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Research Article

PRODUCTION, CRYOPRESERVATION, AND TRANSFER OF MURRAH EMBRYOS THROUGH MULTIPLE OVULATION AND EMBRYO TRANSFER (MOET) TECHNOLOGY IN SRI LANKA

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ABSTRACT: Sri Lanka has approximately 0.32 million buffaloes experiencing stagnant growth over the last few decades. MOET would be a better approach for the rapid multiplication of existing genetically superior buffalos. The objective of the study was conducted over 15 months to establish MOET for buffalos to improve milk production and the genetic value of their population in Sri Lanka. Superovulation (n=10) of buffalos was conducted using a modified, FSH-based standard protocol. Three artificial inseminations (AI) were conducted in 12 hrs. intervals from the estrous with imported Murrah semen. At the embryo flushing, 18 embryos were collected using a retrograde flushing technique. Out of that, Compacted morulae (n=3), Morulae (n=12), Damaged (n=2), and Degenerating oocytes (n=1) were identified while classifying those into 'Excellent' (n=4), 'Good' (n=8), Fair (n=2), Poor (n=3) and Degenerated/Unfertilized (n=1) categories. A set of embryos (n=6) were cryopreserved under vitrification technology for 6-8 months. One pregnancy in each fresh (n=8) and frozen (n=6) embryo transfer was reported at day 56 of post-insemination. However, those were terminated before the term. The study highlights the feasibility of MOET for buffalo population expansion in Sri Lanka. Optimizing MOET would be an important biotechnological tool to expedite the expansion of the buffalo population while providing genetically superior buffalo calves to the farmers in Sri Lanka.

Keywords: Buffalo embryos, Embryo freezing, Embryo transfer, Murrah, Oestrous synchronization, Super ovulation.

INTRODUCTION

Buffalos play a major role in milk production in the world. India is the world's highest milk producer [1], receiving 55% of its milk from buffalos [2, 3]. Buffalos represent the second highest contribution to the national milk production in the dairy industry in Sri Lanka. Sri Lanka has approximately 0.32 million buffaloes [4, 5] of which the majority (0.28 million) are either indigenous or crosses between exotic breeds such as Nilli-Ravi, Murrah, and Surthi.

The growth of the buffalo population has come to a standstill throughout the last few decades owing to several issues with their management and breeding [6]. In addition to the low production capacity, those indigenous buffalos have been expressing low reproductive efficiency [7] due to prolonged postpartum anoestrous and long inter-calving intervals [8]. Furthermore, studies on estrous inductions to increase reproductive efficiency using GnRH and FSH injections have failed [9, 10, 11].

Exotic types of locally available buffaloes, especially Murrah [12], are well adapted to the local environment and the conditions throughout the year. Furthermore, Murrah is conceded as one of the highest potential buffalo milk producers while representing the highest contribution to buffalo milk production which is 80%

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of the total milk production of its home tract in the Haryana province of India. Due to that, out of Asian River types, Murrah is the most popular buffalo breed in the livestock sector even in its original country, India [3]. It is revealed that the value of focusing on the efficient breeding of locally available Murrah and other high-producing exotic buffaloes is a viable means to overcome their low productivity. Under similar situations, many other countries have identified the MOET and other related reproductive biotechnologies as potential solutions for the efficient multiplication of buffalos in the world [13].

In vivo embryo production, surgical embryo transfer [14, 15, 16], embryo splitting [17, 18], and embryo freezing [19, 20] have been established for goats in Sri Lanka. Although imported embryo transfer [21, 22, 23], *in vivo* embryo production, and freezing [24] have been experienced in cattle, none of those have been carried out for buffalos before this study in the country. Under that circumstance, the application of Multiple Ovulation and Embryo Transfer (MOET) technology on existing exotic breeds, especially Murrah and their crosses, would be a better approach for the rapid multiplication of the buffalo population in Sri Lanka. The objective of this study was to establish production, cryopreservation, and fresh and frozen embryo transfer using MOET technology for buffalos in Sri Lanka towards the improvement of their production and genetic value.

MATERIALS AND METHODS

Ethical clearance

All the experiments were carried out according to the ethical guidelines and approval (VERC-20-09) received from the Ethical Clearance Committee, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

Location

The study was carried out at the buffalo unit of the Veterinary Teaching Farm (VTF) (7°15'08.3 N, 80°36'12.0 E) [25], Uda-Peradeniya, Department of Farm Animal Production and Health, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

Selection and management of buffalo cows

A group of potential buffalo cows (n=65) was evaluated for their pedigree, production, reproductive history, and general health status with special attention to the reproductive system.

