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Theriogenology

The Response of Donor Holstein Cows to Ovarian Super-Stimulation Using Two Different Protocols GnRH and Estradiol

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ABSTRACT

The objective of the present study was planned to compare the response of donor Holstein cows to a GnRH-based (GnRH-SOV, n=9) and estradiol-based (E2-SOV, n=10) protocols. The super-stimulation protocols consisted of twice daily decreasing doses of porcine FSH with a total dose of 400 mg and the cows in both groups were sampled three times, before initiation of superovulation, at estrus and at embryo collection from donors. Circulating levels of FSH, LH, progesterone, estrogen and anti-Mullerian hormone (AMH), as well as response to superovulation were compared between the two groups. Results revealed that embryo yield and quality did not differ (P<0.05) between the two groups. When circulating concentrations of hormonal biomarkers were tested for possible correlations with embryo quality or quantity, progesterone was correlated with total embryo (r=0.71, P<0.01), total transferable embryo (r=0.84, P<0.01), second grade and third grade embryo (r=0.72 and 0.73, respectively; P<0.01) regardless of the time of sampling. Strong positive correlations were recorded between circulating levels of AMH prior to superovulation and embryo yield (total embryo, r=0.50; P<0.01), and quality (first grade embryo, r=0.78, P<0.01; second grade and third grade embryo, r=0.47 and 0.50, respectively with P<0.05). In conclusion, donor Holstein cows responded successfully to GnRH-SOV and E2-SOV protocols and circulating levels of progesterone and AMH could predict donor response to superovulation in Holstein cows.

Keywords: *Superovulation, GnRH, Estradiol, AMH, progesterone, Embryo quality*

INTRODUCTION

Embryo transfer (ET) is becoming widely used across the globe to incorporate superior genetics and to enhance reproductive performance of food animals including cows (Alkan *et al.*, 2020). Variability of the response of donor cows to superovulation is still the most limiting factor against widespread application and commercialization of embryo transfer programs (Abdel Aziz *et al.*, 2017).

Although ET has been increasingly advanced in the last few decades, significant increases in number of total embryos recovered by each superovulated donor has not been achieved (Bo *et al.*, 1996). Controlling the estrous cycle of donor cow to recruit a new follicular wave at the beginning of the protocol and exogenous hormonal manipulations led to improved hyperstimulation at time of recovery and an increased recovery rate (Nasser *et al.*, 1993; Bo *et al.*, 1996 and Baruselli *et al.*, 2012). Despite of that, over the last 2 decades, the average number of total embryos recovered remains at an average of 6.5 (AETA, 2017). Recently, hormonal interactions during the superstimulation period and their impacts on embryo production have been extensively studied (Oshba, *et al.*, 2018). Progesterone (P4) not only plays a dominating role after ovulation for maintaining a pregnancy and elongating the conceptus (Forde *et al.*, 2012), it also suppresses the Luteinizing hormone

(LH) surge which important for final maturation and ovulation of dominant follicles (Hatler *et al.,* 2008). The impact of sub-luteal or natural P4 concentrations on the growth of the pre-ovulatory follicular wave and the quality of embryos produced led to conflicting results in literature. Additionally, it is not well-known that sub-luteal concentrations of P4 can improve the quality of embryos produced during superovulation. (Hatler *et al.*, 2008; Cerri *et al.,* 2011; Wiltbank *et al.,* 2014 and Huang *et al.,* 2016).

Follicle stimulating hormone (FSH) is essential to stimulate follicular recruitment (Adams *et al.,* 1992), therefore, it is the foundation for superstimulation of the estrous cycle by exogenous administration. Important factors of exogenous gonadotropins promoting diversification of protocols include: type and concentration of administered gonadotropin, dosage, administration interval, and timing of first treatment (Bo and Mapletoft, 2014).

Previous research literature regarding the original protocol specified to super-stimulate bovine ovaries cite the importance of initiating treatment coinciding with donor's anticipated second follicular wave of the estrous cycle, i.e. on day 8-12 of the cycle, for successful embryo production (Bo and Mapletoft, 2014).

