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Superovulation with a single administration of FSH in aluminum hydroxide gel: a novel superovulation method for cattle

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Abstract. Superovulation (SOV) is a necessary technique to produce large numbers of embryos for embryo transfer. In the conventional methods, follicular stimulating hormone (FSH) is administered to donor cattle twice daily for 3 to 4 days. As this method is labor intensive and stresses cattle, improving this method has been desired. We previously developed a novel and simple SOV method, in which the intramuscular injection of a single dose of FSH in aluminum hydroxide gel (AH-gel) induced the growth of multiple follicles, ovulation and the production of multiple embryos. Here we show that AH-gel can efficiently adsorb FSH and release it effectively in the presence of BSA, a major interstitial protein. When a single intramuscular administration of the FSH and AH-gel mixture was performed to cattle, multiple follicular growth, ovulation and embryo production were induced. However, the treatments caused indurations at the administration sites in the muscle. To reduce the muscle damage, we investigated alternative administration routes and different amounts of aluminum in the gel. By administering the FSH in AH-gel subcutaneously rather than intramuscularly, the amount of aluminum in the gel could be reduced, thus reducing the size of the induration. Moreover, repeated administrations of FSH with AH-gel did not affect the superovulatory response. These results indicate that a single administration of FSH with AH-gel is an effective, novel and practical method for SOV treatment.

Key words: Aluminum hydroxide gel, Cattle, Embryo, Superovulation

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Several decades ago, embryo transfer (ET) methods were established in cattle. Since embryos recovered from females with valuable economical traits can be utilized, this technology facilitates the selection of females. However, cattle produce only a single oocyte per estrous cycle; therefore, the number of embryos that can be recovered from the genetically superior individuals is limited. To solve this problem, superovulation (SOV) methods were developed, which include the stimulation of multiple follicular growth and embryo production through gonadotropin administration.

In the early days of ET, equine chorionic gonadotropin (eCG) was used for SOV [1–3]. In cows, eCG has a half-life of 40 h and persists in the circulation for up to 10 days [4]. This causes prolonged stimulation of the ovaries inducing abnormal endocrine profiles and deterioration of embryo quality [5, 6]. Later, eCG was replaced with follicular stimulating hormone (FSH) because cows were found to respond better to FSH in some respects [7]. As the half-life of FSH is much shorter (approximately 5 h) [8] than that of eCG, current SOV protocols using FSH have consisted of twice daily intramuscular administrations for 3 to 4 days [7, 9–11]. This is labor intensive and causes stress to donor cattle, which results in a decreased superovulatory response [12] and inhibited luteinizing hormone (LH) surge for ovulation [13]. Therefore, there is a need for a simple SOV method. We report here, a novel SOV method that utilizes a single administration of FSH with aluminum hydroxide gel (AH-gel).

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SOV of Cattle by a Reduced Number of or a Single FSH Administration

For the SOV of cattle, it is common to administer FSH twice a day for 3 to 4 days in decreasing doses [10, 11, 14], since the half-life of FSH is short in cattle [8].

A single administration of FSH with agents that slow its release may be used in lieu of multiple administrations. For example, it was shown that mixing FSH with polyvinylpyrrolidone (PVP) slowed its release and kept its concentration in the blood high enough to induce the development of multiple follicles [15–17]. However, it is difficult to homogeneously dissolve FSH in PVP solutions due to the high viscosity of PVP.

Hyaluronan, a glycosaminoglycan that is widely distributed in the body, has also been used to slow the release of FSH [18]. Specifically, it was shown that a singe administration of FSH diluted in 2% of hyaluronan induced a superovulatory response. However, it is also difficult to mix FSH with 2% hyaluronan because like PVP, hyaluronan has a high viscosity. Using a lower concentration of hyaluronan facilitated the mixing, but the mixture was only able to induce SOV when it was given as two administrations 48 h apart. [19].

Characteristics of AH-gel

Recently, various chemicals that can adsorb macromolecules and achieve a sustained release of different compounds *in vitro* have been developed [20–23]. AH-gel is widely used as an adjuvant. It is usually prepared by adding an alkali to a solution of aluminum salt, thereby generating aluminum oxyhydroxide ($AlO(OH)•nH_2O$) [24], which is more commonly known simply, albeit incorrectly, as aluminum hydroxide.

AH-gel is opalescent (Fig. 1) and has a low viscosity. AH-gel has a fibrous morphology (Fig. 2) giving it a high surface area and a high ability to adsorb different substances. The principal binding force of AH-gel is electrostatic. Other types of binding interactions include hydrogen-bonding and hydrophobic bonding [25, 26]. The point of zero charge (PZC) is 11.1, resulting in a positively charged surface at neutral pH [27].

Fig. 1. AH-gel in an ampoule. The gel is opalescent and has a low viscosity.

Ability of Aluminum Hydroxide Gel (AH-gel) to Adsorb and Release FSH

First, we investigated the ability of AH-gel to adsorb and release FSH. This was done in the presence of bovine serum albumin (BSA), which facilitates the release of FSH (see below). Porcine FSH (pFSH, 30 Armour unit (AU), Antrin-R10, Kawasaki Pharmaceutical, Japan) was mixed with AH-gel (5 ml at a concentration of 3 mg aluminum (Al) /ml in saline, pH 7.4, Kyoritsu Pharmaceutical, Japan), and then the supernatant was recovered following centrifugation. The precipitate was resuspended in saline with 1% BSA (pH 7.4) and incubated for 1 h at 37ºC. This suspension was centrifuged again and the supernatant was recovered. The concentrations of pFSH in the supernatants were measured by a radioimmunoassay (RIA). The AH-gel adsorbed almost all of the pFSH (an average of 29.97 mg, 99.9%). Subsequently, 22.24 mg pFSH (74.2% of the adsorbed FSH) was released from the AH-gel in the presence of BSA. These results demonstrated that the AH-gel can effectively adsorb and release pFSH in the presence of BSA.

