 mm petri dish on a warm stage (~38°C), in order to search for COCs. Following OPU, a replacement CIDR® or PRID® Delta was inserted into each donor and the subsequent cycle commenced with DFR 8 days later.

2.4. In vitro embryo production (IVP)

These procedures were also identical between the two experiments and were described previously [8]. COCs were graded on a four-point scale according to [22,23]. Briefly, Grade 1 COCs had >5 layers of compact cumulus cells with a clear, even ooplasm; Grade 2 COCs had <5 layers of compact cumulus cells with a clear, even ooplasm; Grade 3 COCs had <5 layers of cumulus cells which were slightly expanded, and the ooplasm was slightly uneven; Grade 4 COCs had <5 layers of expanding cumulus and uneven ooplasm or were denuded or fully expanded. Only Grade 1-3 COCs were matured (IVM). This was undertaken in 1.8 mL HEPES buffered TCM199-based media in a screw top cryovial (Nunc, ThermoFisher Scientific, Loughborough, UK) at atmospheric CO_2 and 38.5°C, for 23-24 h.

Frozen/thawed semen from a single bull was used for *in vitro* fertilization (IVF). Sperm preparation was by centrifugation through a 45%/90% BoviPure (Nidacon International AB, Mölndal, Sweden) gradient. Fertilization occurred in 50 µl drops of modified Tyrode's lactate fertilization media under oil as used previously [7,8]. Oocytes were washed in fertilization media then placed in drops at a maximum of 5 per drop. 2 µl of sperm preparation media was added to each drop to give a final concentration of 70,000 sperm per drop. Oocytes and sperm were co-incubated for 18-21 h in a humidified environment of 5% CO_2 in air at 38.5°C.

Embryos were cultured in SOF based sequential culture media as described previously [7,8], in a humidified environment under oil at 6.8% CO_2, 5% O_2 and 38.5°C. Briefly, 21 h post fertilization, (a.m. of Day 1), presumptive zygotes were denuded by repeated pipetting, and transferred at no more than 11 per drop to 10 µl drops of the first culture media. Cleavage was assessed 30 h later (p.m. of Day 2) and oocytes classified according to cell number (i.e., 1, 2-3, 4-5 and >6 cells). Zygotes were transferred approximately 42 h later (Day 4) to 10 µl drops of the second culture media. Progression to morula was assessed 48 h later (Day 6), and embryos transferred to 20 µl drops of the third culture media. Embryos were assessed again 48 h later (Day 8), for stage and quality in accordance with the International Embryo Technologies Society (IETS) guidelines for bovine embryo assessment [24].

2.5. Plasma Progesterone (P4)

Blood samples collected into lithium heparin 10 mL vacutainers from the coccygeal vein at DFR and OPU (Experiment 1) and at DFR, OPU, and 24 and 72 h after device replacement (*Expt. 2*). P4 concentrations were analyzed at the Segalab (Laboratório de Sanidade Animal e Segurança Alimentar, 4490-295 Argivai, Portugal) using a chemiluminescent assay (Immulite, Siemen, Wales, UK) as described previously [25]. Limit of assay detection was 0.20 ng/mL and mean intra- and inter-assay coefficients of variation were 7.4 and 8.1% respectively.

2.6. *Statistical analyses*

Analyses were performed using the GenStat statistical package (19th Edition, VSN International, 2018; https://www.vsni.co.uk/). Vaginal-purulent discharge scores were analyzed using Kruskal-Wallis one-way analysis of variance on ranks (H-test). Data are presented as scatter plots around median scores. P4 concentration data were analyzed using REML generalized linear mixed models where, in Experiment 1, 'Donor' formed the random effect and 'Cycle' (i.e., 1st, 2nd, 3rd…) and 'P4 device', together with interactions between these terms, formed the fixed effects. In Experiment 2, 'Cycle stage' (i.e., Day of OPU/DFR, 24 and 72 h later) replaced 'Cycle' in these models. These data are presented as means ± SEM. Data on the number of follicles aspirated and oocytes retrieved were analyzed using REML generalized linear mixed models assuming Poisson errors and used log-link functions. Data are presented as means ± SEM. All proportion

data (relating to embryo development) were also analyzed using REML generalized linear mixed models that on this occasion assumed binomial errors and used logit-link functions. In these models 'Donor' formed the random effect, and 'Cycle' and 'P4 device' were fixed effects. Proportion data are presented as mean percentages ± SEM.

