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SUPEROVULATORY RESPONSE AND EMBRYO YIELD IN PURE FRIESIAN, INYAMBO, AND CROSSBREED COWS UNDER FIELD CONDITIONS IN RWANDA

[*1,4]Bizimana F., [2]Shumbusho F., [2]Hirwa C.A., [3]Båge R. and [4]Ntawubizi M.

[1]Rwanda Institute for Conservation Agriculture, P.O. Box 007, Nyamata, Rwanda
[2]Rwanda Agricultural & Animal Resources Development Board, P.O. Box 5016, Huye, Rwanda
[3]Department of Clinical Sciences, Swedish University of Agricultural Sciences, P.O. Box 7054, SE-750 07, Uppsala, Sweden
[4]School of Veterinary Medicine, University of Rwanda, P.O. Box 57, Nyagatare, Rwanda

[*]Corresponding author's email: ferdinandbizimana77@gmail.com

ABSTRACT

The study aimed to investigate the superovulatory responses and embryo yield in pure Friesian, Inyambo, and crossbreed cows in Rwanda. The research was conducted from 2018 to 2019, using 38 breeding donor cows from the Rwanda Agriculture and Animal Resources Development Board cattle research farms. Donor cows represented five breed genotypes: Inyambo, Friesian, and various crossbreeds (Inyambo × Friesian, Inyambo × Jersey and Inyambo × Sahiwal). Superovulation was induced using two commercial preparations of follicle stimulating hormone (FSH): Stimufol and Pluset, with three inseminations at 12-h intervals. Embryo recovery was conducted on day 7 post-insemination. Factors such as FSH types, donor breed, age, parity, body weight, body condition score, interval from last calving, milk yield, and ovary status were analyzed for their impact on superovulatory responses and embryo yield. A total of 181 embryos were collected, yielding 107 transferable embryos, with a recovery rate of 56.9%. The FSH type and donor body weight significantly ($p < 0.05$) influenced superovulatory responses and embryo yield, the number of developed corpora lutea, total flushed structures (unfertilized ova and embryos), viable embryos, and embryo stages. Stimufol-treated donors contributed 83.8% of viable embryos. Well-conditioned donors weighing 300-400 kg and 500-600 kg yielded higher numbers of viable embryos. This study showed that there is potential application of MOET in Rwanda's dairy genetic improvement and Inyambo local breed genetic conservation through embryo cryopreservation, Further research is recommended to explore factors influencing bovine embryo recovery rates for sustainable practice.

Key words: blastocysts, East Africa, IETS, MOET, reproductive biotechnologies

INTRODUCTION

Agricultural production constitutes approximately 26% of Rwanda's Gross Domestic Product (GDP) and serves as the primary source of employment for nearly 66% of the population. Within the agricultural sector, livestock farming contributes 3% to the overall GDP (NISR, 2021). Notably, the cattle production system in Rwanda predominantly consists of Inyambo (one of the two main Rwandan local cow strains) and their crossbreeds. Inyambo is characterized by their long horns, represent 76% of the national herd. Despite their relatively low milk yield, producing between 1 and 3.5 liters per day, the Inyambo breed exhibits remarkable resilience to harsh environmental conditions and tick-borne diseases that commonly affect dairy breeds in the region (Hirwa *et al*., 2017).

In efforts to increase milk production, the Rwandan government supports the breeding of high-productive foreign cattle, primarily utilizing Friesian and Jersey bulls to improve the genetics of Inyambo (De Vries *et al.,* 2020). However, this practice of rapid and uncontrolled cattle crossbreeding may pose a threat to the existence of Inyambo long-horned cattle, which are uniquely adapted to the local climate and terrain (Hirwa *et al.*, 2017). Artificial insemination (AI), which results in a hybrid genetic inheritance from both parents, is what is regularly used for breeding of Inyambo. However, this method may necessitate many years to achieve significant genetic improvement in local cow breeds like Inyambo for enhanced milk production (Gebre *et al.,* 2022). An alternative solution is multiple ovulation and embryo transfer (MOET), which involves the dissemination of the entire genetic potential of elite dairy bulls and cows (Norris, 2001; Mapletoft and Hasler, 2005). This process entails inducing superovulation in donor cows to collect multiple embryos, which are usually subsequently transferred to recipient cows with limited genetic potential for milk production (Khan *et al.,* 2022). MOET plays a central role in the global trade of genetic resources, conserving endangered species, maintaining elite dairy herds, minimizing the risk of exotic diseases and the cost of production, and eliminating transportation stress (Moore and Hasler, 2017).

