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Response to GnRH Administration at Artificial Insemination in Crossbred Temperate Dairy Cattle Super-ovulated for Embryo Transfer Program under a Tropical Environment

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Abstract

Multiple ovulation and embryo transfer (MOET) technologies are efficient strategies for multiplying high genetic merit cows. Gonadotrophin releasing hormone (GnRH) plays a key role in the endocrine control of ovulations in cattle. The objective of this study was to assess the effectiveness of exogenous GnRH injections at the time of artificial insemination (AI) in super-ovulated crossbred temperate dairy cows under tropical environments. Super-ovulations (n = 24) were conducted using 12 genetically sound dairy cows with a standard protocol with two AIs in a 12-hour interval. Cows in the treatment group (TG) (n = 12) received GnRH (100µg) at the first AI and cows in the control group (CG) (n = 12) did not. Embryos were collected by the non-surgical retrograde flushing technique on day 7 post-AI. Collected embryos were classified according to the standard FAO guidelines. Ovulation rate, embryo recovery rate, percentage of transferable embryos, and rate of degenerated oocytes were compared between the two groups. The ovulation rate (P = 0.083) tended to be higher while the median numbers of embryos recovered (P = 0.003), embryo recovery rate (P = 0.008), and percentage of transferable embryos (P = 0.019) were significantly higher in the TG. Further, the number of degenerated oocytes was notably lower in the same group. The results of the study revealed that the administration of GnRH at the time of AI in superovulation protocols significantly improved the embryo production, recovery, and the number of transferable embryos in crossbred cows in tropical environments.

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Artificial insemination, cattle, embryo transfer, GnRH, superovulation

Introduction

Multiple ovulation and embryo transfer (MOET) is a process by which in vivo created embryos are harvested from a donor animal and transferred into the uterus of a recipient animal to complete the rest of the pregnancy period (Betteridge, 1981). This technology could be applicable for the exchange of valuable germplasm within a country or internationally without diminishing its gene base in a safe way (Duran, 2000), and could provide reliable breeding material for farmers (Perera *et al.*, 2010). This technology has been developing as an answer for reproductive inefficiencies in livestock animals that have arisen due to heat stress as well (Al-Katanani *et al.*, 2002). After the cleavage stage, developing embryos acquire a thermotolerance capacity with an accumulation of antioxidants in response to the heat-inducible production of reactive oxygen species (Sakatani, 2017). However, the efficiency of ovarian activities that would affect the superovulation of embryo donor cows may rely on several intrinsic and extrinsic factors (Baruselli *et al.*, 2017). Distress, which arises due to an elevated environmental temperature, would be a negative factor for breeding efficacy, especially in temperate cows living in a hot, humid environment such as in Sri Lanka (Soumya *et al*., 2016; Jayathilaka *et al.*, 2019a; Jayathilaka *et al*., 2019b;). High-producing cows are the most susceptible group of animals to such adverse effects (Armstrong, 1994). A lower reproductive efficiency under heat stress would be of the utmost concern to dairy farmers (Hansen, 2009). The impact of elevated environmental temperatures on the reproductive performance of dairy cattle can be reduced by several means (Armstrong, 1994; Soumya *et al.*, 2016; Sakatani, 2017). For this, different hormonal protocols are used to improve breeding with artificial insemination (AI), even during the summer season, in many temperate countries around the world (Wolfenson *et al.*, 2000).

The gonadotropin-releasing hormone (GnRH) has been identified as the primary regulator of the reproductive endocrine pathway (Baba *et al.*, 1971; Schally *et al.*, 1971). In addition to olfactory, optic, and auditory stimulations, light, heat (Volez, 2016), stress, nutrition, and physical stimulations also regulate the secretion of GnRH from the GnRH neurons of the hypothalamus (Stephen, 1971). It enters the hypophyseal portal circulation and acts on the G-protein-linked receptors of gonadotropic cells of the anterior pituitary to produce and secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). GnRH release happens in a pulsatile mode, with low pulse frequencies inspiring more FSH synthesis and high pulse frequencies inducing further LH production (Stamatiades & Kaiser, 2018). The elevated circulatory FSH and LH hormone levels regulate the endocrine function and follicular maturation in the ovaries (Alex, 1988; Thatcher *et al*., 1993; Schally, 2000). Further, injecting more FSH during superovulation in cattle would lead to more follicles developing and a higher ovulation rate (Deguettes *et al*., 2020). Also, it helps to regulate the micro-environmental functions in follicles. However, a surge release of LH is a critical requirement for ovulation in cattle (Volez, 2016). Since there is the presence of a higher number of pre-ovulatory follicles during MOET, final development and ovulation could be enhanced by increasing the levels of both FSH and LH. The administration of exogenous GnRH would be a convenient way to fulfill this requirement. Nevertheless, administration of GnRH is usually considered optional or an additional hormonal stimulation in most of the superovulation and AI protocols available for cattle. Exogenous GnRH or its analogs function like endogenous GnRH in animal reproduction (Uddin *et al*., 2023). The presence of a higher number of developed follicles during MOET means the demand for GnRH would be higher at the releasing of FSH and LH for final development and ovulation. Under these circumstances, the naturally released concentration of GnRH may not be sufficient for the induction of ovulation in a certain number of follicles (Seidel & Seidel, 1991). Therefore, GnRH is an important hormone in superovulation, AI, and synchronization protocols in cattle (Hassanein *et al.*, 2024a; Hassanein *et al.*, 2024b). Furthermore, it is an effective hormone in treating follicular cysts