3. Results

3.1 Vaginal-purulent discharges and Plasma P4 concentrations are increased in PRID® Delta *vs CIDR® treatment groups (Expt. 1 and 2)*

There was clear evidence across the two experiments that, following removal of P4-releasing devices, vaginal-purulent discharge-scores were greater (by between 0.25 to 0.5 units) for the PRID® Delta than the CIDR® (Fig. 1A and B). These differences were consistent for both non-stimulated DFR and FSH-stimulated OPU cycles (Supplementary Fig. S1A and B). However, vaginal-purulent discharge score did not increase significantly over time with successive cycles of DFR and OPU (Supplementary Fig. S2A and B).

Plasma P4 concentrations were greater (P<0.001) when a CL was present than absent, but in Experiment 1 there was no difference in plasma P4 concentrations between the two P4-releasing devices at the point of vaginal device renewal (i.e., on the day of either DFR or OPU; Fig. 3A). This was not surprising given that these P4-releasing devices would have been *in situ* for between 6-8 days prior to P4 determination. In Experiment 2, therefore, P4 concentrations were determined at DFR/OPU, and 24 and 72 h after device renewal. These analyses revealed that plasma P4 concentrations were indeed higher (P=0.017) in PRID® Delta vs CIDR® treated heifers, and that these elevated concentrations were maintained for at least 72 h following device insertion (Fig. 4).

3.2*. OPU-IVP is enhanced in the presence of a CL or treatment with a PRID® Delta (Expt. 1).*

The number of follicles aspirated and oocytes retrieved was similar for both CIDR® and PRID® Delta treatment groups either in the presence or absence of a donor CL (Table 1A), and did not vary significantly between cycles (data not presented). Similarly, there was no difference between treatment groups in the number or grade of oocytes that went into maturation (Table 1B). By Day 2 following IVF, embryo stage was generally similar between treatment groups although the percentage of 5-6 cell embryos was greater (P<0.001) when oocytes were retrieved from donors in the presence than absence of a CL (Table 1Bi). By Day 6, however, there was an indication that the percentage. of viable embryos of oocytes inseminated was less (P=0.072) for the CIDR® than PRID® Delta treatment groups, particularly in the absence of a CL (Table 1Bii). Indeed, by Day 8 this difference between P4-releasing devices was significant (P<0.05), with clear evidence of an interaction (P<0.05) between CL presence at the time of aspiration and P4-releasing device on the percentage Day 8 blastocysts of oocytes that were inseminated and cleaved following insemination (Table 1Biii); as well as the percentage of advanced (IETS Stages 7 to 9, Grade 1-

2) blastocysts of oocytes matured (Figure 3B). These interactions indicate that, in the absence of a CL, Day 8 blastocyst development was impaired for the CIDR® relative to the PRID® Delta treatment group.

3.3. Modest differences between PRID® Delta *and CIDR® treatments in 'CL-free' cycles of OPU (Expt. 2)*

Working with a younger group of donor heifers, and in the absence of a functional CL, more modest differences in OPU success were observed between PRID® Delta and CIDR® supported cycles in the second experiment (Table 2). In these younger animals, ovarian response to the same regime of FSH treatment and withdrawal prior to OPU led to a greater (P<0.001) number of follicles available for aspiration compared to *Expt. 1* (35.7 ± 1.54 vs 19.9 ± 1.93). Furthermore, of these follicles, a greater percentage (56.8 ± 1.07, *Expt. 2* vs 39.9 ± 1.78, *Expt. 1;* P = 0.006) were in the smallest of the three size categories aspirated (i.e., 5-6 vs 7-10 and >10 mm diameter).

The number of follicles aspirated and oocytes retrieved in *Expt. 2* was similar for both CIDR® an PRID® Delta treatment groups (Table 2A). However, the percentage Grade 1 oocytes was lower (P = 0.039) whilst the percentage Grade 2 oocytes greater (P = 0.052) for the PRID® Delta than the CIDR® treatment groups (Table 2B). In keeping with *Expt. 1*, there was an indication that blastocyst development by Day 8 was improved in PRID® Delta treated compared to CIDR® treated heifers, although this only reached statistical significance (P = 0.047) for the percentage of Day 8 blastocysts that developed from Day 6. In this experiment the number and grade of oocytes that were matured, and the percentage that cleaved following insemination, did not vary between cycles (Supplementary Table S1). However, embryo development to Days 6 and 8 did vary (P<0.05) between cycles, although there was no consistent progressive trend that would indicate a cumulative effect.

