Effect of Gonadotropin Hormones on Bovine *in Vivo* Embryo Production and Subsequent Conception Rates Following Embryo Transfer

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Abstract

Multiple ovulation and embryo transfer (MOET) has been established to mass produce in vivo embryos from high quality donor cows. Inducing superovulation using either follicle-stimulating hormone (FSH) or equine chorionic gonadotrophins (eCG) influences the number and quality of in vivo embryos that may be recovered from a single donor cow, making it an essential stage in the MOET process. Therefore, the objective of this study included (1) comparing the superovulatory responses of donor cows, the embryos production rate and quality of embryos collected from Charoke cows treated with either FSH or eCG hormones, and (2) evaluating conception and calving rates following embryo transfer. All donor cows (n=33) with at least one parity were hormonally treated either with FSH or eCG. All of the parameters in the FSH- and eCG-treated groups did not differ significantly ($p > 0.05$) in the current investigation. However, cows treated with FSH had a larger percentage of donors with positive superovulatory response (56%) than the eCG group (44%). An average of three transferable embryos was collected per animal receiving either FSH or eCG hormones. The production of one life calf in the embryo transfer demonstrated that adequate progesterone levels during embryo development. Additionally, performing embryo transfer on day 6 following artificial insemination, with more than one embryo per recipient, could enhanced the conception rates. In conclusion, this study indicated that both FSH and eCG can be utilized to superovulate donor cows under local conditions, resulting in the birth of live calves.

Keywords: Superovulation, FSH, eCG, MOET, Charoke cow.

Introduction.

Reproductive biotechnologies such as MOET have the potential to significantly enhance animal breeding programmes in Malaysia. In vivo embryo production system is recognised as remarkable biotechnologies in cattle due to their ability to generate enhanced genetic gains in dairy and beef cattle herds. Consequently, in vivo embryo

production using the MOET technique accounts for the majority of cattle embryos produced worldwide, despite the high costs and the often-variable number of embryos produced (Galli et al., 2003). This reproductive biotechnology can accelerate cattle genetic improvement programmes, thereby boosting animal production.

Superovulation is a crucial step in the MOET technique. It is defined as the hormonal manipulation designed to enhance ovulation during the ovulatory cycle by stimulating the ovaries to release multiple oocytes (Lai et al., 2019). Inducing superovulation in cows allows for the development of several oocytes into embryos that would otherwise degenerate under normal conditions. Gonadotropins such as FSH or eCG are routinely used to induce follicular growth and achieve multiple ovulations in cows. Hormones such as FSH and Luteinizing hormone (LH), extracted from the pituitary glands of swine and sheep (Hesser et al., 2011), is short half-life molecule that has become the preferred method for commercial-scale induction of superovulation in cows. FSH is primarily responsible for initiating follicle development, while eCG exhibits both FSH - and LH-like activities and has a high affinity for FSH and LH receptors in the ovaries, making it widely used for superovulation in various mammalian species (Lunenfeld, 2004). Furthermore, eCG a long half-life molecule produced by endometrial cup cells of mares (Murphy and Martinuk, 1991; De Rensis and Lopez-Gatius, 2014; Vilanova et al., 2019) is often preferred by farmers to reduce animal handling on the farm (Fernando et al., 2020).

In the MOET technique, fertilization of oocytes by sperm is a crucial step in the production of embryos following superovulation. Fertilization occurs in the oviduct, specifically in the ampulla where sperm swim against the current in the uterus to the site of fertilization. The opportunity for sperm to fertilize the ovum is very brief making it essential for sperm to be present at the fertilization site when the ovum is ovulated. The fusion of ovum and sperm forms a diploid zygote (2n) which develops in the oviduct until it reaches the blastocyst stage before implanting into the uterine wall. This embryonic development progresses start from a 2-cell stage until blastocyst (comprising more than70 cells) over a period of 5 – 7 days. The blastocyst is the final stage of the embryo that is suitable for transfer into the uterus of the recipient dam or for freezing in liquid nitrogen at a temperature of –196 °C for future use.

