Research Article

Effect of Superovulation Number and Interval on Cattle: A Managemental View

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Abstract | The current study was conducted to assess the effect of superovulation number and interval on superovulation outcomes and hormonal profile of Chinese Holstein heifers. About 97 healthy Chinese Holstein heifers underwent three consecutive superovulation treatments. The interval between the first and second treatments was 43–58 days (long interval), while the interval between the second and third treatments was 28 days (short interval). The superovulation outcomes including the numbers of unfertilized ova, degenerated embryos, available embryos, and total embryos were recorded. Hormonal profiles of the heifers were evaluated at three phases during each superovulation round. The first phase was assessed on day 0 (the time of CIDR insertion for intravaginal progesterone). The second phase was assessed on day 9 of the superovulation protocol, at the end of FSH administration and the first artificial insemination (AI). The third phase was assessed on day 16 of superovulation during embryo flushing. The results revealed that repeated superovulation significantly decreased the numbers of available embryos and total embryos (P = 0.002). Repeated superovulation also reduced the concentrations of FSH and AMH, particularly at the time of embryo flushing (P = 0.001). These findings suggest that repeated superovulation has adverse effects on ovarian activity, ovarian function, and hormonal profiles in Chinese Holstein heifers. Repeated superovulation negatively affected embryo yield and several reproductive hormones. From a management perspective, repeated superovulation may not be beneficial for cow performance or welfare.

Keywords | Holstein cows, Superovulation number and interval, Ovarian activity, Reproductive performance, Farm management, Animal production

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INTRODUCTION

Superovulation allows female animals to generate an increased number of oocytes and embryos, significantly enhancing the probability of successful pregnancies compared to the standard reproductive cycle, hence maximizing output (Bó and Mapletoft, 2014; Çizmeci and Güler, 2018). Superovulation can improve fertility and

enhance genetic traits of cattle (Hasler, 2014; Zago *et al*., 2023). Superovulation in cattle was first documented in United States by administration of gonadotropins several days prior to the expected estrus to stimulate numerous ovulations (Gordon, 1975). The efficiency of producing transferable embryos following superovulation in Japanese Black (JB) cattle is a key determinant of the economic viability of embryo production for both farmers and veterinarians (Okawa *et al*., 2025).

More than ten viable oocytes can be obtained with each estrus from heifers and cows that have undergone superovulation. The primary tenet of superovulation involves the provision of follicular stimulation through the infusion of follicle-stimulating hormone (FSH) and equivalent substances (Hussein *et al*., 2014). Multiple factors influence the results of superovulation and embryo retrieval. These encompass factors linked to animals, the environment, and management (Mikkola, 2017). Animal related factors include age, breed, parity, and hormones. Early reports about superovulation in cattle revealed the impact of age on superovulation and embryo transfer results. The quantity of ova, embryos, and transferable embryos obtained was additionally impacted by the donor's cow age (Breuel *et al*., 1991). Recently, superovulation response isn't significantly affected by the age of the animal due to the improvement of genetic selection (Mikkola, 2017). Breed differences affect superovulation in cattle through the number of oocytes and embryos that were recovered, as recorded by Breuel *et al*. (1991) or due to the variation in the incidence of ovulation or may be attributed to the hormone concentrations difference between breeds (Pfeiffer *et al*., 2014). The parity number significantly impacted the response of superovulation in Changbaishan black cattle as the numbers of available embryos and the numbers of total embryos were higher in multiparous animals than nulliparous animals (Deng *et al*., 2015).

The most common hormones that are used in cattle superovulation are follicle stimulating hormone (FSH), and pregnant mare serum gonadotropin (PMSG). Early trials in embryo transfer in cattle were carried out using PMS and FSH (Hasler, 2014). The superovulation response, oocyte quality, ovulation rate, and quantity of embryos obtained are enhanced by adequate levels of follicle-stimulating hormone (FSH) and the absence of luteinizing hormone (LH) (Moor *et al*., 1984). PMSG is usually called equine chorionic gonadotrophin (eCG) and extracted from pregnant mares serum. It has a significantly extended biological half-life compared to FSH hence, single dose of it is sufficient for superovulation and it is cost effective (Alfuraiji *et al*., 1993; Slenning and Wheeler, 1989). Recently, anti-Müllerian hormone (AMH) has emerged as a promising reproductive biomarker with the ability to predict the ovarian follicle pool in donor cows. It plays an important role in fertility and the superovulatory response of cows (Monniaux *et al*., 2010). Variation in AMH levels among cows has been well documented, making it a reliable marker for predicting superovulation response in animals (Fushimi *et al*., 2019).

