

—Technology Report—

## Effect of a single epidural administration of follicle-stimulating hormone via caudal vertebrae on superstimulation for *in vivo* and *in vitro* embryo production in Japanese black cows

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**Abstract.** Here, we describe a simplified procedure for embryo production in the Japanese black cow that uses a single caudal epidural injection of follicle-stimulating hormone (FSH). First, we compared the efficiency of superovulation for *in vivo* embryo production between conventional multiple FSH treatment (control, n = 10) and single epidural administration (epidural, n = 5). The number of transferable blastocysts was similar between control and epidural groups ( $4.7 \pm 3.5$  and  $9.0 \pm 6.0$ , respectively). Next, we compared *in vitro* embryo production by ovum pick-up and *in vitro* fertilization (OPU-IVF) between control (n = 12) and epidural groups (n = 12). The rate of development to transferable blastocysts was higher in the epidural group than in the control (23.3 vs. 10.5%,  $P < 0.001$ ). In conclusion, a single epidural administration of FSH can induce follicular development comparable to that of the conventional superovulation protocol and may improve the productivity of OPU-IVF.

**Key words:** Epidural administration, Follicle-stimulating hormone (FSH), Ovum-pick up, Superovulation  
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In the cattle industry, superstimulation by treatment with follicle-stimulating hormone (FSH) is widely used to induce follicular growth and to improve the efficiency of *in vivo* embryo production and of *in vitro* embryo production (IVP) using ovum-pick up (OPU) followed by *in vitro* fertilization (IVF) [1]. OPU-IVF with FSH treatment increases the numbers of embryos from poorly productive donor cows for *in vivo* embryo production [2, 3]. However, conventional FSH treatment, which consists of multiple intramuscular injections, is stressful for the animals and time-consuming for veterinarians. Therefore, many studies have sought to simplify FSH treatment with a single subcutaneous high-dose of FSH dissolved in saline [4–6], or in a solvent that enables FSH to be released slowly, such as polyvinylpyrrolidone (PVP) [4, 7], aluminum hydroxide gels [8], or hyaluronan-based slow-release formulations [9, 10]. However, the effectiveness of these different treatments varies considerably, probably because of differences in the amount of subcutaneous fat tissue in the animals [4–6].

Burm *et al.* [11] reported that alfentanil (an opioid analgesic drug) was slowly absorbed into the general circulation after epidural administration in humans. In cattle, epidural anesthesia is routinely

performed to prevent contraction of the rectum and facilitate uterine flushing for embryo collection and embryo transfer [12]. If FSH injected into the epidural space is absorbed slowly and can induce follicular development, it will become a simple alternative method for the superstimulation of follicular development in cattle. In the present study, we examined the effect of epidural FSH administration via caudal vertebrae on *in vivo* embryo production and IVP followed by OPU-IVF in Japanese black cows.

To investigate the effect of epidural FSH administration on *in vivo* embryo production, we collected embryos from cows given twice-daily intramuscular FSH administration for 3 days (control) or a single epidural FSH injection (epidural). As shown in Table 1, the number of large follicles ( $\geq 10$  mm in diameter) at estrus and corpora lutea at the time of embryo collection did not differ between treatments. The number of collected oocytes/embryos and transferable blastocysts after epidural treatment was higher than in the control group (collected oocytes/embryos;  $P = 0.08$ , transferable blastocysts;  $P = 0.10$ ). These results indicate that epidural treatment was as effective as the conventional treatment for inducing superovulation in cattle. One caveat is that we used a total of 20 AU for the control, as described elsewhere [13], and 30 AU for the epidural treatment, based on a single FSH subcutaneous administration in a previous report [8]. We need to investigate the optimal FSH dose to induce ovulation after an epidural injection in future work.

To investigate the effect of epidural FSH administration on IVP followed by OPU-IVF for cattle with low productivity by *in vivo* embryo production, we conducted control or epidural FSH treatment before OPU. The animals produced an average of one or fewer

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**Table 1.** Superovulatory response induced by twice-daily intramuscular administration for 3 days (control) or a single epidural administration of FSH

Treatment (replicates)	Dose of FSH (AU)	No. of follicles at estrus ( $\geq 10$ mm)	No. of corpora lutea at embryo collection	No. of oocytes or embryos	No. of transferable embryos
Control (10)	20	19.4 $\pm$ 5.4	11.9 $\pm$ 6.3	10.9 $\pm$ 7.6	4.7 $\pm$ 3.5
Epidural (5)	30	22.6 $\pm$ 6.0	14.4 $\pm$ 5.0	18.3 $\pm$ 5.4	9.0 $\pm$ 6.0
P value		0.31	0.46	0.08	0.10

Values are mean  $\pm$  SD. Five cows were treated on control twice and then on epidural once.