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Embryo transfer (ET) was initially realized in a rabbit, 130 years ago (Adams, 2018), and the first successful attempt in a bovine was reported sixty years ago (Moore and Hasler, 2017). There is evidence suggesting that around 313,780 and 1,166,034 bovine embryos are respectively, worldwide produced using *in vivo* and *in vitro* techniques per year (Joao and Viana, 2021). Currently, the United States and Canada are leading exporters of *in vivo* bovine embryos, with over 9,000 embryos per year (Joao and Viana, 2021). Although MOET activities on the African continent are poorly reported to the International Embryo Transfer Society (IETS), South Africa itself was reported to export 1103 *in vivo* embryos in 2016, representing 4.4% of the world export share (Perry, 2017). Despite its potential benefits, MOET technology remains relatively undocumented and under-explored in Rwanda and neighboring African countries. Therefore, the present study aims to investigate the superovulatory response and embryo yield in pure Friesian, Inyambo, and crossbreed cows under field conditions in Rwanda.

MATERIALS AND METHODS

Study Site and Duration

The research was conducted in Rwanda over the period of 2018 to 2019. Donor cow superstimulation, embryo collection, and data collection occurred at three research stations used by the Rwanda Agriculture and Animal Resources Development Board (RAB): Songa (2° 24′ S, 29° 46′ E) in the Southern Province (altitude: 1500-1600 m asl), Kinigi Station (01° 43′ S, 29° 54′ E) in the Northern Province (altitude ranging from 1892-2400 m above sea level, and Nyagatare (2° 30′ S, 30° 25′ E) in the Eastern Province (average altitude of 1425 m asl). Despite the geographic differences among the three RAB farm stations, the animals were experiencing similar farming practices, utilizing a natural pasture grazing system: The region experiences a bimodal rainfall pattern, characterized by short rainy seasons from September to December and long rainy seasons from March to May. Conversely, long dry seasons extend from June to August, with short dry seasons occurring from January to February.

Experimental Design and Treatments

Twenty, 12 and 6 donor cows from, respectively Songa, Kinigi, and Nyagatare research stations were selected for superovulation to provide embryos. Before selection, thorough examination of genital organs, including the ovaries, uterus, and cervix status was conducted by manual palpation and transrectal ultrasonography using a portable ultrasound machine equipped with a 7.5-MHz linear probe (Easi-Scan 3, BCF Technology Ltd, Livingston, UK) to ensure the absence of pathological disorders including abnormal uterine structure, and pathological discharge.

Estrus synchronization of donors was performed using an intravaginal device impregnated with 1.55 g of progesterone (PRID Delta, CEVA Santé Animale, Libourne-France), followed by super-stimulation with porcine FSH (Stimufol, Reprobiol, Liège, Belgium) administered intramuscular (i.m.) in decreasing doses (100-100 µg, 75-75 µg, 50-50 µg, 25-25 µg) at 12-h intervals, starting on the 5th day after PRID insertion. Additionally, 750 µg of D-cloprostenol (Enzaprost® D-C, Biogénesis, Bagó, Argentina) were injected i.m. on day 6 and 200 µg of GnRH (Cystorelin®, Merial, Duluth, USA) i.m. on day 8. The superovulation protocol was adapted and used as described in Hasler (2004) and Silva *et al.* (2009). Donors underwent three inseminations within 12-h intervals, commencing 48 h post-PRID removal.