Donor cows were also super-stimulated with techniques to appropriately time endogenous release of FSH followed by a new follicular wave with proven effectiveness and similar numbers and quality of embryos at recovery (Mapletoft and Bo, 2013). These approaches included exogenous GnRH (Martinez *et al.*, 1999; Bo *et al.,* 2006; Bo and Mapletoft, 2014); administration of estradiol-17β with P4 (Bo *et al.,* 1995, 1996), and ultrasound guided follicle ablation (DFA) of all follicles > 5mm (Bergfelt *et al.,* 1997; Baracaldo *et al.,* 2000) to induce a new follicular wave.

Anti-Müllerian hormone (AMH) is a 140-kDa glycoprotein belonging to the transforming growth factor b (TGFb) family (Knight and Glister, 2006). It is expressed in the gonads, especially in Sertoli cells of the testis and granulosa cells of the ovary which localized to the granulosa cells of small growing antral follicles ((Monniaux *et al.,* 2012 and Rico *et al.,* 2011). This demonstrates that cows with a greater antral follicle population have much higher AMH concentrations than cows with a smaller antral follicle population. A strong positive relationship has been observed between circulating AMH and in vivo embryo production following superovulation of lactating dairy cows (Abdel Aziz *et al.,* 2017). In addition, the circulating AMH is a good predictor of superstimulation, superovulation, and embryo production, (Abdel Aziz *et al.,* 2017).

This study was designed to compare response of donor Holstein cows to two different super-stimulation protocols GnRH and estradiol. In addition, the relationship between various endocrine biomarkers including FSH, LH, P4, Estrogen (estradiol) as well as AMH and superovulatory response of embryo donor cows were studied.

MATERIALS AND METHODS

Animals

The current study was carried out on nineteen pluriparous donor Holstein cows average age 4.6 ear in a commercial embryo transfer unit at a well-managed private Holstein herd (Sapphire Dairies ltd, Lahor-Punjab, Pakistan). The farm is about 4000 animals. All animals were proven free from any infectious diseases and were routinely vaccinated gainst (FMD, BVD, IBR, Brucella, BEF, Clostridia and Pasteurella).

Animals were housed in a free stall barn and were milked three times daily and fed a totally mixed ration which was constructed to meet or exceed the nutrient requirements of dairy cattle according to the NRC recommendations (NRC, 2001).

The experiment was carried out during the period from beginning of January to end of February 2018 during which the average daily temperature was 15 degree Celsius and the average relative humidity was 80 degree.

Donor cows were selected based on their previous health, production and reproduction records by analyzing available data records from the farm software system. (dairy comp DC305 management system). Total 305-day milk for the previous lactations, services per conception, interval from calving to first estrus, days to conception, and regularity of cyclicity and incidence of infertility problems in the current and previous seasons were the criteria used to select donor cows.

Donor scanning

All donors were scanned before superovulation program with linear transducer Portable Ultrasonic Diagnostic System (SonoScape Co. Ltd., China) provided with trans-

rectal linear transducer (5 MHZ) for rectal scanning and to evaluate the genital tract status and ensure the soundness of the tract without any pathological problem. Follow up the superovulation response after each injection of pFSH (Folltropin® 35 IU FSH per mL Vetoquinol USA Inc.). Moreover, last scanning was done on the day of insemination and on the day of the embryo recovery

Superovulation protocols

The first group (GnRH-SOV, n=9) was submitted to a gonadotropin releasing hormone-based superovulation protocol according to Abdel Aziz *et al.,* (2017) as shown in Figure (1)**.** This protocol beginning by insertion of CIDR (Controlled Internal Drug Releasing device) each one contain 1.38 g progesterone zoetis USA) then injection of one dose of GnRH im (12 µg Buserelin, Receptal® "4µg/ml" MSD, USA) then injection of pFSH (Folltropin® 35 IU FSH per mL Veto quinol USA Inc.). Also, injection of $PGF_2\alpha$ (500 µg Cloprostenol, Estrumate®"250µg/ml" MSD, USA).

The second group (E2-SOV, n=10) was submitted to an estradiol-based superovulation protocol according to Oshba (2018) as shown in Table (1). this protocol beginning by insertion of CIDR (Controlled internal drug releasing device, each one contain 1.38 g progesterone zoetis USA) then injection of one dose 3 ml of estradiol (Estradiol® over every 100 ml contain Estradiol Benzoate 0.25 g formulation agents, Argatina) then injection of pFSH (Folltropin® 35 IU FSH per mL Vetoquinol USA Inc.). Also, injection of PGF2 α (500 µg Cloprostenol, Estrumate® "250µg/ml" MSD, USA)

