Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.006

RESEARCH ARTICLE

Effects of Different Sources of FSH, Injection Methods, Embryo Collection Methods and Seasons on the Superovulation Response in Belgian Blue Cattle

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ARTICLE HISTORY (22-392)

Received:	November 23, 2022
Revised:	January 6, 2023
Accepted:	January 8, 2023
Published online:	January 22, 2023

Key words:
Belgian Blue cattle
Superovulation
FSH
Embryo collection methods

ABSTRACT

Superovulation is a key technique in embryo transfer. This study aimed to investigate the effects of two different sources of FSH (Ningbo Second Hormone Factory – NSHF™, China and Vetoquinol, Australia), FSH injection methods (decreasing dose and equal dose), embryo collection methods (injection-type flushing tube and three-channel type flushing tube) and seasons (winter and spring) on superovulation response in Belgian Blue cattle. For this purpose, 80 Belgian Blue cattle (aged 2-3 years, parity=1) were randomly divided into four groups, with each group was further divided into two sub-groups. Superovulation experiments were carried out through Cue-Mate+FSH+PGF2α+GnRH, followed by artificial insemination. The effects of the above four factors on superovulation response were determined by comparing the mean numbers of corpora lutea, total embryos and viable embryos. The results showed non-significant difference in the effects of FSH produced by NSHF™, China and Vetoquinol, Australia on the superovulation response, though the cost of NSHF™ was lower. Moreover, the superovulation response to decreasing-dose injection of FSH was significantly better than that of equal dose injection ($P<0.05$). Regarding embryo collection methods, the results of injection-type flushing tube were significantly better than those of three-channel type flushing tube ($P<0.05$). Similarly, superovulation results during spring season were significantly better than those during winter ($P<0.05$). In summary, the best method of superovulation in Belgian Blue cattle is to inject FSH with reduced dose/s and collect embryos through injection-type flushing tube method during spring season.

To Cite This Article: Huang F, Niu P, Wang J, Liu B, Yue C-G, Fang D, Qian W-X and Gao Q-H, 2023. Effects of different sources of FSH, injection methods, embryo collection methods and seasons on the superovulation response in belgian blue cattle. Pak Vet J, 43(1): 204-208. http://dx.doi.org/10.29261/pakvetj/2023.006

INTRODUCTION

Belgian Blue is a beef breed of cattle from central and northern Belgium, which is characterized by early maturity, high adaptability to environment and increased muscle development with low fat. However, due to the lack of myostatin, increased muscle development (especially the gluteal muscles) squeezes the birth canal, so around 90% calves are delivered through the caesarean section (Tuska *et al.*, 2021 & 2022), which adversely affects their production in countries with underdeveloped veterinary facilities (Kolkman *et al.*, 2010). However, multiple ovulations and embryo transfer (MOET) technology can effectively improve the production capacity of this breed.

Superovulation is a key step during embryo transfer. In cattle, superovulation is induced through the administration of exogenous gonadotropins (FSH or eCG) in the donor cow, which leads to an increase in the number of developed and mature follicles (Kimura, 2016). This increases the discharge of mature oocytes in the donor cow, resulting in increased number of calves following artificial insemination and embryo transfer (Gomez-Leon *et al.*, 2018; Cirit *et al.*, 2019). However, the success of superovulation is affected by individual variations in donor cows, efficacy of gonadotropins, embryo collection methods, seasonal variations and similar other factors (Neto *et al.*, 2005; Mikkola *et al.*, 2019; Khodadadi *et al.*, 2022).

Presently, FSH is commonly used in superovulation (Chacon *et al.*, 2020; Gutiérrez-Reinoso *et al.*, 2022). However, different FSH preparations have FSH in varying amounts. Consequently, the source, dose and method of FSH administration can directly affect the outcome of superovulation in donor cows (Palomino *et al.,* 2017; Mikkola *et al.,* 2019). Previous studies have shown that multiple injections of the total dose of FSH is more effective than single FSH dose (Bó and Mapletoft, 2018; Sakaguchi *et al.,* 2018). Hiraizumi *et al.* (2015) subcutaneously injected FSH in donor cows and achieved a good superovulation response. Embryo collection is also an important part of MOET, and the success of embryo collection can be measured by the quantity and quality of embryos collected (Szabari *et al.,* 2008). There are two main protocols for non-surgical embryo collection: three-channel type and injection-type flushing tubes.

