Intrafollicular LH administration in dairy heifers treated with a GnRH agonist

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ABSTRACT: The aim of this study was to evaluate the effect of intrafollicular treatment (IFT) with different doses of luteinising hormone. Experimental heifers were treated with a single deslorelin implant to desensitise gonadotroph cells of the pituitary gland. Thereafter, follicular development was stimulated by exogenous FSH treatment. Intrafollicular treatment with 10, 5, 1 and 0.01 μg LH was performed on one single follicle while other follicles remained untreated. Human chorionic gonadotrophine (2000 UI) was administered intravenously as a control. Ovulation and development of the corpus luteum occurred after all intrafollicular treatments with 10 and 5 μg LH. After IFT using 1 μg of LH 75% animals (3/4) ovulated. The dose of 0.01 μg was not followed by any ovulation whereas control treatments with hCG were followed by an ovulation of the majority of follicles present in the ovaries. In conclusion, IFT with different doses of LH (greater than 0.01 μg) is capable of inducing ovulation.

Keywords: intrafollicular treatment; double channel system; luteinising hormone; deslorelin; cattle

Abbreviations

CL = *corpus luteum*, **FSH** = follicle stimulating hormone, **hCG** = human chorionic gonadotrophin, **IFT** = intrafollicular treatment, **LH** = luteinising hormone, **OPU** = ovum pick-up, **TVFA** = ultrasound guided transvaginal follicular aspiration

Ultrasound guided transvaginal follicular aspiration (TVFA) in cattle has commonly been used for oocyte collection (ovum pick-up, OPU) (Pieterse et al. 1988). After various modifications of the TVFA equipment it has also been used for intrafollicular treatment (IFT) (Kot et al. 1995). Intrafollicular injections of different substances like hCG, phosphate-buffered saline or insulin-like growth factor-I have been described in cattle (Kot et al. 1995; Bergfelt et al. 1998; Ginther et al. 2004; Shahiduzzaman et al. 2010). Intrafollicular insemination has even been reported in mares (Eilts et al. 2002) as well as in cows (Lopez-Gatius and Hunter 2011). However, to our knowledge, no report describes IFT using a luteinising hormone (LH).

Because of the reports describing the influence of the aspiration procedure on the formation of luteal tissue in ovaries (Bergfelt et al. 1998, Amiridis et al. 2000, Petyim et al. 2001), the elimination of a possible influence of endogenous LH release on ovulation onset after intrafollicular injection may be of benefit before IFT. Deslorelin implants have been described as causing a desensitisation of the hypophysis without altering the follicular status (Maclellan et al. 1997; Padula and McMillan 2005). For this reason, they have been used in our study.

The aim of the study was to determine an efficient dose of LH administered into the dominant follicle in heifers treated by deslorelin implants using the recently described double channel device for IFT (Cech et al. 2013).

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MATERIAL AND METHODS

IFT equipment

A real-time B-mode ultrasound machine (SSD-500, Aloka, Japan) equipped with a convex ultrasound transducer (7.5 MHz Aloka UST 9125, Japan) placed in a plastic holder was used to scan the ovaries. Intrafollicular treatment was performed using a newly developed metal needle holder containing two channels (treatment channel and aspiration channel) running to the crest of the conical end of the holder, both into one injection needle. Standard disposable Luer-type needles were used for IFT (0.60 × 60 mm, 23 G). The injection set consisted of two shortened infusion tubes and two 1 ml syringes. One tube, needle and syringe were used for aspiration and the secondary ones were used for treatment. All parts of the new device were originally made by MEDIN, a.s., Nove Mesto, Czech Republic. The new system enables the aspiration of follicular fluid and subsequent IFT with exactly the same amount of solution when the dead volume was only the volume of the needle (Figure 1). Intrafollicular treatment was performed as previously described (Cech et al. 2013).

Figure 1. The double channel equipment for intrafollicular treatment

Animals, treatment and ultrasound schedule

The complete preparation and treatment schedule is presented in Table 1.

Preparation phase. Five heifers of Czech pied cattle breed at the age of 14 months were synchronised by cloprostenol administration 1 (500 µg i.m. pro toto, Oestrophan®, Bioveta a.s., Czech Republic) on D-11. Jugular catheters were fitted in three heifers on day seven of the cycle. Twenty four hours later all heifers were treated with a single deslorelin implant (4.7 mg *s.c. pro toto*, Suprelorin®, Virbac S.A., France) placed on the outer surface of an ear (D0). Cloprostenol administrations 1 and 2 were performed: D2 (2nd administration) and D9 (3nd administration).