Five healthy animals with body condition scores (BCS) of 2.75 -3.5 were selected for the study. They were 3-4 years old and had calved 3-4 months ago for the first time. Selected animals were dewormed with Albendazole ('Analgon', India) and given a dose of multivitamin (Dutch Farm Veterinary Pharmaceuticals, Holland) injection initially. The animals were fed with ad libitum forage (Hybrid Napier variety CO-3 and Guinea grass), 2kg of concentrate mix (Dairy Milk Flush, Sri Lanka.), and 45 g of the vitamin-mineral mixture (Pecutrin, Sri Lanka) daily.

Experimental design

In the first part of the experiment, 2/5 of the animals were recruited for the superovulation as embryo donors while the remaining 3/5 of the animals were used for the estrous synchronization as embryo recipients. In the second part of the experiment, another 2/5 were recruited as the embryo donors while using the remaining 3 animals as the embryo recipients. Accordingly, all the selected buffalo cows were used as both embryo donors as well as recipients in a consecutive rotation until each animal gets two opportunities to be the embryo donor (Table 1).

Superovulation of embryo donors

The superovulation protocol was designed as a modification of Situmorang's [26] protocol. Two embryo donors were subjected to super-ovulation at a time. All five buffaloes were super-ovulated twice according to the experimental design (Table 1) elaborated in Fig. 1. At 12 hrs. from the 5th dose of FSH injection, the ovaries of embryo donors were scanned (6.5MHz, per-rectal linear probe) to detect the synchrony of follicular developments (Fig. 5).

Embryo flushing

Six days post-insemination; embryo flushing was carried out in embryo donors. The number of corpora lutea (CLs) in both ovaries was counted (Table 2) manually, immediately before the flushing and the count was confirmed by trans-rectal ultrasound scanning (6.5 MHz, linear probe) (Fig. 6) at the end of the flushing process.

The flushing was carried out with the induction of posterior epidural anesthesia (4 ml, 2% Lignocaine inj., Sri Lanka) using a pre-warmed (35°C) commercial embryo flushing medium (Vigrotm Complete Flush, U.S.A.) on day 6 from the first AI. Both uterine horns were flushed using standard non-surgical retrograde embryo flushing techniques and devices [27].

Embryo evaluation

Embryo-holding medium (0.5ml/well) (Vigro Holding plus, USA) was filled into the first and second wells of a four-well plate (NunclonTM, Denmark) and embryos were searched under a dissection microscope and transferred using a 5µl micro dispenser 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 keeping 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' [27] and Colorado State University [28], using an inverted light microscope (Olympus CK 2, Japan) with 200 magnifications. Under that, collected embryos were graded as 'Excellent', 'Good', 'Fair', 'Poor', and 'Degenerated/Unfertilized' (Fig. 10). Throughout the evaluation process, embryos were maintained under 35°C [29], using a stage warmer.

Oestroussynchronization of embryo recipients

Three selected buffalo cows were estrous synchronized parallel to each superovulation of this experiment as a modification of Vikash *et al.* [3] and elaborated in Fig. 2.

Fresh embryo transfer

In the beginning, estrous synchronized embryo recipients were per-rectally examined under both palpation and ultrasound scanning technologies (6.5 MHz, leaner rectal prob) for the location, type (Table 3), and approximate size of CLs. Corpora lutea which were equal to or higher than 1cm in diameter were considered transferrable stagers except for cystic corpora lutea (>2.5 cm). The relevant side of the thigh was marked with red color spray paint to identify the side of the CL located.

Selected embryos (n=8) were transferred to the ipsilateral horn-carrying corpus luteum of the recipient animals on day 6 post-estrous synchronization as elaborated in Fig. 3. The animal was kept separately under the same intensive management. The same procedure was applied to all the fresh embryo transfers in this study.

Embryo freezing

A set of 'Excellent' and 'Good' grade embryos (n= 6) were cryopreserved under vitrification technology. At the freezing process, 10% Ethylene glycol containing commercial freezing medium (ViGROT Methylene glycol freeze, Bioniche, Pullman USA) was used. Freezing medium (1ml) was filled into the 3rd well of the 4-well plates. The plate was kept on a stage warmer which was maintained at 35°C. Embryos, that were in the holding medium were transferred into the freezing medium using a micropipette. Those embryos were kept in the freezing medium for 5 minutes under 35°C. Each embryo was loaded into a mini straw separately according to the guidelines of Total Livestock Genetics [29] while leaving two air spaces towards the manufacturer's end and one towards the laboratory end from embryo embryo-rich portion of the medium. The laboratory end was sealed using a straw sealer. The embryo-rich straws were labeled including date of harvest, grade, dam number, species, and breed. Then it was plunged into a goblet which was inside the LN2 tank in a canister, swiftly. At the plunging manufacturer's end the straw was directed into the LN2 and the canister was repositioned immediately. It was maintained until the frozen-thawed embryo which would be transferred 6-8 months later. The same procedure was applied to all the frozen embryo transfers in this study.

