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เรื่อง

การย้ายฝากตัวอ่อน (คัพภะ) ในกระบือปลัก

EMBRYO TRANSFER IN SWAMP BUFFALO

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การย้ายฝากตัวอ่อนในกระบือปลัก

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์ 4 ประการ คือ

1) การตอบสนองของรังไขของกระบือปลักต่อการกระตุ้นด้วยฮอร์โมนโกแนดโดโตรปิน

2) การเจริญเติบโตของตัวอ่อน (ตัพภะ) ระยะแรก

3) การย้ายฝากตัวอ่อนโดยวิธีไม่ผ่าตัด และการเหนี่ยวนำให้เกิดลูกแฝด และ 4) เก็บตัวอ่อนในลักษณะแช่แข็ง

ใช้กระบือปลัก 14 ตัว กระบือนาง 3 ตัว และแม่กระบือ 11 ตัว อายุ 4 ถึง 12 ปี หนัก 315 ถึง 480 กก. กระตุ้นให้ไขตกเพิ่มขึ้น โดยใช้ฮอร์โมนโกแนดโดโตรปิน เอฟ เอสเอช 32 มก. (2 ตัว) และ 50 มก. (4 ตัว) และ พี่เอ็มเอสจี 2500 ไอยู (2 ตัว) และ 2700 ไอยู (3 ตัว) ให้ผลการตอบสนองโดยเฉลี่ย 50% ของกระบือที่ได้รับฮอร์โมน อัตราการเก็บตัวอ่อนได้ 54.5% (6/16), ได้ตัวอ่อนปกติ เสื่อมและไม่ได้รับการผสม 37.5 (6/16), 37.5 (6/16) และ 25% (4/16) ตามลำดับ เก็บตัวอ่อนด้วยวิธีไม่ผ่าตัด รวมทั้งสิ้น 33 ครั้ง เมื่อตัวอ่อนอายุได้ 5.5, 6.0, 6.5, 7.0 และ 7.5 วัน หลังการเป็นสัด ทั้งนี้เพื่อศึกษาการเจริญเติบโตของตัวอ่อนระยะแรก ลักษณะของตัวอ่อนที่พบได้แก่ระยะ 16 เซลล์, คอมแพค มอรูลา, บลาสโตซีส เฮ็ทช์บลาสโตซีส และ เอ็กซ์แพนดิ้ง บลาสโตซีส ตามลำดับ อัตราการเก็บตัวอ่อนจะได้สูงกว่าเมื่อเก็บจากกระบือเป็นสัดตามธรรมชาติ เทียบกับการเก็บหลังการเหนี่ยวนำให้เป็นสัด