Several studies have shown that transferring embryos created via the MOET process can successfully result in the birth of healthy kids. Embryo transfer is the process by which an embryo is collected from a donor female and subsequently implanted into a recipient female where it can complete its development (Stroud, 2012). This practice often leads to successful pregnancies and the birth of live calves. However, the conditions that influence embryo production and pregnancy rates following embryo transfer can vary between laboratories. An embryo

transfer programme involves a series of steps that depend on factors associated with the embryo, the donor and recipient, as well as the interaction among these factors (Faizah et al., 2018). Therefore, the objective of this study included (1) comparing the superovulatory responses of donor cows, as well as the embryos production rate and quality of embryos obtained from Charoke cows treated with either FSH or eCG hormones, and (2) evaluating conception and calving rates following embryo transfer.

Materials and Methods

Animal Ethics

This study was conducted with the approval of the MARDI Animal Ethics Committee, (Approval number: 20201130/R/MAEC00085).

Animal management treatment

This study was conducted at the National Animal Embryo Centre (NAEC) Complex, MARDI Kluang, Johore. All the animals included in the study were selected based on their normal estrus cycle and healthy good reproductive tracts, with each having at least one parity (previous calving). The adult animals were of various ages (more than 5 years old), and had a good body condition score averaging 4 and 5 on a scale of 1 to 5. Deworming and multivitamin were given at least two weeks before the experiment. Two or three animals were placed in the pens at the NAEC Complex where they were fed with grass and concentrate according to their dietary requirements, ad libitum access to drinking water. All pens were cleaned every morning before feeding time.

Estrus Synchronization and Superovulation

In this study, thirty-three Charoke cows used as donor cows. All donor cows were hormonally treated according to an established protocol (Habsah et al., 2016) as shown in Figure 1. For 12 days, each cow received 1.9 g of progesterone using a controlled internal drug release device (CIDR-B; pfizer®, Eazy-Breed, New Zealand). The day CIDR-B was inserted was designated as day 0. Before the removal of CIDR-B on day 13, the donors were administered a total of 10 ml FSH (ovine pituitary extract, ICPbio limited, Auckland, New Zealand) or 10 ml of eCG (Folligon, Intervet International B.V. Ireland) intramuscularly, administrated as either a decreasing dose or a single dose, respectively, for superovulation hormone treatment. This was followed by 2 ml of intramuscular injection of Prostaglandin-F2α (PGF2: Estrumate, Schering-Plough) on day 13. Signs of estrus were observed 36-72 hours after the removal of CIDR-B. During this period, estrous cows received 2 ml of Receptal, 6 hours of standing heat detection and fixed-time artificial insemination was performed 48 and 60 h after CIDR removal. Embryo's recovery was carried out on day 6 after the last insemination.