Although the effects of different methods and other influencing factors affecting the outcome of bovine superovulation have been investigated, the mean number of viable embryos obtained following superovulation could not be improved (Bó and Mapletoft, 2014). Therefore, the main aim of this study was to compare the effects of FSH from different sources, hormone injection methods, embryo collection methods and season on the bovine superovulation response, with a view to determine the optimal scheme with low cost and good results in terms of quality embryos.

MATERIALS AND METHODS

Experimental animals and feeding management: A total of 80 healthy Belgian Blue cows (BCS=5~7, aged 2-3 years, parity=1 and body weight=600±50Kg) were selected from the farm of Aral City, Xinjiang, China (40°54′N, 81°30′E). Rearing conditions for these cows were quite identical. B-mode ultrasound and rectal examination of these cows revealed normal reproductive organs, with no obvious cervical curvature, uterine horn symmetry, normal ovarian function and no reproductive diseases. During the experiment, donor cows were semi-housed and fed with Total Mixed Ration (TMR) diet. Roughage was mainly straw, which was fed at 10:00 and 17:00 hours daily. Adequate clean drinking water was supplied *ad lib*. The forage, feed and feeding procedures were relatively stable throughout the study.

Main reagents: The reagents used in this study were obtained from the following companies: Controlled internal drug release vaginal device containing 1.56g progesterone (Cue-Mate) was obtained from Vetoquinol™ Australia; FSH was obtained from Vetoquinol™ (700 IU/box, 150$/box, 20mL/box) and Ningbo Second Hormone Factory–NSHF™, China (1000 IU/box, 59.32$/box, 10mL/box); Semen straws, 0.25 mL/straw was obtained from Qinhuangdao, China (the same batch of frozen semen from the same Belgian Blue bull); Prostaglandin F2α (PGF2α, 10 bottle/box, 0.2mg/bottle) and synthetic gonadotropin-releasing hormone (GnRH, 100μg/bottle) were purchased from NSHF™, China.

Experimental design: In this study, Cue-Mate+FSH+PGF2α+GnRH protocol was used for the superovulation of donor cows (Mikkola *et al.,* 2019). The experimental scheme is shown in Fig. 1. The experimental cows were divided into four main groups based on different FSH sources, FSH injection methods, embryo collection methods and seasons (winter - November & December, 2021 and spring – March, 2022), as shown in Table 1. The Cue-Mate was inserted on day 0 and respective FSH preparation was intramuscularly injected twice daily at 12h interval from day 9 to day 12. The injection methods were equal-dose injection (200μg for each injection for 4 days) and reduced-dose injection (200μg twice on the 9th day, 150μg twice on the 10th day, and 100μg twice on the 11th day, with 50μg twice on the 12th day). On day 11, 30μg PGF2α were injected following FSH injection in the morning, and Cue-Mate was removed after FSH injection in the afternoon. After injecting 30μg GnRH on day 13, follicles development was monitored by using rectal examination and B-mode ultrasound examination. The cows were inseminated at 8-12h after the onset of estrus, followed by one insemination at 12h interval, and the third insemination if estrus persisted. On the 19th day, the number of corpora lutea formed on ovaries was detected and recorded by rectal examination and B-mode ultrasound, followed by non-surgical embryo collection.

Embryo collection and grading: Firstly, the donor cows were injected with 150μg lidocaine hydrochloride for local anesthesia. After proper disinfection, an expansion rod was inserted into the cervix, followed by insertion of an embryo collection tube into the uterus. According to the size of the uterine horn, the balloon of the tube was inflated so that the uterine horn is plugged.

For injection-type embryo flushing tube (Fig. 2a), a 50ml syringe was used to extract phosphate buffer solution (PBS). The PBS was injected from the tube into the uterine horn, the uterine horn was gently massaged through the rectum, and PBS was extracted into the embryo collection cups. The uterine horn (on each side) was washed with 500 mL of PBS. For the use of Three-channel type embryo flushing tube (Fig. 2b), the uterine horn (at each side) was washed with 500mL of PBS, and washed PBS was collected into the embryo collection cups. The total time taken for embryo collection by each method was also recorded.

The collected embryos were evaluated under the microscope. According to the morphology and development status, embryos were categorized into four grades: 1, 2, 3 and 4, as described earlier (Bó and Mapletoft, 2013). Embryos of grades 1 and 2 were defined as viable embryos for this study.