The increase in corticosteroids caused by stress during the preovulatory period might result in a decrease in the release of gonadotropins and the suppression of the LH surge, ultimately leading to the failure of ovulation (Stoebel and Moberg, 1982). To achieve a better superovulation response, cows should be reared under suitable management conditions, must be free from lameness, disease , and injuries (Thomsen and Houe, 2018). It is well known that ration has a significant role in ET recipients' success rates. Studies have been conducted on the effects of several nutritional parameters on reproduction, including feed consumption, energy, protein source and amount, and the addition of fatty acids, vitamins, and minerals. Energy and dry matter intake (DMI) are the two most important nutritional parameters for superovulated cattle (Chorfi *et al*., 2007). The incorporation of protein, fatty acids, or minerals as nutritional supplements did not lead to an increase in the number of viable embryos in super ovulated cattle; however, research indicates that vitamin supplements may improve the average yield of transferable embryos (Velazquez, 2011). The number and interval of superovulation considered as one of the managemental factors affecting superovulation results in cattle, few studies reported the effect of repeated superovulation in cattle meanwhile these studies did not display the effect in detail. No previous reports about the effect of repeated superovulation and superovulation intervals on hormonal assay of cows were found. Therefore, the current study was conducted to evaluate the effects of superovulation number with short or long interval on superovulation outcome and hormonal assay of cows.

MATERIALS AND METHODS

Animals and experimental design

A total of 97 healthy Chinese Holstein heifers aged 12-13 months with an average body weight of 360±7.07 kg were kept in indoor houses and were fed TMR two times per day.

The heifers in the current study were superovulated for 3 consecutive times. The intervals between superovulation times were different. The interval between the first and the second superovulation was 43-58 days (long interval) while the interval between the second and the third superovulation was 28 days (short interval).

Superovulation protocol

The superovulation protocol used in this trial was (CIDR-

E2-FSH-PG-GnRH) following the procedures of (Gutiérrez-Reinoso *et al*., 2022) with some modifications. At 0-day CIDR was inserted intravaginally (CIDR-1.38 g- DEC international, NZ, Ltd.). One day after CIDR insertion, each heifer was injected with estradiol benzoate 2 mg (4mg/2ml) intramuscular. In the 5th day post CIDR insertion, FSH was started to be injected for 4 days in decreasing doses (FOLLITROPIN-V, Canada). FSH powder was diluted with 20 ml of diluent and administered intramuscularly on the 5th, 6th, 7th, and 8th days after CIDR insertion. It was injected twice daily at doses of 2.5, 2, 1, and 0.5 ml, respectively, for a total of eight injections. On the evening of day 7 of the superovulation protocol, PG was administered (2 vials, 4 mg/2 ml per vial). A second PG injection at the same dose was given on the morning of day 8, and the CIDR was then removed. Heat was detected 24 hours after CIDR removal, and 12 hours after heat detection the first AI was performed, accompanied by a GnRH injection (100 µg per vial). The second AI was carried out 12 hours after the first AI was conducted.

Embryo flushing and evaluation

On 16th day of the protocol the embryos were flushed non-surgically. The donor heifers were injected with lidocaine 2% epidurally, the dosage of lidocaine was 5 to 10 ml per animal. The cervix was dilatated with dilating rod, and then the flushing tube (#16 or #18, Silicone ET Catheter Luer Lock, 2- way) with a rustless steel inner core was slowly inserted into the uterine horn. When the flushing tube reaches the bend of the uterine horn, pull out the inner core about 5cm, and then send the flushing tube to the front of the uterine horn. When the inner core reaches the bend of the uterine horn again, pull out the inner core 5-10cm outward until the flushing tube reaches the front end of the uterine horn. Draw out the inner core from the flushing tube when the air sac of it is filled with about 8-12ml. Using a 50 ml syringe inject phosphate buffer saline (PBS) into the uterine horn for 4 times until the total volume of flushing for each horn was 200ml, the same was done with the other horn. Inject the recovered solution from the 8 X 50ml syringes for embryo flushing into a filter (Filter Coletor Para TE, WTA) at room temperature (18°C-22°C) and filter. When about 20ml of the recovered solution remains in the filter, shake the filter and pour it into a 90mm petri dish with a 1cm cross on the outside bottom. Wash the wall and bottom of the filter with PBS solution until there is no mucus and pour the remaining solution into the petri dish. The petri dishes were put on the stage of a type of stereomicroscope at 50 to 100X magnification for checking all the embryos as recommended by Bó and Mapletoft (2013). The number of unfertilized ova, degenerated embryos, available embryos, and total embryos were recorded.