Frozen embryo transfer

During that process, mini straws that contained embryos (n= 6) at -196°C (LN2), were transferred into a thawing flask that contained 37°C water and was kept for 30 seconds. Then each straw was taken out and the surface was wiped using dry tissue paper. After the insertion of the manufacturer's end into the embryo transfer gun, the tip of the laboratory end was cut perpendicularly using a straw cutter. The rest of the procedure was like the fresh embryo transfer mentioned previously [29].

Pregnancy diagnosis

Pregnancy diagnosis in both fresh and frozen embryo transfer was carried out at day 50 of post-embryo transfer (day 56 of the fetus) using transrectal ultrasound scanning (6.5 MHz with per-rectal linear probe) technology and it was further confirmed at day 90 from the embryo transfer under the same technology.

RESULTS AND DISCUSSION

All the embryo donors (n=10) and recipient (n = 15) animals showed estrus 24 - 36 hrs. after the removal of CIDR. The common signs of the estrus were clear, thick colorless ropy mucous hanging from the vulva or pasted on the tail and/ or thigh (Fig. 4) and the swollen nature of the vulva.

Table 1. Roster of buffalo cows used for embryo flushing and transfer. (D-Embryo donor, R-Embryo recipient).

	Embryo transfer cycle					
	No	**1**	**2**	**3**	**4**	**5**
Buffalo cow	1	D	D	R	R	R
	2	R	R	D	D	R
	3	D	R	R	R	D
	4	R	D	D	R	R
	5	R	R	R	D	D

Table 3. Location and type of corpora lutea in embryo recipient buffalo cows.

Type of the CL	Ovary	
	Left	**Right**
Bud shape	05 (4)	07(5)
Crown and neck shape	00	00
Embedded	01 (1)	02(2)
Cystic	00	00

[Presence of bud shaped CL revealed as the dominant type and more activities or the follicular waves in the right ovary in oestrous synchronized Murrah buffalos. Numbers of transferrable level CLs are given in brackets].

The superovulation protocol explained for buffalos by Situmorang [26], was modified to have more potentiated stimulation at the superovulation in this experiment. At that modification, 500 µg of PGF2α was administered 14 days before the commencement of the FSH injection schedule which would be supportive to luteolysis of the probable mid-phase CL or cystic corpora lutea which can adversely affect the effectiveness of the protocol. Furthermore, luteolysis would provide stimulation for activation of the next estrous cycle leading to a functional CL at the period of FSH administration. Further, 7 days before the commencement of the administration of FSH, a CIDR was introduced intravaginally and removed at the last date of FSH administration. It would further accelerate the effect of probable natural CL in that stage. Progestogen and progesterone released by CIDR, and CL respectively have the same negative feedback to the hypothalamus and the anterior pituitary with higher effectiveness [30]. With that, a higher concentration of GnRH in the hypothalamus and FSH with LH in the anterior pituitary would be accumulated. Release in a higher concentration of those hormones secondary to removal of the CIDR and luteolysis with a second PGF2α injection may lead to a more effective super-ovulatory process in embryo donors [31].

Table 2. Location and type of corpora lutea in embryo donor buffalo cows.

Type of CL	Location of CL		
	Left ovary	**Right ovary**	**Total**
Bud	7	11	18
Crown and neck shape	0	0	0
Embedded	2	3	5
Total	9	14	23

[Presence of bud shaped CL revealed as the dominant type and more activities or the follicular waves in the right ovary in super ovulated Murrah buffalos].