Statistical analysis: In this experiment, SAS® 9.4 software was used to analyze the data obtained from each group (the number of corpora lutea, total embryos, viable embryos, and the time of flushing embryos for embryo collection methods). Comparisons between groups were made using independent t-tests. The experimental results were expressed as mean±standard error (SE) and $P<0.05$ was used as the criterion for significant difference.

RESULTS

Effect of FSH injection from two sources on superovulation: The superovulation results of FSH from two sources in Belgian Blue cattle are shown in Table 2. Superovulation was recorded in all 10 (100%) cows of Vetoquinol™ group against 90% (9/10) cows in NSHF™ group. The mean number of corpora lutea formed, total embryos and viable embryos collected by using FSH from Australian Vetoquinol™ were slightly higher than those

Table 1: Detail of different schemes adopted for the assessment of superovulation response in Belgian Blue cattle

	Number of donors	FSH source	FSH injection method	Method of collecting embryos	Starting date	Season
Scheme	10	Vetoquinol	equal-dose injection	Injection type	November 10, 2021	Winter
I	10	Ningbo Second Hormone Factory				
Scheme	10	Ningbo Second Hormone Factory	equal-dose injection	Injection type	December 5, 2021	Winter
II	10		decreasing-dose injection			
Scheme	10	Ningbo Second Hormone Factory	decreasing-dose injection	Injection type	March 5, 2022	Spring
III	10			three-way type		
Scheme	10	Ningbo Second Hormone Factory	decreasing-dose injection	Injection type	December 13, 2021	Winter
IV	10				March 15, 2022	Spring

Table 2: Effects of two different FSH sources on superovulation response in Belgian Blue cattle (mean±SE)

FSH source	Number of donors	Mean number of corpora lutea	Mean number of embryos	Mean number of viable embryos
Vetoquinol™	10	7.70±2.45[ns]	5.30±1.88[ns]	3.70±1.63[ns]
NSHF™	10(9)	7.20±3.32[ns]	4.80±2.20[ns]	3.20±1.68[ns]

Values within parentheses indicate number of cows which showed superovulation response: ns = Non-significant difference between values within a column.

Table 3: Effects of two FSH injection methods on superovulation response in Belgian Blue cattle (mean±SE)

FSH injection method	Number of donors	Mean number of corpora lutea	Mean number of embryos	Mean number of viable embryos
Identical dose	10	7.60±1.26[a]	5.00±1.24[a]	2.80±1.13[a]
Decreasing dose	10	9.10±1.72[b]	6.40±1.64[b]	4.10±1.52[b]

Values with different superscripts within a column differ significantly (P<0.05).

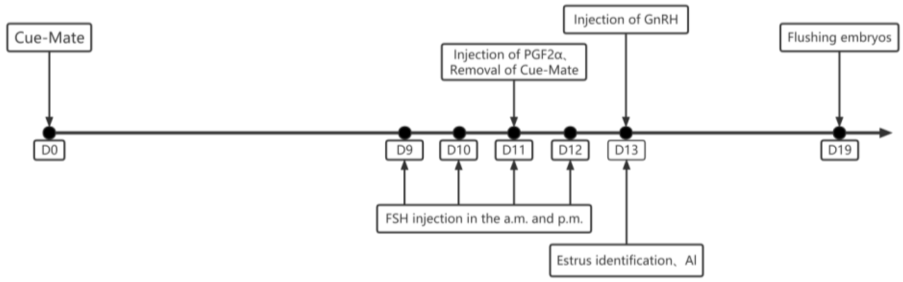

Fig. 1: The scheme of protocol for induction of superovulation in Belgian Blue donor cows.

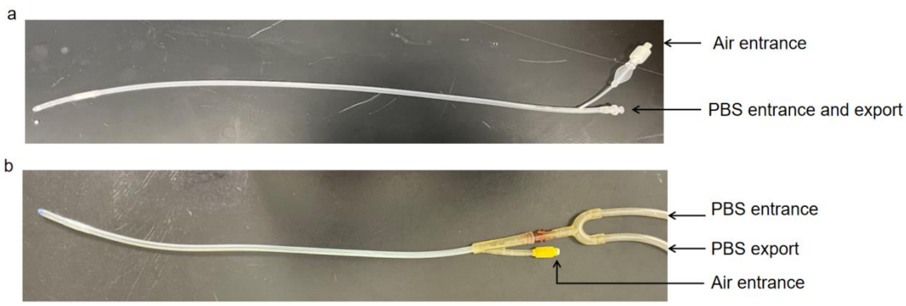

Fig. 2: Embryo collection/flushing tubes of Injection-type (a) and Three-channel type (b).

collected following the use of FSH from NSHF™. However, the differences in these parameters following the use of FSH obtained from the two sources were statistically non-significant.