Blood sampling and hormonal test

Blood samples were collected from each heifer at 3 phases in each superovulation round. The first phase of blood samples collection was carried out on day 0 (time of CIDR insertion), while the second blood collection phase was conducted on the 9th day of superovulation protocol at the end of FSH injection and first AI. The third blood sampling was conducted on the 16th day of superovulation (embryo flushing). In each blood sampling phase, 5 ml of blood was collected from tail vein of each cow in clean sterilized tube, serum was obtained and stored at -20°C until analysis. Serum FSH, Estradiol (E2), and LH concentrations were evaluated using radioimmunoassay, while AMH levels were determined by ELISA.

Statistical analysis

SPSS Statistics for Windows, version 23.0 (IBM Corp; Armonk, NY, U.S.A.) was used for data analysis. The results from the current study were expressed as means ± SEM. The results obtained from the effect of repeated superovulation were analyzed by using one- way ANOVA and means were compared by Duncan's multiple range test. The normality of the data distribution was evaluated by a Shapiro-Wilk test. The results obtained from the interval of superovulation were analyzed by using an independent t- test. Differences were declared significant when P≤0.05.

RESULTS

Table 1 shows the impact of superovulation numbers on the unfertilized ova, degenerated embryos, available embryos, and the total embryo numbers. The results revealed that the number of unfertilized ova and the number of degenerated embryos decreased in the second superovulation when compared to the first and the third superovulations

Table 1: Effect of the number of superovulation treatments on superovulation outcomes in heifers.

Item	Superovulation			
	First	Second	Third	P value
Number of unfertilized ova	1.22±0.22^{b}	0.89±0.22^{b}	1.93±.22^{a}	0.004
Number of degenerated embryos	1.56±0.16^{a}	0.90±0.16^{b}	1.15±0.16^{ab}	0.01
Number of available embryos	5.31±0.36^{a}	3.85±0.36^{b}	3.54±0.36^{b}	0.002
Total number of embryos	8.09±0.48^{a}	5.64±0.48^{b}	6.62±0.48^{b}	0.002

Least square means with different superscript letters at the same row are significantly different at p≤0.05.

(P= 0.004) and (P=0.01) for the number of unfertilized ova and the number of degenerated embryos respectively. The first time of superovulation displayed superior available embryos and total embryo numbers than the second and third times of superovulation (P=0.002).

Superovulation outcomes affected by the interval between the successive superovulation are displayed in Table 2. Long interval 43-58 days decreased the number of unfertilized ova comparing to short interval 28 days (P= 0.002). However, the difference was not significant, long intervals between superovulation time decreased the number of degenerated embryos and total embryo number and improved the number of available embryos.

Table 2: Effect of superovulation interval on superovulation outcomes in heifers.

Item	Superovulation interval		
	Long interval	Short interval	P value
Number of unfertilized ova	0.89±0.20[b]	1.93±0.25[a]	0.002
Number of degenerated embryos	0.90±1.37	1.15±1.57	0.22
Number of available embryos	3.85±0.35	3.54±0.34	0.53
Total number of embryos	5.64±0.47	6.62±0.48	0.15

Least square means with different superscript letters at the same row are significantly different at p≤0.05.

The repeated superovulation affected hormonal assay of heifers in different stages. The results in Table 3 revealed the effect of different superovulation numbers on the hormonal concentrations at the time of CIDR application. AMH and FSH concentrations were increased with repeated superovulation the concentrations of them were higher in the second and the third superovulations than the first one. On the other hand, E2 levels were higher in the first superovulation than the second and the third times. The highest levels of LH were recorded in the first superovulation time when compared with the second and third superovulations.

