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PRESENT STATUS OF EMBRYO TRANSFER IN WATER BUFFALO (A Review)

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The great importance of the water buffalo as a major source of draft power for farm tillage, transport and as a source of supplementary income through milk and meat production has triggered a keen interest in its reproduction. Techniques to generate an economically feasible number of fertilized eggs from one animal (donor) which eventually can be transferred to a number of surrogate mothers (recipients) are being developed. Such techniques are referred to as embryo transfer (ET) technology, which consists of several steps, each of which is critical. These include :

- a) Selection of donor and recipients
- b) Superovulation
- c) Estrous synchronization between donor and recipients
- d) Recovery of embryos
- e) Examination and classification of embryos
- f) Transfer of embryos

Failure in any one of the above steps would result in either a low conception rate or complete failure. This technology has opened exciting new possibilities in enabling the introduction of breeding schemes where a nucleus breeding herd can provide an economical method of interpolating the desired production traits.

While the application of this technology in the water buffalo requires much additional research work, it has already been clearly demonstrated that a highly acceptable product can result. The birth of a 35-kg male buffalo calf 300 days after non-surgical transfer of an expanded blastocyst confirmed this claim (6).

This success has prompted a number of countries such as Bulgaria (1), India (16), Malaysia (31), the Philippines (20), Thailand (21) and the United States (5,6) to develop ET technology in the water buffalo. However, the problems of frequently

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producing a large number of fertilized ova and how to recover them remain to be solved.

This review was therefore undertaken to describe the factors governing embryo transfer in the water buffalo and suggest ways of potentially overcoming difficulties encountered in its implementation.

SELECTION OF DONOR AND RECIPIENTS

Essentially, the prospective donor must be reproductively sound, well-nourished, disease-resistant and viable in terms of superiority for draft-milk-meat for smallholders and draft-meat-milk production for ranch-type systems. Buffalo cows with congenital reproductive defects or other abnormalities such as endometritis and vaginitis, with irregular estrous cycles or those undernourished should not be used as donors. As demonstrated in cattle, donors with such problems either have low ovulation rates and/or low production of normal embryos (7, 8). The ideal breeding age for a donor buffalo cow on the average is 4~9 years. Buffalo cows that are too old would most likely have poor responses to superovulatory treatment.

Recipients must have a proven reproductive performance, free of congenital or infectious diseases to obtain high conception rates and have a sturdy body size to avoid problems of dystocia. They must also be healthy and assured of not being pregnant.

SUPEROVULATION

The production of sufficient number of viable embryos through the use of gonadotropins for embryo transfer and multiple birth has been a subject of interest in buffaloes. In particular, follicle stimulating hormone (FSH) and pregnant mare serum gonadotropin (PMSG) have become the primary superovulatory drugs for donor buffalo cows. FSH is usually given within a 4- to 5-day period at midcycle as in cattle through a series of injections so that follicles are recruited immediately prior to lysis of the existing corpus luteum. A total dose of 40mg to 50mg is given to elicit optimal ovarian stimulation. The series of injections is required because of its short half-life in the circulation. The PMSG is used as a single injection of 2,000~3,000 IU. Its long half-life is said to affect gamete migration and development of early-stage embryos. PMSG still present after ovulation could have a deleterious effect on the quality of embryos by stimulating steroid secretion. However, the development of anti-PMSG, which has been applied in cattle and administered at the time of estrus, can improve the efficiency of PMSG and the quality of transferable embryos (29). Comparatively, FSH stimulation of ovaries results in more ovulation and recovery of embryos of better quality than with PMSG (13). A luteolytic dose of prostaglandin F₂ alpha (PGF₂α) is usually given 48 h after initiation of the treatment.