(Kesler, 1982), delayed ovulation (Gabriel, 2006), sub-estrous conditions, and stimulating estrous (Arthur *et al*., 1982; Youngquist, 1988). However, the use of GnRH at the time of AI to induce ovulation in superovulation protocols has been limited (Chankitisakul *et al*., 2017).

Thus, the objective of this study was to evaluate the effectiveness of administering GnRH at the time of AI in the MOET program in crossbred dairy cows reared in a tropical (hot and humid) environment.

Materials and Methods

Ethical clearance

All experimental protocols were approved (VERC-21-07) by the Committee of Ethical Clearance on Animal Research, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka. Further, all the experiments were performed following the relevant guidelines and regulations stipulated by the ethics committee.

Location

The study was carried out from January to May 2022 at the Veterinary Teaching Farm (7°15'08.3 N, 80°36'12.0 E) (VYMaps.com), Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka. During the experiment period the ranges of average monthly environmental temperatures of the area were 30-32^{o}C and 19-22^{o}C during the daytime and night, respectively. The relative humidity of the area during the same period was 77-80% (World weather online, 2022).

Selection and management of embryo donors

A group of prospective embryo donor cows (n = 23) were evaluated for their pedigree, production, reproductive history, and general health status whilst paying special attention to their reproductive system. The position of the reproductive tract within the pelvic cavity, shape and size of the cervix, length and consistency of the uterus, and ovarian pathology was evaluated by manual and transrectal ultrasonography. Twelve high-producing (25-30 L/day) healthy cows (Friesian × Jersey), which were imported recently from Australia, with body condition scores (BCS) of 2.25-2.5 were selected for the superovulation program. All the selected cows were three years old and had calved for the first time 3-4 months prior to selection. Selected animals were dewormed with Albendazole ('ANALGON', Wockhardt Limited India) and given a multivitamin (Dutch Farm Veterinary Pharmaceuticals, Dutch Farm International BV, Holland) injection initially. The animals were fed daily with ad libitum forage (CO_3 and Guinea grass), 8kg of concentrated mix (CIC, Dairy Milk Flush, Sri Lanka.), and 60 g of a vitamin-mineral mixture (Pecutrin Farm Chemy, Sri Lanka). Cows that achieved a BCS of 3.0-3.5 at the end of the one-month pre-experiment period were subjected to MOET. In the first instance, four cows were used.

Experimental design

A 2×2 crossover experimental study was conducted to evaluate the effects of the GnRH treatment at AI in the MOET program. The embryo donor cows were randomly assigned into two groups (Groups 1 and 2) of six animals each. In the first part of the experiment, cows in Group 1(TG) received a GnRH injection at AI and at superovulation while the cows in Group 2 (CG) received no hormonal treatment. In the second part, the groups of animals were switched for the treatments (i.e., Group 2 [TG] cows received GnRH injections at AI while Group 1 [CG] cows received no hormonal treatment). This treatment allocation was decided on a random basis. All cows were super-ovulated at the beginning of each period as per the protocol described below.

Superovulation of donors

Embryo donors were super-ovulated according to the procedure explained in **Figure 1**.