As a modified protocol, the response of the ovaries in embryo donors was evaluated under per-rectal ultrasound scanning at 12 hrs. from the 5th FSH injection. Asynchrony of follicular developments was detected at 1 super-ovulation (Fig. 5) under ultrasound scanning technology and others were synchronized as expected in the study. Although the provided conditions are equal for all the donors, there could be individual variations in the response to different external factors that may affect the natural synthesis and release of GnRH. An example of such would be distress due to threats from dominant animals or fighting within the group, climatic changes, and surrounding noises among others. However, the presence of synchrony in all other super-ovulations revealed the success of the modified superovulation protocol for buffalos in the local environment. Measurements of the ovaries in super-ovulated buffalo cows were approximately 3-4.5× 2-2.5× 2-2.5 (length × width × thickness) cm and compared to the cattle, all palpable CLs were smaller (≈ 0.75-1.5cm in diameter) (Fig. 6) at the per-rectal palpation in day 6 post-AI. Usually, in buffaloes, both ovaries and ovarian structures are smaller than the counterparts of cattle. All the palpable CLs were in bud-shape and no crown and neck-shaped CLs were detected in embryo donors (Table 2). Compared to most of the superovulation protocols used for cattle, this protocol consists of an additional AI. It would be supportive to minimize the effect of asynchrony in ovulation while providing fertilization opportunities for more ovulated oocytes [32]. As a result of that, a higher number of embryos (n=18) were recovered (Fig.7 and Fig. 8) at day 6 post-AI flushing with a 78% embryo recovery rate.

Furthermore, the study resulted in higher percentages of ideal development stages (83%) (Fig. 9) and transferrable category (78%) (Fig. 10) of embryos. Those considerably higher results revealed the

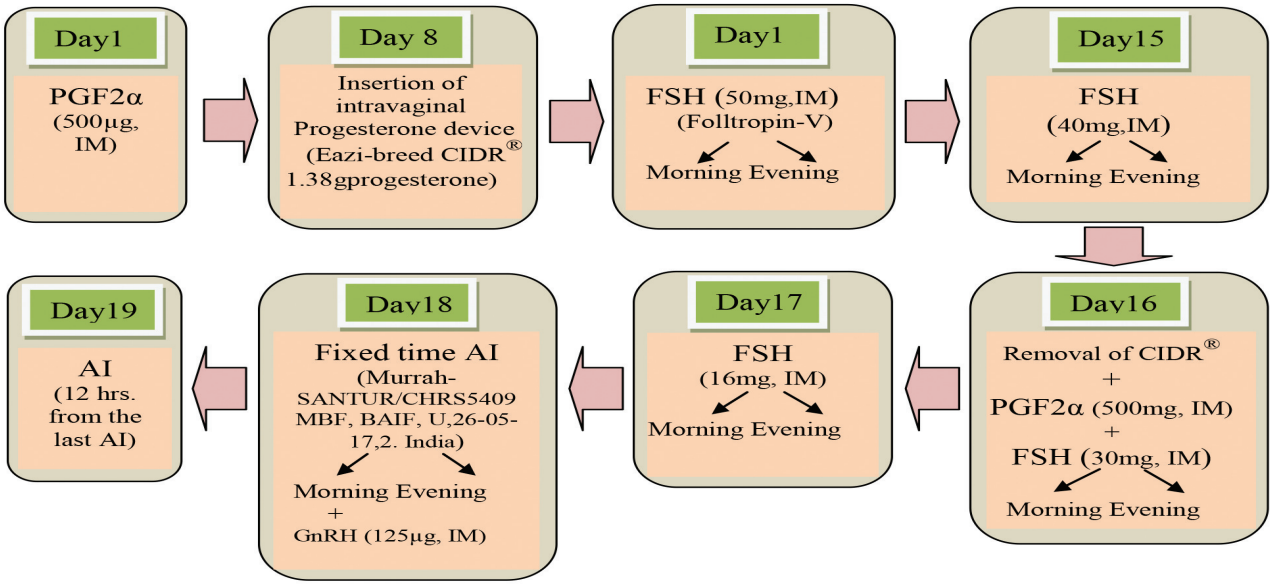

Fig. 1. Modified protocol used for the superovulation of embryo donor buffalos.

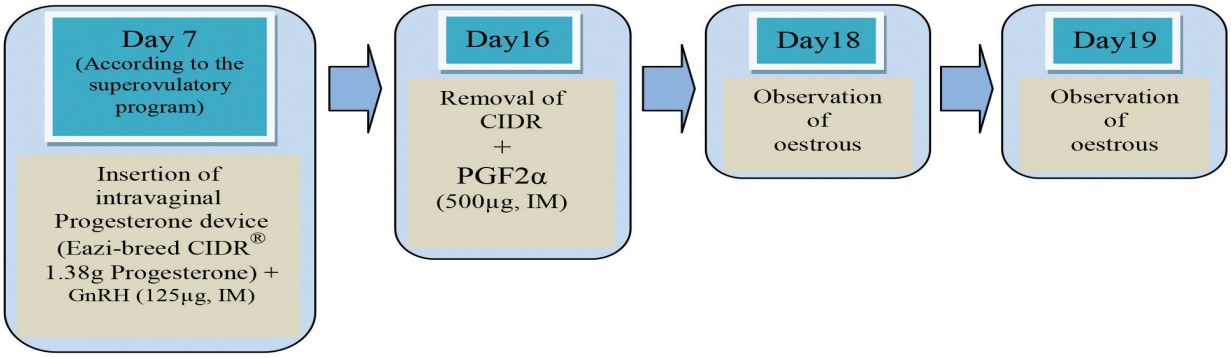

Fig. 2. Modified protocol used for the oestrous synchronization of embryo recipient buffalos.

