

Journal of the Hellenic Veterinary Medical Society

Vol 73, No 4 (2022)



Effects of FSH Administered in Different ways on Superovulation Response and Blood FSH Levels in Cows

SÜ Çizmeci, AD Dinç, M Güler, H Alkan, MB Kivrak, A Takci, MF Çiftçi, ÖF Yeşilkaya, P Bulut, S Bulut, A Zhunushova

doi: [10.12681/jhvms.27334](https://doi.org/10.12681/jhvms.27334)

Copyright © 2023, Sakine Ülküm ÇİZMECI



This work is licensed under a [Creative Commons Attribution-NonCommercial 4.0](https://creativecommons.org/licenses/by-nc/4.0/).

To cite this article:

Çizmeci, S., Dinç, A., Güler, M., Alkan, H., Kivrak, M., Takci, A., Çiftçi, M., Yeşilkaya, ÖF, Bulut, P., Bulut, S., & Zhunushova, A. (2023). Effects of FSH Administered in Different ways on Superovulation Response and Blood FSH Levels in Cows. *Journal of the Hellenic Veterinary Medical Society*, 73(4), 4739–4746.
<https://doi.org/10.12681/jhvms.27334>

Effects of FSH Administered in Different Ways on Superovulation Response and Blood FSH Levels in Cows

S.Ü. Çizmeci^{1*}, D.A. Dinç¹, M. Güler¹, H. Alkan¹, M.B. Kırıak², A. Takci²,
M.F. Çiftçi¹, Ö.F. Yeşilkaya¹, P. Bulut¹, S. Bulut³, A. Zhunushova⁴

¹Selcuk University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Konya, Turkey

²Cumhuriyet University, Faculty of Veterinary Medicine Department of Obstetrics and Gynecology, Konya, 58140, Turkey

³Selcuk University, Faculty of Veterinary Medicine Department of Internal Medicine, Konya, 42130, Turkey

⁴Kyrgyz-Turkish Manas University, Faculty of Veterinary Medicine Department of Pharmacology and Toxicology, Bishkek 720044, Kyrgyzstan

ABSTRACT: This study aimed to compare three ways of FSH administration [single-dose subcutaneous (sc) using an adjuvant, single-dose epidural without an adjuvant and the conventional intramuscular (i.m) repeated decreasing doses] on blood FSH levels and superovulation response. Holstein cows (n:18) were divided into three groups for the superovulation procedure (n=6). The animals in group (1) received intramuscular (im) decreasing doses of FSH (Stimufol, 500 µg porcine FSH and 100 µg porcine LH, Ulg FMV, Liège, Belgium) at 12-h intervals for 4 d. The animals in group (2) received FSH (500 µg) into the epidural space (e). The animals in group (3) received a single subcutaneous (SC) dose of FSH (500 µg) added in 10 mL of Montanide ISA 206 adjuvant. CL count in the ovary was measured by ultrasonography and superovulation was determined based on the presence of >4 CL. For FSH measurement, blood samples were collected from the *V. jugularis* of all the animals. Serum FSH (pFSH) concentration was determined by ELISA. After synchronisation and superovulation procedures, all animals had estrus (100%). Superovulation response (≥ 4 CL) was detected in six animals in group 1 (im), one animal in group 2 (e), and four animals in group 3 (sc). Superovulation responses in e group were lower than those in im group ($P<0.05$), but they were like those in sc group ($P>0.05$). Serum FSH concentrations were similar between groups for all sampling times ($P>0.05$).

Keywords: Epidural; FSH; Holstein cow; Montanide; Superovulation response

Corresponding Author:

S.Ü. Çizmeci, Selcuk University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Konya, Turkey
E-mail address:

Date of initial submission: 21-06-2021
Date of acceptance: 25-09-2022

INTRODUCTION

Superovulation is one of the crucial stages of embryo transfer (Gordon, 2004). This procedure is performed to enhance ova production from a cow in each estrus cycle, inducing ovarian stimulation using gonadotropins and thus increasing the number of embryos, depending on the breed characteristics of the cow (Kanagawa et al., 1995; Bowen, 2003; Son et al., 2007). Superovulation procedure not only increases the number of oocytes released from follicles but also stimulates ovulation and enables maturation of oocytes (Rahman et al., 2008; Sağırkaya, 2009; de Souza et al., 2011).

Animal response to superovulation varies depending on body condition score, age, breed, nutritional status, type of gonadotropin, administration method, climate and other environmental conditions (Mapletoft et al., 2002; Silve et al., 2009; Amiridis and Cseh 2012; Hussein et al., 2017).