Embryo collection

Embryo flushing was conducted using

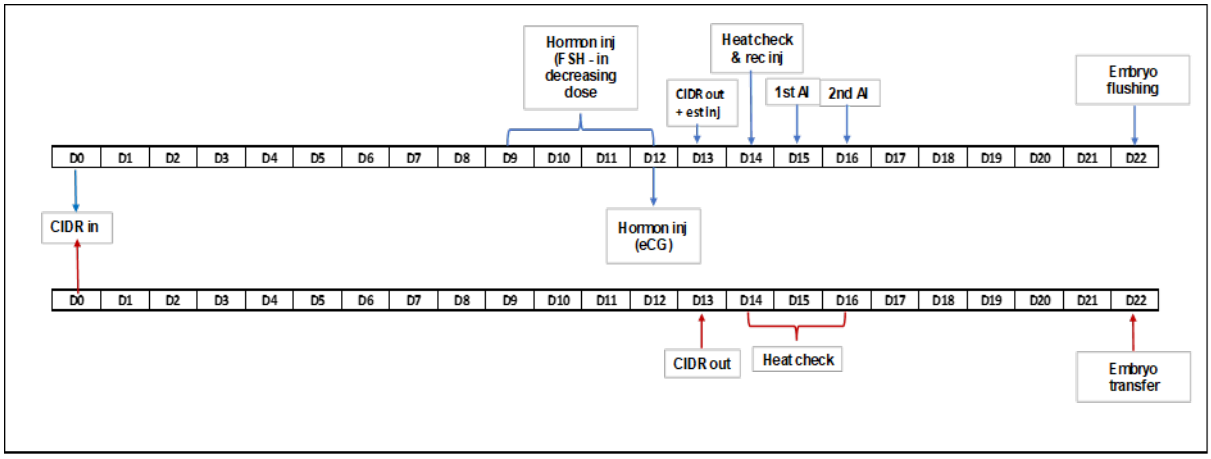

Figure 1: MOET programme schedule used in the study

boviflush solution (Boviflush; Miniture Australia Pty. Ltd., Smythesdale, VIC, Australia) on day 6. During the flushing procedure, a two-way rubber catheter was inserted past the cervix and into the uterus. A 20 ml balloon was utilised to secure the catheter during the procedure in order to collect the flushing media and uterine contents. After the flushing, the media was transported to the laboratory to search for embryos using a stereo microscope. The collected embryos were evaluated and graded before placing in bovihold media ((Bovihold; Miniture Australia Pty. Ltd., Smythesdale, VIC, Australia) and frozen in liquid nitrogen at -196oC for future use.

Embryo transfer

On day eleven before to CIDR-B removal, all recipient dams received Thirty recipient dams were used in three embryo transfer (ET) programs. natoque dictum iaculis litora dapibus. On day eleven before to CIDR-B removal, all recipient dams received On day eleven before to CIDR-B removal, all recipient dams received intramuscular injections of PGF2α after receiving a CIDR-B containing 1.9 g of progesterone for 12 days. After CIDR-B was removed, oestrus was found 36–72 hours later. The frozen embryos from Experiment 1 were non-surgically implanted into the uteri of recipient Charoke cows on day 6 of the oestrous cycle. In the experiments, each recipient dams received one or more embryos of in vivo-derived embryos with the present of corpus luteum. Rectal palpation was used to diagnose pregnancy at 90, 150 and 270 days after embryo transfer.

Experimental design

Two separate experiments were designed to assess the effectiveness of estrus synchronization and superovulation using 2 types of gonadotropins hormone, in vivo embryo production and transfer, and pregnancy rates involving Charoke cows as donor and recipient dams as shown in Figure 2.