Embryo collection and evaluation of embryos

On day 7 after insemination, donor cows in both groups were flushed using a two-way Foley catheter (18 fr balloon 30 cc, Agtech, Inc. Manhattan, USA) and a Y-shape connector tube according to Abdel Aziz ***et al., (2017)***, using Vigro complete flushing medium (1000 ml per horn) (Vigro complete flush medium 1L bag Vetoquinol, USA, Inc.). After flushing, each cow was administered 750 µg Cloprostenol to induce luteolysis and to avoid possible pregnancy if any embryo is left inside the uterus. Individual flushing fluid was searched under the stereomicroscope which have Continuous zoom magnification from 15x to 90x (Meiji Emz-5 Microscope, Japan) using a square search dish and searching was done about 2 to 3 times for each searching dish. Recovered embryos were transferred to holding medium (Vigro holding plus 50 mL bag Vetoquinol USA Inc.) for classification of embryo quality and also for washing. This step was repeated twice time to clean embryos from debris.

Embryo grading

Grading for embryo quality was done according to international embryo transfer society guidelines (IETS, 2010). Embryos were classified into first grade, second grade, third grade and degenerated embryos (IETS, 2010). Stereomicroscope was used for identifying the stage of each embryo development as early morula, compacted morula, early blastocyst and blastocyst. The total number of recovered structures was evaluated microscopically for Degenerate embryos (embryos at 8-cell stage and earlier stage) or transferable embryos (morphologically intact compact morula, early blastocyst and expanded blastocyst).

Finally, the collected embryos were classified as:

Grade 1: Embryos were morphologically intact and had an even granulation and cell distribution.

Grade 2: Embryos with small deviations, like few excluded blastomeres.

Grade 3: Embryos had an uneven cell organization, loose structures, with numerous free blastomeres.

Blood sampling and Hormonal assay

Three blood samples were obtained from each donor cow from tail vein about (10 ml) by using vacutainer tubes. The first one was obtained prior to initiation of superovulation, the second at estrus and the third at collection of embryos. Serum was separated via centrifugation at 3000 rpm for 30 minutes and serum samples were stored at -80 degree till being assayed for FSH, LH, estradiol, P4 and AMH.

Serum P4, estrogen (estradiol), follicular stimulating hormone (FSH) and LH levels were assayed by Sandwich ELISA Quantitative micro-well technique using kits from Bioneovan. Co., Ltd. (China) and following manufacture instructions

AMH assay

Anti-Mullerian hormone was measured according to (Abdel Aziz *et al.*, 2017) with the active MIS/ AMH ELISA® kit (Cloud-Clone Corp, Houston, TX, USA) with a sensitivity of

177 pg/mL. The AMH was measured in 50 micro-liter undiluted plasma samples following manufacturer protocol. Intra and interassay coefficients of variation were less than 10% and 12%, respectively.

Statistical analysis

Effects of the type of the superstimulation protocol on the outcome were examined using the *t* test. Pearson's correlation coefficients were determined to examine the possible relationships between donor hormonal variables and outcomes of superovulation.

Altogether, a p value <0.05 was considered significant, while a p value from above 0.05 to 0.1 was considered a statistical tendency (SPSS, 2007).

RESULTS

The obtained results showed in Figure (2) indicates that the two protocols were effective and did not vary significantly in embryo yields. The mean number of Total collected embryos (TE) was numerically higher in GnRH-superstimulated cows (10.25±2.12), compared to 9.25±1.77 in estradiol-superstimulated group. The mean numbers of Total transferable embryos (TTE) collected were (6.25±1.47 and 6.13±1.44) respectively.

The percentages of embryo quality grades did not vary significantly between the two superovulation protocols as illustrated in Figure (3) However, the percentage of TTE was numerically higher in GnRH-superstimulated cows accounting for 68.48±10.39% of collected embryos, compared to estradiol-superstimulated cows (59.86±10.32%).

No significant correlations were observed among hormone levels and outcomes of superovulation in donor cows superstimulated using estradiol-based protocol. Nevertheless, progesterone levels were significantly correlated with TE (r=0.71), SGE (r=0.72), TGE (r=0.73) and TTE (r=0.84) in GnRH-superstimulated donors (Table 2).

As presented in Table (3), AMH predicted the superovulatory response of embryo donor Holstein cows. AMH measured in samples obtained prior to superstimulation was highly correlated with TE (r=0.50), TTE (r=0.56), FGE (r=0.78), SGE (r=0.47), TGE (r=0.50). On the other hand, circulating levels of AMH prior to superstimulation were correlated with number of DE (r=0.33).

