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

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16 Running head: Single epidural administration of FSH

17

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26 **Abstract**

27 Here, we describe a simplified procedure for embryo production in the Japanese black cow
28 that uses a single caudal epidural injection of follicle-stimulating hormone (FSH). First, we compared
29 the efficiency of superovulation for *in vivo* embryo production between conventional multiple FSH
30 treatment (control, n = 10) and single epidural administration (epidural, n = 5). The number of
31 transferable blastocysts was similar between control and epidural groups (4.7 ± 3.5 and 9.0 ± 6.0 ,
32 respectively). Next, we compared *in vitro* embryo production by ovum pick-up and *in vitro*
33 fertilization (OPU-IVF) between control (n = 12) and epidural groups (n = 12). The rate of
34 development to transferable blastocysts was higher in the epidural group than in the control (23.3 vs.
35 10.5%, $P < 0.001$). In conclusion, a single epidural administration of FSH can induce follicular
36 development comparable to that of the conventional superovulation protocol and may improve the
37 productivity of OPU-IVF.

38 (150 words)

39 **Key words**

40 Epidural administration, FSH, Ovum-pick up, Superovulation

41 **Text**

42 In the cattle industry, superstimulation by treatment with follicle-stimulating hormone (FSH)
43 is widely used to induce follicular growth and to improve the efficiency of *in vivo* embryo production
44 and of *in vitro* embryo production (IVP) using ovum-pick up (OPU) followed by *in vitro* fertilization
45 (IVF) [1]. OPU-IVF with FSH treatment increases the numbers of embryos from poorly productive
46 donor cows for *in vivo* embryo production [2, 3]. However, conventional FSH treatment, which
47 consists of multiple intramuscular injections, is stressful for the animals and time-consuming for
48 veterinarians. Therefore, many studies have sought to simplify FSH treatment with a single
49 subcutaneous high-dose of FSH dissolved in saline [4, 5, 6], or in a solvent that enables FSH to be
50 released slowly, such as polyvinylpyrrolidone (PVP) [4, 7], aluminum hydroxide gels [8], or
51 hyaluronan-based slow-release formulations [9, 10]. However, the effectiveness of these different
52 treatments varies considerably, probably because of differences in the amount of subcutaneous fat
53 tissue in the animals [4, 5, 6].

54 Burm *et al.* [11] reported that alfentanil (an opioid analgesic drug) was slowly absorbed into
55 the general circulation after epidural administration in humans. In cattle, epidural anesthesia is
56 routinely performed to prevent contraction of the rectum and facilitate uterine flushing for embryo
57 collection and embryo transfer [12]. If FSH injected into the epidural space is absorbed slowly and can
58 induce follicular development, it will become a simple alternative method for the superstimulation of
59 follicular development in cattle. In the present study, we examined the effect of epidural FSH
60 administration via caudal vertebrae on *in vivo* embryo production and IVP followed by OPU-IVF in
61 Japanese black cows.

62 To investigate the effect of epidural FSH administration on *in vivo* embryo production, we
63 collected embryos from cows given twice-daily intramuscular FSH administration for 3 days (control)
64 or a single epidural FSH injection (epidural). As shown in Table 1, the number of large follicles (≥ 10
65 mm in diameter) at estrus and corpora lutea at the time of embryo collection did not differ between

66 treatments. The number of collected oocytes/embryos and transferable blastocysts after epidural
67 treatment was higher than in the control group (collected oocytes/embryos; $P = 0.08$, transferable
68 blastocysts; $P = 0.10$). These results indicate that epidural treatment was as effective as the
69 conventional treatment for inducing superovulation in cattle. One caveat is that we used a total of 20
70 AU for the control, as described elsewhere [13], and 30 AU for the epidural treatment, based on a
71 single FSH subcutaneous administration in a previous report [8]. We need to investigate the optimal
72 FSH dose to induce ovulation after an epidural injection in future work.