The manner in which proteins bind to AH-gel is complicated. Some studies have reported that the electrostatic attractive force is important for the adsorption of proteins by AH-gel [26, 27]. As mentioned above, AH-gel has a point of zero charge (PZC) of 11.1 [27, 28] and thus has positive charge at pH 7.4. In contrast, pFSH has an isoelectric point (IEP) of 4.5, giving it a negative charge at pH 7.4 [29]. Therefore, AH-gel may adsorb this protein through the electrostatic force at pH 7.4. Furthermore, it has been reported that adsorbed proteins can be displaced from the gel by interstitial proteins [30]. In our experiment, the adsorbed pFSH was released in the presence of BSA *in vitro*. BSA is a major protein of bovine interstitial fluid and has an IEP of 5.0 [29]. Accordingly, BSA is also negatively charged at pH 7.4 and thus may be adsorbed to AH-gel. This property of BSA may induce the displacement of the adsorbed pFSH from the AH-gel both *in vitro* and *in vivo* (Fig. 3).

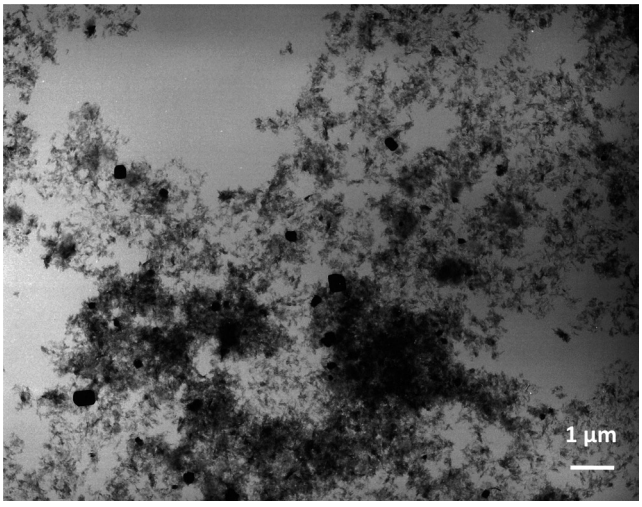

Fig. 2. Microphotograph of AH-gel. The structure is fibrous and does not form crystals.

SOV with a Single Administration of FSH in AH-gel to Cattle via the Intramuscular Route

To evaluate the effect of a single administration of FSH in AH-gel, a mixture of pFSH (30 AU) and AH-gel (5 ml, containing 3 mg Al /ml) was administered intramuscularly to Japanese Black cows at days 9 to 12 (estrus = day 0). As a control, cows were given multiple administrations of pFSH twice daily for 4 days (5, 5, 4, 4, 3, 3, 2 and 2 AU in saline). The pFSH administration schedules are shown in Fig. 4. At 48 h after the initiation of treatment, the cows were treated with 750 µg of a prostaglandin F2α ($PGF_{2\alpha}$) analogue (cloprostenol) to induce corpus luteum (CL) regression and estrus. Cows were inseminated with frozen-thawed semen at 12 and 24 h after the detection of estrus. Seven days after artificial insemination, embryos were collected non-surgically, counted and classified to determine the number of transferable embryos (Grade 1 and 2, according to the International Embryo Transfer Society manual [31]). The numbers of CLs and large follicles (> 8 mm) were also estimated by ultrasonography. No significant differences were found between the two FSH administration methods for any

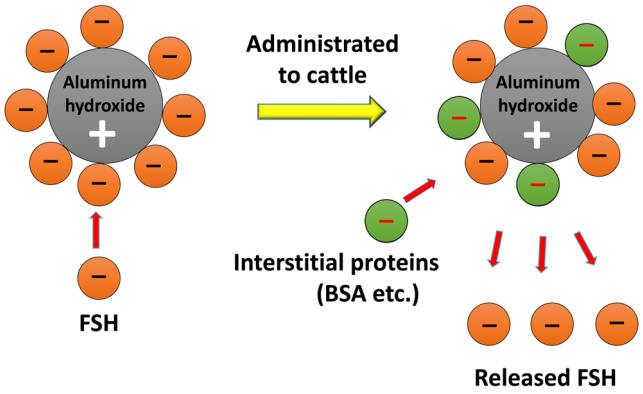

Fig. 3. Schematic diagram of the manner of adsorption of FSH to AH-gel and its release. At physiological pH (7.4), the gel is positively charged. Since FSH is negatively charged at this pH, it is adsorbed to the gel. In the presence of BSA, which is also negatively charged at this pH (both *in vivo* and *in vitro*), the adsorbed FSH is replaced and released.