**Table 2.** The number of follicles and collected oocytes at OPU after twice-daily intramuscular administration for 3 days (control) or a single epidural administration of FSH

Treatment (replicates)	Dose of FSH (AU)	No. of follicles at OPU		No. of collected oocytes at OPU	
		Small (< 6 mm)	Large ( $\geq 6$ mm)	Total	Good quality
Control (12)	30	23.3 $\pm$ 8.9	1.8 $\pm$ 5.4	16.5 $\pm$ 7.3	11.8 $\pm$ 6.2
Epidural (12)	30	22.1 $\pm$ 10.5	1.2 $\pm$ 2.1	17.7 $\pm$ 9.7	13.9 $\pm$ 6.4
P value		0.77	0.77	0.74	0.41

Values are mean  $\pm$  SD.

**Table 3.** *In vitro* production of oocytes collected after twice-daily intramuscular administration of FSH for 3 days (control) or a single epidural administration of FSH

Treatment	Dose of FSH (AU)	No. of oocytes (replicates)	% of cleaved (n)	% of blastocysts (n)	% of transferable blastocysts (n)	No. of transferable blastocysts/OPU session
Control	30	181 (12)	44.2 (80)	10.5 <sup>a</sup> (19)	10.5 <sup>a</sup> (19)	1.6 $\pm$ 1.9 <sup>x</sup>
Epidural	30	210 (12)	43.3 (91)	26.2 <sup>b</sup> (55)	23.3 <sup>b</sup> (49)	4.1 $\pm$ 3.6 <sup>y</sup>

<sup>a, b</sup> Different superscripts indicate significant differences within a column ( $P < 0.0001$ ). <sup>x, y</sup> Different superscripts indicate significant differences within a column ( $P < 0.05$ ). Values of no. of transferable blastocysts/OPU session are presented as mean  $\pm$  SD.

transferable blastocysts by uterine flushing after conventional FSH treatment in the previous three embryo collections within 8 months. After conventional or epidural treatment, most follicles were less than 6 mm in diameter, and the number of follicles and collected oocytes was similar between treatments (Table 2). The proportion of cleaved oocytes after IVF was also similar between treatments (Table 3). However, the rate of blastocysts and transferable blastocysts in the epidural group was higher than that of the control (Table 3,  $P < 0.0001$ ). The number of transferable blastocysts per OPU-IVF session in the epidural group was also higher than in the control (Table 3,  $P < 0.05$ ). The rate of pregnancy after transfer of *in vitro* derived blastocysts was comparable between control (8/8) and epidural (3/4) groups, with an overall success rate of 91.7%. The diameter of follicles [14, 15] and the morphological quality of oocytes [16] are correlated with the developmental competence of oocytes. In the present study, there were no differences in those parameters between the two treatments. Moreover, the diameter of most follicles was less than 6 mm. The cause of the higher developmental competence of oocytes in the epidural group is unclear; however, we speculate that FSH activates P450 aromatase and promotes estradiol production from granulosa cells [17]. Such a change would result in

improved developmental competence of oocytes, because granulosa cells surrounding *in vitro*-grown oocytes with higher maturational competence tend to secrete more  $E_2$  than those surrounding less competent oocytes [18]. It will also be necessary to carry out studies of blood FSH concentrations after epidural FSH administration and to examine the effect of FSH on development of small follicles and on estradiol production. Sugimura *et al.* [19] recently showed that twice-daily intramuscular FSH administration for 4 days (total 30 mg) in cattle increased the diameter of follicles and improved the developmental competence of oocytes without any effect on the morphology of the cumulus-oocyte complexes. Transcriptome analysis has shown that genes related to cell movement and migration showed down-regulated expression in FSH treated cattle, which could prevent the disruption of cell-to-cell connections. The genes that show up-regulation in the cumulus cells of cattle without FSH are similar to those in the granulosa cells of atretic follicles [19]. Although the reason for the differences in follicular diameters between the present and previous studies is unclear, FSH administration into the epidural area may improve the competence of oocytes, as reported by Sugimura *et al.* [19].