FSH is a gonadotropic hormone used for superovulation. It is usually extracted from the pituitary gland of sheep and pigs. It exerts its effect by binding to and activating FSH 2 receptors in small- and medium-sized follicles stimulating their development (Rahman et al., 2008).

Exogenous FSH administration in cows or mature heifers stimulates the development of multiple follicles, rescuing follicles that would normally be lost to atresia during the estrus cycle. FSH stimulation is needed until oocytes reach the size to respond to LH, and this lasts for approximately 4 d (Bo and Mapletoft, 2014; Deguettes et al., 2020).

The half-life of FSH is approximately 5 h, and it is usually necessary to be administered at 12-h intervals for 3 to 4 d, to maintain adequate FSH levels in the blood and stimulate multiple follicular growth. Repeated FSH administration is a very demanding procedure. Common errors during its application are not uncommon resulting in decreased superovulation response or no response at all. In addition, repeated administrations may result in stress in donor cattle, thus reducing the ovarian response and inhibiting the LH surge required for ovulation (Bo et al., 2017).

Superovulation application's other goal is to reduce the stress for females and to simplify the superovulation protocols while collecting a lot of good-quality embryos from the donor (Ochea et al., 2015). It has been reported that in limited studies on epidural FSH

application, superovulation results similar to traditional applications have been obtained (Taşdemir et al., 2012; Chumchai et al., 2021). However, it is also reported that the embryo quality obtained is lower (Chumchai et al., 2021). It is thought that epidural oils may help slow release of FSH into the peripheral circulation (Lee et al., 2003a). In addition, the success of superstimulation in dairy cattle may have been insufficient, as there were differences in the structure and proportions of epidural fats between different breeds. Similarly, the results are not satisfactory in Holstein cows, which probably have less subcutaneous adipose tissue (Bo et al., 1994).

The epidural application, which is an alternative to repeated FSH applications, has been tried by taking local anesthetics as a role model. It is reported that the blood FSH level rises 3 hours after the application and decreases to basal levels at the 36th hour (Sakaguchi et al., 2018). However, there is also a study stating that blood FSH level increases very rapidly within 2 hours and decreases to basal level at the end of 10 hours (Chumchai et al., 2021). Studies have reported that superovulation responses close to the classical method are obtained after epidural application (Taşdemir et al., 2012; Satılmış et al., 2021).

Owing to the difficulty of the conventional superovulation procedures, researchers have been trying to develop simpler protocols such as FSH subcutaneously (SC) in single-dose by using adjuvant. An alternative way of prolonging FSH release following a single im administration is to combine it with certain vehicles that lead to slow release over a few days. These substances are often polymers; they are biodegradable and do not react with body tissue (Bo et al., 2017).

When agents that slow down FSH release are used for superovulation in cattle, FSH can be administered as a single dose. Therefore, the administration of FSH in combination with vehicles, such as Montanide, aluminium hydroxide (AH) (Kimura, 2016), polyvinylpyrrolidone (PVP) (Takedomi et al., 1995), or hyaluronan (Tribulo et al., 2012), has been reported to slow down FSH release, ensuring that FSH reaches the concentration required for superovulation in blood and remains at a high level for sufficient time.

Montanide ISA 206 (Seppic, France) is a vaccine adjuvant with water, oil, water (W/O/W) properties, which is used in the testing and administration of many vaccines in the veterinary field. It is preferred as it provides a long release time.

This study aimed to compare three ways of FSH administration [single-dose subcutaneous (sc) using an adjuvant, single-dose epidural without an adjuvant and the conventional intramuscular (i.m) repeated decreasing doses] on blood FSH levels and superovulation response.

MATERIAL AND METHODS

The study was conducted with the approval of Selçuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee (SÜVDAMEK) (2018/19).

In total, 18 Holstein cows aged 4 to 6 y and having a milk yield > 35 to 40 L were included in the study. Cows that did not have a postpartum uterine infection, did not have any pathology in their genital organs and were between 90-130 days after calving were used in the study. Animals were selected based on clinical examination (i.e., general examination, rectal examination, and ultrasonography) results. Animals were divided into three groups for superovulation procedures (n = 6). In all animals, controlled internal drug release, which was silicone impregnated with 1.38 g progesterone (CIDR 1380®, Pfizer/Zoetis), was introduced intravaginally in the evening of the beginning day (day 0) and 100 µg GnRH (Gestavet® GnRH, HIPRA) was administered (im) in the evening of Day 2.