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1 ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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หรือหลังกระตุ้นให้ไข่ตกเพิ่มขึ้น 78% (7/9) เทียบกับ 46% (6/13) เทียบกับ 54.5% (6/11) ตามลำดับ ทำการยายฝากตัวอ่อนโดยวิธีไมผาตัด 3 ครั้ง ครั้งแรกเมื่อตัวอ่อนอายุ 7.5 วัน ลักษณะเป็นเอ็ทช บลาสโตซีส ฝากไปยังตัวรับ ไมพบการตั้งท้อง ครั้งที่ 2 เก็บตัวอ่อนอายุ 7.0 วัน ระยะเอ็ทช บลาสโตซีส และฝากไปยังตัวรับ ไมพบการตั้งท้องเช่นกัน สวนครั้งที่ 3 เก็บตัวอ่อนอายุ 6.5 วัน เป็นระยะมอรูลา 2 ตัว จากตัวให้ ซึ่งได้จากการกระตุ้นให้ไข่ตกเพิ่มขึ้น แล้วฝากไปยังตัวรับที่มีระยะวงจรสัดใกล้เคียงกับตัวให้ ตัวรับตัวทั้ง 2 ตั้งท้อง แม่ตัวรับตัวหนึ่งซึ่งได้รับการผสมตามธรรมชาติขณะเป็นสัด คลอดลูกแฝดผู 1 คู่ และแม่อีกตัวคลอดลูกเพศผู 1 ตัว ระยะอุ้มท้องทั้ง 329 และ 332 วัน ตามลำดับ นอกจากนี้ยังได้ศึกษาการเก็บตัวอ่อนแช่เย็นที่ -196° ซ จำนวน 9 ตัว ระยะต่าง ๆ คือ 2 เซลล์, 16 เซลล์ คอมเพค มอรูลา บลาสโตซีส และเอ็ทช บลาสโตซีส ใช้วิธีอัตโนมัติ และวิธีใช้มือปรับเครื่องมือ หลังแช่เย็นศึกษารูปร่างของตัวอ่อนในแบบต่าง ๆ ได้แก่ เกรด-เอ ปกติ, เกรด-บี บางส่วนถูกทำลาย และเกรด-ซี ตัวอ่อนถูกทำลายทั้งหมด พบได้ 22.2 % (2/9), 22.2 % (2/9) และ 55.6 % (5/9) ตามลำดับ ซึ่งลักษณะต่าง ๆ ดังกล่าวนี้ พบได้ใกล้เคียงกันในทั้ง 2 วิธีที่ใช้

ผลการศึกษาครั้งนี้ชี้ให้เห็นถึงการตอบสนองที่ต่ำของรังไข่ ต่อการกระตุ้นด้วยฮอร์โมนโกนาโดโตรปิน รวมทั้งตัวอ่อนที่ผลิตได้ การเจริญเติบโตของตัวอ่อนระยะแรกใช้เป็นแนวทางในการยายฝากตัวอ่อนให้ประสพความสำเร็จได้ นอกจากนี้ยังสามารถเก็บตัวอ่อนในลักษณะแช่เย็นที่ -196° ซ ได้อีกด้วย

EMBRYO TRANSFER IN THAI SWAMP BUFFALO (BUBALUS BUBALIS)

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ABSTRACT

The present investigation was aimed at 4 objectives

1) responses of buffalo cows to superovulation using gonodotrophin

2) early embryonic development

3) embryo transfer and induction of twinning

4) cryo preservation of embryos using manual and automatic methods. Fourteen Thai swamp buffalo (3 heifers and 11 cows, 4 to 12 years old, weighed 315 to 480 kg were used in this study. Superovulation by using FSH 32 mg (n = 2) and 50 mg. (n = 4) and PMSG 2500 i.u. (n = 2) and 2700 i.u. (n = 3) resulted in average of 50% of treated animals responsed to the treatment. Recovery rate of embryos was 54.5% (6/11) while for normal embryo, degenerating embryos and unfertilized eggs, they were 37.5% (6/16), 37.5% (6/16) and 25% (4/16) respectively. A total of 33 nonsurgical embryo collections was carried out on Days 5.5, 6.0, 6.5, 7.0 and 7.5 to investigate early development. The

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different stages were the 16-cell stage, compacted morula, blastocyst, hatched blastocyst and hatched expanding blastocyst, respectively. A higher recovery rate was obtained with single embryo collection after natural estrus than after induced estrus or superovulation, 78% (7/9) vs 46% (6/13) vs 54.5% (6/11), respectively. Nonsurgical embryo transfer was carried out using 3 donors and 4 recepients. Single embryo recovery was performed in one donor on Day 7.5 after estrus and the embryo found was a hatched blastocyst which was transfered to a recipient. Pregnancy diagnosis performed 2 months later to be nonpregnant. A hatched blastocyst and a degenerated embryo were recovered from a superovulated donor on Day 7.0 and 2 morulae were recovered from a second superovulated donor on Day 6.5 the hathed blastocyst and the 2 morulae were transfered to 3 recipients and the 2 recipients which received the morulae were subsequently diagnosed pregnant and calved 3 bull calves with gestation period of 329 days and 332 days for the twin bull and a single bull calf respectively. A total of 9 embryos at stage of 2 cell embryo, 16-cell embryo, compacted morula, blastocyst and hatched blastocyst were frozen to -196° C with manual and automatic methods. Post thawed morphology showed undamage embryo, (grade-A) partial damage (grade-B) and total damaged (grade-C) embryos could be obtained by both methods embryos. They were 22.2 % (2/9), 22.2 % (2/9) and 55.6 % (4/9) respectirely.