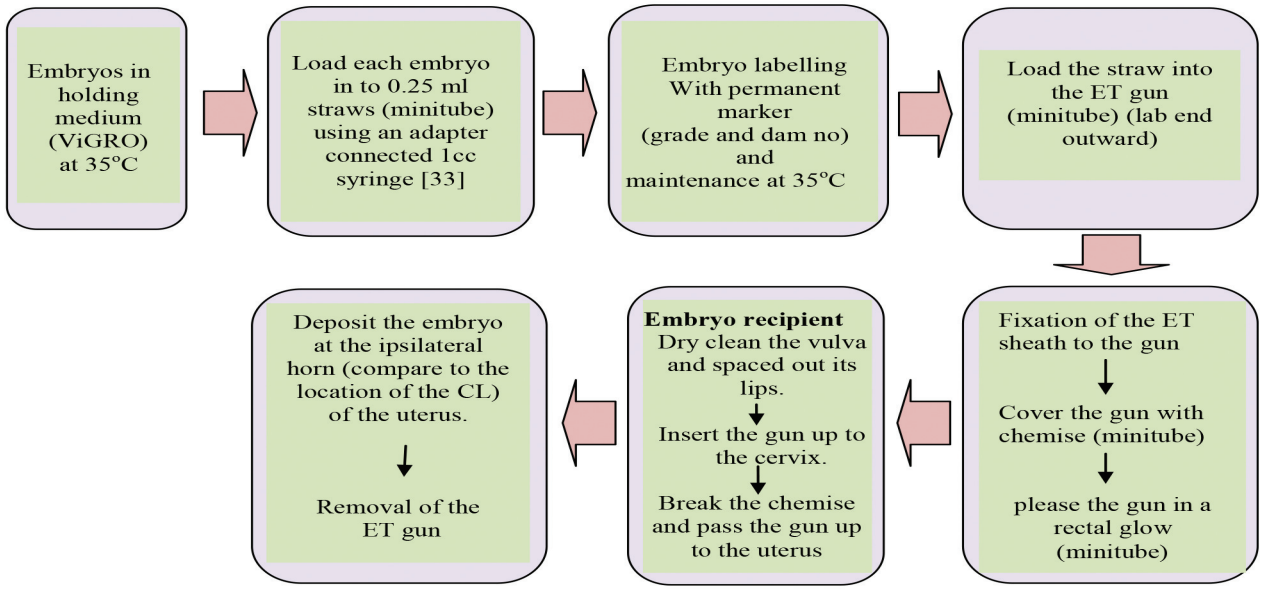

Fig. 3. Procedure of fresh embryo transfer practised in the study.

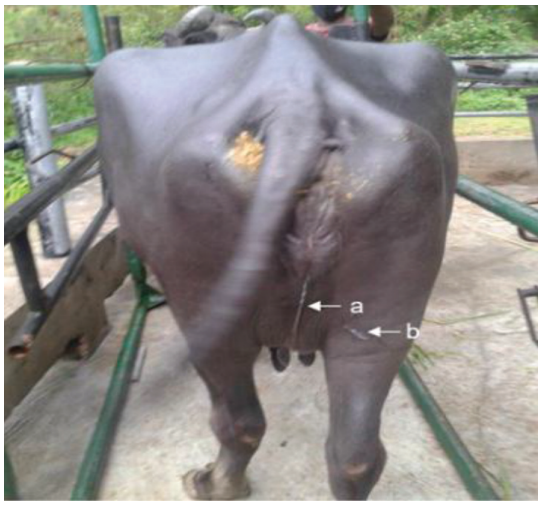

Fig. 4. Response to superovulation of an embryo donor buffalo. [a - Thick, colourless ropy mucus hanging from the vulva, b- Pasted mucus on the lower part of the caudal thigh].