Embryo flushing

Seven days post insemination, embryo flushing was carried out in the animals of both groups. The number of corpora lutea (CLs) (**Table 1**) in both ovaries was counted manually immediately before the flushing and the count was confirmed by trans-rectal ultrasound scanning (6.5 MHz, Bondway - China) (**Figure 2**) at the end of the flushing process. Embryo

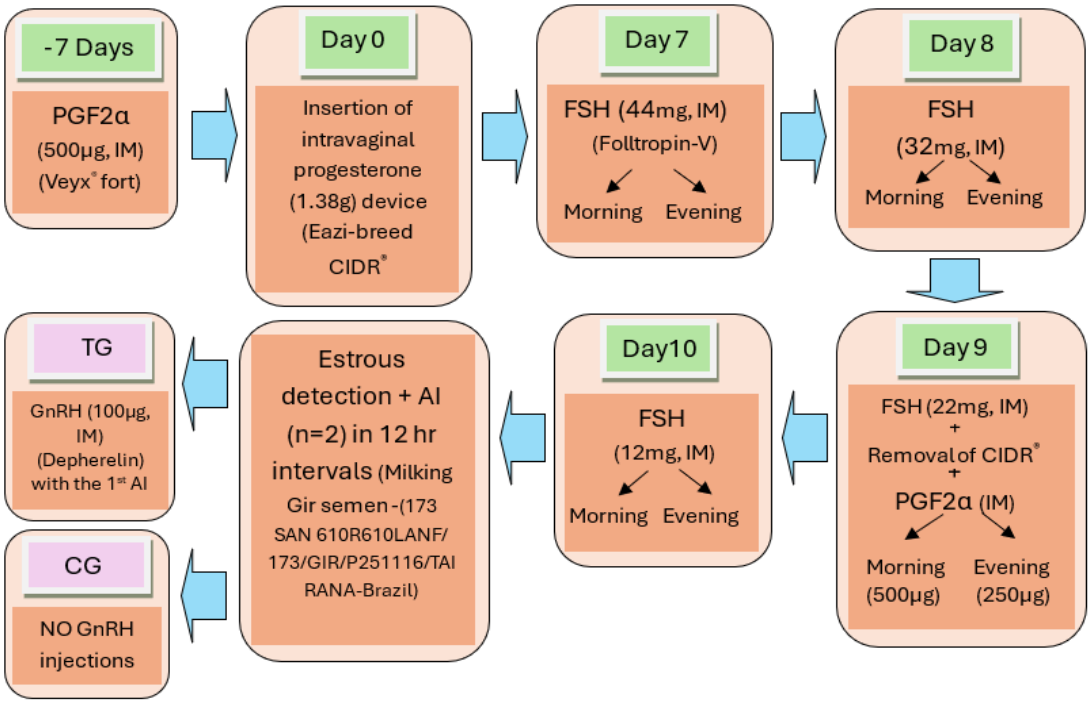


Figure 1. Superovulation process of embryo donors. The figure indicates the administering of $PGF_{2\alpha}$ 7 days prior to the onset of the proper super-ovulatory protocol. AI- artificial insemination, IM- intramuscular route, TG- treatment group, CG- control group

flushing was carried out with the induction of posterior epidural anesthesia (6mL, 2% LIGNOCAINE INJ., SPC, Sri Lanka) using a pre-warmed (35°C) commercial embryo flushing medium ('VIGRO™ COMPLETE FLUSH' Vetoquinol, U.S.A.) on day 26 of the program. Both uterine horns were flushed by using the standard retrograde embryo flushing technique (Seidel & Seidel, 1991). At the end of the flushing, the embryo-rich flushing medium, which remained in the filter, was used for the isolation of embryos. The mucus and the debris adhering to the filter membrane were rinsed into a searching dish with 20mL of the commercial rinsing medium ('BoviRinse', Minitube, Germany) for the isolation of any embryos trapped in the debris. At the end of flushing, 500µg of $PGF_{2\alpha}$ was administered to each embryo donor, intramuscularly (IM).

Embryo handling and evaluation

Embryo-holding medium (0.5mL/well) ('ViGRO HOLDING plus', BIONICHE Animal Health, USA) was added into the first and second wells of a four-well plate ('Nunclon™, Kmstrupvej 90', Denmark) and embryos were searched for under a dissection microscope (SZ–ST Olympus, Japan), and transferred using a 5-µL micro dispenser (The Drommond Scientific Co, U.S.A.) into the first well of the four-well plate for the first washing. Then, the embryos were transferred to the second well for the next washing. While in the second well, embryo evaluation and classification were carried out according to the guidelines given by the 'Training manual for embryo transfer in cattle, FAO animal production and health paper 77' (Seidel & Seidel, 1991) and Colorado State University (Takeda, 1986) using an inverted microscope (Olympus CK 2, AB technology, Japan). Under these guidelines, the collected embryos were graded as 'Excellent', 'Good', 'Fair', 'Poor', and 'Degenerated oocytes' (**Figure 5**).

Two more replicates of the experiment were carried out using the remaining pre-conditioned embryo donor cows (n = 8). The experiment was repeated using the same 12 cows according to the experimental design.