The hormone concentrations affected by repeated superovulation after the end of FSH injection and at the time of first AI are displayed in Table 4. The level of AMH, FSH, and E2 concentrations were lower in the first superovulation time than other times. LH concentrations were decreased with repeated superovulations.

At the time of embryo flushing, the lowest concentration of AHM was recorded at the second superovulation while the highest level was reported at the first superovulation. FSH concentrations were higher in the first and the second superovulations than the third one. LH was not significantly affected by repeated superovulation at the time of embryo flushing. E2 level was increased with repeated superovulation (Table 5).

DISCUSSION

The findings indicated that the second superovulation reduced the counts of unfertilized eggs and degenerated embryos in comparison to the first and third superovulation.

Table 3: Effect of the number of superovulation treatments on reproductive hormone concentrations at the time of CIDR insertion.

Hormones	Superovulation			
	First	Second	Third	P value
AHM (Pg/mL)	53.45±1.41[c]	113.51±2.65[b]	124.88±4.16[a]	<0.001
FSH (mIU/ mL)	3.95±0.96[b]	4.47±0.10[a]	4.40±0.13[a]	0.003
LH (mIU/ mL)	7.16±0.20[a]	6.12±0.15[b]	6.80±0.18[a]	0.001
E2 (Pg/mL)	12.79±0.36[a]	10.56±0.40[b]	9.29±0.40[c]	<0.001

Least square means with different superscript letters at the same row are significantly different at p≤0.05. AMH: Anti- Müllerian hormone, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, and E2: Estradiol.

Table 4: Effect of the number of superovulation treatments on reproductive hormone concentrations at the first artificial insemination (after completion of FSH injections).

Hormones	Superovulation			
	First	Second	Third	P value
AHM (Pg/mL)	82.36±4.12[b]	86.26±3.73[b]	121.51±3.42[a]	0.001
FSH (mIU/ mL)	3.94±0.11[b]	4.57±0.14[a]	4.39±0.11[a]	0.001
LH (mIU/ mL)	7.27±0.21[a]	6.51±0.20[b]	6.08±0.20[b]	0.001
E2 (Pg/mL)	10.98±0.40[b]	12.03±0.56[ab]	12.67±0.50[a]	0.05

Least square means with different superscript letters at the same row are significantly different at p≤0.05. AMH: Anti- Müllerian hormone, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, and E2: Estradiol.

Table 5: Effect of the number of superovulation treatments on reproductive hormone concentrations at the time of embryo flushing.

Hormones	Superovulation			
	First	Second	Third	P value
AHM (Pg/mL)	116.22±3.50[a]	50.40±1.66[c]	87.45±4.30[b]	0.001
FSH (mIU/ mL)	4.36±0.11[a]	4.63±0.15[a]	3.83±0.08[b]	0.001
LH (mIU/ mL)	7.13±0.22	7.45±0.25	7.17±0.21	0.57
E2 (Pg/mL)	5.73±0.29[c]	6.85±0.29[b]	14.09±0.42[a]	0.001

Least square means with different superscript letters at the same row are significantly different at p≤0.05. AMH: Anti- Müllerian hormone, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, and E2: Estradiol.

This may be ascribed to the diminished reproductive capacity and efficacy of heifers in generating additional fertilized eggs and the prevalence of damaged embryos during the initial superovulation. The elevated quantity of unfertilized eggs and deteriorated embryos may result from the ovaries' inadequate response to superovulation and subsequent ovarian fatigue. Conversely, heifers subjected to a single superovulation exhibited a greater quantity of available and total embryos compared to those superovulated two or three times.

This may be due to an increase in the number of superovulations, which resulted in decreased fertilization, ovulation, and reproductive performance. The interval between 2 consecutive superovulations affected significantly the number of unfertilized ova. Long interval 43-58 days decreased the number of unfertilized ova compared to short interval 28 days, perhaps due to the longer interval enhancing ovarian recovery relative to the shorter one. The results agree with (Bastidas and Randel, 1987) who reported in cows subjected to repeated superovulation, both the ovarian response and the quantity of transferable embryos diminished. Bos taurus beef cows that were superovulated a single time produced a greater number of transferable embryos compared to cows that underwent superovulation two or three times (Donaldson and Perry, 1983). In contrast, the available and the total embryo numbers were not significantly affected by repeated superovulation (Yadav and Purohit, 2015). The evaluation of the outcomes from the 204 donors, that subjected to superovulation multiple times with a 60-day interval between treatments, indicated that this factor had no effect on embryo production (Silva *et al*., 2009).