Table 1: Estradiol-based superstimulation protocol for donor Holstein cows

DAY	TIME	DONOR COWS
Day 0	am	Insert CIDR + 2.5 ml Estradiol Benzoate
Day 4	7am	40 mg FSH
	5pm	40 mg FSH
Day 5	7am	30 mg FSH
	5pm	30 mg FSH
Day 6	7am	20 mg FSH + 500 ug cloprostenol
	5pm	20 mg FSH + 500 ug cloprostenol
Day 7	7am	10 mg FSH + Remove CIDR
	5pm	10 mg FSH
Day 8	7am	150 ug receptal
	5pm	AI Donor
Day 9	7am	AI Donor
	5pm	X
Day 15	10am	FLUSH

CIDR: Controlled internal drug release; **FSH:** Follicle stimulating hormone; **ET:** Embryo transfer.

Table 2: Pearson correlation coefficients (r) between hormonal biomarkers and superovulatory response of donor Holstein cows.

	Protocol	TE	FGE	SGE	TGE	DE	TTE
FSH	1	0.34	-0.13	0.21	0.07	0.48	0.05
	2	-0.13	-.29	-0.19	0.09	0.06	-0.21
LH	1	-0.17	-0.34	-0.35	0.13	-0.01	-0.25
	2	-0.16	-0.15	-0.49	-0.41	0.24	-0.36
Estrogen	1	0.42	-0.09	0.44	0.42	0.34	0.32
	2	-0.13	-0.18	0.03	0.29	-0.27	-0.02
Progesterone	1	*0.71**	*0.45*	*0.72***	*0.73***	*0.25*	*0.84***
	2	-0.18	-0.34	0.07	0.13	-0.17	-0.15

TE: Total embryos, **FGE:** First grade embryo, **SGE:** Second grade embryo, **TGE:** Third grade embryo, **TTE:** Total transferable embryos, **DE:** Degenerated embryo, **FSH:** Follicle stimulating hormone**, LH:** Luteinizing hormone.
Protocol 1: GnRH-based superovulation; protocol 2: estradiol-based superovulation.
* P<0.05; ** P<0.01

Table 3: Pearson correlation coefficient (r) between circulating anti-Mullerian hormone prior to superstimulation and response of donor Holstein cows.

Item	Pearson correlation coefficient	P value
Total structures (TE)	0.50	<0.05
Transferable embryo (TTE)	0.56	<0.05
First grade embryo (FGE)	0.78	<0.01
Second grade embryo (SGE)	0.47	<0.05
Third grade embryo (TGE)	0.50	<0.05
Degenerated embryo (DE)	0.33	<0.05

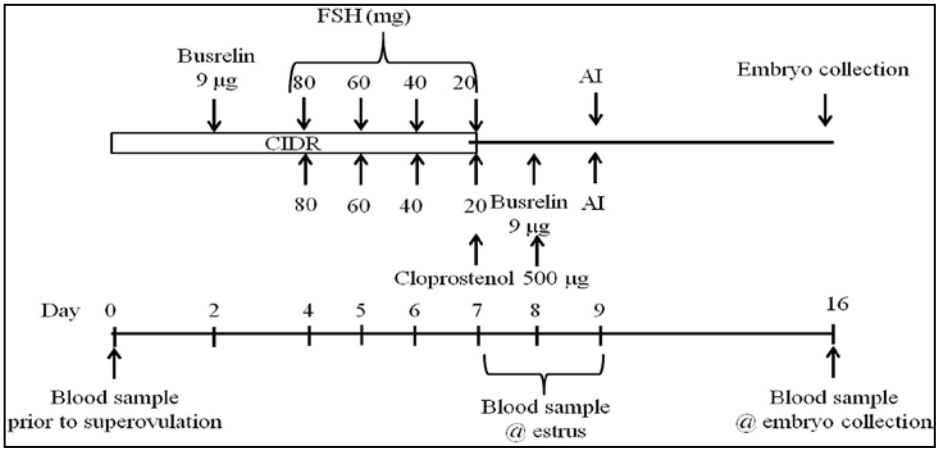

Figure (1): GnRH-based superovulation protocol with sampling times.