Treatment phase. In order to simplify the chronology of manipulation with animals, days corresponding to treatment phase will be indicated by asterisks.

TVFA of all follicles larger than 5 mm was performed six days before IFT (D13 = D-6*). A stimulation was performed between D-4* and

Table 1. The preparation and treatment schedule

Preparation phase				Treatment phase			
Clop 1	Deslo	Clop 2	Clop 3	TVFA	FSH	IFT	Clop + TVFA
D-11	D0	D2	D9	D13 = D-6*	D-4*–D-1*	D0*	D7*

Clop = cloprostenol treatment, Deslo = deslorelin treatment, TVFA = aspiration of all follicles exceeding 5 mm, FSH = superstimulation using FSH, IFT = intrafollicular treatment with saline

D-1* with eight decreasing doses of FSH (together 130–275 µg, Stimufol®, ULg, FMV, Belgium).

Intrafollicular LH administration (LH, prof. Beckers, ULG, FMV) was performed 12 h after the last FSH injection into one of the largest follicles in each heifer (D0*). Other follicles remained untreated. Cloprostenol administration was performed on D7* as well as TVFA of all remaining follicles. Two days later further FSH stimulation and the new treatment phase was initiated. The treatment phase was performed 1–5 times in individual heifers. Thirteen IFT sessions and four control sessions were performed in all heifers.

Four different LH doses were administered in IFT sessions: 10 µg (n = 3), 5 µg (n = 3), 1 µg (n = 4) and 0.01 µg (n = 3). Human chorionic gonadotrophin (2000 UI *i.v. pro toto*, Pregnyl®, Organon, Netherlands) was administered in control sessions (n = 4).

The whole experimental period lasted 12 weeks.

Ovaries were scanned daily using a real-time B-mode ultrasound machine (SSD-500, Aloka, Japan) equipped with a linear ultrasound transducer (7.5 MHz Aloka UST 5561, Japan). Ovulation and development of CL was considered as a positive response after IFT.

Blood sampling, LH and progesterone assays

Serial blood samples for the LH assay were taken during two distinct intervals after deslorelin treatment: Period 1 (3 h prior until 9 h after) and Period 2 (26 h prior to 29 h after). These samples were collected at 15 min intervals into heparinised tubes. Samples for the progesterone assay were taken before IFT (D0*) and subsequently on D3* and D7*. Plasma was immediately separated by centrifugation before being stored at –20 °C.

LH concentrations were determined in plasma by using a double antibody radioimmunoassay procedure, as previously described (Ayad et al. 2007). The minimum detection limit of the LH-RIA technique was 0.35 ng/ml. The intra-assay and inter-assay coefficients of variation in the LH radioimmunoassay were 3.3% (3.8 ± 0.1 ng/ml) and 10.2% (4.1 ± 0.4 ng/ml), respectively.

Plasma progesterone concentrations were determined by using a direct radioimmunoassay (RIA) method (without extraction), as previously described in detail (Lopez-Gatius et al. 2007). The minimum detection limit of the P4-RIA technique used was 0.15 ng/ml. The intra-assay and inter-assay coefficients of variation of the LH radioimmunoassay were 13.8% (3.1 ± 0.4 ng/ml) and 19% (2.7 ± 0.5 ng/ml), respectively.

Statistical analysis

The results are presented as mean value (± SD). Differences between groups were analysed using Student's paired t-test, calculated using Excel software.

RESULTS

Preparation phase

Ovulation was observed in four heifers (80%, 4/5) after the deslorelin treatment. The second cloprostenol administration (D2) caused luteolysis of the *corpus luteum* that had been present after the oestrus on D-8; however, it was not followed by ovulation. Cloprostenol administration 3 (D9) caused luteolysis of the corpus luteum that had developed as a result of the deslorelin treatment and was not followed by an ovulation. Follicles present on ovaries after deslorelin treatment did not show progressive development.