In group 1 (im), starting on the evening of day 4, FSH (Stimufol, 500 µg porcine FSH and 100 µg porcine LH, Ulg FMV, Liège, Belgium) was administered (im) in decreasing doses (1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, and 1 mL) at 12-h intervals, for 4 d to induce superovulation. In group 2 (e), a single injection of FSH (500 µg) was administered into the epidural space in the evening of Day 4. In group 3 (sc), a mixture prepared with FSH (500 µg) added to 10 mL of Montanide ISA 206 (Seppic, France) adjuvant was administered as a single sc injection, in the evening of Day 4.

Preparation of the adjuvant: Stimufol (6 U) to be used for the six animals in group 3 was prepared by diluting it with 10 cc dilution solution included in its kit. Montanide ISA 206 vehicle (60 cc) was filtered into sterile 50 cc Falcon tubes a 40 µm injector filter and sterilised. Montanide and drug solution (60 cc each) were added into a previously autoclaved glass bottle. It was homogenised using a magnetic stirrer for 3 h in the cooling cabinet at $\geq 4^{\circ}\text{C}$. After the homogenisation, the drug was dosed into previously autoclaved glass bottles (20 cc each). The bottles were

sealed with sterile rubber cap and metal cap and kept at 4°C until used.

The cows in all groups were treated with 35 mg PGF2α (Estrumate, MSD, 263 mcg of Cloprostenol sodium, India) on Day 7 and 25 mg PGF2α on Day 8 in the morning, and the CIDR was removed from the vagina. Estrus were determined using a pedometer. On the 16th day of the experiment CL count in the ovary was measured by ultrasonography (Mindray DP 50, linear probe) and superovulation was determined based on the presence of 4 CL. For FSH measurement, blood was collected from the *v. jugularis* of all the animals before FSH administration and at h 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 18, 24, 36, 42, 48, 54, 60, 66, 72, and 96 after FSH administration. The collected blood was centrifuged at 2,000 rpm for 15 min to separate their serums. Serum samples were kept at -20°C until the assayed. Serum FSH (pFSH) concentration was determined by ELISA using a commercial kit (Porcine Follicle Stimulating Hormone Elisa Kit, Bioassay Technology Laboratory, Shanghai, China) with a detection limit of 0.017 ng/mL and a measurement range of 0.26 to 8.33 ng/mL. The sensitivity of the kit has been reported to be 0.28 U/L were read (Bioassay technology Laboratory Cat. No. E0183Po).

Homogeneity of variance test was used to determine the distribution of data. The normality of the data distribution was assessed with the Shapiro–Wilk test and the homogeneity of variance with the Levene's test. The data did not display normal distribution. Mann–Whitney U test was used for statistical analysis of superovulation responses. Mean serum FSH concentrations at each sampling time between groups were also evaluated with Mann–Whitney U test ($P>0.05$). SPSS-Statistics-22 package software was used for the tests. A P-value of 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

It was determined that all of the cows that underwent synchronization and superovulation procedures showed oestrus (100%). As a result of the ovarian examination performed on the 16th day, superovulation response (≥ 4 CL) was detected in 6 animals in Group 1 (im), 1 animal in Group 2 (e), and 4 animals in Group 3 (sc) (Table 1), total CL counts, and left ovary CL count in group 2 (e) were lower than in group 1 (im) ($P<0.05$), and similar to group 3 (sc) ($P>0.05$). Serum FSH concentrations were similar between groups for all sampling times (Table 2) ($P>0.05$).

Table 1. Superovulatory response in the three groups of the trials

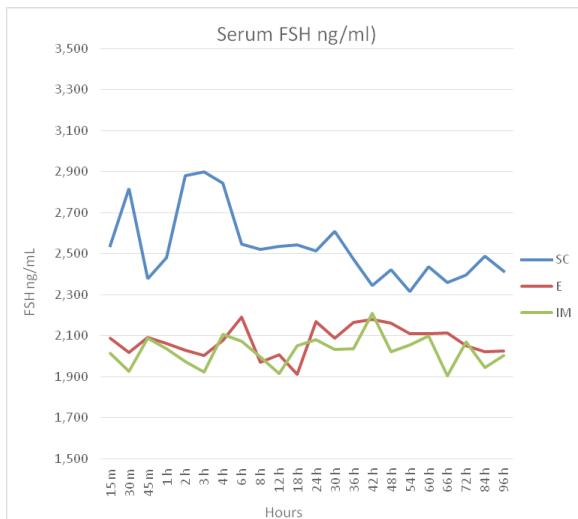
PARAMETER	Group 1 (im) (n:6)	Group 2(e) (n:6)	Group3 (sc) (n:6)
Superovulated animals (number)	6 ^a	1 ^b	4 ^{ab}
Superovulated animals (ratio - Mean+SD)	1,00±0,0 ^a	0,17±0,41 ^b	0,67±0,52 ^{ab}
CL/Right Ovary (mean)	2,33	1	1,67
Cl/Left Ovary (mean)	4,33 ^c	0,66 ^d	3,33 ^{cd}
TotalCL (mean)	6,67 ^e	1,66 ^f	5,13 ^{ef}
Follicle (total)	3	1	20