Flushing was performed on day 7 after insemination. Prior to uterine flushing, the number of corpora lutea (CL) on the ovary was recorded using the transrectal ultrasound machine described above. Embryo flushing and recovery were conducted under epidural anesthesia using 5 ml of 2% lidocaine hydrochloride (Xylestesin, Cristália, Itapira, Brazil) on day 7 post-insemination using a uterine catheter (two-ways Foley catheter size 20, 30 ml, Maersk Medical A/S, Lynge, Denmark) and a 60 ml plastic syringe. Uterine flushing was performed using 1000 ml of flushing media (Dulbecco's PBS medium, Gibco Life Technologies Corporation, Grand Island, NY, USA). Each uterine horn received 500 ml of flushing solution. Following positioning of the Foley catheter balloon in the uterine horns, each horn was filled with 50-100 ml of solution using a 3-prong Y-catheter, and the recovered fluid was collected in a sterile embryo filter (Agtech Inc. Zona™ Filter, Radiated, CAT. #D03, USA) with pores of 70 µm wide for filtration. Subsequently, the uterine flushing liquid was evaluated in the embryo laboratory to identify and assess embryos based on quality and developmental stage.

Identification and manipulation of transferable fresh embryos were conducted using a stereomicroscope, following procedures outlined in (Chumchai *et al*., 2021). After washing the filters with holding solution (TCM-199 supplemented with 200 mM L-glutamine, 10 mg/ml gentamicin and 20% FCS), the liquid was transferred into Petri dishes for embryo classification using a stereo microscope, based on quality and developmental stages following the IETS criteria as described in Bó and Mapletoft (2013).

Factors Considered

The study examined nine independent variables, including types of FSH, donor breed, age, parity, body weight, BCS, interval from last calving, milk yield, and ovary status, for their influence on superovulatory responses and embryo yield (as dependent variables). These dependent variables included estrus signs, the number of developed corpora lutea, collected embryos, their stages and grades, and unovulated follicles (UOF).

The five donor breeds were determined using the RAB herd record book, showing Inyambo (pure Rwandan local longhorn cow): II (n = 6), Friesian: FF (n = 12), and various crossbreeds, namely Inyambo × Friesian: IF (n = 9), Inyambo × Jersey: IJ (n = 5), and Inyambo × Sahiwal: IS (n = 6). The donor age (years), parity number, interval from last calving (months), and peak milk yield (liters) were reported from the RAB record book as the highest quantity yielded during lactation. The body weight (kg) was estimated using a tape measure (RONDO), while BCS was estimated according to the method described by Edmonson *et al.* (1989) on a scale of 1-5. Ovary status (having a CL or not on the ovary on PRID insertion day determined during rectal palpation and ultrasound scanning) was examined for its influence on superovulatory responses and embryo yield. The dependent variables included estrus signs (vaginal mucus; mounting other cows; standing to be mounted; silent), number of developed corpora lutea on right and left ovaries within 7 days after superovulation, number of collected embryos, their stages (morulae, blastocysts, and unfertilized oocytes-UFO), grades (1, 2, 3, and 4), and non-ovulatory follicles (NOF).

Total recovery refers to the retrieval of all substances or structures (UFO and embryos) in relation to the total ovulations, considering the combined count of CL and NOF. On the other hand, total flush output represents the average of recovered substances (UFO and embryos) per individual donor cow. The superovulatory response parameter assesses the extent of ovulation in preovulatory follicles, considering both CL and NOF.

In the evaluation of superovulation responses and embryo yield, various parameters were defined and computed as described in Vieira *et al.* (2014). The response rate was determined as a percentage, calculated by dividing the number of cows with at least three CL and/or NOF by the total number of treated cows, then multiplying by 100. Similarly, the recovery rate was derived as a percentage, obtained by dividing the total recovery or flush output (including UFO and embryos) by the sum of the total number of CL and NOF, then multiplied by 100. Additionally, the proportion of UFO was defined as a percentage, achieved by dividing the number of UFO by the total recovery or flush output, then multiplied by 100, while the rate of transferable embryos was calculated as the ratio of the total number of transferable embryos to the total recovery or flush output, multiplied by 100.