As shown in Figure 2, a significant LH release was observed in Period 1. Concentrations of LH were higher than the mean values measured before deslorelin treatment (1.79 ng/ml), reaching mean maximal levels (58 ng/ml) 5 h after deslorelin treatment. Thereafter, concentrations decreased to 2.7 ng/ml at 9 h after deslorelin treatment. A slow decrease of

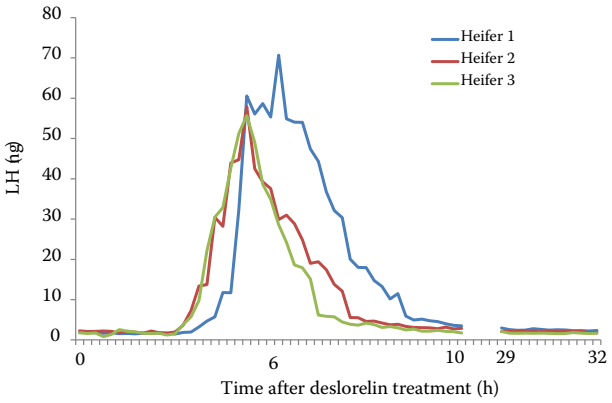

Figure 2. Concentrations of luteinising hormone after deslorelin treatment in heifers

Table 2. Progesterone concentrations on D0, D3 and D7 in positively responding heifers after IFT using different doses of LH and after *i.v.* hCG treatment

Day	Treatment			
	IFT 10 µg LH	IFT 5 µg LH	IFT 1 µg LH	2000 UI hCG *i.v.*
D0	0.25 ± 0.07	0.21 ± 0.04	0.33 ± 0.13	0.15 ± 0.0
D3	0.26 ± 0.05	0.29 ± 0.14	0.52 ± 0.25	2.02 ± 1.7
D7	2.66 ± 1.38	1.66 ± 0.62	1.43 ± 0.91	28.43 ± 17.37

mean LH values was observed in Period 2 (concentrations ranging from 2.37 to 1.92 ng/ml).

Treatment phase

The number of follicles developing after FSH treatment varied ranged from 1 to 31. One of the heifers showed a repeatedly weak response (one to five follicles).

A positive ovarian response (ovulation and corpus luteum development) was observed in all cases after IFT using doses of 10 and 5 µg LH. In one heifer two ovulations occurred after IFT using 10 µg of LH. After IFT using 1 µg of LH 75% of animals (3/4) ovulated. The dose of 0.01 µg was not followed by an ovulation. Control treatments with hCG were followed by an ovulation of the majority ovarian follicles present in the ovaries (Figure 3 and 4).

Progesterone concentrations increased gradually from D0 to D7 with the development of CLs in positively responding heifers. However, the differences among Days 0, 3 and 7 in separate groups were not significantly different due to the limited number of animals (Table 2).

DISCUSSION

Ultrasound-guided intrafollicular injection of substances has been reported as a useful research tool (Kot et al. 1995). In this study the effects of different doses of intrafollicularly administered hCG as well as the technical aspects of IFT have been described (Kot et al. 1995). However until now no study has been published regarding intrafollicular treatment with LH. Therefore the aim of our study was to determine an efficient dose of intrafollicularly administered LH.

The process of follicular aspiration has been reported to be sufficient to induce partial luteinisation and development of a morphologically normal secondary *corpus luteum* (CL) in the presence of natural cyclic CL, however, under circumstances which have not yet been determined (Amiridis et al. 2000). Similarly, in another study (Petyim et al.

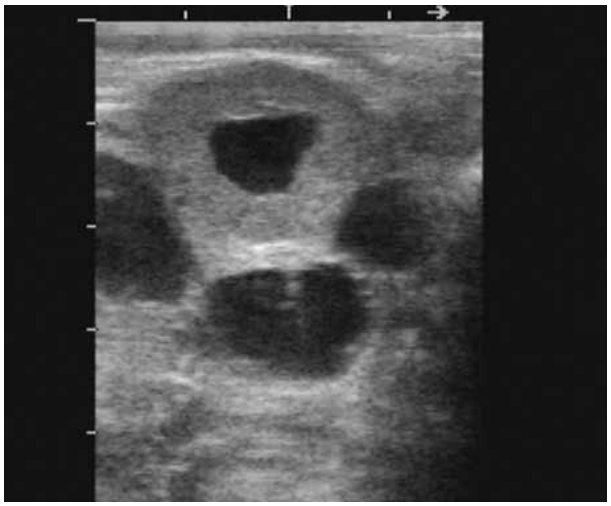

Figure 3. A positive response seven days after intrafollicular treatment using 10 µg of LH. *Corpus luteum* with cavity, four follicles