<0.05 was considered statistically significant. The difference between group im and group e was statistically significant.

Table 2. SerumFSH concentration(ng/ml) in intramusculer,epidural and, subcutaneus groups (mean ±SD)

Time	Group 1 (im) (n:6)	Sd	Group 2 (e) (n:6)	Sd	Group 3 (sc) (n:6)	Sd
0.25	2,015±0,388		2,087±0,517		2,539±1,760	
0.5	1,925±0,468		2,020±0,804		2,819±2,159	
0.75	2,089±0,357		2,091±0,630		2,379±0,994	
1	2,037±0,542		2,062±0,925		2,480±1,279	
2	1,973±0,363		2,031±0,451		2,881±1,847	
3	1,922±0,262		2,005±0,645		2,901±2,168	
4	2,107±0,543		2,077±0,679		2,844±2,204	
6	2,074±0,483		2,191±0,636		2,545±1,665	
8	1,996±0,400		1,971±0,542		2,522±1,734	
12	1,915±0,355		2,007±0,558		2,536±1,898	
18	2,051±0,374		1,913±0,539		2,543±1,956	
24	2,079±0,449		2,170±0,736		2,515±1,584	
30	2,032±0,406		2,089±0,731		2,611±1,549	
36	2,038±0,455		2,165±0,718		2,474±1,444	
42	2,208±0,436		2,182±0,675		2,346±1,031	
48	2,021±0,401		2,163±0,700		2,420±1,842	
54	2,055±0,394		2,111±0,780		2,314±1,423	
60	2,098±0,448		2,111±0,616		2,438±1,280	
66	1,905±0,331		2,115±0,664		2,36±1,069	
72	2,068±0,464		2,052±0,621		2,397±1,211	
84	1,945±0,544		2,022±0,554		2,487±1,428	
96	2,005±0,499		2,025±0,631		2,417±1,569	

<0.05 was considered statistically significant. No significant differences were noticed between groups in any of the sampling times.

**Figure 1.** Blood FSH concentration in the three groups of the trial.

The primary purpose of superovulation in cattle is to obtain many transferable embryos and to ensure acceptable pregnancy rates after transfer (Bo et al., 2002). However, the response of donor cows to gonadotropin administration, which is an important cost factor for the embryo transfer industry, is highly variable. The superovulation response is affected by several factors such as genetics, physiological characteristics (age, ovarian condition, and the follicle population), nutritional status, health conditions, season, farm management, different commercial preparations of FSH, dose, and route of administration (Mapletoft et al., 2002; Silve et al., 2009; Amiridis and Cseh 2012; Hussein et al., 2017).

For the stimulation of follicular development in donor cattle, conventionally, FSH extracted from the pituitary or produced as recombinant is administered twice a day, while eCG is administered (im) as a single-dose. Mapletoft et al., (2002). However, due to its long half-life, large follicles may remain on the ovary even after ovulation. Estrogen released from these follicles can adversely affect the number of embryos collected (Forcada et al., 2011). Another important disadvantage of eCG is that it causes antibody formation in the donor animal (Dieleman et al., 1993). During the embryonic development period, high blood estradiol levels can cause nucleus anomalies (Kanagawa 1995).

A single-dose FSH protocol was developed to be an alternative to conventional twice-daily treatment protocols for the superovulation of cows. The single-dose protocol is expected to increase usability in farms that lack adequately trained personnel.

It was reported that the administration (sc) of a single dose of pFSH was highly effective in inducing superovulation in cattle but was not as effective as the conventional method in cattle with lower body condition scores (Bo et al., 2017). It was notified that may be associated with low superovulation response slow absorption, the amount of adipose tissue, or the application site after single-dose sc pFSH administration (Bo et al., 1994).