3.4. Combined analyses of Expt. 1 and 2 confirms PRID® Delta enhancement of embryo development

A key metric in bovine OPU-IVP is the percentage blastocysts of oocytes inseminated and particularly advanced (potentially transferrable) blastocysts of oocytes inseminated. The following analyses were on combined data for the last three cycles of Expt. 1 (coinciding with CL absence) with data for all cycles from *Expt. 2*. The statistical model employed was that used for the percentage data analyzed separately for *Expt.1* and *2* described earlier, but with the inclusion of the term 'Experiment' in the fixed model. This term was included to account for between study variance.

These combined analyses confirmed that the percentage Day 8 blastocysts of oocytes inseminated was greater (P = 0.022) for the PRID® Delta relative to the CIDR® treatment group

(50.9 ± 4.00 vs 38.7 ± 3.27). Similarly, the percentage advanced Day 8 blastocysts (IETS Stage 7 [Grade 1&2] to 8/9 [all Grade 1]) of oocytes inseminated was also greater (P = 0.021) for the PRID® Delta treatment group (39.2 ± 3.82 vs 27.3 ± 2.91).

4. Discussion

The key finding to emerge from this study is that enhanced peripheral P4 concentrations (originating from either an endogenous CL and/or progesterone releasing device) during FSH-stimulated and 'coasted' cycles of OPU in cattle improves subsequent embryo development during IVP. This finding has important practical implications for protocol design in stimulated cycles of OPU in cattle and/or choice of intra-vaginal P4 releasing device. For example, luteal-phase follicle aspiration could obviate the need for exogenous P4 support following initial induced ovulation (and estrus) for up to two cycles of OPU (Fig. 1A and Fig. 3) whilst ensuring optimal levels of embryo development. This would have beneficial effects in terms of animal welfare (i.e., reducing vaginal discharges (Fig. 2)) and risk of vulvo-vaginitis that can arise with vaginal implants [17,18], as well as minimising cost (i.e., reduced usage of P4 releasing devices). When stimulated cycles of OPU are undertaken in the absence of an endogenous CL then choice of P4 releasing device becomes important. In the current study, the PRID® Delta released more P4 into peripheral circulation than the CIDR® (Fig. 4), an observation consistent with previous studies in ovariectomized and/or non-lactating Holstein cows [15,16], and this was associated with improved *in vitro* embryo production. At present, however, mechanisms underlying the beneficial effects of enhanced peripheral P4 on subsequent embryo production are unclear and consequences for pregnancy outcome following embryo transfer unknown. Both await further investigation.

4.1. Progesterone concentrations during antral-follicle development and oocyte quality

Increased concentrations of P4 in peripheral circulation during antral follicle development are generally associated with enhanced oocyte quality leading to improved embryo development and fertility in induced and/or stimulated, as well as in spontaneously ovulating, cycles [26,27,28,29,30,31]. The general thinking from these studies is that these benefits arise in part as a consequence of P4-mediated suppresion of LH pulse frequency and reduced duration of follicle dominance, consequently limiting the extent of oocyte ageing [32]. There could also be localized, but as yet poorly understood, actions of P4 on the follicle-enclosed oocyte. However, much of the evidence supporting such actions is conflicting, being based on non-FSH stimulated abattoir derived ovaries with little or no clinical history of the donor. In such studies both positive [33,34] and negative [35] effects of CL presence on oocyte quality and *in vitro* embryo development have been reported. Similarly, the effects of CL proximity (i.e., ipsi- vs contra-lateral ovary) are variable, with evidence of both positive [33,36] and negative [37] effects of CL location on subsequent *in*

vitro embryo production. It is unlikely that information arising from such studies could shed much light on the current set of observations.