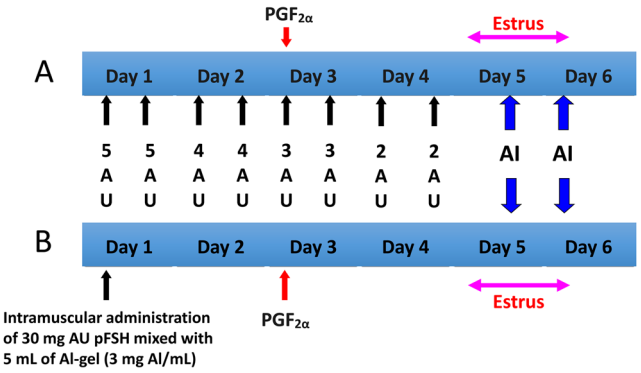

Fig. 4. pFSH administration schedules. Methods for multiple administrations of FSH (A) and a single administration of FSH with AH-gel (B) are shown.

Table 1. Comparison of ovarian responses and embryo production between a conventional multiple administration method and a single intramuscular administration of pFSH in AH-gel given to Japanese black cows

Treatment	No. of				
	Cows treated	CLs	Large follicles	Eggs recovered	Transferable embryos
AH-gel	15	11.0 ± 1.4	3.7 ± 0.8	11.1 ± 2.5	9.1 ± 2.3
Multiple	15	11.7 ± 1.8	4.0 ± 0.3	9.3 ± 1.7	8.0 ± 1.8

Values are shown as mean ± SEM.

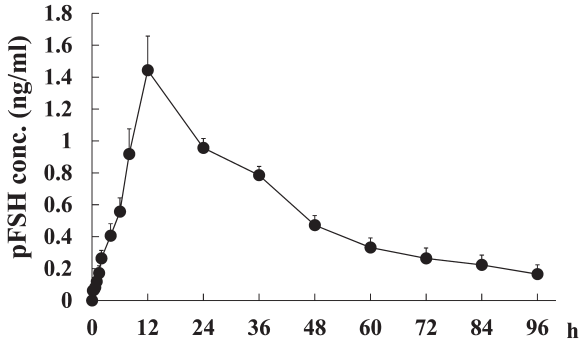

Fig. 5. Changes in plasma FSH concentrations in cows after a single intramuscular administration of FSH with AH-gel. Values are shown as means ± standard errors of the means (SEM).

of the measurements (Table 1), indicating that SOV with a single administration of FSH in AH-gel is comparable to the conventional multiple administration method.

The half-life of FSH in the body is relatively short. Since it is important to keep the concentration of FSH high to induce multiple follicular growth for successful SOV, two FSH administrations per day for 3 to 4 days are usually necessary for successful SOV in cattle. Importantly, it was shown that a single administration of FSH dissolved in saline did not induce multiple follicular growth in cattle [17].

After a single intramuscular administration of FSH in AH- gel, FSH was first detected in the blood at 2 h, peaked at 12 h and was still detectable after 3 days (Fig. 5). This result indicates that the AH-gel released FSH gradually *in vivo* as well as *in vitro*. Therefore, the ovarian responses and embryo collections from the two FSH treatments were not significantly different (Table 1).

Since AH-gel is an exogenous substance, it is very important to determine whether it is eliminated from or accumulates in the body, and whether it damages tissue at the site of administration. In rabbits, when AH-gel is administered as an adjuvant for vaccines, the gel is rapidly absorbed and eliminated in the urine [32, 33]. However it has been suggested that if AH-gel is not rapidly eliminated from the site of administration, the remaining AH-gel induces lesions such as granulomas, lesional macrophage accumulation, or macrophagic myofasciitis [34–37]. In cows that were administered AH-gel intramuscularly, the site of administration was characterized by the appearance of foreign body granulomas, macrophages, foreign body giant cells and monocytes (Fig. 6).

SOV with a Single Administration of FSH in AH-gel to Cattle via the Subcutaneous Route

As mentioned above, lesions occurred at the site of intramuscular administration of the FSH and AH-gel mixture. Injecting meat cattle with vaccines, antibiotics, and hormones causes lesions that require trimming, devaluation of cuts, and consumer dissatisfaction from tough meet [38, 39], which results in economic losses in meat production [40]. Thus, many attempts have been made to avoid the formation of such lesions including their mitigation by subcutaneous administration [40–42]. Specifically some reports indicate that the subcutaneous administrations of antibiotics, vaccines, hormones, and vitamins effectively mitigates the formation of lesions [40–42]. Therefore, we hypothesized that by subcutaneously administering

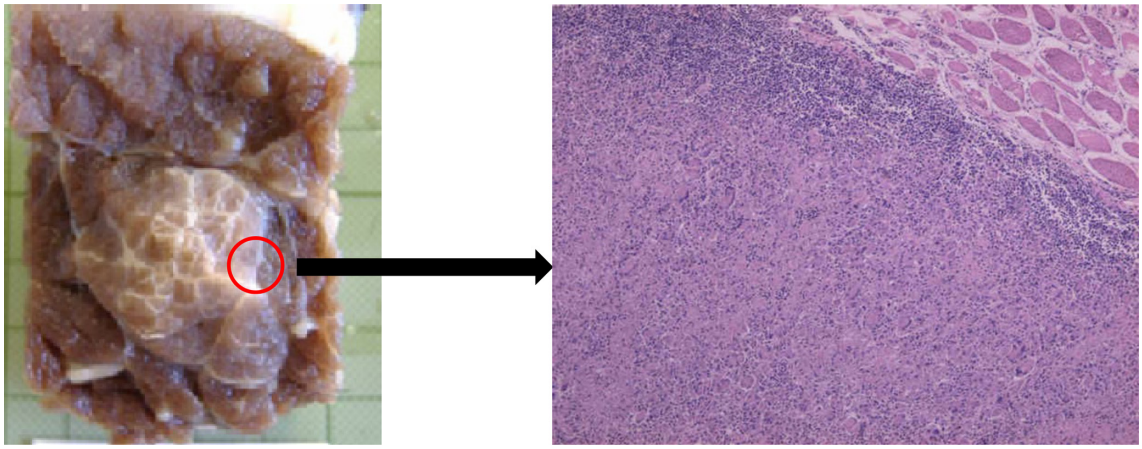

Fig. 6. Lesions occurred at the site of intramuscular FSH administration with AH-gel. An example of a foreign body granuloma that was formed at the administration site (left). An accumulation of macrophages, foreign-body giant cells, and monocytes observed at the administration site (right).