In many studies, superovulation response has been achieved in cows with single-dose FSH treatment involving different adjuvants. In these studies, hyaluronic acid (HA, wt 2%) (Tribulo et al., 2011; Tribulo et al., 2012), PVP (wt 25% to 50%) (Takedomi et al., 1995; Yamamoto et al., 1995) or gelatin (wt 3.2%) (Hill et al., 1985) have been used. It has also been re-

ported that polyethylene glycol (PEG, 3% to 140%) supplemented with Novocain can be used (Kosovskij et al., 2016). Novocaine allows the blood supply to increase at the injection site by dilating the vessels. While PEG or PVP allows injection both SC and IM, good results can be obtained when HA is administered IM only. Gelatin is applied SC only in the neck area (Deguettes et al., 2020).

A study comparing the use of AH-gel with the conventional method reported that there was no difference in superovulation response and transferable embryo rates. After the im administration of a single-dose FSH dissolved in AH-gel, FSH was detected in the blood in the first 2 h, peaked in 12 h, and detected even after 3 d (Kimura, 2016).

In superovulation studies using PVP, results similar to the conventional method have been reported showing that it can be used successfully (Takedomi et al., 1995; Yamamoto et al., 1995; Tribulo et al., 2011; Chasombat et al., 2013). Hyaluronan is a polymer found in the reproductive organs of most animals and does not react when administered parenterally. It is a simple glycosaminoglycan with high biocompatibility, and when used as a diluent, it facilitates the continuous release of drugs (Oh et al., 2010). Studies using hyaluronan have reported similar results to those of the conventional method (Tribulo et al., 2010; Tribulo et al., 2011; Rogan et al., 2010). No superovulation study has been found reporting Montanide ISA 206. In the presented study, CL response close to the conventional method was produced in the group receiving FSH with Montanide ISA 206. Montanide ISA 206 was found to be as effective as other adjuvants used for slow release of FSH.

In a study, there was no statistically significant difference in superovulation response of Ongole cattle after im FSH administration at equal doses twice a day or single-dose epidural (Imron et al., 2016).

In another study, researchers compared that were two various amounts of decreasing doses, epidural, and im single doses FSH applications, and as a result, reported no significant statistical difference between the groups. Researchers reported that the reasons for this result are unclear however, may be dependent on due to the slow absorption of FSH (Taşdemir et al., 2012).

It has been reported that the distribution of drugs in the epidural space is affected by epidural fats. After injection, the drugs diffuse into the dura mater, epi-

dural veins, and epidural fat. Drugs absorbed from epidural fats can then gradually re-distribute into the dura mater and epidural veins (Lee et al., 2003 a, b). Therefore, it is thought that epidural fats contribute to the slow release of FSH into the peripheral circulation and that the FSH concentration can be maintained above baseline for more than 72 hours (Sakaguchi et al., 2018).

In the present study, no statistical difference was found between the conventional group administered with two doses per day for 4 d (Group 1) and the group 3 (sc) administered with FSH diluted in Montanide. There was no statistical difference between the epidural and sc administration groups (groups 2 and 3). A study comparing SC and epidural application could not be found. But, this result is consistent with the literature that compared sc or epidural FSF to the conventional method (Devroey et al., 2004; Chumchai et al., 2021; Sakaguchi et al., 2022). There was a difference between the conventional method and epidural administration ($P < 0.05$). The superovulation response obtained after epidural (Group 2-e) administration were found to be lower than classical administration (Group 1-im).

In a study, three different protocols (protocol 1: pFSH/im 4 times a day for 2 d, protocol 2: LH/iv 6 times a day for 6 d, and a combination of protocols 1 and 2) were applied to cross-bred beef heifers and blood FSH levels were measured. It was reported that FSH concentration increased continuously for the first 24 h, gradually decreased until h 60, and then decreased to the baseline level in Groups 1 and 3, and there was no change in FSH level in Group 2 (Crowe et al., 2001).

Coke et al. (1997) reported that in heifers treated with FSH (Group 1, ovine FSH/im 1 mg every 6 h for 30 h; Group 2, FSH and pulsatile LH 50 mg pig LH every h for 96 h; Group 3, saline) an increase of blood FSH concentration was observed up to thrice of that of the control group in Groups 1 and 2 (Coke et al., 1997).

Another study compared a control group with no stimulation, a group administered with 200 mg FSH twice a day (a total of 200 mg FSH), and groups administered (sc) with single-dose 200 and 300 mg FSH with hyaluronan. The results of this study revealed that, although the blood FSH remained at the baseline (0.5 ng/mL) in the control group, it reached 1 to 1.2 ng/mL in the group receiving FSH twice a day and re-

mained at these levels until h 60. (Vieira et al., 2015).