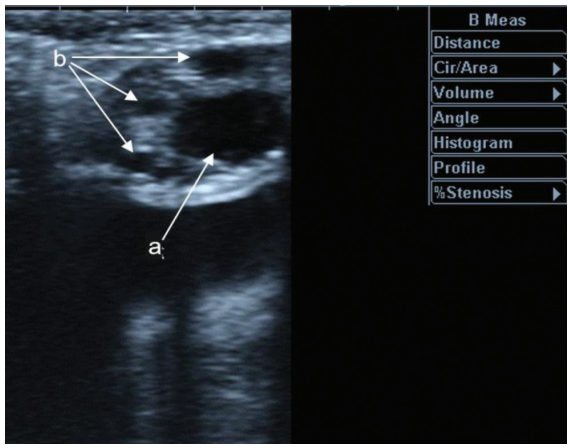

Fig. 5. Ultrasound scanning image (6. 5 MHz) of the right ovary at 12 hrs from the 5th dose of FSH injection in the super ovulatory process of buffalos. [Single development of a pre-ovulatory level follicle (a) while several other follicles remain in different developing stages (b)]

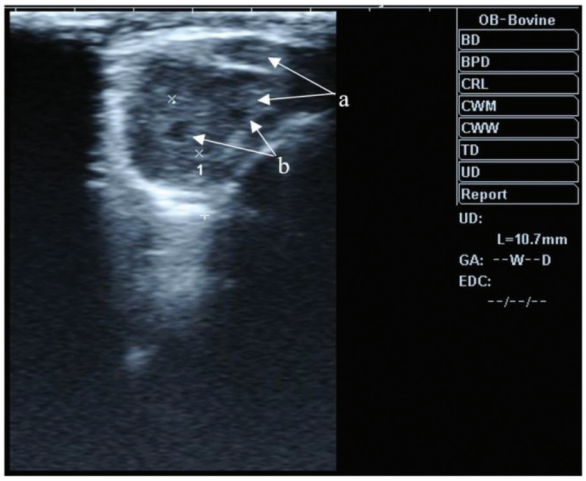

Fig. 6. Scanning image of a super ovulated ovary in a buffalo embryo donor at day 6 post-AI. [The gap in between two '×' marks (1) indicates the diameter (10.7 cm) of the largest CL of the animal. a- Visible parts of two other corpora lutea. b- Lacunae of CLs].

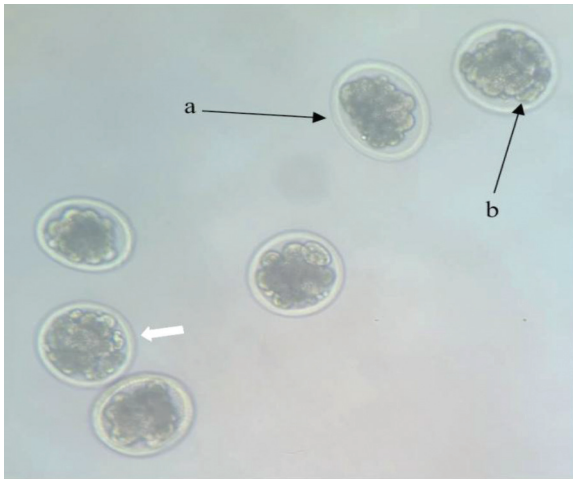

Fig. 7. A set of embryos (Morulae) harvested on day 6 post AI at the embryo production process of the MOET program in buffalos (×200). [White arrow: indicated a compacted morula which consists of comparatively thinner zona pellucida without clear perivitelline space. a- Elliptical-shaped morula stages misshapen cellular mass, b- Morula with a set of excluded cells].

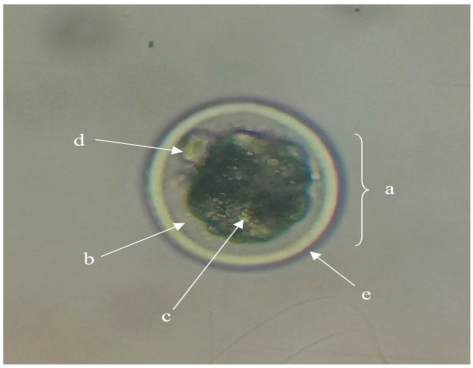

Fig. 8. Degenerating morula stage of buffalo embryo, harvested at day 6 post-AI. (×200). [Shrunken cellular mass (a) with proportionately higher perivitelline space (b). Vacuolated cytoplasm of the blastomeres (c). an excluded blastomeres (d), zona pellucida (e)].

applicability of the modified super ovulatory protocol for Murrah buffalos under local conditions.