Data analysis

The following equation was employed to calculate the embryo recovery rate (ERR) using

the number of embryos recovered (NER) and the number of CLs counted (NCC) for each group.

$$ERR = \frac{NER}{NCC} \times 100\%$$

Firstly, the number of embryos, the number of corpora lutea, embryo recovery rate, and the number of transferable embryos were descriptively analyzed and reported, and then their respective arithmetic means (± standard deviation), 25^{th}, 50^{th} (i.e., median), and 75^{th} percentiles, and ranges were determined. Secondly, to determine if the GnRH treatment at AI significantly affected the above variables, data were analyzed using a one-sample t-test modified for 2×2 crossover experiments (Senn, 2002; Jones & Kenward, 2015). In this statistical procedure, the difference of the respective variables (e.g., difference in the number of embryos = number of embryos of the GnRH treatment – number of embryos of the control) between the GnRH treatment and the control was compared, and the mean difference was estimated. The Shapiro–Wilk normality test was used to check the assumption of the data normality (i.e., the normality of differences). The period effect was estimated using a one-way ANOVA modified for 2×2 crossover experiments (Senn, 2002). The carry-over effect of the GnRH treatment between periods was assumed negligible and not estimated because of the known short half-life of the hormone (Ursula, 2017). Significance was declared at $P < 0.05$, and the tendencies were discussed if $0.05 \leq P < 0.10$. Data analysis was performed using commercial statistical software (Stata SE version 14, Stata, College Station, Texas, USA).

Results and Discussion

All the super-ovulated embryo donors expressed the signs of estrous from 24-36 hrs from the removal of the CIDR devises. The common signs of estrus were a swollen, hyperemic vagina, clear, colorless vaginal mucus discharge, and the standard to be mounted by other cows. However, all the embryo donors didn't exhibit all the signs at any given time. Furthermore, thick, colorless ropy mucus discharges were the most common estrous signs reported in the experiment.

The number of corpora lutea in the embryo donors was counted to represent their ovulations in this study. The ranges of corpora lutea in the embryo donors of the TG and CG were 1-12 and 1-5 per cow, respectively (**Table 1**).

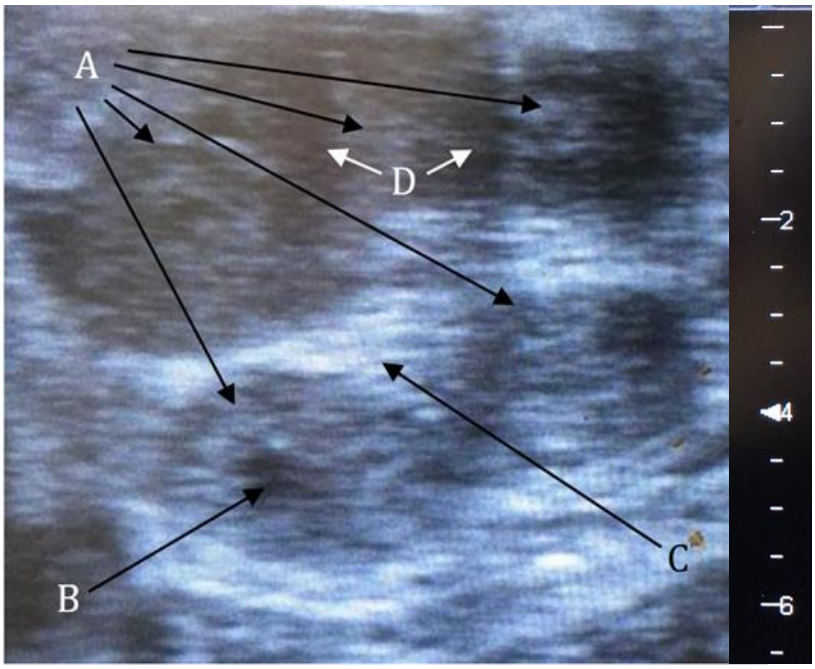
Early CLs were softer with a higher risk of having a false negative count during rectal palpation in the cows. At day 7 or during the mid-phase of the CLs, it was easier to identify them during palpation due to their firm nature at that stage. On day 7, one CL was missed during the rectal palpation technique in an embryo donor of the TG in this study. The correct number (**Table 1**) was achieved at the second counting, which could identify the missed embedded CL using ultrasound scanning technology. Although ultrasound scanning was a useful aid to accurately identify the number of CLs (**Figure 2**), the rectal palpation method could also accurately count the number of CLs when performed by an experienced person, particularly if the embedded CLs are not present and when counting is done in the middle of the diestrus period. Usually, embedded CLs could be identified due to enlargement of the located area of the ovary and the increased firmness (from the mid-phase) of the region compared to the surrounding parenchyma of the ovary.