In a study, was reported that where a single dose of sc FSH was administered to the different areas in the body of Holstein cattle, the blood FSH level increased to 1 to 1.5 ng/mL in 12 h and then decreased to the baseline level. In the same study, researchers reported after a single-dose im and a single-dose sc, blood FSH levels were increased up to 2.5 ng/mL and 1.2 ng/mL in the first 12 h and were decreased to the baseline level after 24 h in all groups (Bo et al., 2017).

It was reported that following single-dose sc administration of two different doses of FSH (20 and 30 AU-armour units) diluted in 10 or 50 mL of normal saline, blood FSH concentrations reached a maximum of 1.5 ng/mL after 20 AU, and 3.5 ng/mL after 30 AU (Hirazumi et al., 2015).

In the present study, serum FSH values increased above 1.9 ng/mL in all three groups. Serum FSH values were found to be higher than in the other groups and remained above 2.5 ng/mL until the 36th hour

Epidural administration was insufficient in creating a superovulation response. This result, which is not consistent with other studies. Studies should be carried out by using other solvents or adjuvants instead of the solvent used during the dissolution of lyophilized FSH. After sc application with Montanide ISA 206, blood FSH levels were higher than other administration routes and the superovulation response was close to that of the traditional method, suggesting that the use of adjuvant may be advantageous. Due to the ease of application, it was concluded that the number of studies should be increased by using different adjuvants and FSH preparations.

CONCLUSIONS

In conclusion, sc single-dose administration of FSH with adjuvant (Montanide ISA 206) yields results similar to the multiple in administration and optimised protocols can be pursued by testing the other Montanide species or using adjuvants.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

FUNDING

The presented study was supported by ***** University Scientific Research Projects Coordinator (BAP) (Project no18401139).

REFERENCES

Amiridis GS and Cseh, S 2012. Assisted reproductive technologies in the management of small ruminants. *Anim Reprod Sci*, 130:152-161. <https://doi.org/10.1016/j.anireprosci.2012.01.009>

Bo GA, Hockley DK, Nasser LF, Mapleton RJ, 1994 Superovulatory response to a single subcutaneous injection of Folltropin-V in beef cattle. *Theriogenology*, 42:963e75.

Bo GA, Baruselli PS, Moreno D, CutaiaL, Caccia M, Tríbulo R, Tríbulo H, Mapleton RJ, 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology* 2002, 57:53-72. doi: 10.1016/s0093-691x(01)00657-4.

Bo GA and Mapleton RJ, 2014. Historical perspectives and recent research on superovulation in cattle. *Theriogenology*, 81:38-48. doi: 10.1016/j.theriogenology.2013.09.020.

Bo GA, Rogan DR, Mapleton RJ, 2017. Pursuit of a method for single administration of pFSH for superstimulation in cattle: What we have learned. *Theriogenology*, 112:26-33. doi: 10.1016/j.theriogenology.2017.09.034.

Bowen RA, 2003. Embryo transfer in domestic animals. In: *Veterinary endocrinology and reproduction*. 5th ed. USA, Blackwell Publishing Company, pp.547-571.

Chasombat J, Sakhong D, Nagai T, Parmpai R, Vongpralub T, 2013 Superstimulation of follicular growth in Thai native heifers by a single administration of follicle stimulating hormone dissolved in polyvinylpyrrolidone. *J Reprod Dev*, 59:214-218. Doi: 10.1262/jrd.2012-119.

Chumchai R, ratsiri T, ratchamak R, vongpralub T, Boonkum W, Chankitisakul V, 2021. Ovarian responses and FSH profiles at superovulation with a single epidural administration of gonadotropin in the Thai-Holstein crossbreed. *Anim Reprod*, 18: e20210053.

Crowe MA, Kelly P, Driancourt MA, Boland MP, Roche JF, 2001 Effects of FSH with and without LH on serum hormone concentrations, follicle growth and intrafollicular estradiol and aromatase activity in GnRH-immunized heifers. *Biology of Reproduction*, 64:368-374. doi: 10.1095/biolreprod64.1.368.

de Sousa FC, Melo CHS, Filho AT, Avelar SRG, Moura A, Martins JAM, Freitas VJ, Teixeira DIA, 2011 Ovarian Follicular Response to different hormonal stimulation treatments in caninde goats. *Anim Reprod Sci*, 125:88-93. doi: 10.1016/j.anireprosci.2011.02.015. Epub 2011 Feb 18.