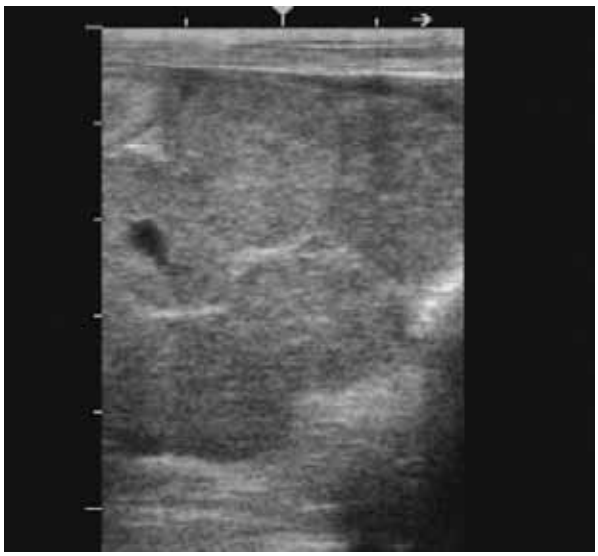

Figure 4. A positive response seven days after intravenous treatment using 2000 IU of hCG. Multiple ovulation, five CLs

2001) CL-like structures were described. According to the authors, a follicular puncture at OPU could be interpreted as an artificial ovulation, and the mechanisms behind the formation of CL-like structures at the point of a puncture mimics the naturally occurring luteinisation subsequent to ovulation (Petyim et al. 2001). Ovum pick-up is an activity slightly different from IFT, because the effect on the follicle is stronger and it is usually repeated, resulting in a significant response by the endocrine system of donor animals. This response is not fully present during the IFT; however, there is some evidence concerning possible hastening or induction of ovulation by follicle puncture (Bergfelt et al. 1998). Nevertheless, the exact reasons have not yet been elucidated.

Therefore, in our study the deslorelin implants have been used to eliminate the possible influence of IFT on endogenous LH action. A desensitisation of the pituitary gland without effect on follicle number and growth after deslorelin treatment has been described (Maclellan et al. 1997). Deslorelin implants induce a substantial but temporary release of LH, and 21 days later there was an observed marked suppression of pulsatile LH release and an absence of response to oestradiol in ovariectomised dairy cows (Padula and McMillan 2005). In our study activity of the deslorelin implants has been demonstrated clearly by the LH profile within 29 h after the deslorelin treatment and by subsequent frequent ultrasound examination. Only follicles with a diameter smaller than 3 mm were present in the ovaries without exogenous FSH stimulation.

Stimulation with FSH was necessary to stimulate follicular development. Although there were demands for the presence of only one follicle for each IFT session, the ovarian response varied from 1 to 31 follicles even if the used FSH dose was very low. Intrafollicular injection was performed on one of the largest follicles at random. However, all follicles were able to ovulate as demonstrated by an identical reaction in control sessions after intravenous hCG administration. The time limitation for intrafollicular treatment with LH in experimental animals and intravenous treatment with hCG in control animals was appointed according to a study describing the new model for superovulation using injection of exogenous LH to induce ovulation in donors treated with deslorelin implants (D'Occhio et al. 1997). In our study, the preparation phase of the experiment created a follicular status in which only small follicles sensitive to FSH were presented on ovaries. Concurrently, pituitary action was eliminated by deslorelin treatment. In the treatment phase all follicles growing after FSH stimulation were able to ovulate as demonstrated by the multiple ovulation after intravenous hCG administration in control sessions. We can conclude that the response to the IFT using LH was the result of the local activity of intrafollicularly injected LH.

The range of LH doses was appointed at approximately 1/1000 of the systemic dose of LH with regard to an earlier study (Kot et al. 1995) in which precise calculations of hCG doses for IFT have been described. According to our results doses of 10, 5 and 1 μg LH were able to induce ovulation whereas the dose of 0.01 μg LH was not followed by an ovarian response. Ultrasound appearance of CLs after IFT was typical as was their diameter which varied between 15–22 mm. One double ovulation after IFT with 10 μg LH was a coincidence. A minimal amount of hormone administered intrafollicularly has been reported to induce ovulation, even the hCG which remains after inadequate post-treatment needle flushing (Kot et al. 1995). In our study, taking into account the results obtained, the dose of 10 μg LH was very high and the ovulation of two adjacent follicles could probably be caused by the leakage of a minimal amount of LH from the needle during its removal after IFT. The development of unovular cystic follicles after IFT has also been described (Kot et al. 1995). However in our study the outcome of individual follicles was not observed because they could not be recognised among other untreated follicles. Even the growing *corpus luteum* on D3 after IFT was difficult to observe.

Plasma progesterone concentrations exceeded 1 ng on D7 in almost all positively responding heifers. After intravenous treatment with hCG and when high number of follicles ovulated (15–20) the progesterone concentrations exceeded 40 ng. However, the differences in separate groups were not statistically different due to the limited number of animals and large variability in progesterone values.