The presence of misshapen and multiple corpora lutea or embedded CL can be missed under per the rectal palpation technique in a super-ovulatory program in cows. Although a higher number of follicles is recruited at the beginning of a follicular wave, the majority could undergo atresia before reaching the preovulatory stage. However, 1-2 follicles may develop up to the preovulatory stage after 2-3 follicular waves in cows (Rhodes,1995). The main goals of super-ovulatory protocols are to increase the number of recruitments of follicles and increase the number of follicles that reach the preovulatory stage leading to more ovulations. However, depending on the efficacy, dose, frequency, and type of different hormones used, the time taken to develop the preovulatory stages and ovulations varies. According to the statistical analysis, a tendency ($P = 0.083$) for the number of corpora

Table 1. The numbers of CLs and embryos (including unfertilized oocytes) recorded in the treatment and control groups.

Group	No. of CL			No. of embryos
	Left	Right	Total	
TG	32	33	65	53
CG	24	23	47	13

*Note: Data from **Table 1** revealed more or less similar ovulations in both the left and right ovaries while resulting in the highest ovulation and embryo production in the treatment group.*



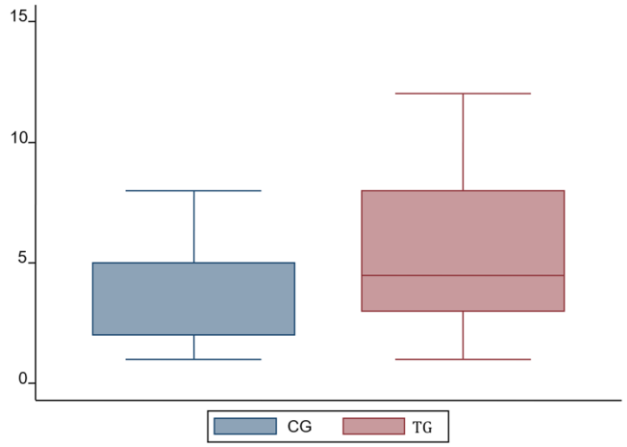
Note: D – Anechoic vertical patches due to improper conductivity.

Figure 2. Trans-rectal ultrasound scanning (Lenoir rectal probe, 6.5 MHz, Bondway - China) image of an ovary in an embryo donor cow during the superovulation process; A – Hypoechoic circular areas represent CLs while the anechoic center of the CL represents the lacunae (B) of it; C – Indicates the hyperechoic interstitial tissue of the ovary.

lutea to be higher in the TG than in the CG was identified (**Figure 3**) in this study. Further, the range of embryos recovered from the TG was 0-12 while carrying 0-5 in the CG.

Embryo production was significantly (P = 0.003) higher in the TG compared to the CG (**Figure 4**). This revealed the ability of the GnRH injection to stimulate and trigger the final advancement of additional developing stage follicles towards the preovulatory stage and ovulation during the expected period. The number of embryos compatible with their age at flushing and the growth stage in the TG was 26 (49%) and it was 4 (31%) in the CG. As embryo flushing was carried out at day 7 post-AI, compacted morulae, early blastocysts, and late blastocysts were the expected compatible growth stages of the embryos (Seidel & Seidel, 1991).

However, different developmental stages, ranging from late blastocysts to unfertilized/degenerating oocytes, were noted in the embryos recovered on day 7 post-AI in both groups, and embryonic developments are attributed to the asynchrony of the ovulation and fertilization of oocytes (Cognie *et al*., 2003). Although under this circumstance, the observed embryo morphologies in the TG were well correlated with post-fertilization development indicating more synchronized ovulation caused by GnRH administration. In the CG, the ovulation was poorly synchronized, and a comparatively low number of oocytes were fertilized. The distribution of different embryonic development stages in both groups followed a similar pattern (**Figure 5**). Interestingly, in the CG, there was a considerably fewer number of damaged embryos that were



Note: The median ± SD number of the CLs was counted in both groups.

Figure 3. Comparative mean values of corpora lutea counted in the treatment (TG) and control (CG) groups of the study.