73 To investigate the effect of epidural FSH administration on IVP followed by OPU-IVF for
74 cattle with low productivity by *in vivo* embryo production, we conducted control or epidural FSH
75 treatment before OPU. The animals produced an average of one or fewer transferable blastocysts by
76 uterine flushing after conventional FSH treatment in the previous three embryo collections within 8
77 months. After conventional or epidural treatment, most follicles were less than 6 mm in diameter, and
78 the number of follicles and collected oocytes was similar between treatments (Table 2). The proportion
79 of cleaved oocytes after IVF was also similar between treatments (Table 3). However, the rate of
80 blastocysts and transferable blastocysts in the epidural group was higher than that of the control (Table
81 3, $P < 0.0001$). The number of transferable blastocysts per OPU-IVF session in the epidural group was
82 also higher than in the control (Table 3, $P < 0.05$). The rate of pregnancy after transfer of *in vitro*
83 derived blastocysts was comparable between control (8/8) and epidural (3/4) groups, with an overall
84 success rate of 91.7%. The diameter of follicles [14, 15] and the morphological quality of oocytes [16]
85 are correlated with the developmental competence of oocytes. In the present study, there were no
86 differences in those parameters between the two treatments. Moreover, the diameter of most follicles
87 was less than 6 mm. The cause of the higher developmental competence of oocytes in the epidural
88 group is unclear; however, we speculate that FSH activates P450 aromatase and promotes estradiol
89 production from granulosa cells [17]. Such a change would result in improved developmental
90 competence of oocytes, because granulosa cells surrounding *in vitro*-grown oocytes with higher

91 maturational competence tend to secrete more E₂ than those surrounding less competent oocytes [18]. It
92 will also be necessary to carry out studies of blood FSH concentrations after epidural FSH
93 administration and to examine the effect of FSH on development of small follicles and on estradiol
94 production. Sugimura *et al.* [19] recently showed that twice-daily intramuscular FSH administration
95 for 4 days (total 30 mg) in cattle increased the diameter of follicles and improved the developmental
96 competence of oocytes without any effect on the morphology of the cumulus-oocyte complexes.
97 Transcriptome analysis has shown that genes related to cell movement and migration showed
98 down-regulated expression in FSH treated cattle, which could prevent the disruption of cell-to-cell
99 connections. The genes that show up-regulation in the cumulus cells of cattle without FSH are similar
100 to those in the granulosa cells of atretic follicles [19]. Although the reason for the differences in
101 follicular diameters between the present and previous studies is unclear, FSH administration into the
102 epidural area may improve the competence of oocytes, as reported by Sugimura *et al.* [19].

103 The results of the present study support previous studies [20-23] that showed the
104 effectiveness of epidural administration of FSH to induce superovulation. FSH solution dissolved in
105 saline is easy to prepare and epidural administration with local anesthesia is a common veterinary skill
106 to facilitate reproductive examination and treatment in cattle. Takedomi *et al.* [4] reported that when
107 FSH dissolved in saline was subcutaneously injected into Holstein heifers, the plasma concentration of
108 FSH markedly increased within 3 h and was maintained until 9 h after administration. FSH decreased
109 to the basal level after 36 h, and superovulation was not induced. However, an FSH solution dissolved
110 in PVP or aluminum hydroxide gel [4, 8] results in a gradual increase in FSH plasma concentrations
111 that peak 12 h after administration; these gradually decrease but are maintained at a concentration
112 higher than the basal level for more than 48 h. Bó *et al.* [24] suggested that circulating FSH levels
113 must be maintained above baseline for at least 72 h to induce follicular growth. They also suggested
114 that the subcutaneous area behind the shoulder, which contains a fat tissue pad, was the optimal area
115 for a single FSH administration, as the fat caused the FSH to be released gradually. It has also been

116 reported that epidural fats affect the distribution of drugs in the epidural space [25, 26]. After injection,
117 drugs diffuse into the dura mater, epidural veins, and epidural fat; drugs absorbed in epidural fats
118 could then re-diffuse to the dura mater and epidural veins gradually [25, 26]. Therefore, we speculate
119 that epidural fats contribute to the slow movement of FSH into the peripheral circulation, and that FSH
120 concentration may be maintained for more than 72 h at higher than basal level. Although epidural
121 administration with local anesthesia has been used widely in bovine management [12], there are large
122 individual variations in onset, duration, and extent of anesthesia [27], which may result from epidural
123 fat [25, 26]. Future studies need to examine the dynamics of peripheral FSH concentration after
124 administration into the epidural area.