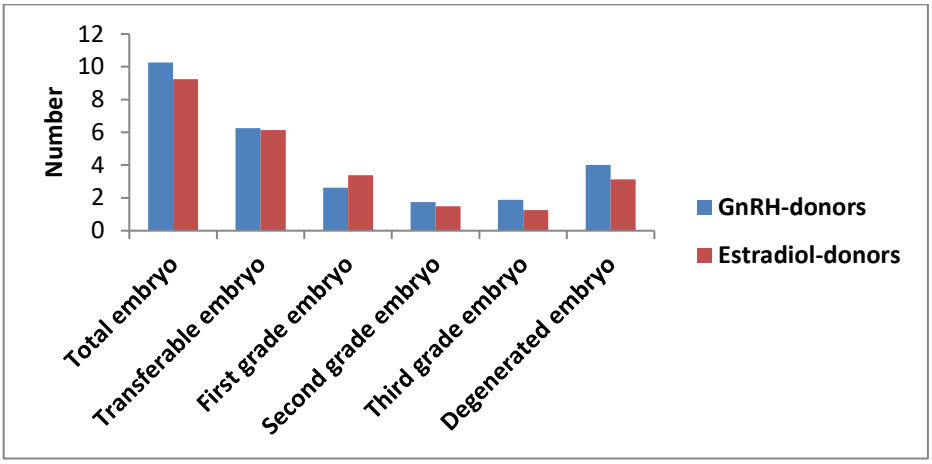

Figure (2): Effect of superovulation protocol on quantity and quality of embryos collected from superovulated donor Holstein cows.

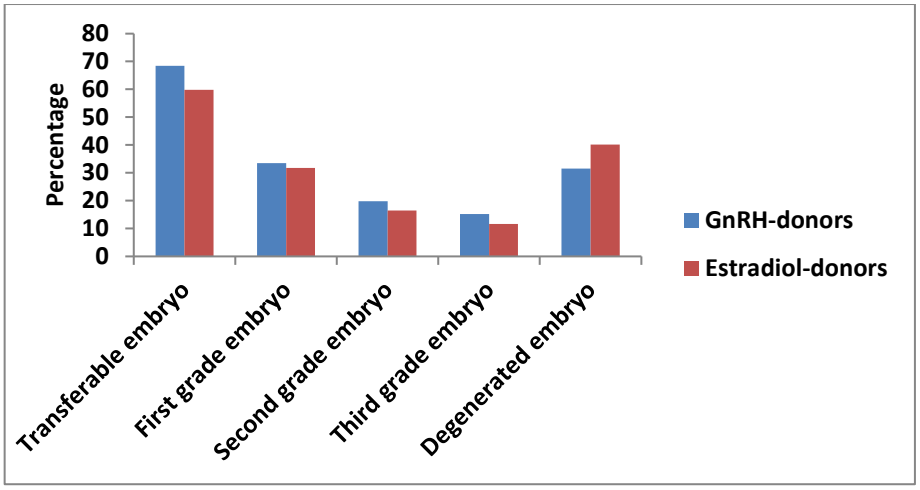

Figure (3): Percentages of embryo quality grades obtained from donor Holstein cows superovulated using estradiol-based or GnRH-based protocols.

DISCUSSION

The present work clarified that both GnRH-based and estradiol-based super-stimulation protocols were effective and produced embryos of comparable quantity and quality (P>0.05). It is important to notice that the hormonal profiles in individual donor cows of the two protocols were similar at different sampling times except for estradiol prior to superovulation which was significantly higher in GnRH donors.

In the present study, the estradiol-based protocol produced comparable embryo quantity and quality to GnRH-based protocol. The ability to induce follicular wave emergence ensures initiation of superstimulation regardless of the stage of the estrous cycle and alleviates the need for estrus detection and waiting for days 8 to 12 of the cycle to initiate FSH treatments (Mapletoft *et al.*, 2002). The use of progestins and estradiol to synchronize new follicular wave emergence in the cow has been reviewed extensively (Bo *et al.*, 2002; Mapletoft *et al.*, 2002; Mapletoft and Bo, 2012). It has been employed by practitioners around the world and has recently been incorporated into protocols that permit fixed-time AI (FTAI) of donors (Baruselli *et al.*, 2006; Bo *et al.*, 2006).