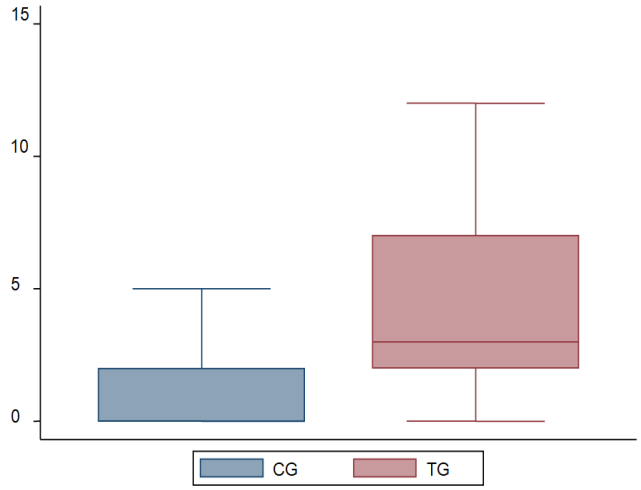


Figure 4. Comparative mean values of the embryos recovered in the treatment (TG) and control (CG) groups of the study. Median ± SD number of the embryos harvested in the control and treatment groups.

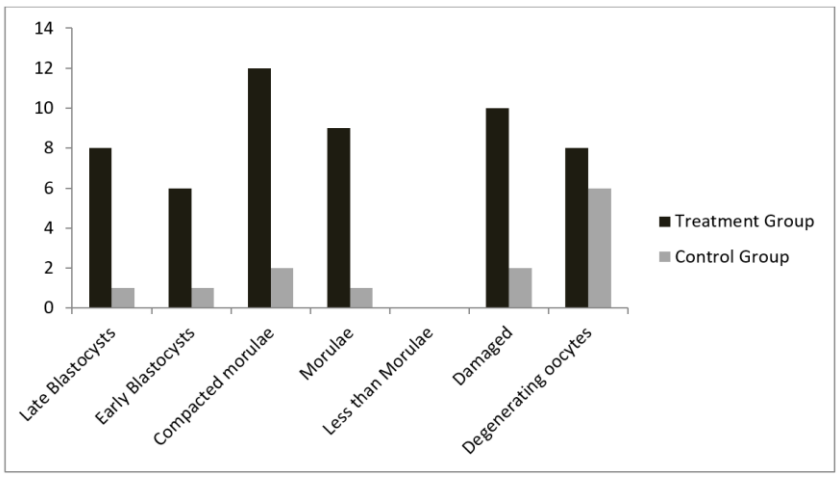


Figure 5. Classification of embryos according to their developmental stage and the physical damage in the TG and CG of the experiment. It revealed a comparatively lower production of compatible (Late blastocyst, Early blastocyst, and Compacted morulae) embryos in the CG, a lack of 'less than morula stage' of embryos in both groups, and a higher production of compacted morulae to late blastocyst stages in the TG.

identified. However, it was not a remarkable difference compared to the recovered number of embryos (TG- 18%, CG-15%) in each group. This damage could have been due to friction, pressure, and vibration of embryos, especially at the flushing and handling steps of the procedure. Thirty-five (66%) transferable embryos were recovered in the TG while 5 (38%) embryos were recovered from the CG. Usually, the embryos that fall into the categories of 'Excellent', 'Good', and 'Fair' are considered for the transferable stages (**Figure 6**) (Seidel & Seidel, 1991). Although a recovered embryo was of typical appearance, it was not placed into the 'Excellent' category if its development was more than 24 hrs. retarded from its ideally expected day 7 growth stage (**Figure 6 B**). In addition, although an embryo was ideally compatible with the day 7 growth stage, it was not categorized into the 'Excellent' category unless it carried the typical appearance due to changes in shape, presence of excluded and/or degenerated blastomeres, etc.

The embryo recovery rates of the TG and CG were 82% and 28%, respectively. This showed the significantly (P = 0.008) higher embryo recovery rate in the TG compared to the CG (**Figure 7**). It can be speculated that timely ovulation following GnRH administration could have resulted in the fertilization of an increased number of oocytes, yielding a better harvest of embryos in the TG.