In all studies published to date, the superovulatory responses have varied, with

low fertilization rates (Table 1). This could be due to the poor nutritional status of the donor (19). Usually, buffaloes are allowed only to graze in pastures without concentrate supplementation, thus taking in more carbohydrates and fiber than protein. Rations with a higher level of protein influence the animal's level of luteinizing

TABLE 1. Preliminary data on superovulation response of water buffalo cows treated with FSH and/or PMSG

References	No. of Buffalo	Drug	Breed	No. of ovulations (mean)	No. of embryos recovered
Alexiev et al. (1988)	126	FSH	MB	4.0	176
“	75	PMSG		3.4	
Chantaraprateep et al. (1989)	5	PMSG	SB	—	16
“	4	FSH		—	
Drost et al. (1983)	8	FSH	JB	2.0	1
Drost et al. (1988)	34	FSH	MB	4.0	41
Karaivanov et al. (1986)	19	FSH	MB &	4.3	24
“	19	PMSG	F1 x F2 (MBxBB)	1.9	11
Karaivanov et al. (1987)	71	—	MB & F1 x F2 (MBxBB)	—	76
Kurup et al. (1988)	145	—	MB	—	165
Ocampo et al. (1988)	4	PMSG	MB & SB	3.0	—
Parnpai et al. (1985)	3	PMSG	SB	9.0	1
Sharifuddin & Jainudeen (1987)	77	—	SB	3.9	15
Sharifuddin & Jainudeen (1988)	17	FSH-P	SB	3.6	1
“	32	PMSG	—	4.3	

JB — Jafarabadi buffalo

MB — Murrah buffalo

SB — Swamp buffalo

F1 x F2 — Crossbreeds of Murrah and Bulgarian buffaloes

(—) — Data not available

hormone (LH) and response to gonadotropin releasing hormone (GnRH), which would eventually affect the ovulation response of the donor. In addition, the season during superovulation treatment influences the rate of ovulation. A marked seasonality in the ovulation response following superovulation treatment has been reported in bubaline (2, 13) and in cattle (37).

The use of either pure FSH or a controlled FSH-LH mixture plus GnRH and its analogues and/or an anti-PMSG, if PMSG is to be used, may improve the donor ovulation response.

ESTROUS SYNCHRONIZATION OF RECIPIENTS

Estrous synchronization has become a beneficial tool in the buffalo industry as it facilitates artificial insemination. It is achieved by using $\text{PGF}_2\alpha$ or its analogues through double injection at 11-day intervals (10, 11, 12, 15, 16, 22, 23, 26, 27) or exogenous progesterone given in the form of a progesterone releasing intravaginal device (PRID) (26, 27, 28). The first approach involves the advanced regression of the corpus luteum, which is accompanied by a sudden decline in progesterone concentration in the peripheral circulation within 24 h. This is followed by estrus characterized by growth of the follicles and an increased estrogen concentrations in the blood. In contrast, the luteal phase in the second approach is prolonged by giving exogenous progesterone. A pseudoluteal phase is created following the natural regression of the corpus luteum, and this normally occurs even in the presence of exogenous progesterone at levels identical to or close to the physiological concentration. In this case, the buffalo will have a suppressed gonadotropin secretion even after the regression of the corpus luteum, thereby inhibiting follicular development.

After 8~12 days *in situ*, withdrawal of the PRID will allow follicular development through the removal of the negative effect on the gonadotropin secretions. Thus, all treated buffaloes are expected to start the cycle in a synchronized fashion. Although the degree of synchronization of estrus was reportedly excellent using both $\text{PGF}_2\alpha$ and its analogues, fertility was not particularly high (2, 9, 10, 23). Some of the factors which might be implicated are: derangement of hormonal patterns after synchronization of estrus, poor nutrition, climatic stress, lactational stress during the early post-partum period, delay in functional involution of the uterus, subclinical uterine or ovarian diseases and improper timing of insemination. Moreover, buffaloes exhibit a weak estrus phenomenon in comparison to other species. It has also been demonstrated that the synergistic effect of progesterone and estradiol- 17β potentiates the ovulatory release of LH. In this species, the high rate of infertility and weak estrus phenomenon may be due to the inefficient synergistic effects of progesterone and estradiol- 17β before the LH surge.