4.1.1. Chromatin compaction of GV oocytes during FSH-stimulated cycles

The FSH-stimulated and 'coasted' cycles of the current study are based on protocols described previously [6,7], and reported more recently by our group in the context of screening for chromosomal abnormalities in both stimulated and non-stimulated cycles of OPU [8]. The >40 h period of FSH withdrawal prior to OPU is critical in determining oocyte developmental competence. It results in a series of intrically choreographed changes in transcript expression between the granulosa, cumulus and oocyte, which ultimately determines post-fertilization developmental competency of the egg [38]. The importance of LH pulsatile release during this period was highlighted by the fact that its inhibition (by treatment with the GnRH antagonist cetrorelix) altered the transcription of genes involved in chromatin organization, chromosome segregation and RNA translation in the oocyte, and genes supporting cell survival, proliferation, transcription and protein synthesis in cumulus cells; with an indication of negative consequences for subsequent *in vitro* embryo development [39,40]. At present the effect of peripheral P4 concentration in regulating LH-mediated actions on COCs, including putative effects on chromosomal error rate reported previously with this system [8,9], are not known and await investigation. A direct effect of P4 on the COC, however, cannot be ruled out, although effects on COC grade were inconsistent between the two experiments (absent in Experiment 1 but present in Experiment 2). There is no clear explanation for this inconsistency, other than a possible donor effect in the younger group of animals within Experiment 2. As COC grade is predictive of subsequent post-fertilization development *in vitro* [23], differences in grade observed between treatments in Experiment 2 could have diminished, to an extent, the positive effects of the PRID® Delta over the CIDR® on embryo development observed in Experiment 1.

Chromatin compaction (i.e., progression from GV0 to GV3; [41]) and cytoplasmic/molecular maturation [42,43] are key events that occur in oocytes during exogenous FSH-stimulated follicle development. It is possible that the benefits of increased P4 concentrations observed in the current study are attributable, at least in part, to its effect (direct or otherwise) in regulating these processes. Indeed, 83% of oocytes recovered at OPU were at the GV2 stage when a similar (to that of the current study) P4-supported and FSH-stimulated protocol was implemented; this in turn led to improved blastocyst yields per cycle [12]. Their protocol of P4 support (offered by a CIDR® inserted for 5 days preceding OPU), together with four injections of FSH over two days prior to 48 h of 'coasting', was similar to that of the current study, and increased the percentage of GV2 stage oocytes at OPU compared to their earlier study of just two FSH injections and no P4 support [44].

Whilst exogenous P4 support (provided by a CIDR® increasing plasma P4 from 0.32 to 2.31 ng/mL) in non-stimulated cycles of OPU in *Bos indicus* cattle increased oocyte recovery rate and oocyte quality (morphological grade), subsequent *in vitro* embryo development was not improved [31]. There is also evidence that high (3.0 to 4.5 ng/mL) rather than low (1.25 to 1.75 ng/mL) concentrations of plasma P4 (arising following the insertion of two new CIDR®s vs one used CIDR®) can impair blastocyst development in non-stimulated cycles of OPU [13]. Plasma concentrations of LH were supressed by elevated P4 (i.e., two CIDR®s) in that study. Pulse frequency of LH was also supressed under high vs low P4 (i.e., 3.8 vs 1.3 ng/mL), arising from the insertion of a new CIDR® in the presence of a CL compared to a used CIDR® in the absence of a CL, in a single cycle of non-stimulated OPU in crossbred taurine cows [45]. However, basic markers of oocyte competence in that study (i.e., morphological grade, Brilliant cresyl blue staining and follistatin transcript expression) were unaffected by P4 concentration; embryo development was not assessed.

The foregoing discussion suggests that P4-regulated synchrony of chromatin compaction in GV oocytes ahead of follicle aspiration and subsequent IVM may be an important contributing factor determining IVP success. However, other as yet unknown modes of action of P4 cannot be discounted, and there doesn't appear to be a cumulative effect of P4 level across successive cycles of OPU. Effects of P4 may be mediated in part via its regulation of pulsatile LH release; whether the action of P4 is beneficial or detrimental may depend on the specific protocols employed (e.g., stimulated vs non-stimulated cycles) for oocyte collection.

4.1.2. Choice of progesterone releasing device

Although it is not possible to elucidate the mechanism(s) by which elevated P4 concentrations enhanced *in vitro* embryo development in the current study, in the absence of a functional CL, the higher concentrations of peripheral P4 generated by the PRID® Delta over the CIDR® led to improved *in vitro* embryo development. Over the years, refinements to estrous synchronization protocols led to shorter treatment programmes which meant that the P4 content of devices such as the PRID® Delta and CIDR® could be reduced, thus minimising unit price and remnant drug content [14]. Whilst both devices are used successfully in cycles of OPU-IVP and fertility management more generally [12,46], the current study indicates that, at least under the stimulated cycles of OPU described herein, IVP outcomes are improved with the greater P4 support provided by the PRID® Delta.