The present findings indicate the low responses of buffalo to gonadotrophin treatment and production of embryos. Embryo transfer and cryopreservation of embryo is this specis are possible.

INTRODUCTION

Biotechnological techniques especially embryo transfer is now routinely used for the purposes of genetic improvement and diseases control in cattle. Furthermore, superovulation of the donor can significantly increase the reproductive rate and preservation of embryos in the frozen form enhances the application of this technique. There are a few reports on superovulation in buffalo and they obtained low rate of ovarian responses (Nittayawattana et al., 1982 ; Drost et al., 1983; Karaivanov et al., 1987). However, Drost et al. (1983, 1988) employed technique of embryo transfer sucessfully in water buffalo of Jaffarabadi and Mediteranean breeds. It's reasonable to investigate the ovarian response of swamp buffalo in the mean time technique of non surgical embryo collection and transfer will be also investigated. If embryo transfer will be employed more efficiently, information on early embryonic development in this species is critical. In addition, preservation of embryos in frozen form will be more economic and applicable than fresh one. The objectives of the present research are to investigate

1) Ovarian responses to gonadotrophin treatment

2) technique of non surgical embryo collection and transfer and induction of twinning.

3) early embryonic development and

4) freezing of embryos.

OVARIAN RESPONSES TO GONADOTROPHIN STIMULATION IN SWAMP BUFFALO (BUBALUS BUBALIS)

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ABSTRACT

Eight swamp buffalo cows (7 cows and 1 heifer) age between 4 to 12 years old and weight between 357-482 kg. were subjected to superovulation by using follicle stimulating hormone (FSH) or pregnant mares serum gonadotrophin (PMSG). The dosage used were 32 (n = 2) and 50 mg (n = 4) for FSH and 2500 (n = 2) or 2700 IU. (n = 3) for PMSG. Overall results revealed 50% of the treated animals response to the treatment used. Recovery rate of embryos was 54.5% (6/11) while for normal embryo, undeveloped and unfertilized eggs, they were 37.5% (6/16), 37.5 (6/16) and 25% (4/16) respectively. The present findings indicated the potential production of swamp buffalo embryos by superovulation with FSH or PMSG.

INTRODUCTION

The technique of biotechnology particularly embryo transfer is now routinely used in several species with different purposes. However, embryo transfer combines with superovulation

of the donor can significantly increase the female reproductive rate and it has a greatest application in farm animals both in research and commercial production (Sreenan, 1988). Superovulation is the most common technique for supplying embryos and data have been accumulated from cattle. At present, the most efficient procedure is using a gonadotophin during mid-cycle and followed 48 h with prostaglandin F2 alpha to induce luteolysis, oestrus and ovulation. Only a few reports on superovulation in swamp buffalo (Nittayawattana et al., 1982 ; Drost et al., 1983 ; Karaivanov et al., 1987) and they obtained low rate of ovarian response, about 2 embryos per collection. In addition, Sharifuddin and Jainudeen (1984) could not recover ova from 10 cows superovulated with PMSG plus GnRH or PMSG. Our laboratory experienced similar findings. However, as technique of embryo transfer was employed successfully in this species by Drost et al. (1983). It's reasonable to reinvestigate the ovarian response of the swamp buffalo to the stimulation by gonadotrophin and further application may be possible.