RECOVERY OF EMBRYOS

Several non-surgical and surgical techniques of embryo collection have been reported in the bovine. Unfortunately, these techniques have not been well developed in the bubaline. Although successful recoveries have been reported (6, 20, 21, 31, 32), basically the techniques used are all modifications developed in cattle. Buffalo embryos enter the uterus on day 4~5 post estrus (Table 2). The number of embryos that are expected to be present can be estimated by counting the corpora lutea by rectal palpation. Prior to flushing, 10~15 ml of 25% lidocaine HCl is administered epidurally between the 2nd and 3rd coccygeal vertebrae. The perineum and vulva are thoroughly cleaned and disinfected by using 70% alcohol. Thereafter, the cervical canal is dilated by using a cervical expander to facilitate the insertion of the catheter. A 14-gauge Foley catheter or similar catheter with an inflatable cuff is inserted into the uterine horn and the catheter is secured in place by inflating the cuff with about 14~16 ml of air, depending on the horn's diameter. Uterine flushing can be done between day 4 and 6 (when the embryos are still freely floating within the uterus) by using phosphate-buffered saline (PBS) plus 2mg of bovine serum albumin per ml concentration. Collection of the flushed medium is carried out by gravity flow into a 1-liter graduated cylinder. The collected medium is allowed to settle for about 30 minutes and the supernatant siphoned off until about 100 ml is left. This is then swirled and decanted into scored plastic petri dishes for examination of embryos under a stereomicroscope.

TABLE 2. Location and developmental stages of superovulated buffalo embryos in relation to the stages of the estrous cycle (1, 6, 14, 21)

Day of cycle	Location of embryos	Stage of embryos
2	oviduct	2-cell
3	oviduct	2- and 4- cell
4	uterus	4-cell and morula
5	uterus	morula and blastocyst
6	uterus	blastocyst and hatched blastocyst
7	uterus	hatched blastocyst

0 - Day of estrus

In bubaline, it is hard to accurately assess the recovery efficiency of non-surgical uterine flushing techniques. Although the recovery rate of a given volume of medium flushed into the uterine horns can be determined, embryo recovery is low. The

reasons for the apparent difficulty in water buffalo embryo recovery have been ascertained to be as follows: sharp coiling of the uterine horns (19, 32), excessive pressure applied on the uterine horn during flushing that causes the fluid to leak through the utero-tubal junction (UTJ) into the ovarian end of the oviduct (32), overstimulation of the ovaries that leads to a failure of fimbriae to envelop the ovary at the time of ovulation (31) and tissue erection at the UTJ and its adjacent structures during the early part of the luteal phase that could block the entry of embryos into the uterus (19).

Surgical recovery of embryos through the midline- and flank-approach is hindered by the small size of the uterine horns in relation to the depth of the abdominal cavity. Further study is needed on the implementation of both recovery techniques in the buffalo.

EXAMINATION OF EMBRYOS

To facilitate the examination of embryos from the supernatant of the medium collected, a grid is made at the bottom of the scored petri dish before the flushing medium is poured into it. Initially, searching for embryos is done under a stereomicroscope at low magnification. After the embryos are found, more detailed studies on the stage of development and quality can be done under higher magnification. The embryos are then aspirated using a sterile Pasteur pipette connected to a syringe with an adapter and transferred to a smaller culture dish with fresh medium (PBS+20% calf serum+100 IU penicillin, 100 μ g/ml dihydrostreptomycin and 0.25 ml amphotericin B). The embryos are washed 3~4 times, evaluated and stored in a controlled CO₂ incubator at 37°C until the transfer.

Normally, the unfertilized ovum is characterized by the failure of cleavage, with only a single mass of vitellus surrounded by the zona pellucida. The presence of blastomeres of equal size, numbering 4, 8, 16 or 32, within the zona pellucida is a clear indication that fertilization and normal cleavage have occurred. The morula's individual blastomeres are difficult to distinguish from one another and the cellular mass of the embryo occupies most of the perivitelline space. The compacted morula's individual blastomeres coalesce, forming a compact mass to differentiate it from the morula. In the early blastocyst, the embryos form a fluid-filled cavity or blastocoele and has the general appearance of a signet ring. Visual differentiation between the trophoblast and the inner cell mass is possible. The blastocyst is characterized by the pronounced differentiation of the outer trophoblast layer and a darker, more compact inner cell mass is evident. The blastocoele is highly prominent with the embryo occupying most of the perivitelline space. The expanded blastocyst, is characterized by a complete or partial loss of the blastocoele and thinning of the zona pellucida. The hatched blastocyst may have completely shed the zona pellucida and may be collapsed or spherical in shape with a well-defined blastocoele. Blastomeres

with lipid droplets of varying sizes and distribution may be an indication of undergoing degeneration. The quality of individual embryos in this case, could be classified according to the following criteria used in the bovine (17): Excellent- an ideal embryo, spherical, symmetrical with cells of uniform size, color and texture. Good- trivial imperfections such as a few extruded blastomeres, irregular shape and a few vesicles are present. Fair- there are extruded blastomeres, vesiculation and a few degenerated cells. Poor- composed of numerous extruded blastomeres, degenerated cells, cells of various sizes, and numerous large vesicles but a viable appearing embryo mass.