Deguettes Q, Fattal E, Moreau M, Lego E, Bochet A, 2020 Controlled delivery of follicle-stimulating hormone in cattle. *International Journal of Pharmaceutics*, 590:119904. <https://doi.org/10.1016/j.ijpharm.2020.119904>

Devroey B, Fauser BC, Platteau P, Beckers NG, Dhont M, Mannaert BM, 2004. Induction of multiple follicular development by a singledose of longacting recombinant follicle-stimulatinghormone (FSH-CT-P,corifollitropin alfa) for controlledovarian stimulation beforein vitro fertilization. *The Journal of Clinical Endocrinology and Metabolism*, 89:2062-2070.

Dieleman SJ, Bevers MM, Vos PLAM, De Loos FAM, 1993 PMSG/anti-PMSG in cattle: a simple and efficient superovulatory treatment? *Theriogenology*, 39: 25e41.

Gordon I, 2004. Reproductive technologies in farm animal. 1st ed. CABI International publishing, Wallingford, UK.

Hill KG, McFarland CW, Rorie RW, Viker SD, Godke RA, 1985 A single 50 mg injection of follicle stimulating hormone (FSH) for superovulation of embryo donor cattle. *Theriogenology*, 23,196.

Hirazumi S, Nishinomiya H, Oikawa T, Sakagami N, Sano F, Nishino O, Kurahara T, Nishimoto N, Ishiyama O, Hasegawa Y, Hashiyada Y, 2015. Superovulatory response in Japanese Black cows receiving a single subcutaneous porcine follicle-stimulating hormone treatment or six intramuscular treatments over three days. *Theriogenology*, 83:466-473. doi: 10.1016/j.theriogenology.2014.09.012. Epub 2014 Sep 21.

Hussein AM, Al-Shakaili YO, Al-Ismaily AN, Al-Alawi HH, 2017 Effect of different doses of FSH on superovulation, production and quality of embryo in North Omani Cattle breed. *Indian J Anim Res*, 51:8-14.

doi: 10.18805/ijar.v0i0F.7003

Imron M, Supriatna I, Amrozi, Setiadi Ma, 2016 Superovulation Responses in Ongole Cattle Crossbreed Treated with a Single Epidural Injection of Follicle Stimulating Hormone. *Journal Veteriner Maret*, 17:78-87. doi: 10.19087/jveteriner.2016.17.1.78

Kanagawa H, Shimohira I, Saitoh N, 1995 Manual of Bovine Embryo Transfer. 2nd ed. National Livestock Breeding Center MAFF, JAPAN.

Kimura K, 2016 Superovulation with a single administration of FSH in aluminum hydroxide gel: a novel superovulation method for cattle. *Journal of Reproduction and Development*, 62:423-429. doi: 10.1262/jrd.2016-066

Kosovskij GY, Popov DV, Brigida AB, 2016 Method of induction of superovulation in cows-donors embryos with prolongation of activity of hypophysial gonadotropins. RU2617042C2.

Lee I, Yamagishi N, Oboshi K, Yamada H, 2003a. Effect of epidural fat on xylazine-induced dorsolumbar epidural analgesia in cattle. *Vet J*, 165: 330-332.

Lee I, Yamagishi N, Oboshi K, Yamada H, 2003b. Antagonistic effects of intravenous or epidural atipamezole on xylazine-induced dorsolumbar epidural analgesia in cattle. *Vet J*, 166: 194-197.

Mapleton RJ, Kristina BS, Gregg PA, 2002 Recent advances in the superovulation in cattle. *Reprod Nutr Dev*, 42:601-611. doi: 10.1051/rnd:2002046.

Ochea M, Pascal M, Şanea A, Birtoiu AI, 2015. The effect of epidural administration of FSH in bovine superovulation protocol. *Scientific Papers Series D Animal Science*,58: 217-220.

Oh EJ, Park K, Kim KS, Kim J, Yang J-A, Kong J-H, Lee My, Hoffman AS, Hahn SK, 2010 Target specific and longacting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. *J Control Release*, 141:2-12. doi: 10.1016/j.jconrel.2009.09.010.