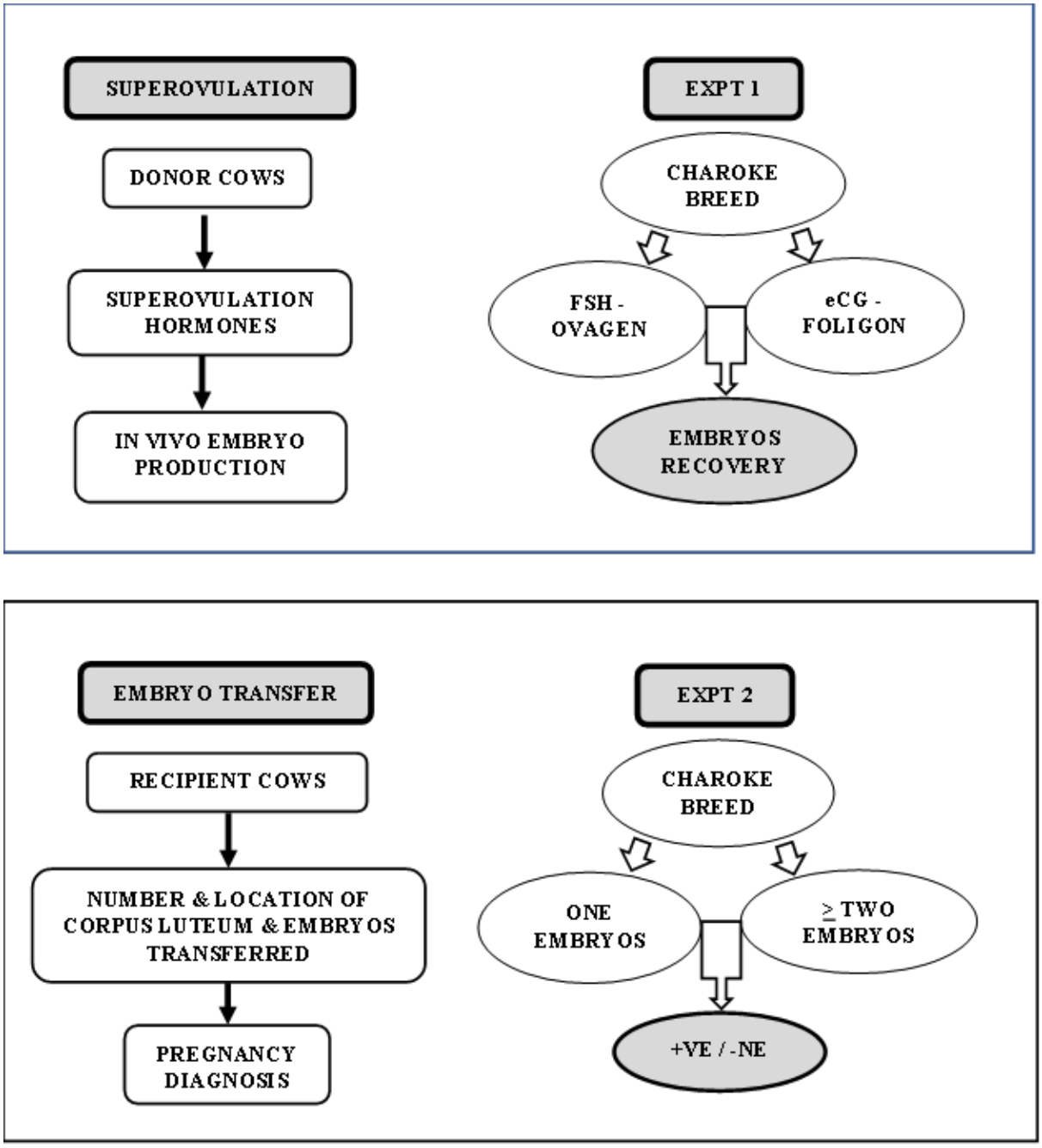

Figure 2: Illustration of experimental design involved in the study

Experiment 1

In Experiment 1, a total of thirty-three Charoke cows were selected as donors to produce in-vivo embryos. The estrus synchronization and superovulation of Charoke cows were performed according to the procedure described above. The Charoke donor cows was treated either with FSH – Ovagen (n=18) or eCG – Foligon (n=15) hormones to produce in-vivo embryos and frozen in liquid nitrogen at -196oC for embryo transfer.

Experiment 2

Three embryo transfer programmes were conducted using 30 recipient dams. Estrus synchronization of a recipient cows was performed according to the

procedures described above. On day 6 of the estrous cycle, each responding recipient dam that had a corpus luteum received one or more in vivo-derived embryos. Only recipients with CL were used in the study. The number of embryos transferred was based on availability and were placed in the uterine horn ipsilaterally based on the detection of corpus lutea. Rectal palpation was used to diagnose pregnancy at 90, 150 and 270 days after embryo transfer.

Statistical Analysis

In Experiment 1, proportional data on the superovulatory response, embryos production rates, and embryo quality from Charoke cows treated with FSH or eCG hormones were analyzed using Analysis of Variance and Duncan's Multiple Range Test to assess the significance of the subsequent development between treatments at $p<0.05$ (SPSS, Version 17). In Experiment 2, descriptive data on conception and pregnancy rates at 90, 150 and 270 days after embryo transfer were collected and discussed.