MATERIALS AND METHODS

Experimental Animals

Eight swamp buffaloes, 7 cows age between 6 to 12 years and 1 heifer age 4 years old weight between 357-482 kg. were used Two teaser bulls a white and a polled have been operated for deviation of the penis and also a fertile bull was included in the experiment. They were raised under similar menagement conditions at Research and Training Centre, Faculty of Veterinary Science, Chulalongkorn University, Nakhon-Pathom. They received concentrate 1 kg in the morning and evening and grazed during the day time.

PRODUCTS

- Prostaglandin F2 alpha analog (Prosolvin^{R} Intervet) contains 7.5 mg/ml.
- Follicle stimulating hormone (FSH - P) Burns Biotech, Labs, Nebraska, USA contains 50 mg).
- Pregnant mares' serum gonadotrophin (PMSG) (Folligen^{R}, Intervet) contains 5000 IU./25 ml.
- Gonadotropin Releasing Hormone (GnRH) (Receptal^{R}, Hoescht) contains 0.004 mg Buserclin.
- Neutra - PMSG (Intervet) contains 5 ml

METHODS

When animals were in heat whether by natural or induced it's designated to be day 0 and during mid-cycle between days 8 to 12 they were subjected to rectal examination for the corpus luteum and treated with one of 4 regimens for superovulation, FSH - P 32 or 50 mg or PMSG 2500 or 2700 IU followed by the administration of 2 doses of PGF 2 alpha 2 ml 48 and 60 h later. In addition, GnRH 2.5 ml was administered intramuscularly at the time of first mating in both FSH regimens. Whereas Neutra - PMSG 5 ml was administered intravenovsly at the first mating for PMSG regimen only.

The FSH - P regimen was given 5 mg morning and evening for 5 days or a total dose of 32 mg and splitted into decreasing doses for 5 days started on day 8 of the cycle. The PMSG regimes of 2500 or 2700 IU. was given once intramuscularly on day 11 of the cycle.

When the treated animals came in heat and confirmed by the behaviour of the teaser bulls, they were mated twice according to the acceptance of the cows. Technique of embryo

recovery have been described previously (Betteridge, 1977 ; Nittayawattana et al., 1982 ; Drost et al., 1983, Sharifuddin and Jainudeen, 1984, Bodhipaksha, 1987 ; Chantaraprateep et al., 1987 ; Lohachit et al., 1987 ; Chantaraprateep et al., 1988). Schedules for non surgical embryo recovery were on days 5.5 to 7.5 using lactate ringer solution as a flushing medium 500 ml per horn. After collection, the effluent in the cylinder were left in the room at 31^{o}C for 30 min and drained out about 400 ml the sediment was then poured into a petri dish and examined under a stereomicroscope with 10x to 40x magnification. Evaluation of the embryos were made and photograph was taken. If appropriate, good quality embryos were subjected to freezing for further investigation.

RESULTS

Ovarian responses of swamp buffaloes to different regimens of superovulation by FSH-P and PMSG were shown in Table 1.

Table 1 Ovarian responses of swamp buffalo cows to superovulation by a total dose of 32 mg or 50 mg of FSH-P or PMSG 2500 - 2700 IU.

Donor no.	Hormone used and ovarian response				Embryos recovery
	FSH-P				
	32 mg		50 mg		
	LO F/CL	RO F/CL	LO F/CL	RO F/CL	
C55	-	-	0/4	0/2	NF
C57	-	-	0/2	0/0	HB and UND
C8	3/2	2/1	-	-	NF
H2	-	-	2/1	3/2	NF
C116	-	-	0/2	0/3	3UND and 2UNF
C8	1/0	2/3	-	-	2EB and UNF
	PMSG				
	2500 iu		2700 iu		
	LO F/CL	RO F/CL	LO F/CL	RO F/CL	
C5	0/1	2/0	-	-	NF
C8	-	-	0/2	0/3	2M
C107	-	-	3/2	0/1	NF
H2	3/1	2/2	-	-	2UND
C376	1/3	1/1	-	-	EB and UNF