Table 2. Effect of various Al concentrations of AH-gel on superovulatory responses in cattle after a single subcutaneous administration of pFSH

Al conc. (mg/ml)	No. of			
	Cows	CLs	Recovered eggs	Transferrable embryos
3	6	10.7 ± 2.9	7.5 ± 2.9	4.8 ± 1.6 [a]
0.3	6	11.7 ± 3.8	9.3 ± 2.9	4.8 ± 1.6 [a]
0.15	6	9.7 ± 3.7	6.7 ± 2.9	1.2 ± 0.5 [b]

Values with different superscripts within the same column are significantly different ($P < 0.05$).

FSH in AH-gel, the amount of AH-gel could be reduced, thereby reducing the size of the lesion.

FSH in AH-gel was administered subcutaneously in a single dose at the base of the neck. The number of recovered transferable embryos was relatively high with Al concentration of 3 and 0.3 mg/ml, but decreased significantly when the Al concentration was 0.15 mg/ml (Table 2). However, all cows developed indurations at the site of injection. The size of induration increased with increasing Al concentration and decreased with time (Table 3). A similar finding was reported for monkeys injected with Al-containing vaccines [36].

We also investigated the effect of various volumes of AH-gel on the superovulatory response and the size of induration at the site of administration, while keeping the total amount of Al constant. The total Al content in a single administration was fixed at 1.5 mg in this experiment. Changing the volume of the gel but keeping the total amount of Al constant at 1.5 mg had little effect on the superovulation responses (number of CLs and recovered eggs), although the number of transferable embryos decreased when 1 ml of the gel was used, though this decrease was not significant (Table 4). The sizes of the indurations are shown in Table 5. The relationship between the days after administration and the volume of the gel was not statistically significant. The sizes of the indurations decreased with time and with decreasing gel volume (Table 5). At 5 days post treatment, when 1 ml of 1.5 mg Al/ml AH-gel was used, the sizes of the indurations were significantly smaller than those that occurred with 5 ml of AH-gel with 0.3 mg Al/ml. Although the sizes of the indurations significantly decreased with time when 5 ml of 0.3 mg Al/ml gel was used, they did not significantly change with time in the other two groups. After subcutaneous administration, a small induration (approximately 15 mm) was detected on the surface of the muscle (Fig. 7), but not inside the muscle as in the case of intramuscular administration (Fig. 6, left photograph). These results suggest that the indurations at the site of administration were located at the surface

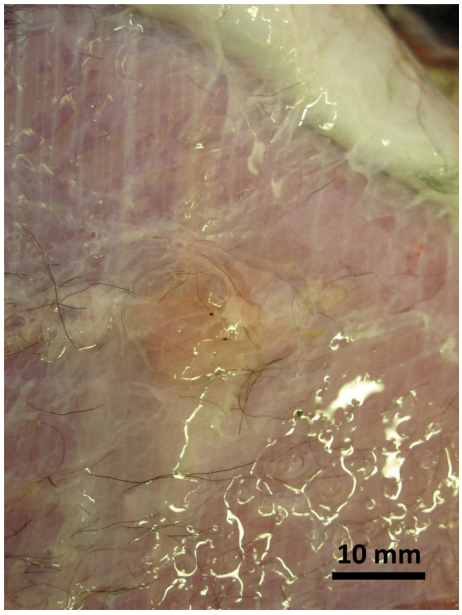

Fig. 7. Lesions occurred at the site of subcutaneous FSH administration with AH-gel. An example of an induration (located at the center of the photograph as brown-yellow tissue) observed on the surface of the muscle at the base of neck.

Table 3. Effect of various Al concentrations of AH-gel on the sizes of indurations after a single subcutaneous administration of pFSH

Al conc. (mg/ml)	No. of cows	Size of induration (mm)					
		5 days *		10 days *		20 days *	
		Length	Width	Length	Width	Length	Width
0.3	6	84.4 ± 9.8^{ax}	32.2 ± 8.0^{l}	73.2 ± 7.4^{axy}	30.8 ± 6.8^{al}	64.6 ± 5.4^{ay}	17.6 ± 2.0^{am}
0.5	6	36.7 ± 7.0^{b}	21.2 ± 1.7	42.4 ± 3.8^{b}	12.0 ± 2.1^{b}	29.8 ± 4.0^{b}	11.5 ± 2.4^{ab}
1.5	6	35.2 ± 7.8^{bx}	18.0 ± 5.3^{l}	21.8 ± 7.4^{bxy}	9.2 ± 3.2^{blm}	12.0 ± 5.4^{by}	6.2 ± 2.6^{bm}

* Days after initiation of FSH treatment. ^{a,b} The values with different superscripts within the same column are significantly different ($P < 0.05$). ^{x,y} The length values with different superscripts within the same line are significantly different ($P < 0.05$). ^{l,m} The width values with different superscripts within the same line are significantly different ($P < 0.05$).