4.2. Vaginal discharges

Based on previous accounts [17,18,47] and the authors' own experience, mild irritation, localized inflamation and vaginal discharges were expected; although in this study these conditions were mild and there was little indication that they increased over time (Fig. 2; Supplemental Fig. S1 and

S2). Interestingly, previous accounts indicate that attached tags protruding from the vulva, intended to facilitate device removal, can increase the severity of vaginal discharge [17]. In the current study all tags were removed from both PRID® Delta and CIDR® devices prior to insertion, as they have been found to lead to a greater incidence of premature loss [17]. Perhaps as a consequence of this, median scores ranged between 1.0 and 1.5 units, and individual scores never exceeded 2.0 units on the scale of Williams et al. [21] (Fig. 2; Supplemental Fig. S1 and S2).

A further factor that may have contributed to low vaginal discharge scores in the current study was the fact that, upon device renewal, the vulva was cleansed and vagina irrigated using a weak solution of potassium peroxy-monosulfate and sodium chloride followed by rinsing with physiological saline. Nevertheless, vaginal discharge scores were greater for the PRID® Delta than CIDR® (Fig. 2; Supplmental Fig. 1). This probably reflects a greater degree of contact and associated irritation with the vaginal wall. However, the difference in discharge scores between the two devices, whilst statistically significant, was small and wouldn't be considered unacceptable with regard to level of irritation and animal welfare [14].

4.3. Conclusions

Enhanced peripheral P4 concentrations (originating from either an endogenous CL and/or progesterone releasing device) during FSH-stimulated and 'coasted' cycles of OPU in cattle can improve subsequent *in vitro* embryo development. In the absence of a CL, choice of P4 releasing device becomes important. On the basis of the current findings, those devices that release greater quantities of P4 into perpheral circulation, such as the PRID® Delta, should lead to improved blastocyst yields during IVP. Currently, the mechanisms underlying these beneficial effects are unclear. They could arise as a consequence of improved synchrony of developmentally competent GV oocytes that are better placed to complete meiosis during subsequent IVM. Enhanced subsequent embryo development, however, indicates that other as yet undefined aspects of 'molecular' and/or 'cytoplasmic' maturation within the oocyte may also be enhanced. These aspects, together with pregnancy establishment following embryo transfer, await further investigation. Finally, we found no evidence that extended use (over several cycles) of either of the two P4-releasing devices evaluated in the current study leads to a pathological response related to vaginitis and associated discharges.

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Declaration of interest

Federico Randi is an employee of Ceva Santé Animale

Data statement

Data can be made available upon reasonable request

CRediT authorship contribution statement

Rob Simmons: Funding acquisition, Conceptualization, Project administration, Investigation, Writing - Review & Editing. **Des Tutt:** Investigation, Formal analysis, Writing - Review & Editing. **Wing Yee Kwong:** Methodology, Investigation. **Gizem Guven-Ates:** Investigation, Writing - Review & Editing. **Remi Labrecque:** Resources, Writing - Review & Editing. **Federico Randi:** Funding acquisition, Conceptualization, Writing - Review & Editing. **Kevin Sinclair:** Funding acquisition, Conceptualization, Resources, Project administration, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing.

Acknowledgements

Progesterone analyses by radioimmunoassay undertake at the Segalab (Laboratório de Sanidade Animal e Segurança Alimentar, 4490-295 Argivai, Portugal).

Appendix A. supplementary data

Uploaded separately

Highlights

- Enhanced progesterone support during FSH-stimulated OPU improves embryo development
- PRID® Delta matches the *corpus luteum* in terms of supporting *in vitro* embryo development
- PRID® Delta superior to CIDR® in terms of supporting *in vitro* embryo development
- Neither PRID® Delta nor CIDR® leads to pathological vaginal discharges upon extended use

List of Figure legends

Fig. 1. Schematic representations of ovarian-cycle regulation at the commencement of procedures in Experiments 1 (**A**) and 2 (**B**). See text for full details. Following the first session of FSH-stimulated OPU in each experiment (OPU 1), heifers underwent DFR eight days later followed by an FSH-stimulated OPU cycle six days later. Five such stimulated cycles were undertaken in Experiment 1 and six were undertaken in Experiment 2. Each experiment involved ten heifers. GnRH, Gonadotrphin Releasing Hormone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DFR, Dominant Follicle Removal; FSH, Follicle Stimulationg Hormone; OPU, transvaginal follicular aspiration. CIDR®, Controlled Internal Drug Release; PRID® Delta, Progesterone Releasing Intravaginal Device.