Legend :	F	Follicle		CL	Corpus luteum
	NF	Not found		B	Blastocyst stage of embryo
	HB	Hatched blastocyst		EB	Early blastocyst
	UNF	Unfertilized egg.		M	Morula
	C	Cow		UND	Undeveloped embryo
	LO	Left ovary		H	Heifer
	RO	Right ovary			

Two, 4, 2 and 3 animals were subjected to FSH-P 32 mg, 50 mg, PMSG 2500 and 2700 IU. respectively. On average 54.4 % (6/11) of animals responsed to the treatment in term of embryo recovery rate.

While for normal embryo, undeveloped and unferfilized eggs, they were 37.5 (6/16), 37.5 (6/16) and 25% (4/16) respectively. The high rate of embryo recovery failure was 45.5% (5/11). One cow was subjected to superovulation 3 times whereas another one was subjected to 2 times.

DISCUSSION

The low rate of embryo recovery in this species has been demonstrated previously (Drost et al., 1983). They reasoned that due to small genital tract and several other factors such as age, parity, nutritional states etc. While for Sharifuddin and Jainudeen (1984), they could not recover any ova from the flushing of seven cows. They stated that the causes were over stimulation of the ovaries together with poor recovery rate of effluent which was only 20-60% the rest may leak through ovarian tube and caused failure to recover embryos. Ovarian responses were noted on examination per rectum, the low rate of embryo recovery was due partly to time of gonodotrophin administration as in some cases we found a few anovulatory follicles on day 6

after estrus.

Several factors exert influence on such recovery rate of embryo. In superovulated cattle even 7 and 8 days after estrus some eggs are still retained within the oviduct as shown by Moore (1975). Furthermore, it's possible that a proportion of egg is expelled shortly after entering the uterus which is around Day 4.5 in this species (Karaivanov et al., 1987) and in superovulated cows estrogen remains at high levels until Day 6 (Booth et al., 1975). It is also well established that with higher levels of protein in the ration, levels of LH and response to gonadotrophin releasing hormone are greater (Jordan and Swanson, 1979).

Furthermore, over stimulation of ovarian tissue with the hormone while the animals were not in a healthy condition rendered poor responses or obtained poor quality embryos or eggs as shown in table 1.

Apparently, both FSH-P and PMSG produced similar ovulatory responses in this species in terms of normal embryos (n = 3). Although small genital tract in this species was informed by Drost et al. (1983) and Sharifuddin and Jainudeen (1984) which caused difficulties for passing Foley cathetor and flushing medium was always disturbed. We encountered such problem during our early work but later on it was solved and flushing was almost 100% of recovery. One cow was subjected to superovulation 3 times the last 2 treatments produced very good quality embryos. This shows that it is possible to superovulate swamp buffalo cows 3 times during 6 months and still obtain a superovulation response. While for another cow subjected to 2 superovulation, one collection could not find the embryo and another collection obtained 2 undeveloped eggs, these findings indicated individual variation of ovarian response to stimulation by gonadotrophin.

Application of GnRH at the time of mating would help augment the concentration of endogenous luteinizing hormone (LH) in turn will induce ovulation while for Neutra-PMSG with anti PMSG activities when given intravenously will act constantly on action of PMSG as this gonadotrophin has prolonged half life up to 5-7 days following the treatment. Given PMSG alone may cause inappropriate time of ovulation and mating. The findings suggested that in this species superovulation is possible by both FSH-P and PMSG. However, a reliable superovulation regimen with a predictable response should be developed.

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REFERENCES

Betteridge, K.J. 1977. Superovulation. In : Embryo transfer in farm animal. A review of techniques and application. Betteridge (ed). Agriculture Canada. Monograph No. 16.p.1-9.