Statistical Analysis

Descriptive statistics were performed, and random effects associated with cow superstimulation response and embryo yield were analyzed using one-way ANOVA in SPSS Statistics, version 23, with significance determined at $p < 0.05$.

RESULTS AND DISCUSSION

The study revealed that the mean number of CL per superstimulated donor stood at 8.30 ± 6.80, and the mean number of NOF recorded per animal was 0.30 ± 0.10 (Table 2). After flushing, a mean of 1.90 ± 0.40, UFO and 3 fertilized embryos per donor were recovered (Table 3).

The present results were similar to the findings of Vieira *et al.* (2014) from 1562 multiple ovulation procedures in commercial dairy cows in southwestern Brazil and from a study on 60 cows of three different genetic groups in Bangladesh Agricultural University (Ali *et al.,* 2011). However, our results showed fewer fertilized embryos than Silva *et al.* (2009) in Brazil on 884 superovulations in 318 Nelore donor cows (*Bos taurus indicus*) and a higher number of transferable embryos compared to Say *et al.* (2022) in Türkiye on 10 South Anatolian red donor cows.

In this study donor genotype (breed) did not significantly ($p > 0.05$) affect the number of CL developed on both ovaries, ovulation rate, number of embryos flushed from both horns, number of viable embryos and unfertilized oocytes, embryo stages ages, total numbers of morulae, early blastocysts and blastocysts, and embryo grades. The results were similar to the findings in an Ethiopian study (Jemal *et al.,* 2021), where the embryo flush outputs did not show a significant difference between Boran and Holstein/Friesian × Boran cross cattle breeds.

In the current study, the research stations (RAB Songa, Kinigi, and Nyagatare) did not show significant ($p > 0.05$) effect on superovulatory responses and embryo yield, except for the number of CL developed ($p = 0.02$). Specifically, 11, 6, and 21 donor cows superovulated in Kinigi, Nyagatare, and Songa respectively developed 10, 5, and 3 CL per donor cow. Notably, cows from Songa station exhibited a lower number of CL, indicating a higher proportion of donors who did not respond optimally to the superovulatory protocols, with underlying causes not being thoroughly identified.

Regarding the production of viable embryos per donor cow, the IF and IJ breeds of cow emerged as the most successful, yielding 5 and 4 embryos per donor, respectively. In contrast, II, IS, and FF breeds of cow produced an average of 2 viable embryos per donor. On average, each donor cow was able to produce three viable embryos.

Table 1: Descriptive statistics characterizing the observed donor cows and studied variables

Variables	Min.	Max.	Mean ± SE
Body weight (kg)	309	690	469.70 ± 16.40
Body condition score (scale of 1-5)	3	4	3.30 ± 0.60
Age (year)	3	11	5.60 ± 0.34
Number of parities	1	8	2.50 ± 0.30
Interval from last calving (months)	2	28	10.40 ± 1.20
Peak milk (liter)	1	22	9.40 ± 1.00

SE - standard error

Table 2: Descriptive statistics of superovulatory response and embryo yield per donor cow

Variables	Min.	Max.	Mean ± SE
Total CL	1.00	20.00	8.30 ± 0.60
Total NOF	0.00	2.00	0.30 ± 0.10
Total flushed structures	0.00	19.00	4.80 ± 0.70
Viable embryos	0.00	11.00	2.80 ± 0.50
Unfertilized oocytes	0.00	11.00	1.90 ± 0.40
Total morula	0.00	8.00	2.10 ± 0.30
Total early blastocysts	0.00	3.00	0.50 ± 0.10
Total blastocysts	0.00	2.00	0.20 ± 0.70
Grade 1 embryos	0.00	7.00	2.10 ± 0.30
Grade 2 embryos	0.00	4.00	0.50 ± 0.20
Grade 3 embryos	0.00	2.00	0.20 ± 0.10

SE - standard error, CL - corpora lutea, NOF - non-ovulatory follicles

The type of FSH (Stimufol and Pluset) utilized had a significant ($p < 0.05$) effect on several key parameters within the study. Specifically, it influenced ($p < 0.01$) the number of CL developed within 7 days post-superovulatory estrus, the number of flushed structures ($p < 0.01$), the count of unfertilized oocytes ($p = 0.04$), and the total number of morula embryos ($p = 0.04$).