Results and discussion

In the present study, the number of Charoke donors with a positive superovulatory response was higher in the FSH-Ovagen group than in the eCG-foligon group, as demonstrated in Table 1. The effects of superovulation with FSH treatments were at least similar to, if not greater than those achieved with eCG as reported in a previous study (Purohit et al., 2006). In this study, 56% of Charoke donor cows responded to FSH-treatment, compared to 44% in the eCG-treated groups. Previous studies indicated that 70-94% of donor cows responded to FSH treatment (Quaresma

Table 1: Effect of gonadotropins on superovulatory response (SR) and embryo production in Charoke cows.

Parameter	Gonadotropin treatments			
	FSH-Ovagen		eCG-Folligon	
Number of donors	18		15	
Positive SR	$2.50 \pm 1.19^{a, xy}$	10 (56%)	$1.50 \pm 0.29^{a, xy}$	6 (44%)
Total ova recovered -n	$6.00 \pm 1.41^{a, y}$	30	$2.75 \pm 0.75^{a, y}$	19
Viable embryos	$4.50 \pm 2.06^{a, xy}$	18	$2.75 \pm 0.75^{a, y}$	11
Degenerated embryos	$1.00 \pm 0.58^{a, x}$	8	$0^{a, x}$	0
Non-fertilized oocytes	$2.00 \pm 0.76^{a, xy}$	4	$2.00 \pm 0.82^{a, y}$	8

Values for recovery of embryos are mean + standard error of the mean, per cow with positive SR.
[a] mean between column within the same row were not significantly different at $p>0.05$.
[x, y] means within same column with different superscripts were significantly different at $p<0.05$

et al., 2003; Baruselli et al., 2006; Purohit et al., 2006). These results may be attributable to the type and dose of hormones used (Baruselli et al., 2011). A significant difference ($p<0.05$) was observed in the number of degenerated embryos compared to the total number of ova recovered in the FSH-treated group. In contrast, the eCG-treated group showed a significantly higher ($p<0.05$) total number of ova, viable embryos and non-fertilized oocytes compared to the group with degenerated embryos. The incidence of degenerated embryos was absent in eCG-treated Charoke cows' group but was higher in the FSH-treated group.

The number of embryos harvested per animal did not differ between the FSH- and eCG treated groups with an average of three embryos collected per animal using either FSH or eCG hormones. The quantity of transferable quality embryos obtained in a prior study varied from 3.0 to 8.2, which was thought to be connected to the kind and amount of superovulation hormones used (Baruselli et al., 2011; Unnikrishnan et al., 2014). Variability in donor cows' superovulatory responses after gonadotropin treatments could be the cause of the discrepancies between our findings and those previously published, as well as other factors such as breed and age of donor cows, and local environmental conditions.

Apart from FSH, eCG has been used in fixed-time artificial insemination procedures in cattle to enhance fertility rates due to its ability to promote follicular growth, among other effects (Murphy, 2012; Nunex-Olivera et al., 2014). In the current investigation, there were no deteriorated embryos and a greater number of unfertilized eggs were linked to the usage of eCG hormones. The application of eCG has proven to be an effective treatment for increasing fertility, particularly in beef breeds (Nogueira et al., 2014); however, results have been inconsistent in dairy cattle (Pulley et al., 2013). Experiments worldwide have been used dosages of eCG ranging from 300 to 3000 IU producing varying outcomes (Unnikrishnan et al., 2014; Fernando et al., 2020; Minguez and Calvo, 2020; Lonergan & Sanchez, 2022). In addition to the type and dose of hormones used in superovulation, factor such as the age and breed of donor cows may also influence outcomes, especially since older cows were included in this study as suggested by Macmillian et al. (2018).