Table 4. Effects of various volumes of AH-gel with constant total Al content on superovulatory responses to by a single subcutaneous administration of pFSH in cattle

AH gel			No. of			
Al conc. (mg/ml)	x	Volume (ml)	Cows	CLs	Recovered eggs	Transferrable embryos
0.3	x	5	6	8.5 ± 4.9	7.5 ± 4.4	5.8 ± 3.5
0.5	x	3	6	10.7 ± 2.9	7.5 ± 2.9	6.2 ± 2.9
1.5	x	1	6	10.5 ± 4.1	6.0 ± 2.7	2.8 ± 1.3

Table 5. Effects of various volumes of AH-gel with constant total Al content on the sizes of indurations after a single subcutaneous administration of pFSH in cattle

Al conc. (mg/ml)	Volume (ml)	No. of cows	Sizes of induration (mm)					
			5 days *		10 days *		20 days *	
			Length	Width	Length	Width	Length	Width
0.3	5	6	45.5 ± 5.2^{ax}	19.3 ± 3.7^{al}	41.0 ± 5.2^{xy}	12.8 ± 1.3^{m}	31.8 ± 1.2^{y}	11.8 ± 1.0^{m}
0.5	3	6	29.7 ± 3.4^{ab}	12.3 ± 1.4^{ab}	30.5 ± 2.6	10.7 ± 1.4	26.7 ± 2.2	9.2 ± 1.7
1.5	1	6	28.0 ± 2.3^{b}	10.0 ± 1.8^{b}	29.8 ± 2.9	12.3 ± 2.6	25.5 ± 1.5	10.0 ± 1.2

* Days after initiation of FSH treatment. ^{a,b} The values with different superscripts within the same column are significantly different ($P < 0.05$). ^{x,y} The length values with different superscripts within the same line are significantly different ($P < 0.05$). ^{l,m} The width values with different superscripts within the same line are significantly different ($P < 0.05$).

of the muscle and their sizes were influenced by the volume of the AH-gel administered.

After subcutaneous administration of FSH in AH-gel, the concentration of FSH in the blood gradually increased and peaked at 8–12 h, and was still detectable at 96 h (Fig. 8). An average of 9.0 ± 3.8 transferable embryos were recovered, whereas no transferable embryos were recovered following intramuscular administration of the same mixture (Table 6). The release of hormones into the circulation is slower by subcutaneous administration than by intramuscular administration [43–45]. Thus, even the lower concentration of AH-gel can retain FSH and release it gradually. On the other hand, in the case of intramuscular administration, the AH-gel with lower aluminum concentration released FSH rapidly.

As an adjuvant, AH-gel was shown to enhance the uptake of antigens by antigen-presenting cells *in vitro* [46], and had a direct effect on the accessory properties of human monocytes in an interleukin-4-dependent manner [47]. These results raise the possibility that repeated SOVs using AH-gel may eventually induce

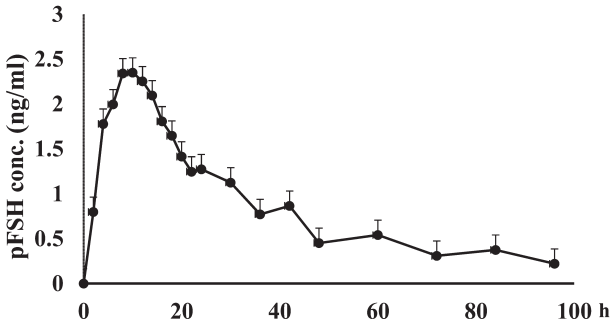

Fig. 8. Changes in plasma FSH concentrations in cows after a single subcutaneous FSH administration with AH-gel. Values are shown as means ± SEM.

Table 6. Superovulatory response of a single administration of FSH in AH-gel by the subcutaneous or intramuscular routes

Administration route	No. of			
	Cows	CLs	Recovered eggs	Transferrable embryos
Subcutaneous	4	17.7 ± 5.2	11.0 ± 4.0 [a]	9.0 ± 3.8 [a]
Intramuscular	4	2.7 ± 2.7	0 [b]	0 [b]

[a,b] The values with different superscripts within the same column are significantly different ($P < 0.05$).

Table 7. Effects of repeated single subcutaneous administration of FSH in AH-gel on the superovulatory responses in cattle

SOV period	No. of		
	CLs	Eggs recovered	Transferable embryos
1	15.1 ± 2.7	11.2 ± 2.1	6.8 ± 1.9
2	14.0 ± 1.9	11.4 ± 1.9	5.6 ± 2.1
3	10.6 ± 1.5	8.1 ± 1.9	4.1 ± 0.9
4	12.6 ± 1.9	9.6 ± 2.3	6.1 ± 2.5

immune responses against FSH, which could interfere with the desired response to FSH. However, for reasons that are unclear, the SOV responses using AH-gel did not decrease after successive administrations (Table 7), indicating that high-value donors may be used repeatedly. With conventional multiple administrations of pFSH for SOV in cattle, repeated treatments do not affect the number of embryos collected or the ovarian responses [48]. The homology of the amino acid sequence of FSH among species may be important. For example, pFSH is not expected to generate an immune response in cattle as the amino acid sequences of porcine and bovine FSH are 96.9% identical in the α subunit [49, 50] and 88.1% identical in the β subunit [51, 52]. Additionally, AH-gel does not appear to be a strong immunogen because in rabbits immunized by ovine FSH, the titer of ovine FSH antiserum was lower when AH-gel was used as a vehicle than when Freund's complete adjuvant was used [53]. Together, these findings suggest that pFSH in AH-gel for SOV does not induce a notable immune response, and consequently, the ovarian response does not appear to be diminished.