Bodhipaksha, P. 1987. Embryo transfer attempts in the swamp buffalo In : Swamp Buffalo Reproduction. P.Chantaraprateep P. Virakul, C. Lohachit and A. Kunavongkrit. (eds). 2nd edition. Chulalongkorn University Press. pp. 341-348.

Booth, W.D., Newcomb, I.R., Strange, H., Ravson, L.E.A. and Sacher, H.B. 1975. Plasma oestrogen and progesterone in relation to superovulation and egg recovery in the cow. Vet. Rec. 97, 366.

Chantaraprateep, P., Lohachit, C., Virakul, P., Kunavongkrit, A., Prateep, P. and Bodhipaksha, P. 1987. Embryo Transfer in Swamp Buffalo. In : In Vitro Fertilization and Embryo Transfer. M. Kamonpatana. (ed). Chulalongkorn University Press. pp. 321-330.

Chantaraprateep, P., Lohachit, C., Kobayashi, J., Virakul, P., Kunavongkrit, A., Techakumphu, M., Prateep, P., Pisornsarakit P. and Limskul, A. 1988. Early embryonic development in Thai Swamp Buffalo. Theriogenology. 31(6) : 1131-1139.

Drost, M., Wright, J.M., Cripe, W.S. and Richter, A.R. 1983. Embryo Transfer in Water Buffalo (Bubalus Bubalis). Theriogenology. 20(5) : 579-584.

Jordan, E.R. and Swanson, L.V. 1979. Serum progesterone and luteinizing hormone in dairy cattle fed varying levels of crude protein. J. Anim. Sci. 48, 1154.

Karaivanov, C., Ylahov, K., Petrov, M., Kacheva, D., Stojanova, M., Alexiev, S., Polihronov, O. and Danev, A. 1987. Studies on preimplantation development of buffalo embryo. Theriogenology. 28(5) 747-753.

Lohachit, C., Chantaraprateep, P., Virakul, P., Kunavongkrit, A. and Bodhipaksha, P. 1987. Technique of Embryo Transfer in Swamp Buffalo. In : In Vitro Fertilization and Embryo Transfer. M. Kamonpatana. (ed). Chulalongkorn University Press. pp. 185-200. (in Thai).

Moore, N.W. 1975. The control of time of estrus and ovulation and the induction of supperovulation in cattle. Aust. J. Agric. Res. 26, 295.

Nittayawattana, S., Chatchaidej, S., Tantisuntikorn, K., Lohachit, C., Chantaraprateep, P. and Boodhipaksha, P. 1982. Non-surgical Embryo Transfer in the Buffalo. Proc. 9th Vet. Association Conference. Bangkok Palace Hotel, 2-3 December. pp. 36-53.

Sharifuddin, W. and Jainudeen, M.R. 1984. Superovulation and non-surgical collection of ova in the water buffalo (Bubalus bubalis). Proc. 10th Internat. Cong. Anim. Reprod. and AI. University of Illinois II : 240.

Sreenan, J.M. 1988. Embryo transfer : Its uses and recent development. Vet. Rec. 624-629.

EARLY EMBRYONIC DEVELOPMENT IN THAI SWAMP BUFFALO (Bubalus bubalis)

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ABSTRACT

A total of 33 nonsurgical embryo collections was carried out to investigate early embryo development in Thai swamp buffalo. Collections were performed on Days 5.5, 6.0, 6.5, 7.0 and7.5. The different stages of embryo development on these days were the 16-cell stage, compact morula, blastocyst, hatched blastocyst and

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hatched expanding blastocyst, respectively. In addition,some degenerating embryos and unfertilized ova were also recovered. A higher recovery rate was obtained with single embryo collection after natural estrus than after induced estrus or superovulation, 78%(7/9) % vs 46% (6/13) vs 54.5% (6/11), respectively. A higher percentage of normal embryos was also obtained with single embryo collection after either natural or induced estrus than after superovulation, 71% (5/7), 83% (5/6) and 38% (6/16), respectively.