The results of the embryo transfer on Charoke cows as recipient dams and using in vivo embryos recovered from Experiment 1 were shown as in Table 2 and Figure 3. In the study, only 47% (14 out of 30) of the recipient dams had corpus luteum and included in the embryo transfer programme. The presence of corpus lutea is one of the indicators to achieving a full-term pregnancy. Embryo transfer programmes that produced positive results were further elaborate in Table 3 and Table 4.

Table 2: The results of the embryo transfer on Charoke cows.

ID	Embryo transferred (no / stages)	CL numbers		Pregnancy diagnosis	Remark
		Right	Left		
NT2	1 morula	2	X	positive	Abortion at 5 months
A5028	1 compact morula	1	X	negative	-
B5029	3 (1 blastocyst + 2 compact morula)	1	X	positive	1 live calf
A8009	2 compact morula	1	X	positive	Abortion at 7 months
A017	3 (2 blastocyst + 1 morula)	1	X	negative	-

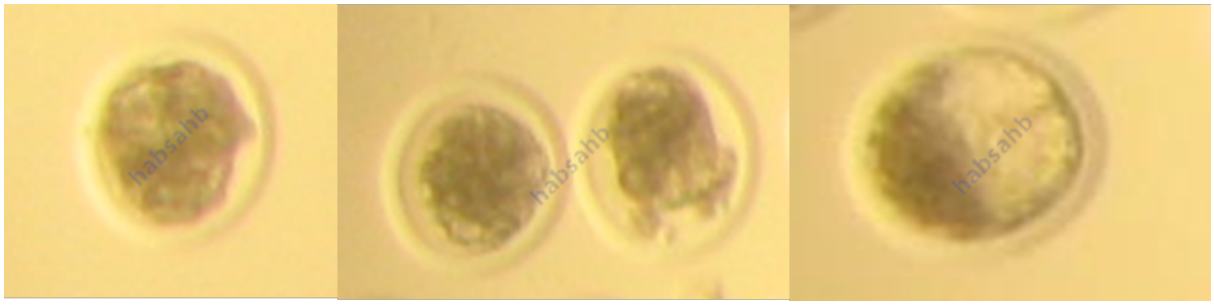

Figure 3: Recovery of in vivo embryos from MOET programme.

Our results from the initial embryo transfer programme showed that when PD was conducted 90 days after embryo transfer, 50% of pregnancies were detected in the recipient dams.

However, by day 150 after embryo transfer, the number of pregnancies in all recipients had decreased compared to day 90, ultimately resulting in total foetal loss by day 270 as shown in Table 3. Additionally, the recipient dams failed to conceive entirely during the second embryo transfer programme. In cattle, it is estimated that approximately 40% of conception loss to occurs between days 8 and 16 of pregnancy (Sreenan and Diskin, 1986; Diskin et al., 2006; Diskin and Morris, 2008). Despite a fertilization rate of approximately 90% after artificial insemination, calving rates range from 30 to 50%, indicating significant embryonic and foetal losses (Sofia et al., 2018). Early embryonic loss was estimated at 29%, accounting for the majority of pregnancy losses, while late embryonic and foetal loss were estimated at 14%, and 13%, respectively (Sofia et al., 2018). Additionally, the freezing and thawing process is complex and typically results in a 10 to 20% reduction in pregnancy rates compared to those observed with fresh embryos (Siedel & Siedel,1991; Thalkar, 2018).

Animal pregnancy rates, uterine environment management, pregnancy maintenance, embryonic growth rates, and embryo survival are all correlated with circulating progesterone levels following fertilization (Lonergan et al., 2013; Lonergan, 2015). Consequently, insufficient levels of progesterone in the uterine lumen may have contributed to pregnancy loss and the fetal loss seen in the study (Madureira et al., 2021: Thanh et al., 2023). Additionally, circulating progesterone levels are known to be associated with embryo viability and early embryo loss (Morris & Diskin, 2008; Lonergan & Sánchez, 2020). Higher progesterone levels stimulate embryo growth during the critical period of maternal recognition of pregnancy (Starbuck et al., 2004; Fair and Lonergan, 2012; Karen et al., 2014).