Effect of two FSH injection methods on superovulation: The effects of two injection methods of FSH (equal-dose injection for four days and decreasing-dose injection for four days) on superovulation response are shown in Table 3. It can be seen from Table 3 that all 20 Belgian Blue cattle of this group showed superovulation reaction. The mean number of corpora lutea in the decreasing-dose injection group was significantly higher than that in the equal-dose injection group ($P<0.05$). Similarly, the mean numbers of total embryos and viable embryos of decreasing-dose injection method were significantly higher than those of equal-dose injection method.

Effect of embryo collection methods on embryo collection: Out of 10 cows subjected to injection-type and three-channel type embryo collection method each, 9 cows (90%) exhibited superovulation (Table 4). There was non-significant difference in the mean number of corpora lutea between the injection-type and the three-channel type embryo collection methods. However, the mean number of total embryos and the mean number of viable embryos collected by the injection type method were significantly higher than those collected by three-channel type collection method (P<0.05). On the other hand, the mean time required for embryo collection per head by injection type was significantly shorter than that for three-channel type method (P<0.05).

Effects of seasons on superovulation: Superovulation tests of Belgian Blue donor cows were conducted during winter and spring seasons in this study (Table 5). It can be seen from Table 5 that superovulation response was recorded in all 10 (100%) cows during spring against 90% (9/10) cows in winter season. The mean number of corpora lutea formed, total embryos and viable embryos collected

Table 4: Effects of different embryo collection methods on embryo collection in Belgian Blue cattle (mean±SE)

Method of collecting embryos	Number of donors	Mean number of corpora lutea	Mean number of embryos	Mean number of viable embryos	Mean time per head (min)
Injection-type	10(9)	8.50±3.30[ns]	6.50±2.41[a]	4.00±1.49[a]	14.91±1.05[a]
Three-channel type	10(9)	7.40±2.98[ns]	4.40±1.95[b]	2.70±1.25[b]	20.86±2.54[b]

Values within parentheses indicate number of cows that showed superovulation response: Values with different superscripts within a column differ significantly (P<0.05): ns = Non-significant difference between values within the same column.

Table 5: Effects of different seasons on superovulation response of Belgian Blue cattle (mean±SE)

Season	The number of donors	Mean number of corpora lutea	Mean number of embryos	Mean number of viable embryos	Weather temperature range (°C)
Winter	10(9)	6.80±2.69[a]	5.20±1.98[a]	3.30±1.33[a]	-8 ~ -1
Spring	10	8.80±1.22[b]	6.70±0.82[b]	4.50±1.08[b]	4 ~ 12

Values within parentheses indicate number of cows showed superovulation response: Values with different superscripts within a column differ significantly (P<0.05)

during spring season were higher than those collected during the winter season. Statistical analysis of the data revealed that the differences in these parameters between winter and spring seasons were statistically significant (P<0.05), indicating that spring season was more favorable than winter season for superovulation in Belgian Blue cows.

DISCUSSION

FSH is the most widely used gonadotropin in bovine superovulation with a decisive role in the superovulation of donor cows (Chacon *et al.,* 2020; Gutiérrez-Reinoso *et al.,* 2022). It increases the number of mature follicles (Kimura, 2016) and the discharge of multiple eggs (Cirit *et al.,* 2019). However, previous studies have suggested that different FSH preparations can affect the superovulation response and the number/quality of viable embryos obtained from donor cows (Sendag *et al.,* 2008). Since FSH has no unified international unit, the activity of FSH produced by different companies could be variable. FSH supplied by different companies, even different batches of the same company, can vary in purity, titer and activity, which can affect superovulation response of donor cows (Palomino *et al.,* 2017). In this study, FSH produced by NSHF™ of China and Vetoquinol™ company of Australia showed non-significant difference in the superovulation response monitored in terms of number of corpora lutea formed, numbers of total and viable embryos collected. However, the FSH produced by NSHF™ of China had a lower cost, being more easily accessible from an economic point of view. The FSH produced by NSHF™ costs $94.91 per head when identical dose injection method is used, while decreasing-dose injection method costs $59.32 per head. The corresponding values of the cost for FSH produced by Vetoquinol™ in Australia are $120 and $75 per head, respectively.