The results of the present study support previous studies [20–23]

that showed the effectiveness of epidural administration of FSH to induce superovulation. FSH solution dissolved in saline is easy to prepare and epidural administration with local anesthesia is a common veterinary skill to facilitate reproductive examination and treatment in cattle. Takedomi *et al.* [4] reported that when FSH dissolved in saline was subcutaneously injected into Holstein heifers, the plasma concentration of FSH markedly increased within 3 h and was maintained until 9 h after administration. FSH decreased to the basal level after 36 h, and superovulation was not induced. However, an FSH solution dissolved in PVP or aluminum hydroxide gel [4, 8] results in a gradual increase in FSH plasma concentrations that peak 12 h after administration; these gradually decrease but are maintained at a concentration higher than the basal level for more than 48 h. Bó *et al.* [24] suggested that circulating FSH levels must be maintained above baseline for at least 72 h to induce follicular growth. They also suggested that the subcutaneous area behind the shoulder, which contains a fat tissue pad, was the optimal area for a single FSH administration, as the fat caused the FSH to be released gradually. It has also been reported that epidural fats affect the distribution of drugs in the epidural space [25, 26]. After injection, drugs diffuse into the dura mater, epidural veins, and epidural fat; drugs absorbed in epidural fats could then re-diffuse to the dura mater and epidural veins gradually [25, 26]. Therefore, we speculate that epidural fats contribute to the slow movement of FSH into the peripheral circulation, and that FSH concentration may be maintained for more than 72 h at higher than basal level. Although epidural administration with local anesthesia has been used widely in bovine management [12], there are large individual variations in onset, duration, and extent of anesthesia [27], which may result from epidural fat [25, 26]. Future studies need to examine the dynamics of peripheral FSH concentration after administration into the epidural area.

In conclusion, a single epidural FSH administration via the caudal vertebrae induced superovulation in Japanese black cows. Epidural administration of FSH also appeared to improve embryonic development after OPU-IVF. Most veterinarians skilled with local anesthesia techniques can apply epidural administration for superstimulation of cows because of the relatively simple protocol for preparation and injection of FSH.

## Methods

### *Animal care*

The Committee for Experimental Animals of Zen-noh Embryo Transfer Center approved all animal procedures in this study. Donor cows and recipient heifers were fed similar food, and water was supplied *ad libitum*. Herds were based on body constitution and social hierarchy.

### *Chemicals*

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### *Collection of in vivo produced embryos*

Five Japanese black cows were used in this study. First, the cows were subjected twice to the control treatment ( $n = 10$ ). Subsequently, we performed the epidural treatment once ( $n = 5$ ). The durations

between each embryo collection were 84 to 91 days. In all treatments, FSH injection began at the mid-luteal period (days 8 to 12) after confirmation of corpora lutea using a portable ultrasound imaging device equipped with a transrectal probe (HS-101V; Honda Electronics, Aichi, Japan). In the control group, FSH treatment consisted of twice-daily (morning and afternoon) intramuscular injections for 3 days with a decreasing dose (5, 5, 3, 3, 2, and 2 AU) per injection for a total of 20 AU of Antrin R-10 (Kyoritsu Seiyaku, Tokyo, Japan). At the fifth FSH treatment, 2 ml cloprostenol (0.25 mg/ml, Resipron-C, ASKA Animal Health, Tokyo, Japan) was injected intramuscularly. In the epidural group, 30 AU of FSH dissolved in 5 ml of saline was administered to the epidural area of caudal vertebrae; 48 h after FSH treatment, cloprostenol was injected intramuscularly. Twelve hours after the onset of estrus, the number of large follicles ( $\geq 10$  mm in diameter) was counted. All cows were then artificially inseminated with frozen-thawed semen from Japanese black bulls. Two cows were also inseminated 24 h after the onset of estrus. The number of inseminations in each cow was identical between FSH treatments ( $1.4 \pm 0.5$ ). Seven days after estrus, embryos were collected under epidural anesthesia using procaine hydrochloride (Enpro injection KS, Kyoritsu Seiyaku); the uteri were flushed using Ringer's solution (Terumo, Tokyo, Japan) supplemented with 0.1% fetal calf serum (FCS) via a multi-eye 16-French embryo collection catheter (Nipro, Osaka, Japan). After embryo collection, corpora lutea were counted by rectal palpation. Collected oocytes and embryos were classified according to the International Embryo Technology Society (IETS) classification system [28]. Grade 1 to 2.5 blastocysts or compacted morulae were classified as transferable blastocysts.