Table 3: The results of the first embryo transfer programme on Charoke cows

ID	Embryo transferred (no. / stages)	CL numbers		Preganncy diagnosis	Remark
		Right	Left		
NS123	2 compact morula	1	X	positive	Abortion at 5 months
24023	1 early blastocyst	1	X	negative	-
K5023	2 (blastocyst + morula)	X	1	positive	Abortion at 9 months
22565	1 early blastocyst	1	X	negative	-

Table 4: The results of the third embryo transfer programme on Charoke cows.

ID	Embryo transferred (no / stages)	CL numbers		Pregnancy diagnosis	Remark
		Right	Left		
NT2	1 morula	2	X	positive	Abortion at 5 months
A5028	1 compact morula	1	X	negative	-
B5029	3 (1 blastocyst + 2 compact morula)	1	X	positive	1 live calf
A8009	2 compact morula	1	X	positive	Abortion at 7 months
A017	3 (2 blastocyst + 1 morula)	1	X	negative	-

Figure 4: A calf born from an embryo transferred to the Charoke cow recipient with ID B5029.

In the present study, embryos were transferred on day 6 post-artificial insemination in all three programmes. Progesterone levels on day 6 following artificial insemination have been shown to positively correlate with pregnancy rates (Thanh et al., 2023). The production of one life calf in the third embryo transfer programme indicates that adequate progesterone levels were maintained during embryo development in the uterus. Furthermore, progesterone levels are considered crucial for enhancing the success of embryo transfer (Budiyanto et al., 2022). The pregnancy rate in cows following non-surgical embryo transfer was lowest when progesterone levels on day 7 were less than 2.0 ng/ml, highest when levels were between 2 and 5 ng/ml, and decreased once more when levels exceeded 5 ng/ml, according to Niemann et al. (1985). Additionally, some recipients in the present study were transferred with more than one embryo, which may improve conception and survival rates of the newborn calf (Davis et al., 1989). Another study found that multiparous animals exhibited higher calving rates but also experience greater mortality compared to nulliparous animals (Gebbels et al., 2023). This suggests that younger recipients should receive more attention than older cows when evaluating embryo transfer success rate.

The reproductive processes involved in the initiation and maintenance of pregnancy are known to be significantly influenced by progesterone. In the present study, 80% (4 out of 5) of the pregnant cows did not carry their pregnancies to full term, suggesting that progesterone levels may have been insufficient. The growth and development of the conceptus rely on progesterone's action on the uterus to control endometrial function, which includes pregnancy recognition, conceptus-maternal interactions, and uterine receptivity for implantation. Progesterone creates optimal uterine conditions for embryo development through its effects on the uterine endometrium.

Conclusion

This study proved that, under local settings, donor cows can be successfully superovulate using FSH and eCG. An average of three transferable embryo per donor cow was produced in Charoke cows. Variability in outcomes may be

attributed to differences in the superovulatory responses of donors following gonadotropin treatments such as FSH or eCG, as well as factors such as the breed and age of donor cows, and local environmental conditions. The production of one viable calf in the third embryo transfer programmes demonstrated that adequate progesterone levels were maintained during embryo development in the uterus. Progesterone levels on day 6 following artificial insemination for embryo transfer have been shown to positively correlate with pregnancy rates. However, 80% (4 out of 5) of the pregnant cows in this study did not carry their pregnancies to full term. Additionally, some recipients were transferred with more than one embryo, which may enhance the conception rate. This MOET programme can be improved through careful preparation of both donors and recipients, including selection based on historical performance, and the administration of FSH and eCG hormones that stimulate the growth of follicles in the ovaries. Monitoring the number of *corpus luteum* observed during embryo transfer, and along with employing skilled technicians are also essential prerequisites for establishing a high success rate and efficiency in MOET technologies.

Acknowledgement

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Conflict of interest

The authors state that there are no conflicts of interest in this study.

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