Application of a Single Administration of FSH in AH-gel to Other Mammalian Species

A single FSH administration in AH-gel is also effective for SOV in other mammalian species. For the SOV of rabbits, FSH is commonly used for follicular growth [54, 55]. A single administration of FSH in PVP has been used to induce SOV in rabbits [56, 57]. We previously used AH-gel as an adsorbent for FSH for the SOV in rabbits [58]. We found that the numbers of total and fertilized eggs recovered from rabbits treated with FSH in AH-gel were similar to multiple FSH injections and were significantly greater than the numbers obtained from a single injection of FSH with PVP.

Moreover, the effect of administering FSH in AH-gel on multiple follicular development and induction of ovarian weight gain was investigated in female immature rats *in vivo* [59]. The ovarian weight of rats given a single administration of FSH in AH-gel was significantly higher compared to that of rats given multiple administrations of the hormone dissolved in saline [60]. Therefore, we expect that a single administration of FSH in AH-gel would be useful for the SOV of various mammalian species.

Conclusions

We have developed a single administration method of FSH using AH-gel for SOV in cattle. Through subcutaneous administration, we could reduce the total amount of Al in the gel, minimizing the damage at the administration site. This method is not only simple and user-friendly, but also can reduce the stress to cattle.

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References

1. **Hafez ES, Sugie T, Gordon I.** Superovulation and related phenomena in the beef cow. I. Superovulatory responses following PMS and HCG injections. *J Reprod Fertil* 1963; **5**: 359–379. [Medline] [CrossRef]
2. **Hafez ES, Sugie T, Hunt WL.** Superovulation and related phenomena in the beef cow. II. Effect of oestrogen administration on production of ova. *J Reprod Fertil* 1963; **5**: 381–388. [Medline] [CrossRef]
3. **Edelman R, Wasserman SS, Bodison SA, Perry JG, O'Donnoghue M, DeTolla LJ.** Phase II safety and immunogenicity study of type F botulinum toxoid in adult volunteers. *Vaccine* 2003; **21**: 4335–4347. [Medline] [CrossRef]
4. **Murphy BD, Martinuk SD.** Equine chorionic gonadotropin. *Endocr Rev* 1991; **12**: 27–44. [Medline] [CrossRef]
5. **Jensen AM, Greve T, Madej A, Edqvist LE.** Endocrine profiles and embryo quality in the PMSG-PGF2alpha treated cow. *Theriogenology* 1982; **18**: 33–44. [Medline] [CrossRef]
6. **Moor RM, Kruip TAM, Green D.** Intraovarian Control of Folliculogenesis - Limits to Superovulation. *Theriogenology* 1984; **21**: 103–116. [CrossRef]
7. **Monniaux D, Chupin D, Saumande J.** Superovulatory Responses of Cattle. *Theriogenology* 1983; **19**: 55–81. [CrossRef]
8. **Demoustier MM, Beckers JF, Van Der Zwalmen P, Closset J, Gillard JL, Ectors F.** Determination of porcine plasma follitropin levels during superovulation treatment in cows. *Theriogenology* 1988; **30**: 379–386. [Medline] [CrossRef]
9. **Looney CR, Bondioli KR, Hill KG, Massey JM.** Superovulation of Donor Cows with Bovine Follicle-Stimulating-Hormone (Bfsh) Produced by Recombinant DNA Technology. *Theriogenology* 1988; **29**: 271. [CrossRef]
10. **Hasler JF, McCauley AD, Schemerhorn EC, Foote RH.** Superovulatory responses of Holstein cows. *Theriogenology* 1983; **19**: 83–99. [CrossRef]
11. **Herrler A, Elsaesser F, Parvizi N, Niemann H.** Superovulation of dairy cows with purified FSH supplemented with defined amounts of LH. *Theriogenology* 1991; **35**: 633–643. [Medline] [CrossRef]
12. **Edwards LM, Rahe CH, Griffin JL, Wolfe DF, Marple DN, Cummins KA, Pitchett JF.** Effect of transportation stress on ovarian function in superovulated Hereford heifers. *Theriogenology* 1987; **28**: 291–299. [Medline] [CrossRef]
13. **Stoebel DP, Moberg GP.** Repeated acute stress during the follicular phase and luteinizing hormone surge of dairy heifers. *J Dairy Sci* 1982; **65**: 92–96. [Medline] [CrossRef]
14. **Wubishet A, Graves CN, Spahr SL, Kesler DJ.** Continuous subcutaneous infusion of follicle-stimulating hormone as a method of superovulating dairy cows. *Theriogenology* 1986; **25**: 809–812. [CrossRef]
15. **Smith LE Jr, Sitton GD, Vincent CK.** Limited injections of follicle stimulating hormone

for multiple births in beef cattle. *J Anim Sci* 1973; **37**: 523–527. [Medline]