Rahman ANMA, Abdullah RB, Khadijah WEW, 2008 A Review of Reproductive Biotechnologies and Their Application in Goat. *Biotechnology*, 7:371-384. doi: 10.3923/biotech.2008.371.384

Rogan D, Tribulo A, Tribulo H, Tribulo R, Guerrero CD, Tribulo P, Mapleton RJ, Bo G, 2010 Dose titration for superovulation of Brangus, Bonsmara and Braford donors with Folltropin-V by a single intramuscular injection. *Reprod Fertil Dev*, 22:365. doi: 10.1071/RD-v22n1Ab416

Sağırkaya H, 2009 Embryo transfer in cattle and the importance of embryo transfer for Turkey. *Uludag Univ J Fac Vet Med*, 28:11-19.

Satılmış M, Yılmaz MA, Sevgi R, Karşahan T, Kızıl SH, Okuroğlu A, Ertem TB, Yıldırır ZT, 2021. The effect of epidural application of fsh on superovulatoryresponse in eastern anatolian red cow. *Livestock Studies*, 61:82-87.

Sakaguchi K, Ideta A, Yanagawa Y, Magano M, Katagiri S, Konishi M, 2018. 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. *J Reprod Dev*, 64:451-455

Sakaguchi K, Suda T, Nimpetch N, Kawano K, Yanagawa Y, Katagiri S, Yoshioka K, Nagano M, 2022. Plasma profile of follicle-stimulating hormone and sex steroid hormones after a single epidural administration of follicle-stimulating hormone via caudal vertebrae in Holstein dry cows. *Anim Sci J*, 93: e13696. <https://doi.org/10.1111/asj.13696>.

Silva JJ, Alvarez RH, Zanenga CA, Pareira GT, 2009 Factors affecting embryo production in superovulated Nelore cattle. *Anim Reprod*, 6:440-445,

Son DS, Choe CY, Choi SH, Cho SR, Kim HJ, Han MH, Ryu IS, Suh GS, Kim UH, Kim IH, 2007 Effect of estradiol benzoate or GnRH treatment prior to superstimulation in CIDR-treated, Korean native cows (Bos taurus). *Anim Reprod Sci*, 100: 14-21. doi: 10.1016/j.anireprosci.2006.06.005. Epub 2006 Jul 28.

Takedomi T, Aoyagi Y, Konishi M, Kishi H, Taya K, Watanabe G, Sasamoto S, 1995 Superovulation of Holstein heifers by a single subcutaneous injection of FSH dissolved in polyvinylpyrrolidone. *Theriogenol-*

ogy, 43:1259-1268. doi: 10.1016/0093-691x(95)00097-r.

Taşdemir U, Satılmış M, Karaşahin T, Kızıl SH, Kaymaz M, Imai K, 2012 The effect of single epidural plus intramuscular injection of FSH on superovulatory response in Anatolian Black cow. Ankara Univ Vet Fak Derg, 59:211-216. https://doi.org/10.1501/Vetfak_0000002527

Tríbulo A, Rogan D, Tribulo H, Tribulo R, Alasino RV, Beltramo D, Bianco İ, Mapleton RJ, BO GA, 2011 Superstimulation of ovarian follicular development in beef cattle with a single intramuscular injection of Folltropin-V. Anim Reprod Sci, 129:7-13. doi: 10.1016/j.anireprosci.2011.10.013

Tríbulo A, Rogan D, Tríbulo H, Tríbulo R, Mapleton RJ, Bo GA, 2012 Superovulation of beef cattle with a split-single intramuscular administration of Folltropin-V in two concentrations of hyaluronan. Theriogenology, 77:1679-1685. DOI: 10.1016/j.theriogenology.2011.12.013

Tríbulo A, Tríbulo H, Tríbulo R, Guerrero CD, Tríbulo P, Rogan D, Mapleton RJ, Bo G, 2010 Superovulation of Angus donors with a single intramuscular injection of Folltropin- V. Reprod Fertil Dev, 22:367. doi: 10.1071/RDv22n1Ab420

Vieira LM, Rodrigues CA, Castro Netto A, Guerreiro BM, Silveira CRA, Freitas BG, Bragança LGM, Marques KNG, Sa Filho MF, Bo GA, Mapleton RJ, Baruselli PS, 2015 Efficacy of a single intramuscular injection of porcine FSH in hyaluronan prior to ovum pick-up in Holstein cattle. Theriogenology, 84:1-10. doi: 10.1016/j.theriogenology.2015.10.036. Epub 2015 Nov 6.

Yamamoto M, Ooe M, Kawaguchi M, Suzuki T, 1995 Dose Response to a Single Intramuscular Injection of FSH Dissolved in Polyvinylpyrrolidone for Superovulation in Cows. J Reprod Dev, 41,93-96. <https://doi.org/10.1262/jrd.41.93>