Repeated superovulation had great effects on hormonal assay of heifers at the time of CIDR insertion (day 0). The AHM and FSH concentrations were increased with repeated superovulation while E2 and LH were decreased. This may be attributed to the effect of superovulation number on the ovarian activity as the CIDR insertion had no effect on plasma hormonal concentrations of Holstein cows at (day 0) time of CIDR insertion as mentioned by (Ohsaki *et al*., 1994). High AHM and FSH concentrations means reservation of follicle activity and increase the number of ova with repeated superovulation, in cattle the number of follicles can be predicted through the AHM concentrations (Batista *et al*., 2014).

After the end of FSH injection and at the time of AI with increased superovulation times, AHM, FSH, and E2 levels were enhanced while LH was decreased. As the same superovulation protocol was used and FSH stimulates follicular growth and activity accompanied with E2 release, the elevation of AHM, FSH, and E2 from the first to the consecutive times may be because of superovulation effect on the size, activity, and the response of the ovary to the superovulation impact. There is a limitation in discussion of the effect of repeated superovulation on blood profile of heifers after FSH injection as the previous studies mainly evaluated the effect of FSH dose or route of administration during superovulation not the effect of repeated superovulation (Chumchai *et al*., 2021; Karl *et al*., 2021). With increase the number of superovulation FSH, AMH, and E2 concentrations were increased which may be attributed to the effect of exogenous FSH injected during superovulation protocol as administration of low doses of FSH improved follicular activity and enhanced estradiol concentrations (Karl *et al*., 2021). At the flushing day the superovulation time had a noticeable effect on hormonal assay of heifers. The AHM level was higher in the first superovulation time than the second and the third times. The third time superovulation showed the lowest FSH concentration among all times. There is no significant effect of repeated superovulation on LH while E2 increased significantly with superovulation numbers. Repeated superovulation may decrease the number of follicles resulting in reduction of AHM concentrations. Reduction of FSH with repeated superovulation may be attributed to follicular exhaustion. With multiple superovulation the AHM concentrations were decreased due to the inhibition of primordial follicle activity (Wang *et al*., 2023). Contrary to the current result, there was no significant difference in E2 concentrations in animals that were subjected to superovulation 1, 3, and 5 times. Also, LH reduced significantly 3 times superovulated animals than animals that were super ovulated for one time as

reported by (Wang *et al*., 2023).

CONCLUSION

It is concluded that repeated superovulation had adverse effects on the ovarian activity and functions. Multiple superovulation reduces the number of available and total embryos and decreases the level of the main reproductive hormones (AHM and FSH), especially at the flushing time. From the managemental view, repeated superovulation is not useful for cows' performance and welfare.

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NOVELTY STATEMENT

The current evaluates the effects of superovulation number with short or long interval on superovulation outcome and hormonal assay of cows.

AUTHOR'S CONTRIBUTION

Conceptualization, Ahmed Sabek, Wenli Yu, Shujun Zhang and Shujing Li; data curation, Ahmed Sabek, Han Jiang and Wenyuan Gu; formal analysis, methodology and validation, Ahmed Sabek, Hang Jiang, Wenyuan Gu, Hongbo Zhu , Eman Elgazzar , Pu zhang and Shuoqing Su; writing—original draft preparation, Ahmed Sabek; writing—review and editing, Wenli Yu , Shujun Zhang.

Ethics statement

The study was conducted at Shijiazhuang Tianquan Elite Dairy Cattle LTD, Luquan District Shijiazhuang City, Hebei Province, China. The study was carried out according to the guidelines for the care and use of animals. The study protocol was approved by the Animal management and Ethics Committee of Huazhong Agriculture University (HZAUCA-2017-009).

Generative AI and AI-assisted technology statement

No artificial intelligence tools were used in the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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