16. **Yamamoto M, Ooe M, Kawaguchi M, Suzuki T.** Superovulation in the cow with a single intramuscular injection of FSH dissolved in polyvinylpyrrolidone. *Theriogenology* 1994; **41**: 747–755. [Medline] [CrossRef]
17. **Takedomi T, Aoyagi Y, Konishi M, Kishi H, Taya K, Watanabe G, Sasamoto S.** Superovulation of Holstein heifers by a single subcutaneous injection of FSH dissolved in polyvinylpyrrolidone. *Theriogenology* 1995; **43**: 1259–1268. [Medline] [CrossRef]
18. **Tríbulo A, Rogan D, Tribulo H, Tribulo R, Alasino RV, Beltramo D, Bianco I, Mapletoft RJ, Bó GA.** Superstimulation of ovarian follicular development in beef cattle with a single intramuscular injection of Folltropin-V. *Anim Reprod Sci* 2011; **129**: 7–13. [Medline] [CrossRef]
19. **Tríbulo A, Rogan D, Tríbulo H, Tríbulo R, Mapletoft RJ, Bó GA.** Superovulation of beef cattle with a split-single intramuscular administration of Folltropin-V in two concentrations of hyaluronan. *Theriogenology* 2012; **77**: 1679–1685. [Medline] [CrossRef]
20. **Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM.** Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. *Clin Exp Immunol* 1985; **61**: 143–151. [Medline]
21. **Peppas NA, Sahlin JJ.** Hydrogels as mucoadhesive and bioadhesive materials: a review. *Biomaterials* 1996; **17**: 1553–1561. [Medline] [CrossRef]
22. **Yewey GL, Duysen EG, Cox SM, Dunn RL.** Delivery of proteins from a controlled release injectable implant. *Pharm Biotechnol* 1997; **10**: 93–117. [Medline] [CrossRef]
23. **Sharma S, Nijdam AJ, Sinha PM, Walczak RJ, Liu X, Cheng MM, Ferrari M.** Controlled-release microchips. *Expert Opin Drug Deliv* 2006; **3**: 379–394. [Medline] [CrossRef]
24. **Shirodkar S, Hutchinson RL, Perry DL, White JL, Hem SL.** Aluminum compounds used as adjuvants in vaccines. *Pharm Res* 1990; **7**: 1282–1288. [Medline] [CrossRef]
25. **al-Shakhshir RH, Regnier FE, White JL, Hem SL.** Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminium-containing adjuvants. *Vaccine* 1995; **13**: 41–44. [Medline] [CrossRef]
26. **Chang MF, White JL, Nail SL, Hem SL.** Role of the electrostatic attractive force in the adsorption of proteins by aluminum hydroxide adjuvant. *PDA J Pharm Sci Technol* 1997; **51**: 25–29. [Medline]
27. **al-Shakhshir R, Regnier F, White JL, Hem SL.** Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. *Vaccine* 1994; **12**: 472–474. [Medline] [CrossRef]
28. **Hem SL, White JL.** Structure and properties of aluminum-containing adjuvants. *Pharm Biotechnol* 1995; **6**: 249–276. [Medline] [CrossRef]
29. **Bhow AS.** Handbook of proteins. Birmingham, Alabama: A&M Publications; 2001.
30. **Heimlich JM, Regnier FE, White JL, Hem SL.** The in vitro displacement of adsorbed model antigens from aluminium-containing adjuvants by interstitial proteins. *Vaccine* 1999; **17**: 2873–2881. [Medline] [CrossRef]
31. **Stringfellow DA, Seidel SM.** Manual of the International Embryo Transfer Society : a procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures. International Embryo Transfer Society; 1998.
32. **Flarend RE, Hem SL, White JL, Elmore D, Suckow MA, Rudy AC, Dandashli EA.** In vivo absorption of aluminium-containing vaccine adjuvants using 26Al. *Vaccine* 1997; **15**: 1314–1318. [Medline] [CrossRef]
33. **Hem SL.** Elimination of aluminum adjuvants. *Vaccine* 2002; **20**(Suppl 3): S40–S43. [Medline] [CrossRef]
34. **Gherardi RK, Coquet M, Cherin P, Belec L, Moretto P, Dreyfus PA, Pellissier JF, Chariot P, Authier FJ.** Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. *Brain* 2001; **124**: 1821–1831. [Medline] [CrossRef]
35. **Valtulini S, Macchi C, Ballanti P, Cherel Y, Laval A, Theaker JM, Bak M, Ferretti E, Morvan H.** Aluminium hydroxide-induced granulomas in pigs. *Vaccine* 2005; **23**: 3999–4004. [Medline] [CrossRef]
36. **Verdier F, Burnett R, Michelet-Habchi C, Moretto P, Fievet-Groyne F, Sauzeat E.** Aluminium assay and evaluation of the local reaction at several time points after intramuscular administration of aluminium containing vaccines in the Cynomolgus monkey. *Vaccine* 2005; **23**: 1359–1367. [Medline] [CrossRef]
37. **Israeli E, Agmon-Levin N, Blank M, Shoenfeld Y.** Macrophagic myofaciitis a vaccine (alum) autoimmune-related disease. *Clin Rev Allergy Immunol* 2011; **41**: 163–168. [Medline] [CrossRef]
38. **George MH, Morgan JB, Glock RD, Tatum JD, Schmidt GR, Sofos JN, Cowman GL, Smith GC.** Injection-site lesions: incidence, tissue histology, collagen concentration, and muscle tenderness in beef rounds. *J Anim Sci* 1995; **73**: 3510–3518. [Medline]
39. **George MH, Cowman GL, Tatum JD, Smith GC.** Incidence and sensory evaluation of injection-site lesions in beef top sirloin butts. *J Anim Sci* 1996; **74**: 2095–2103. [Medline]