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

130

131 **Methods**

132 **Animal care**

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

136 **Chemicals**

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

139 **Collection of *in vivo* produced embryos**

140 Five Japanese black cows were used in this study. First, the cows were subjected twice to the
141 control treatment (n = 10). Subsequently, we performed the epidural treatment once (n = 5). The
142 durations between each embryo collection were 84 to 91 days. In all treatments, FSH injection began
143 at the mid-luteal period (days 8 to 12) after confirmation of corpora lutea using a portable ultrasound
144 imaging device equipped with a transrectal probe (HS-101V; Honda Electronics, Aichi, Japan). In the
145 control group, FSH treatment consisted of twice-daily (morning and afternoon) intramuscular
146 injections for 3 days with a decreasing dose (5, 5, 3, 3, 2, and 2 AU) per injection for a total of 20 AU
147 of Antrin R-10 (Kyoritsu Seiyaku, Tokyo, Japan). At the fifth FSH treatment, 2 ml cloprostenol (0.25
148 mg/ml, Resipron-C, ASKA Animal Health, Tokyo, Japan) was injected intramuscularly. In the epidural
149 group, 30 AU of FSH dissolved in 5 ml of saline was administered to the epidural area of caudal
150 vertebrae; 48 h after FSH treatment, cloprostenol was injected intramuscularly. Twelve hours after the
151 onset of estrus, the number of large follicles (≥ 10 mm in diameter) was counted. All cows were then
152 artificially inseminated with frozen-thawed semen from Japanese black bulls. Two cows were also
153 inseminated 24 h after the onset of estrus. The number of inseminations in each cow was identical
154 between FSH treatments (1.4 ± 0.5). Seven days after estrus, embryos were collected under epidural
155 anesthesia using procaine hydrochloride (Enpro injection KS, Kyoritsu Seiyaku); the uteri were
156 flushed using Ringer's solution (Terumo Corp., Tokyo, Japan) supplemented with 0.1% fetal calf
157 serum (FCS) via a multi-eye 16-French embryo collection catheter (Nipro Corp., Osaka, Japan). After
158 embryo collection, corpora lutea were counted by rectal palpation. Collected oocytes and embryos
159 were classified according to the International Embryo Transfer Society (IETS) classification system
160 [28]. Grade 1 to 2.5 blastocysts or compacted morulae were classified as transferable blastocysts.

161 **OPU for *in vitro* embryo production**

162 We used three Japanese black cows for this experiment. Each cow was subjected to the
163 control and epidural FSH treatments four times (n = 12 in each group). The order of control and
164 epidural treatments was random and the time between each OPU was 7 to 35 days (total period = 119

165 days). First, the follicular wave in the cows was synchronized by a 1-ml intramuscular injection of
166 gonadotrophin-releasing hormone analogue (Consultan injection containing 50 µg/ml fertirelin acetate,
167 ASKA animal health), or intravaginal insertion of a progesterone device (1.9 g, CIDR 1900, Zoetis
168 Japan, Tokyo, Japan) and a 1-ml intramuscular injection of estradiol-benzoate solution (Ovahormone
169 injection containing 2 mg/ml estradiol-benzoate, ASKA animal health). FSH treatment began 64–66 h
170 after the synchronization treatment. In the control group, FSH treatment consisted of twice-daily
171 (morning and afternoon) intramuscular injection for 3 days of a decreasing dose of FSH (7, 7, 5, 5, 3,
172 and 3 AU) for a total of 30 AU. In the epidural group, 30 AU of FSH dissolved in 5 ml saline was
173 injected into the epidural area of the caudal vertebrae. OPU was conducted with an ultrasound imaging
174 device (ProSound 2, Hitachi-Aloka Medical, Tokyo, Japan), equipped with a 7.5-MHz long-handled
175 convex transducer (UST-994P-5, Hitachi-Aloka Medical), at 75–78 h after FSH treatment. The
176 number of follicles in the ovaries was counted, and follicles were classified by their diameter (small:
177 <6 mm and large: ≥6 mm) because oocytes derived from larger (≥6-mm) follicles have higher
178 developmental competence [14, 15]. Follicles were aspirated using a single-lumen needle (17-gauge,
179 600-mm long; Misawa Medical, Ibaraki, Japan) connected to a 50-ml tube (Falcon 2070; Becton
180 Dickinson, Franklin Lakes, NJ, USA) via a silicone tube (100-cm long, 1-mm internal diameter). The
181 collection tube was warmed at 37°C in a portable incubator (FV-5; Fujihira Industry, Tokyo, Japan)
182 and the other silicone tube was connected to a vacuum pump with a foot-pedal switch (MODEL 4,
183 Fujihira Industry).