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Fig. 3. Experiment 1. Plasma progesterone (P4) concentrations (**A**) and percentage of matured oocytes that gave rise to advanced (IETS Stages 7 to 9, Grade 1-2) Day 8 blastocysts (**B**). Blood sampling commenced 3 days after an initial reference oestrus (Day 0), and was undertaken at the time of dominant follicle removal and ovum pick-up (downward facing arrows) 6 and 8 days after P4 device insertion. Corpora lutea (CLs) were visible during the first two cycles of OPU but not thereafter.

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Table 1. Expt. 1 - Effect of stage of cycle (corpus luteum present or absent) and intravaginal device on oocyte recovery and *in vitro* embryo production over five successive cycles of transvaginal follicular aspiration (OPU) (5 heifers per treatment group).

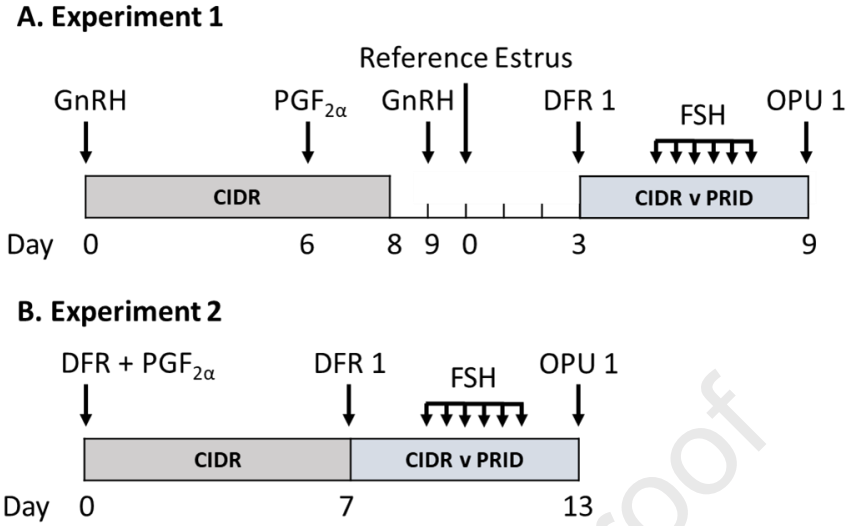
Corpus luteum (CL)	**Present**		**Absent**		**Significance (P)**		
Vaginal device (P4)	**CIDR®**	**PRID® Delta**	**CIDR®**	**PRID® Delta**	**CL**	**P4**	**CL x P4**
A. Aspiration							
Follicles aspirated, n	18.5 ± 1.83	18.6 ± 1.96	16.7 ± 1.42	22.3 ± 1.64	-	-	0.068
Oocytes retrieved, n	12.3 ± 1.70	12.4 ± 1.83	9.8 ± 1.24	14.7 ± 1.52	-	0.052	-
Retrieved of aspirated, %	66.2 ± 5.74	66.9 ± 6.09	58.5 ± 5.15	65.7 ± 4.28	-	-	-
B. Culture							
Oocytes matured, n	12.1 ± 1.69	11.9 ± 1.78	9.8 ± 1.23	14.4 ± 1.50	-	0.078	-
Grade 1 of matured, %	50.0 ± 7.84	54.2 ± 8.40	59.0 ± 6.99	49.7 ± 5.88	-	-	-
Grade 2 of matured, %	14.6 ± 5.82	18.1 ± 6.83	12.0 ± 4.85	10.5 ± 3.79	-	-	-
Grade 3 of matured, %	35.4 ± 9.37	27.7 ± 9.43	29.1 ± 8.06	39.8 ± 7.19	-	-	-
Bi. Day 2 embryos							
Oocytes inseminated, n	12.1 ± 1.68	11.7 ± 1.77	9.3 ± 1.21	13.7 ± 1.46	-	-	-
Cleaved of inseminated, %	88.7 ± 5.18	93.9 ± 4.24	87.5 ± 5.03	91.5 ± 3.51	-	-	-
2 cells of cleaved, %	2.3 ± 1.70	2.6 ± 1.90	6.1 ± 2.54	4.7 ± 1.80	-	-	-
3-4 cells of cleaved, %	16.3 ± 6.58	11.7 ± 6.05	35.7 ± 8.00	16.7 ± 5.03	0.077	0.095	-
5-6 cells of cleaved, %	38.4 ± 5.86	41.6 ± 6.28	23.5 ± 4.79	20.7 ± 3.69	<0.001	-	-
>6 cells of cleaved, %	43.0 ± 10.7	44.2 ± 11.33	34.7 ± 9.63	58.0 ± 8.07	-	-	-
Bii. Day 6 embryos							
Day 6 of inseminated, %	73.2 ± 6.90	74.4 ± 7.40	55.4 ± 7.21	74.4 ± 5.23	-	0.072	-
Biii Day 8 blastocysts							
Day 8 of matured, %	65.0 ± 4.84	63.9 ± 5.27	35.0 ± 4.41	59.5 ± 3.73	0.029	0.027	0.052
Day 8 of inseminated, %	65.0 ± 6.77	64.6 ± 7.38	36.6 ± 6.37	62.8 ± 5.28	0.060	0.020	0.049
Day 8 of cleaved, %	73.3 ± 6.64	68.8 ± 7.25	41.8 ± 6.94	68.7 ± 5.27	0.049	0.037	0.028
Day 8 of Day 6, %	85.1 ± 6.00	82.8 ± 68.5	65.1 ± 8.73	83.1 ± 4.89	-	-	-
Stage 7(1&2‡) to 9† of Day 8, %	68.3 ± 7.88	64.2 ± 8.85	48.8 ± 10.49	70.9 ± 6.01	-	-	0.077