The events of the developmental stages in the embryo may vary between individuals and according to the mode of ovulation, specifically for superovulated animals. Thus, to find embryos of different stages in the uterine flushing is normal (1, 3, 6, 14, 21). This can be attributed to the wide variation in the times of ovulation of different follicles.

TRANSFER OF EMBRYOS

For successful manipulation and conception, the embryos should be kept *in vitro* under a suitable medium (PBS+20% CS+antibiotic) before transfer. Under normal transfer conditions, embryos classified as excellent or good can be transferred as fast as possible without culturing, as in the case of bovine.

The success of embryo transfer is also dependent on the synchronized donor-recipient pattern. The grade of synchronization of the recipient with the donor should be limited to -24 h, -12 h, +12 h or +24 h differences, though a 0 h difference is more likely to yield a better result. Recent reports in cattle have indicated that such differences in synchronous transfer can be tolerated without a significant reduction in the pregnancy rate (30). Basically, only the non-surgical approach for ET in the buffalo has been reported to be successful (Table 3). The technique is similar to that of artificial insemination (AI). The embryo is aspirated into a 0.25ml or 0.5ml French straw in such a way that it is locked between two air bubbles then is loaded into the AI gun. Under epidural anesthesia, and after thorough cleansing of the vulva and perineum, the AI gun is introduced into the *os cervix* and threaded through the cervical canal into the uterine horn ipsilateral to the functional corpus luteum. The embryo can be deposited either in the middle part of the uterine horn or approximately one third of the way up to the uterine horn or placed as deep as possible, avoiding damage to the mucosa, and the gun slowly withdrawn.

The surgical approach is less compelling because of the small size and sharp coiling of bubaline uterine horns. They are located deep in the pelvic cavity as compared to cattle, thus exteriorizing them through a flank or midline incision is more difficult.

TABLE 3. Summary of published reports on ET in the water buffalo with resulting live births

References	Method of		Medium for		No. of embryos transferred	Percent pregnancy (%)	Remark
	recovery	transfer	recovery	transfer			
Alexiev et al. (1988)	Non-surgical and from slaughtered buffalo	Non-surgical and from slaughtered buffalo	—	—	99	13/71 (18.3)	7 live births
Drost et al. (1983)	Non-surgical	Non-surgical	PBS + 1% bovine serum + antibiotic	PBS+20% bovine serum + antibiotic	1	1/1 (100.0)	1 live birth
Drost et al. (1988)	"	"	"	"	41	5/28 (17.9)	4 live births
Kurup et al. (1988)	"	"	—	—	87	8/87 (9.2)	2 live births

(") — The same

(—) — Data not available

CONSTRAINTS ON ET IN WATER BUFFALO

Some of the factors which tend to limit the development and implementation of ET technology in the water buffalo are as follows :

1) Low ovulation rate

At present, there is no concrete information available regarding the low responses of water buffaloes to superovulatory treatments. However, factors such as nutrition, season, improper timing of insemination / mating, stress and derangement of hormonal pattern have been implicated (23, 36).

Another factor of utmost importance is the number of primordial follicles and the frequency of atresia in the water buffalo. It has been reported that the number of primordial follicles is higher and the frequency of atresia is lower in cattle than in water buffaloes (4, 20). These factors could account for the lower response of the buffalo to superovulatory gonadotropins.

2) Low fertility rate

Delay in the functional involution of the uterus, climatic stress, poor levels of nutrition and management, lactational stress during the early post-partum period and derangement of the hormonal patterns after the synchronization of estrus contribute to the low fertility rate in the water buffalo (23).

3) Low recovery rate

The problem of the low recovery rate has been ascribed to various factors mentioned before and difficulties in the identification of hatched blastocyst in the flushings (1). Another possible explanation might be an overstimulation of the ovary resulting in a large number of abnormal ova.