In the process of superovulation in cattle, relatively high levels of gonadotrophins are required to stimulate the resting primordial follicles to become mature follicles (Reddy *et al.,* 2010). Studies have also shown that FSH has to be injected twice-daily for four consecutive days, so that the superovulation response is enhanced. In this study, two FSH injection methods were compared (an equal-dose injection for four days and decreasing-dose injection for four days). The results showed that the superovulation response of decreasing-dose injection was significantly better than that of equal-dose injection method (P<0.05). The decreasing-dose injection method seems to be more in line with the physiological changes of FSH in the ovary and blood of donor cows (Facioli *et al.,* 2020). According to Tríbulo *et al.* (2012), decreasing-dose injection method can reduce the utilization of FSH, which will prevent FSH waste, thereby reducing the total cost.

Embryo collection is an important part of the superovulation and embryo transfer process. The three-channel type and the injection type flushing tube methods are widely used for non-surgical embryo collection (Neto *et al.,* 2005). However, for embryo collection one of these methods has to be selected (Szabari *et al.,* 2008), and there is almost no comparative study between the three-channel embryo collection and the injection-type embryo collection methods. In this study, we compared the three-channel embryo flushing and the injection-type embryo flushing tube methods for embryo collection. The results showed that the number of total and viable embryos collected by the injection type method were significantly higher than the three-channel type method (P<0.05). This could have been due to the fact that the mean time required for completion of embryo collection process by the injection type method in this study was significantly shorter (14.91±1.05 minutes) than that for the three-channel type method (20.86±2.54 minutes).

The effect of season on superovulation response of donor cows looks to be mainly due to the temperature (Seifi-Jamadi *et al.,* 2020). Mikkola *et al.* (2019) found that temperature significantly affected the superovulation response of donor cows. In contrast, Barati *et al.* (2006) recorded non-significant variations in the superovulation response between winter and summer seasons in Sistani cattle. The results of the present study showed that the superovulation response in spring season was significantly better than that in winter (P<0.05). This is consistent with the results of Hansen *et al.* (2001), indicating that the season had a significant impact on the superovulation response of Belgian Blue cattle, which are native to Belgium. The mean temperature in Belgium during winter is 1~6ºC, while in spring it is 7~13ºC. However, the mean temperature in winter in the experimental area was -8~-1ºC, and in spring it was 4~12ºC. It is possible that the too low mean temperature in the test area during winter has adversely affected the superovulation response of Belgian Blue cattle.

Conclusions: This study showed that use of FSH produced by Vetoquinol™, Australia and NSHF™, China had no difference in superovulation response in Belgian Blue cows, though NSHF™ was more cost-effective. Decreasing-dose of FSH and spring season were more conducive to superovulation in cattle breed. Similarly, injection-type embryo flushing tube was more conducive to embryo collection compared to three-channel type. In conclusion, the decreasing-dose injection method for superovulation in spring, together with superovulation through injection-type embryo flushing method, renders superovulation strategy quite cost-effective in Belgian Blue cattle.

Ethical approval: All animal experiments in this study were conducted according to the Regulations and Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China, revised in 2004). The present study was also approved by the Institutional Animal Care and Use Committee of Tarim University, Beijing, China.

Acknowledgements: This study was supported by the Key Technology Research Project for Population Quality Improvement of Southern Xinjiang Beef Cattle (No. S2020BB287), and by the Demonstration Project for Hybrid Breeding of Belgian Blue Cattle (No. TDZKJS202002).

Authors contribution: Fei Huang and Qing-hua Gao conceived the idea and drafted the project, while Qing-hua Gao and Wen-xi Qian arranged necessary funding. Qing-hua Gao, Fei Huang and Di Fang carried out experimental work; Fei Huang and Cheng-guang Yue statistically analyzed the data. Fei Huang, Qing-hua Gao, Peng Niu, Jie Wang and Bo Liu prepared the original draft and conducted necessary editing. All authors interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

Declaration of Interest: The authors declare that there are no competing interests associated with this manuscript.

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