‡ Refers to IETS morphological grade [24]; † All Stage 9 blastocysts were Grade 1 (IETS)

Table 2. Expt. 2 - Effect of intravaginal device on oocyte recovery and *in vitro* embryo production over five[#] sequential cycles of transvaginal follicular aspiration (OPU) in the absence of an endogneous corpus luteum (5 heifers per treatment group).

Vaginal device	CIDR®	PRID® Delta	Significance
A. Aspiration			
Follicles aspirated, n	41.1 ± 2.66	30.2 ± 2.27	-
Oocytes retrieved, n	26.4 ± 1.98	20.6 ± 1.73	-
Retrieved of aspirated, %	64.6 ± 1.36	68.1 ± 1.55	-
B. Culture			
Oocytes matured, n	26.2 ± 0.94	19.6 ± 0.81	-
Grade 1 of matured, %	61.2 ± 0.0173	42.6 ± 2.03	0.039
Grade 2 of matured, %	17.4 ± 0.0135	23.6 ± 1.74	0.054
Grade 3 of matured, %	21.5 ± 0.0146	33.9 ± 1.94	-
Bi. Day 2 embryos			
Oocytes inseminated, n	25.5 ± 1.01	16.2 ± 0.89	0.110
Cleaved of inseminated, %	90.3 ± 0.0192	87.1 ± 0.0295	-
2 cells of cleaved, %	4.3 ± 0.0106	4.5 ± 0.0156	-
3-4 cells of cleaved, %	15.6 ± 0.0213	16.4 ± 0.0308	-
5-6 cells of cleaved, %	22.7 ± 0.0190	27.5 ± 0.0280	-
>6 cells of cleaved, %	57.5 ± 0.0354	51.6 ± 0.0503	-
Bii. Day 6 embryos			
Day 6 of inseminated, %	68.5 ± 3.38	67.3 ± 4.68	-
Biii. Day 8 blastocysts			
Day 8 of inseminated, %	37.4 ± 3.46	45.2 ± 4.90	-
Day 8 of cleaved, %	41.5 ± 3.68	51.9 ± 5.26	0.108
Day 8 of Day 6, %	55.1 ± 2.33	66.3 ± 3.15	0.047
Biv. Advanced Day 8 blastocysts			
Stage 7(1&2[‡]) to 9[†] per cycle, n	7.1 ± 0.54	5.9 ± 0.53	-
Stage 7(1&2[‡]) to 9[†] of matured, %	35.2 ± 3.38	44.2 ± 4.92	0.102
Stage 7(1&2[‡]) to 9[†] of inseminated, %	37.4 ± 3.46	45.2 ± 4.90	0.154
Stage 7(1&2[‡]) to 9[†] of cleaved, %	31.1 ± 3.21	41.7 ± 4.78	0.104
Stage 7(1&2[‡]) to 9[†] of Day 6, %	41.3 ± 3.54	53.4 ± 4.99	0.056
Stage 7(1&2[‡]) to 9[†] of Day 8, %	74.7 ± 3.89	80.5 ± 4.23	-

A technical glitch with our low-oxygen incubator during Cycle 3 meant that data from that cycle was discounted in the final analysis. ‡ Refers to IETS morphological Grade [24]; † All Stage 9 blastocysts were Grade 1 (IETS)