40. **Van Donkersgoed J, Dubeski PL, Aalhus JL, VanderKop M, Dixon S, Starr WN.** The effect of vaccines and antimicrobials on the formation of injection site lesions in subprimals of experimentally injected beef calves. *Can Vet J* 1999; **40**: 245–251. [Medline]
41. **Van Donkersgoed J, Dubeski PL, VanderKop M, Aalhus JL, Bygrove S, Starr WN.** The effect of animal health products on the formation of injection site lesions in subprimals of experimentally injected beef calves. *Can Vet J* 2000; **41**: 617–622. [Medline]
42. **Colazo MG, Martínez MF, Kastelic JP, Mapletoft RJ.** Effects of dose and route of administration of cloprostenol on luteolysis, estrus and ovulation in beef heifers. *Anim Reprod Sci* 2002; **72**: 47–62. [Medline] [CrossRef]
43. **Burrows GE, Griffin DD, Pippin A, Harris K.** A comparison of the various routes of administration of erythromycin in cattle. *J Vet Pharmacol Ther* 1989; **12**: 289–295. [Medline] [CrossRef]
44. **Bo GA, Hockley DK, Nasser LF, Mapletoft RJ.** Superovulatory response to a single subcutaneous injection of Folltropin-V in beef cattle. *Theriogenology* 1994; **42**: 963–975. [Medline] [CrossRef]
45. **Knox RV, Tudor KW, Rodriguez-Zas SL, Robb JA.** Effect of subcutaneous vs intramuscular administration of P.G. 600 on estrual and ovulatory responses of prepubertal gilts. *J Anim Sci* 2000; **78**: 1732–1737. [Medline]
46. **Baylor NW, Egan W, Richman P.** Aluminum salts in vaccines—US perspective. *Vaccine* 2002; **20**(Suppl 3): S18–S23. [Medline] [CrossRef]
47. **Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LA.** The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect Immun* 2001; **69**: 1151–1159. [Medline] [CrossRef]
48. **Lubbadeh WF, Graves CN, Spahr SL.** Effect of repeated superovulation on ovulatory response of dairy cows. *J Anim Sci* 1980; **50**: 124–127. [Medline]
49. **Goodwin RG, Moncman CL, Rottman FM, Nilson JH.** Characterization and nucleotide sequence of the gene for the common alpha subunit of the bovine pituitary glycoprotein hormones. *Nucleic Acids Res* 1983; **11**: 6873–6882. [Medline] [CrossRef]
50. **Kato Y, Ezashi T, Hirai T, Kato T.** The gene for the common alpha subunit of porcine pituitary glycoprotein hormone. *J Mol Endocrinol* 1991; **7**: 27–34. [Medline] [CrossRef]
51. **Maurer RA, Beck A.** Isolation and nucleotide sequence analysis of a cloned cDNA encoding the beta-subunit of bovine follicle-stimulating hormone. *DNA* 1986; **5**: 363–369. [Medline] [CrossRef]
52. **Kato Y.** Cloning and DNA sequence analysis of the cDNA for the precursor of porcine follicle stimulating hormone (FSH) beta subunit. *Mol Cell Endocrinol* 1988; **55**: 107–112. [Medline] [CrossRef]
53. **Westhoff WE, Slootstra JW, Puijk WC, Kuperus D, Flinterman JF, Schaaper WM, Oonk HB, Meloen RH.** Detection of epitopes on follicle-stimulating hormone and FSH-antiserum-induced suppression of bioactivity of follicle-stimulating hormone and luteinizing hormone. *J Reprod Immunol* 1996; **30**: 133–149. [Medline] [CrossRef]
54. **Treloar AF, Schabdach DG, Sansing S, Keller LS.** Superovulation of New Zealand white rabbits by continuous infusion of follicle-stimulating hormone, using a microosmotic pump. *Lab Anim Sci* 1997; **47**: 313–316. [Medline]
55. **Kauffman RD, Schmidt PM, Rall WF, Hoeg JM.** Superovulation of rabbits with FSH alters in vivo development of vitrified morulae. *Theriogenology* 1998; **50**: 1081–1092. [Medline] [CrossRef]
56. **Hirabayashi M, Hirao M, Takahashi R, Kimura K, Hirasawa K, Ueda M, Hochi S.** Production of transgenic rabbits using centrifuged pronuclear zygotes. *J Vet Med Sci* 2000; **62**: 1047–1052. [Medline] [CrossRef]
57. **Mehaisen GMK, Viudes-de-Castro MP, Vicente JS, Lavara R.** In vitro and in vivo viability of vitrified and non-vitrified embryos derived from eCG and FSH treatment in rabbit does. *Theriogenology* 2006; **65**: 1279–1291. [Medline] [CrossRef]
58. **Hashimoto S, Kimura K, Kuramochi T, Aoyagi K, Hirako M, Kawaguchi M, Iwata H, Hirao M, Kitada K, Hirasawa K, Ueda M.** Responsiveness of rabbits to superovulation treatment by a single injection of follicle-stimulating hormone with aluminum hydroxide gel. *Mol Reprod Dev* 2007; **74**: 1208–1212. [Medline] [CrossRef]
59. **Steelman SL, Pohley FM.** Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin. *Endocrinology* 1953; **53**: 604–616. [Medline] [CrossRef]
60. **Kimura K, Hirako M, Iwata H, Aoki M, Kawaguchi M, Seki M.** Successful superovulation of cattle by a single administration of FSH in aluminum hydroxide gel. *Theriogenology* 2007; **68**: 633–639. [Medline] [CrossRef]