IFT using LH should be performed in the future in a larger number of animals to assess the minimal effective dose of LH (expected to be between 0.01 to 1 μg), as well as a comparative study using IFT with LH and hCG.

Our study demonstrated a positive ovarian response to intrafollicular treatment with LH in a range of doses 1 and 10 μg. Further experiments are necessary to determine the minimum dose which effectively induces ovulation.

REFERENCES

Amiridis GS, Robertson L, Jeffcoate IA, Belibasaki S, Boyd JS, O'Shaughnessy PJ (2000): Formation of a secondary corpus luteum after ultrasound-guided follicular aspiration in cows. Acta Veterinaria Hungarica 48, 193–198.

Ayad A, Sousa NM, Sulon J, Hornick JL, Watts J, Lopez-Gatius F, Iguer-Ouada M, Beckers JF (2007): Influence of progesterone concentrations on secretory functions of trophoblast and pituitary during the first trimester of pregnancy in dairy cattle. Theriogenology 67, 1503–1511.

Bergfelt DR, Brogliatti GM, Adams GP (1998): Gamete recovery and follicular transfer (GRAFT) using transvaginal ultrasonography in cattle. Theriogenology 50, 15–25.

Cech S, Mala J, Indrova E, Lopatarova M, Dolezel R, Dluhosova H, Zilka L (2013): The introduction of a double-channel system for the intrafollicular treatment of cattle. Veterinarni Medicina 58, 10–15.

D'Occhio, MJ, Sudha G, Jilella D, Whyte T, Maclellan LJ, Walsh J, Trigg TE, Miller D (1997): Use of GnRH agonist to prevent the endofenous LH surge and injection of exogenous LH to induce ovulation in heofers superstimulated with FSH: A new model for superovulation. Theriogenology 47, 601–613.

Eilts BE, Pinto CR F, Paccamonti DL, Niemanstverdreit AS, Garcia E, Godke RA (2002): Transvaginal intrafollicular sperm cell injection with concomitant artificial insemination in the cyclic mare. Theriogenology 58, 631–633.

Ginther OJ, Bergfelt DR, Beg MA, Meira C, Kot K (2004): In vivo effects of an intrafollicular injection of insulin-like growth factor 1 on the mechanism of follicular deviation in heifers and mares. Biology of Reproduction 70, 99–105.

Kot K, Gibbons JR, Ginther OJ (1995): A technique for intrafollicular injection in cattle: Effects of hCG. Theriogenology 44, 41–50.

Lopez-Gatius F, Hunter RHF (2011): Intrafollicular insemination for the treatment of infertility in the dairy cow. Theriogenology 75, 1695–1698.

Lopez-Gatius F, Garbayo JM, Santolaria P, Yaniz J, Ayad A, de Sousa NM, Beckers JF (2007): Milk production correlates negatively with plasma levels of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. Domestic Animals Endocrinology 32, 29–42.

Maclellan LJ, Bergfeld EGM, Earl CR, Fitzpatrick LA, Aspden WJ, Kinder JE, Walsh J, Trigg TE, D'Occhio MJ (1997): Influence of the luteinizing hormone-releasing agonist, deslorelin, on patterns of estradiol-17 β and luteinizing hormone secretion, ovarian follicular responses to superstimulation with follicle-stimulating hormone, and recovery and in vitro development of oocytes in heifer calves. Biology of Reproduction 56, 878–884.

Padula AM, Macmillan KL (2005): Oestradiol-17 β responsiveness, plasma LH profiles, pituitary LH and FSH concentrations in long-term ovariectomized Holstein cows at 24 h, 48 h and 21 days following treatment with an absorbable GnRH agonist implant. Animal Reproduction Science 85, 27–39.

Petyim S, Bage R, Forsberg M, Rodriguez-Martinez H, Larsson B (2001): The effect of repeated follicular puncture on ovarian morfology and endocrine parameters in dairy heifers. Journal of Veterinary Medicine, Series A 48, 449–463.

Pieterse MC, Kappen KA, Kruip ThAM, Taverne MAM (1988): Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology 30, 751–762.

Shahiduzzaman AKM, Beg MA, Palhao MP, Siddiqui MAR, Shamsuddin M, Ginther OJ (2010): Stimulation of the largest subordinate follicle by intrafollicular treatment with insulin-like growth factor 1 is associated with inhibition of the dominant follicle in heifers. Theriogenology 74, 194–201.

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