In addition to that, the date of embryo flushing was carried out 24 hours earlier than the cattle embryo flushing. In general cattle, embryos are flushed on day 7 post-AI while doing it on day 6 in buffalos. Although cattle embryos are available in the uterus from day 6, it is available from day 5 post-AI in buffalos [33] for flushing. Cattle blastocysts hatched after day 7 while buffalo blastocysts hatch even on day 5 post-AI [34, 35, 36]. Furthermore, when the embryo reaches the uterine horn, gradually, it will start extensive migration throughout the uterus. Hence, more delay from its

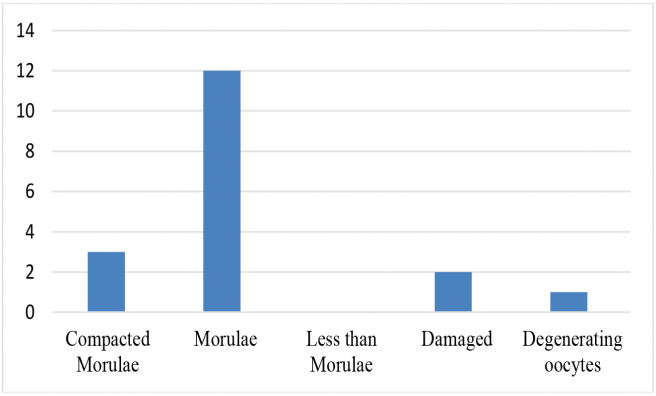

Fig. 9. Classification of harvested embryos according to the developing stages. [The highest number of developing embryos belonged to the morula stage and other embryonic development lesser than morula were not there].

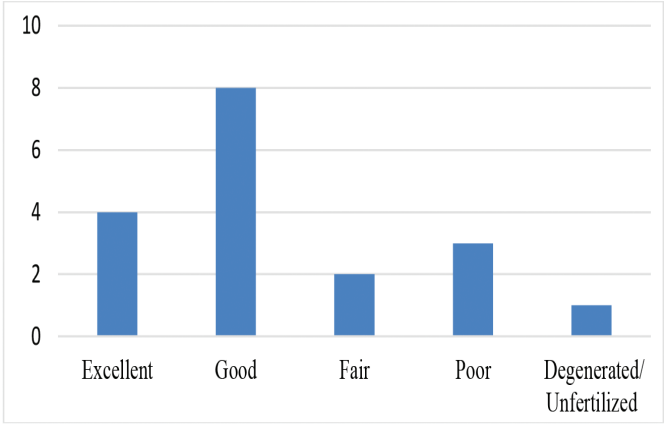

Fig. 10. Classification of embryos according to the quality (Grade) identified at the evaluation. ['Good' grade became the highest while 'Degenerated/ unfertilized' grade became the lowest].

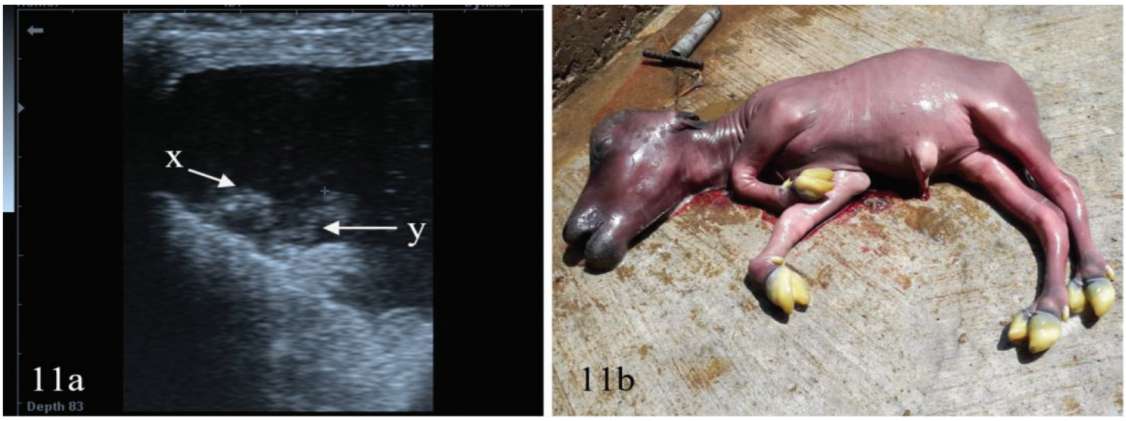

Fig. 11. The first pregnancy reported in fresh embryo transfer for buffalos in Sri Lanka. [(From left to right): 11a: Ultrasound scanning (6.5MHz) image of day 56 pregnancy. x-foetus, y- umbilical cord. 11b: Aborted buffalo (Murrah) foetus (female) at the 8 months after the embryo transfer in the program. Fig. 11b represents the 56 days of pregnancy of this calf. It was aborted two days after an attack on the dam by another buffalo cow in the herd].