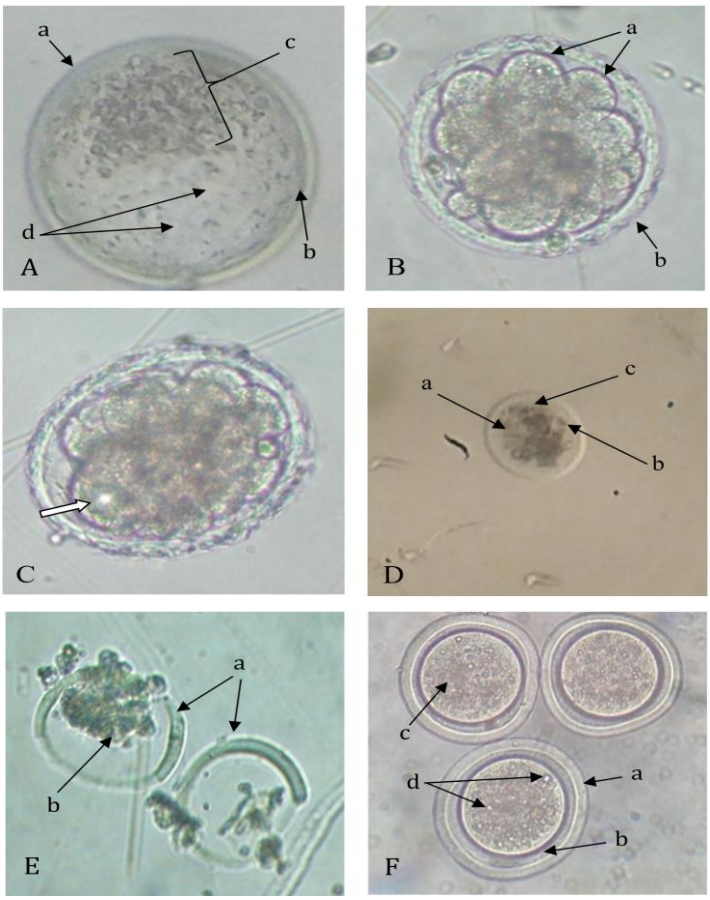


Figure 6. Classification of embryos, harvested at day 7 post-AI; (A) 'Excellent' grade; late (expanded) Blastocysts (×200). a- Well-stretched zona pellucida indicates the closer to hatching of the blastocyst, b- thin trophoblast, c- well-developed polar embryoblast, and d- clearly demarcated blastocele revealed the typical appearance of a late blastocyst. (B) 'Good' grade; Morula (×200) developing towards the compacted stage; a- blastomeres are getting smaller in size and higher in number, b- stretching and thinning of the zona pellucida with the development of the embryo. (C) 'Fair' grade; (×200). Although it has developed up to the compacted morula stage, the changed shape (oval) and presence of degenerating blastomeres (arrow), are classified into the above category. (D) 'Poor' grade embryo with a- degenerated cellular area, b- excluded cells, and c- the disaggregated cellular area of the embryo. (E) Mechanically damaged embryo (×200); a- separated parts of the zona pellucida, b- damaged compacted blastomeres. (F) Degenerating oocytes (×200); comparatively thicker a- zona pellucida, b- larger perivitelline space indicates the shrinkage of the c-cytoplasm. d- Vacuoles in the cytoplasm also provide supportive evidence for the degeneration of oocytes.

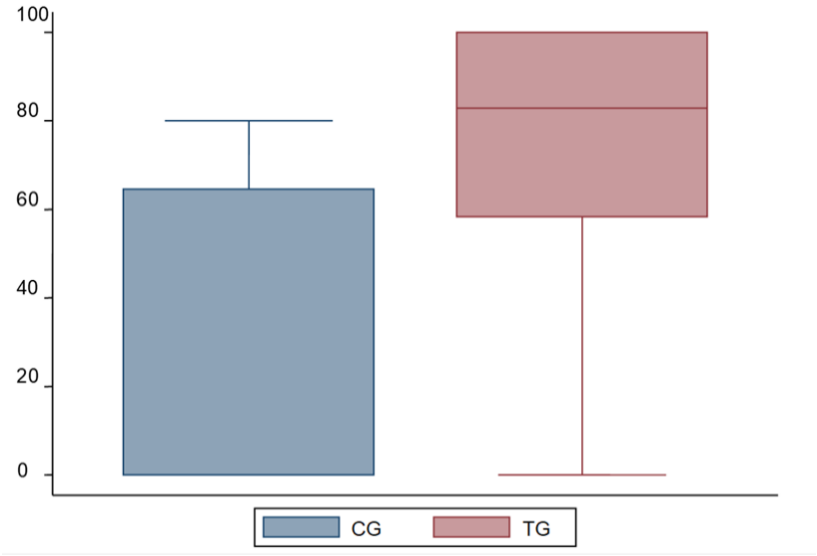


Figure 7. Comparison of the embryo recovery rates in the exogenous GnRH- treated group (TG) and the control group (CG) of cows