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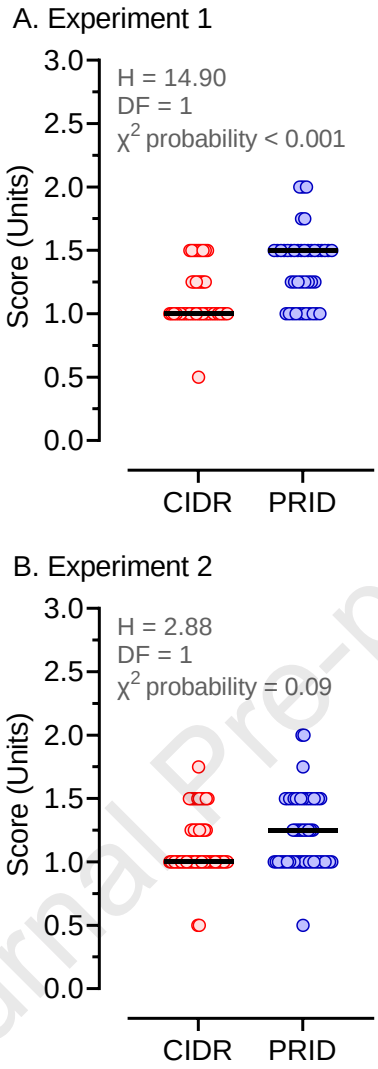

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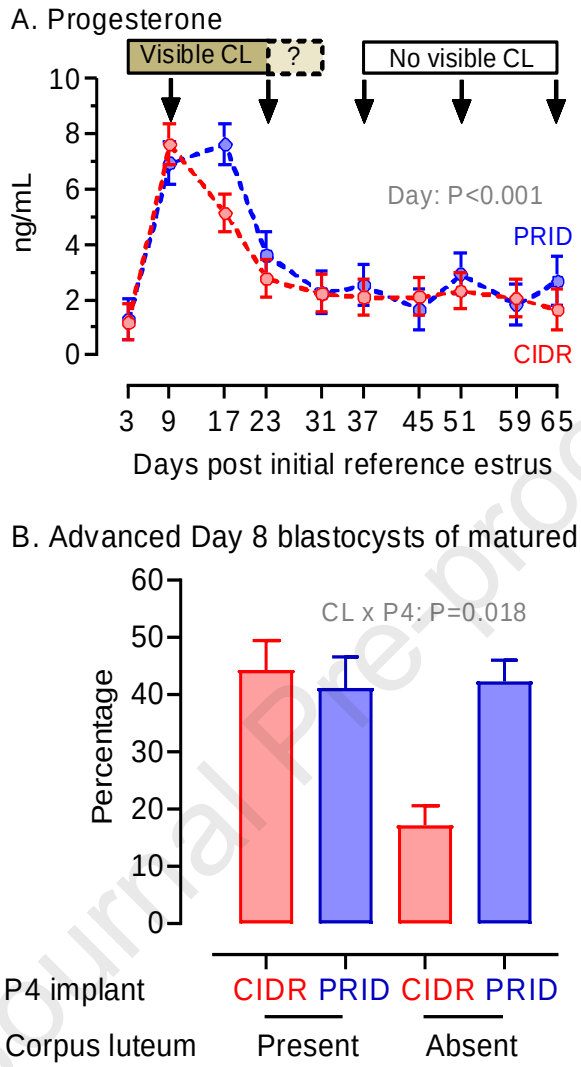

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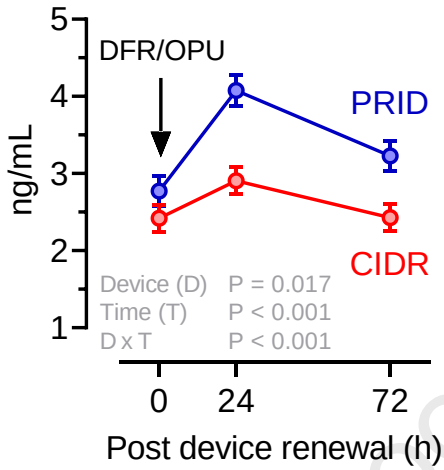

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Highlights

- Enhanced progesterone support during FSH-stimulated OPU improves embryo development
- PRID® Delta matches the *corpus luteum* in terms of supporting *in vitro* embryo development
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