In this study, Stimufol exhibited notable outcomes, contributing to 83.8% of the 107 viable oocytes flushed from a total of 38 donor cows. In contrast, Pluset yielded a higher number of embryos during Simmental cow superovulation in Kazakhstan (Seiteuov *et al.,* 2013). However, Stimufol used in multiparous cow superovulation in Algeria (Adel *et al.,* 2018) yielded a similar embryo count as observed in the present study.

The bodyweight of the donor cows significantly ($p < 0.01$) influenced the total number of flushed structures, viable embryos), and unfertilized oocytes. The body weight of donor cows highly significantly ($p < 0.01$) impacted the total number of flushed structures, viable embryos, and unfertilized oocytes). Donor cows with body weights near the mean (469.70 ± 16.40 kg), as shown in Tables 1 and 4, produced a higher number of viable embryos. Donor cows within the weight ranges of 300-400 kg and 500-600 kg yielded more viable embryos than those in other weight categories, with averages of 3 and 4 embryos per donor, respectively.

The BCS did not exhibit a significant influence on superovulation responses and embryo yield, except for the total number of CL ($p = 0.03$). However, notable trends were observed regarding BCS and viable embryo production. Donor cows with BCS of 3.5 and 4.5 yielded more viable embryos compared to other scores, producing an average of 4 and 3 embryos per donor respectively. This was not contradicting the findings in Lamb *et al.* (2016), which highlighted the role of proper nutrition in potential donor cows.

The age of the donor cow showed no significant ($p > 0.05$) effect on superovulatory responses and embryo yield, except for the count of CL and unfertilized oocytes, where a significance level of $p = 0.03$ was observed for both. Notably, donor cows aged between 3 and 5 years and those over 5 and 7 years old displayed higher ($p < 0.05$) embryo production compared to other age categories, yielding an average of 3 and 4 embryos per donor, respectively. This is not in conflict with the findings in Silva *et al.* (2009), where donor age negatively affected the number and quality of the embryos, with younger donors producing better and more embryos than older animals in *Bos taurus indicus*.

Table 3: Superovulation responses and embryo yield considering donor estrus signs, corpora lutea, nonovulatory follicles, embryo stages and grades among donor breed genotypes

	Donor breeds						
Variables	II ($n = 6$)	IF ($n = 9$)	IJ ($n = 5$)	IS ($n = 6$)	FF ($n = 12$)	Total	(%)
Estrus signs/donor							
Mu & Mo	1	3	0	0	1	5	13.20
Mu, Mo & S	4	2	4	4	7	21	55.30
Mu	0	2	0	2	1	5	13.20
Si	1	2	1	0	3	7	18.40
Total	6	9	5	6	12	38	
Total CL	29	83	45	44	113	314	
NOF	1	2	2	2	3	10	
Flushed structures							
UFO	8	17	7	9	33	74	40.90
Viable embryos	9	44	19	12	23	107	59.10
Total	17	61	26	21	56	181	
Viable embryos/donor	2	5	4	2	2	3	
Embryo stages							
Morulae	5	30	14	11	20	80	74.80
Early blastocysts	3	10	4	1	2	20	18.70
Blastocysts	1	4	1	0	1	7	6.50
Embryo grades							
Grade 1	8	31	16	10	18	83	45.90
Grade 2	1	9	2	2	3	17	9.40
Grade 3	0	4	1	0	2	7	3.90
Grade 4	8	17	7	9	33	74	40.90

II - Inyambo; FF - Friesian; IF - Inyambo × Friesian; IJ - Inyambo × Jersey; IS - Inyambo × Sahiwal; Mu - vaginal mucus (Mu); Mo - mounting other cow; S - standing to be mounted (S); Si - silent heat; CL - corpora lutea; UFO - unfertilized ova; NOF - non-ovulatory follicles