184 **Oocyte maturation and IVF**

185 After collection, oocytes were washed in a filter cup (Em con, Immuno Systems, Spring
186 Valley, WI, USA) with Dulbecco's phosphate buffered saline containing 5% FCS, and transferred to a
187 90-mm plastic dish. Oocytes completely surrounded by cumulus cells were defined as good quality.
188 The oocytes were used for IVP (maturation and IVF of oocytes and culture of embryos) as previously
189 described with a slight modification [13]. Briefly, oocytes were cultured in 700 µl IVM medium (20 or

190 more oocytes) in 4-well tissue culture plates (Nalge Nunc International, Roskilde, Denmark) covered
191 with paraffin oil (Nacalai Tesque, Kyoto, Japan) or in 100- μ l droplets (19 or less oocytes) covered
192 with paraffin oil in a 35-mm plastic dish (Nalge Nunc International). The IVM medium used here was
193 tissue culture media 199 containing 25 mM HEPES (Invitrogen, Carlsbad, CA, USA) and 5% FCS.
194 After IVM, oocytes were co-incubated with frozen-thawed motile sperm (2.5×10^6 /ml) from a bull
195 separated by a Percoll gradient (45% and 90%) in a 100- μ l droplet (≤ 30 oocytes/droplet) of IVF
196 medium (IVF100; Research Institute for the Functional Peptides, Yamagata, Japan) covered with
197 paraffin oil for 6 h at 38.5°C under 5% CO₂ in humidified air. After IVF, presumptive zygotes were
198 removed from cumulus cells by pipetting and cultured in 700 μ l of culture media in 4-well tissue
199 culture plates (20 or more zygotes) or in 100- μ l droplets (19 or less zygotes) covered with paraffin oil
200 in a 35-mm plastic dish. Culture media was CR1aa medium [29] with 2% FCS for 2 days at 38.5°C
201 under 5% CO₂ and 5% O₂ with high humidity. Zygotes were then cultured in USU-6 medium [30]
202 containing 5% FCS for 5 days. Seven days after IVF, blastocysts of grades 1 to 2.5 blastocysts (IETS
203 classification [28]) were used for further study.

204 **Embryo transfer to recipient heifers and pregnancy diagnosis**

205 Each blastocyst was loaded into a clear plastic straw (0.25 cm³) and transferred
206 non-surgically into the uterine horn ipsilateral to the existing corpus luteum of a Holstein heifer using
207 an embryo transfer device (YT GUN, Yamane-teq Co., Ltd., Nagano, Japan) on days 6 to 8 after estrus.
208 Pregnancy diagnosis was performed by a portable ultrasound imaging device equipped with a
209 transrectal probe around 30 and 60 days after estrus.

210 **Statistical analysis**

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

215

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- 307

308 **Tables**

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

Treatment (replicates)	Dose of FSH (AU)	No. of follicles at estrus (≥ 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

311 Values are mean \pm SD.

312 Five cows were treated on control twice and then on epidural once.

313

314

315

316 Table 2. The number of follicles and collected oocytes at OPU after twice-daily intramuscular

317 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 (≥ 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

318 Values are mean \pm SD.

319

320

321 Table 3. In vitro production of oocytes collected after twice-daily intramuscular administration of FSH
322 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 ^a (19)	10.5 ^a (19)	1.6 ± 1.9 ^x
Epidural	30	210 (12)	43.3 (91)	26.2 ^b (55)	23.3 ^b (49)	4.1 ± 3.6 ^y

323 ^{a,b}: Different superscripts indicate significant differences within a column (P < 0.0001).

324 ^{x,y}: Different superscripts indicate significant differences within a column (P < 0.05).

325 Values of no. of transferable blastocysts/OPU session are presented as mean ± SD.