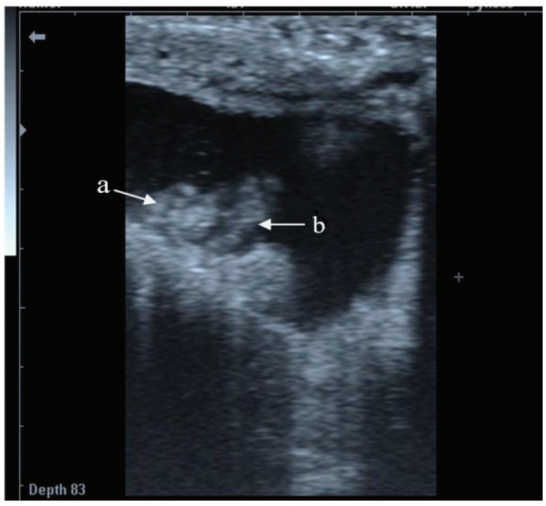

Fig. 12. Ultrasound scanning (6.5MHz) image of day 56 pregnancy reported in frozen embryo transfer for buffalos in Sri Lanka. [a -foetus, b- umbilical cord].

arrival to the uterine horn may cause poor embryo recovery. Considering such a possibility, flushing was conducted on day 6 post-AI in this study as well.

Removal of CIDR and administering of PGF2α on day 8 of an estrous synchronization protocol for Murrah has been reported [3]. However, in this experiment, the estrous synchronization of the embryo recipients should be synchronized with the stage of the estrous cycle of embryo donors. Therefore, with the better practical experience of the authors, removal of CIDR, and administration of PGF2α were practiced on day 9 of the program. Even with that amendment, PGF2α can lead to luteolysis at the probable mid-phase (functional) of those CL without adverse effects on the estrous synchrony. Development of the CLs was examined in both per-rectal palpation and ultrasound scanning on estrous synchronized embryo

recipient animals. At that examination, nine embryo recipients were bearing CLs in the right ovaries while the remaining six animals bore those in the left ovaries. Those results revealed comparatively higher activities or ovarian cycles of the right ovary in embryo recipient animals with estrous synchronization. Furthermore, those were bud-shaped and embedded types of CLs while bud-shaped CLs were dominant. Luteal cysts and crown and neck-shaped CLs were not reported in embryo-recipient animals in this study (Table 3). However, both crown and neck shape and luteal cysts have been reported in recipients of cattle embryo transfer in Sri Lanka [37].

Recipient animals with smaller CLs (<1 cm in diameter) weren't considered for embryo transfer. Those CLs may not be able to produce the required concentration of progesterone to maintain the pregnancy. To have an intermediate conception rate bovines should have 1-3 ng/ml of serum progesterone level [38, 39]. Similarly, it was planned not to use recipient animals that are carrying cystic CLs due to probable conception failure with the production of higher (> 6ng/ml) progesterone levels [40].

Compared to the cattle, ultrasound scanning for pregnancy diagnosis in buffalo embryo recipients was performed 21 days later. The gestation period of cattle and buffaloes is approximately 285 and 310 respectively [41]. It reveals the comparatively delayed growth of the buffalo embryos and fetuses to reach up to detectable levels.

At the pregnancy diagnosis on day 50 post-transfer, one embryo recipient was found to be pregnant in each embryo transfer category (Fig.11A and Fig. 12). However, on day 90 post-transfer, the pregnancy of the recipient who belonged to the fresh embryo transfer was intact while another animal was identified as non-pregnant under the ultrasound scanning. Furthermore, in the eighth month of the pregnancy, it was aborted (Fig. 11B) two days after the hyperthermia (105°C) due to the attack of another cow in the group.

CONCLUSIONS

Expression of estrous (100%) with 23 corpora lutea revealed the possibility of applying the modified super ovulatory and estrous synchronization protocols for Murrah buffalos in Sri Lanka. Furthermore, a 78% embryo recovery rate, 83% ideal development stages, 78% transferrable embryos, and 2 confirmed pregnancies revealed the feasibility of performing MOET technology, embryo freezing, and frozen embryo transfer for buffalos in Sri Lanka. The study will be continued using the same breed and other buffalo breeds available in the country. It would be supportive for validation of the technologies to practice efficiently with higher transferable embryo recovery, pregnancy, and calving rates.

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