### *OPU for in vitro embryo production*

We used three Japanese black cows for this experiment. Each cow was subjected to the control and epidural FSH treatments four times ( $n = 12$  in each group). The order of control and epidural treatments was random and the time between each OPU was 7 to 35 days (total period = 119 days). First, the follicular wave in the cows was synchronized by a 1-ml intramuscular injection of gonadotrophin-releasing hormone analogue (Consultan injection containing 50  $\mu\text{g/ml}$  fertirelin acetate, ASKA animal health), or intravaginal insertion of a progesterone device (1.9 g, CIDR 1900, Zoetis Japan, Tokyo, Japan) and a 1-ml intramuscular injection of estradiol-benzoate solution (Ovahormone injection containing 2 mg/ml estradiol-benzoate, ASKA animal health). FSH treatment began 64–66 h after the synchronization treatment. In the control group, FSH treatment consisted of twice-daily (morning and afternoon) intramuscular injection for 3 days of a decreasing dose of FSH (7, 7, 5, 5, 3, and 3 AU) for a total of 30 AU. In the epidural group, 30 AU of FSH dissolved in 5 ml saline was injected into the epidural area of the caudal vertebrae. OPU was conducted with an ultrasound imaging device (ProSound 2, Hitachi-Aloka Medical, Tokyo, Japan), equipped with a 7.5-MHz long-handled convex transducer (UST-994P-5, Hitachi-Aloka Medical), at 75–78 h after FSH treatment. The number of follicles in the ovaries was counted, and follicles were classified by their diameter (small:  $< 6$  mm and large:  $\geq 6$  mm) because oocytes derived from larger ( $\geq 6$ -mm) follicles have higher developmental competence [14, 15]. Follicles were aspirated using a single-lumen needle (17-gauge, 600-mm long; Misawa Medical,

Ibaraki, Japan) connected to a 50-ml tube (Falcon 2070; Becton Dickinson, Franklin Lakes, NJ, USA) via a silicone tube (100-cm long, 1-mm internal diameter). The collection tube was warmed at 37°C in a portable incubator (FV-5; Fujihira Industry, Tokyo, Japan) and the other silicone tube was connected to a vacuum pump with a foot-pedal switch (MODEL 4, Fujihira Industry).

### Oocyte maturation and IVF

After collection, oocytes were washed in a filter cup (Em con, Immuno Systems, Spring Valley, WI, USA) with Dulbecco's phosphate buffered saline containing 5% FCS, and transferred to a 90-mm plastic dish. Oocytes completely surrounded by cumulus cells were defined as good quality. The oocytes were used for IVP (maturation and IVF of oocytes and culture of embryos) as previously described with a slight modification [13]. Briefly, oocytes were cultured in 700 µl IVM medium (20 or more oocytes) in 4-well tissue culture plates (Nalge Nunc International, Roskilde, Denmark) covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) or in 100-µl droplets (19 or less oocytes) covered with paraffin oil in a 35-mm plastic dish (Nalge Nunc International). The IVM medium used here was tissue culture media 199 containing 25 mM HEPES (Invitrogen, Carlsbad, CA, USA) and 5% FCS. After IVM, oocytes were co-incubated with frozen-thawed motile sperm ( $2.5 \times 10^6$ /ml) from a bull separated by a Percoll gradient (45% and 90%) in a 100-µl droplet ( $\leq 30$  oocytes/droplet) of IVF medium (IVF100; Research Institute for the Functional Peptides, Yamagata, Japan) covered with paraffin oil for 6 h at 38.5°C under 5% CO<sub>2</sub> in humidified air. After IVF, presumptive zygotes were removed from cumulus cells by pipetting and cultured in 700 µl of culture media in 4-well tissue culture plates (20 or more zygotes) or in 100-µl droplets (19 or less zygotes) covered with paraffin oil in a 35-mm plastic dish. Culture media was CR1aa medium [29] with 2% FCS for 2 days at 38.5°C under 5% CO<sub>2</sub> and 5% O<sub>2</sub> with high humidity. Zygotes were then cultured in USU-6 medium [30] containing 5% FCS for 5 days. Seven days after IVF, blastocysts of grades 1 to 2.5 blastocysts (IETS classification [28]) were used for further study.

### Embryo transfer to recipient heifers and pregnancy diagnosis

Each blastocyst was loaded into a clear plastic straw (0.25 cm<sup>3</sup>) and transferred non-surgically into the uterine horn ipsilateral to the existing corpus luteum of a Holstein heifer using an embryo transfer device (YT GUN, Yamane-teq, Nagano, Japan) on days 6 to 8 after estrus. Pregnancy diagnosis was performed by a portable ultrasound imaging device equipped with a transrectal probe around 30 and 60 days after estrus.

### Statistical analysis

All statistical analyses were performed using software (StatView 4.51, Abacus Concepts, Calabasas, CA, USA). The data in Tables 1 and 2 were analyzed by a Student's *t*-test. The data in Table 3 were analyzed by a Chi-square test, except for the numbers of transferable blastocysts which were compared using Student's *t*-test. Data are presented as means  $\pm$  standard deviation.

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