Table 4: Effects of station, FSH type, and donor bodyweight on superovulatory response and embryo yield in the observed cows

Independent factors	Dependent factors	Value	*p*-value
Research stations			
Kinigi	Number of CL per donor	10	0.02
Nyagatare		5	
Songa		3	
FSH			
Stimufol (*n* = 24)	Number of CL per donor	10	< 0.01
Pluset (*n* = 14)		6	
FSH			
Stimufol (*n* = 24)	Total flushed structures (UFO and embryo) per donor	34	< 0.01
Pluset (*n* = 14)		147	
FSH			
Stimufol (*n* = 24)	Morula embryos	17	0.04
Pluset (*n* =14)		63	
Body weight			
300-400 kg	Total flushed structures (UFO and embryo) per donor	43	< 0.01
> 400-500 kg		78	
> 500-600 kg		37	
> 600 kg		23	
Body weight			
300-400 kg	Viable embryos	31	0.03
> 400-500 kg		37	
> 500-600 kg		30	
> 600 kg		9	

FSH - follicle stimulating hormone; CL - corpora lutea; UFO - ova

Parity did not demonstrate a significant effect on superovulatory responses and embryo yield. The same results were found in (Hussein *et al.,* 2014). However, interestingly, older animals with parities ranging from 4 to 8, along with primiparous cows, exhibited a notable trend of higher embryo production. Specifically, cows within the 4 to 8 parity range and first parity cows yielded 4 and 3 embryos per donor, respectively, surpassing other parity categories. While the interval from last calving in donor cows did not show a significant effect on superovulatory responses in this study, it is remarkable that super-ovulated donors stimulated during the period ranging from 4 months postpartum and beyond yielded more embryos compared to those stimulated within the 4-month period following the last calving. Specifically, donors stimulated during the later period produced an average of three embryos per cow, whereas those stimulated earlier yielded only one embryo per cow.

It was revealed that the capacity of a donor cow to produce more milk than average did not exhibit a significant effect on superovulatory responses and embryo yield. However, the results did not conflict with the findings reported by Hussein *et al.* (2014). Donors with a peak milk yield of 4 to 5 liters per day per cow showcased the highest production of viable embryos, yielding an average of 6 embryos per cow. This was followed by cows producing 6 to 9 liters per day, as well as those producing more than 16 liters (indicative of good dairy cows in the Rwandan setting), which produced 3 viable embryos each. Conversely, cows producing less than 3 liters and those producing between 10 and 15 liters yielded fewer viable embryos, with an average of 2 or fewer embryos per cow. The underlying reasons for these variations were not thoroughly explored in the present study.

The presence or absence of a CL in donor cows on the day of progesterone device (CIDR) insertion (day 0) was found to have no significant effect on superovulatory responses and embryo yield. These results were in agreement with the results in Tríbulo *et al.* (2011). However, an interesting observation emerged regarding the number of viable embryos produced. Donors without a CL on day0 yielded a higher number of viable embryos compared to donors with a CL, producing an average of 4 and 2 viable embryos per donor, respectively.

CONCLUSION

The bovine embryo recovery rate was deemed moderately good, suggesting potential application in Rwanda's dairy genetic improvement and Inyambo local breed genetic conservation through embryo cryopreservation. The study recommends further research to explore major factors influencing bovine embryo recovery rates for sustainable application in the mentioned contexts.

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DATA AVAILABILITY

The datasets generated and analyzed during the current study are available and we are currently working on uploading the dataset to a repository once the link provided. The data was curated and managed in Microsoft Excel and analyzed using SPSS Statistics for Windows, Version 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp).

AUTHORS CONTRIBUTIONS

Conceptualization, F.B., F.S. and C.H.; methodology, F.B., FS. and C.H.; validation, C.H., R.B., and M.N.; formal analysis, F.B., and M.N.; investigation, C.H.; resources, S.F. and C.H.; data curation, F.B.; writing—original draft preparation, F.B.; writing—review and editing, M.N and R.B.; supervision, C.H., R.B., and M.N.; project administration, C.H.; funding acquisition, C.H. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts in this paper.

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