4) Low conception rate

Factors such as embryonic and uterine asynchrony, the grade of embryo and environmental conditions could contribute to the survival of embryos after transfer as reported in studies conducted in laboratory and domestic animals (24). Unfortunately, no comprehensive information is available concerning buffaloes.

Nevertheless, synchrony between the requirements of developing embryos and secretions of the uterus has long been recognized as critical to nurturing a successful pregnancy. The factors that may influence the rate of embryonic development include incubation of embryos after *in vitro* culture or fertilization, restriction to the oviduct, inheritance of preimplantation embryonic development genes, sex differences and time of ovulation. Uterine asynchrony on the other hand might have resulted from factors that influence estradiol-to-progesterone levels such as hormonal therapy, immunization against testosterone, stress, variability in progesterone synthesis between animals and nutrition.

Embryos classified as excellent and good will achieve a better result than fair and poor embryos (33), while exposure to elevated environmental temperature will cause a higher incidence of abnormal embryos (25).

POTENTIAL CONSIDERATIONS

1) A priming injection of FSH early in the estrous cycle as a prelude to superovulating gonadotropin administration.

In sheep and cattle, the number of animals that responded to the superovulatory hormonal stimulation by ovulation, number of utilizable ova recovered per animal are increased following early-cycle porcine follicle stimulating hormone (FSH-p) priming (38).

Like water buffaloes, both sheep and cattle have a biphasic periovulatory FSH rise. An increased ovulation rate accompanied by an increase in the fertilization rate is observed in the 2nd round of superovulation. The periovulatory, post-LH surge and FSH rise may be responsible for follicle recruitment.

2) Pressure application on the UTJ area via a rectal approach during the non-surgical flushing of embryos.

Unpublished observations have indicated that pressing the UTJ area during flushing is likely to yield the most number of expected embryos. This procedure can prevent the leaking of flushing fluid through the UTJ into the oviduct as previously observed (32).

FUTURE INVESTIGATIONS

1) Cryopreservation of embryos

The embryos of several mammalian species can now be successfully preserved by freezing and storing at -196°C . Different freezing procedures have been developed, ranging from the conventional method using a freezing machine, quick freezing and vitrification. Although no data on the freezing of buffalo embryos are available, description of procedures is deemed important because ET necessitates the freezing of embryos.

a. Conventional freezing involves the dehydration of embryos in the presence of freezing medium with cryoprotectants by slow cooling to the temperature of -30°C before plunging into liquid nitrogen (LN_2). The inclusion of glycerol has progressed from stepwise to one-step addition and the stepwise removal of the cryoprotectant has been replaced by a one-step method using sucrose.

Cryoprotectants may be classified either as intracellular (e. g., glycerol, dimethylsulfoxide, ethylene glycol and 1, 2 propanediol) or extracellular (sucrose, raffinose, protein, lipoproteins, egg yolk, milk and blood serum). The survival of cryopreserved embryos is dependent on factors such as the stage of the embryo at freezing, the state of the zona pellucida during freezing and thawing, the freezing medium, the cryoprotectant and the cooling, freezing and thawing rates, as well as the concentrations of the cryoprotectants.

b. Quick freezing: The embryo is allowed to be cryopreserved by direct transfer into LN_2 vapor at -170°C to -180°C before plunging into LN_2 or by direct plunging

into LN₂ (35).

c. Vitrification is a process by which the cryoprotectants solidify, assuming an amorphous glass-like solid form during cooling without the formation of crystals (18). Crystallization normally occurs when bulk samples of biological cells and tissues are cooled to low temperatures. Both the extra- and intra-cellular components are vitrified at the same time.

The presence of high concentrations of cryoprotective agents, and high cooling and warming rates are essential to execute vitrification.

2) *In vitro* fertilization

The high cost of drugs used for synchronization and superovulation and the steps in embryo collection and transfer are major constraints in the widescale implementation of ET technology. However, this could be reduced if buffalo oocytes collected from the abattoir could be matured and fertilized *in vitro*, then subsequently transferred to suitable recipients or frozen for future use.

Until now, only limited information has been available regarding *in vitro* fertilization in buffaloes (34). Future researches should focus on the possibility of using this technique as an alternative to superovulation in producing large numbers of embryos for transfer.

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