After estradiol metabolism, a surge of FSH occurs with a new follicular wave emergence, on average 4 days after treatment (Bo *et al.*, 1995). FSH super-stimulatory doses are initiated at that time, i.e., 4 days after treatment, therefore, allow for a new cohort of 3- to 5-mm follicles, to grow at the same time. Although the number of transferable embryos hasn't exceeded that when cows were superstimulated between 8 to 12 days after estrus, the fertilization rate in donor cows treated by estradiol and progestin treatments was significantly higher than in the control cows in two studies (Bo *et al.*, 1991 and 1995). Furthermore, heifers superstimulated 4 days after the insertion of a progestin device without the administration of estradiol also had a lower percentage of fertilized ova and transferable embryos than those treated with estradiol at the time of insertion of the progestin device (Bo *et al.*, 1991). Collectively, these results suggest that the administration of exogenous FSH might not only induce growth of healthy follicles but also those that have already undergone atresia.

According to Goulding et al. (1994), superstimulatory treatment early in the luteal phase in heifers with a progestin device resulted in poor embryo quality which was apparently because of the stimulation of subordinate follicles that were already undergoing atresia, with oocytes that were undergoing degeneration. Hence, estradiol and progestin treatments not only eliminated the need for estrus synchronization and detection before super-stimulation, but also tended to improve ova/embryo quality. Hence, (Bo and Mapletoft, 2014) concluded that the estradiol-based protocol is an efficient method to super-ovulate donor Holstein cows.

The statistical analyses of Pearson's correlations of endocrine biomarkers with outcomes of superovulation in Holstein donor cows revealed that only P4 levels prior to super-stimulation were highly correlated with TE (r=0.71), SGE (r=0.72), TGE (r=0.73)and TTE (r=0.84) and those correlations were observed only in GnRH-superstimulated

donors. On the other hand, no significant correlations could be detected between endocrine biomarkers and the outcome of superovulation in estradiol-based protocol. This significant correlation of circulating P4 with embryo quality in case of GnRH-stimulated donors and the lack of any correlations of circulating P4 with embryo quality in estradiol-superstimulated donors may be due to beneficial effects of P4 on oocyte developmental competence and embryo quality in donors super-stimulated using GnRH protocol (Wiley *et al.*, 2019). The authors document that high P4 donors on days 2 and 3 of the super-stimulation protocol yielded greater number of corpora lueta and total embryos, meanwhile the percentage of embryos classified as grad 1 was higher in low P4 donor cows. Subluteal P4 concentrations, even at 0.1-1.0 ng/ml in a previous study (Hatler *et al.*, 2008) have been reported to block LH and ovulation. Conversely, others have reported increased diameter of dominant follicles (Carvalho *et al.*, 2008; Hatler *et al.*, 2008) for females subjected to subluteal concentrations of P4 during the ovulatory follicle wave. P4 impacts in MOET research suggests subluteal P4concentrations allow for irregular LH pulses, causing disturbance in nuclear maturation of oocytes (Nasser *et al.*, 2011; Baruselli *et al.*, 2012). Previous subluteal P4 studies that mainly focused on the first follicular wave of the estrous cycle, when P4 is still relatively low, observed a decreased TE compared to those supplemented with exogenous P4 (Hatler *et al.*, 2008; Nasser *et al.*, 2011; Rivera *et al.*, 2011), thus further scientific research regarding the impact of various concentrations of P4 on subsequent follicular waves in a cycle on gamete developmental competence and embryonic development should be carried out.

As evident in literature circulating AMH in the current study predicted embryo donor's response to superovulation (Rico *et al.*, 2009; Souza *et al.*, 2015; Abdel Aziz *et al.*, 2017). However, Vernunft et al. (2015) cited that AMH was not correlated to embryo quality or quantity of embryos produced from superovulated cows.

In the present work, circulating AMH was significantly correlated with total structures collected, transferable embryo, first grade embryo, second and third grade embryo. The unusual finding in the present investigation is that circulating AMH was also positively correlated with the number of degenerated embryos collected ($r=0.33$, $P<0.05$). It might be due to the increased response of donor cows with high levels of AMH to superovulation.

CONCLUSION

This study revealed that estradiol-based superstimulation protocol was as effective as GnRH-based protocol for superovulation in embryo donor Holstein cows. In addition, estimation of circulating levels of progesterone and AMH prior to superovulation of embryo donor Holstein cows can be recommended to select best responding donors.

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Conflict of interest

The authors declare they do not have any conflict of interest.

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