Many other factors can contribute to the poor recovery of embryos in MOET and include the use of poor-quality flushing fluid, complete or partial obstructions of oviducts, the occurrence of hemorrhagic anovulatory follicles (HAFs) (Peter, 2004; Bashir *et al*., 2016), the lack of suitable devices, and improper practices, among others. In addition, some embryos may go missing if they are firmly attached to mucus or if there is excess cellular debris in the flushed medium. Harvested embryos could also be misplaced after isolation when being washed and loaded into straws. Therefore, these probable variations were limited by having the same experienced person handle all the key steps of the study. The possible causes for poor embryo recovery listed above were common for all the embryo donors (Seidel & Seidel, 1991; Ashworth, 2013) in both groups in this study. In the TG of the study, a significantly higher number ($P = 0.019$) of embryos were of the transferable grade compared to the CG (**Figure 8**). A higher number of degenerated or unfertilized oocytes and damaged embryos in a super-ovulatory program would lead to both biological and economical losses, which would result in lowering the efficacy of the embryo donor and the increased cost of embryo production. The most possible cause for unfertilized or degenerated oocytes could be the result of delayed ovulations (**Figure 8**). In the present study, although the number of damaged embryos was higher in the TG (18.87%) compared to the CG (15.38%), it cannot be considered a remarkable difference and could vary depending on the practical issues during flushing. Further, the TG (15%) recorded fewer unfertilized/degenerated oocytes compared to the CG (46%). This observation also reiterates that the administration of GnRH at the first AI timely induced ovulation for the subsequent fertilization. There is strong evidence to speculate that the administration of GnRH could also increase the number of ovulations and fertile oocytes in cattle. It is also evident from this study that two AIs performed 12 hours apart were not good enough to fertilize even the ovulated oocytes and the endogenous GnRH drive was not sufficient to elicit complete ovulation in the super-ovulated crossbred cows maintained under hot, humid conditions (Prado *et al*., 1989). As GnRH has a short half-life, studies to identify the optimum dose at the time of AI and the optimum time from AI to administer the hormone would be useful in the validation of the present study under the same hot, humid conditions.

Conclusions

This study provides new information about the role of GnRH administration at AI on the embryo production efficiency and embryo

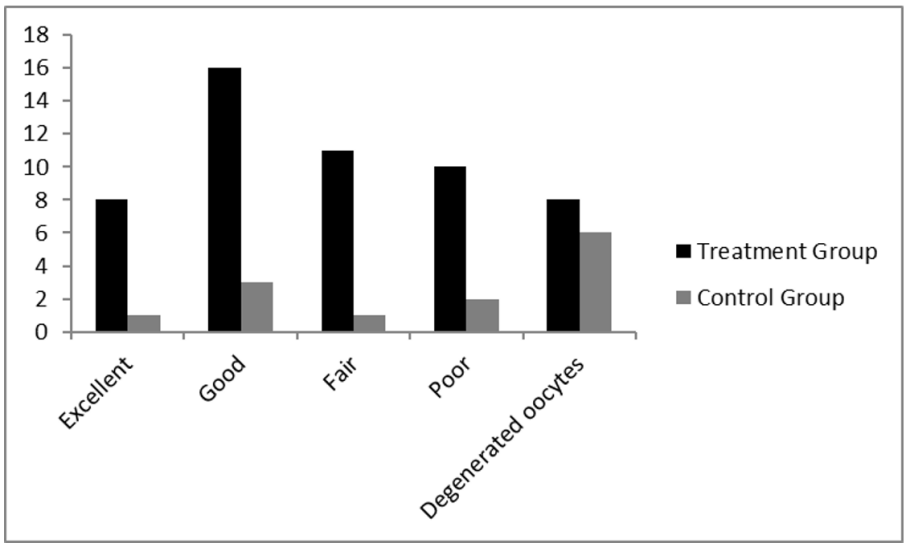


Figure 8. Different categories/grades of embryos harvested in the two groups. The results revealed the comparatively higher production of embryos in the different grades of the TG. The 'Excellent', 'Good', and 'Fair' categories represent the transferable embryos of the experiment.

quality of cows in Sri Lanka. The results of this study are interesting and give new evidence about the effects of GnRH on cattle embryo production. Further, these results revealed the tendency of cows having a remarkably higher number of ovulations with a significantly higher embryo harvest under the IM injection of exogenous GnRH at the time of AI in MOET programs of crossbred Friesian-Jersey cattle under tropical conditions. Furthermore, it may result in a significantly advanced embryo recovery rate as well. Besides, it could be beneficial to have a significantly elevated number of transferable embryos, while decreasing the number of unfertilized or degenerating oocytes in the harvest. The action of the endogenous GnRH can be further enhanced with the administration of exogenous GnRH at the time of AI in superovulation during MOET in crossbred Friesian-Jersey cattle under a tropical environment. A protocol for the administration of GnRH at the time of AI in temperate countries was validated and modified to be practiced in tropical countries like Sri Lanka. It would be beneficial for the rapid multiplication of genetically sound dairy cows towards higher milk production and fulfill the demand of breeding material in the dairy sector. The study will be continued for the validation of the technique for other temperate large and small ruminants as well.

Acknowledgments

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