Key words : embryo, early development, swamp buffalo

INTRODUCTION

In most mammals, the embryo enters the uterus about 3 to 4 d after ovulation. Subsequently, the embryo reaches the morula stage and develops into a blastocyst which later becomes implanted. In cattle, this differentiation occurs about 6 to 7 d after the onset of estrus (estrus= Day 0). During Days 8 to 9,the bovine blastocyst expands and sheds its zona pellucida,and development continues until implantation (1,2). These events seem to occur earlier in Murrah buffalo (3) and in river buffalo (4).

Early embryonic development has not been reported for swamp buffalo, whose reproductive anatomy and physiology are different from those of Murrah buffalo and of cattle (4, 5). Thus, the aim of our research was to study swamp buffalo embryos at different stages of development.

MATERIALS AND METHODS

Fourteen swamp buffalo (3 heifers and 11 cows, 4 to 12 yr old, weighing 315 to 480 kg) were used in this study. Two mature bulls with surgically deviated penises were used as teaser animals for estrus detection, while one fertile bull was used for natural mating. All animals were raised under similar management conditions, were fed 1 kg. of concentrate twice daily and were allowed to graze ad libitum in the field during the day.

Three types of embryo collection were used : single ova collection after natural or induced estrus, or after superovulation. For induction of estrus, buffalo cows or heifers were palpated per rectum to identify a corpus luteum on the ovary. Only buffalo with prominent functional corpora lutea were injected intramuscularly (i.m.) with 15 mg of prostaglandin F 2 alpha (PGF2 Prosolvin^{R}, Intervet, Boxmeer-Holland). Estrus detection was carried out twice daily, in the morning and evening, using teaser bulls. Generally, animals showed the onset of standing estrus (Day 0), and most donors remained in estrus for 12 h. Donors were mated twice with a fertile bull, at the onset of standing estrus and again 10 to 12 h later.

In cases of superovulation, two and three donors were treated with 2500 and 2700 IU of pregnant mares' serum gonadotropin (PMSG; Folligon^{R}; Intervet, Boxmeer-Holland), respectively. Follicle stimulating hormone-pituitary (FSH-P; Armour, Burns-Biotec, Omaha, Nebraska, USA) was given in decreasing doses at 12 h intervals twice daily for 4 days (6-6, 5-5, 3-3 and 2-2 mg) for a total of 32 mg to 2 donors, and

twice daily in equal doses for 5 days (5-5, 5-5, 5-5, 5-5 and 5-5) for a total of 50 mg to 4 donors.

During the mid-cycle, between Days 8 and 12, donors were examined per rectum for the presence of a prominent corpus luteum and treated with one of four regimens. Prostaglandin F 2alpha (15 mg) was administered in the morning and evening at 48 and 60 h after PMSG and on the last day of the FSH treatment. Gonadotropin stimulating hormone (GnRH;1.0mg; Receptal^{R}, Hoechst, Munich, West Germany) was administered i.m. at the time of the first mating.

On Days 5.5, 6.0, 6.5, 7.0 and 7.5 after onset of standing estrus embryos were collected nonsurgically via the cervix as previously reported (8-11). Briefly, the donor was tranquilized with xylazine (20 mg/i.m.), and epidural anesthesia was achieved with xylocaine (3 ml). Embryos were collected with a two-way Foley catheter (French gauge 14 or 16) by flushing each uterine horn with 500 ml of isotonic lactated Ringer'ssolution to which 1,500,000 IU penicillin had been added. Normally, the uterine horn ipsilateral to the corpus luteum was flushed first and embryos were collected into a graduated cylinder.

Evaluation of embryos was performed 30 min after flushing at room temperature (31°C) with a stereomicroscope at 10 to 40 x magnification. The embryos were washed twice in 2 ml of Whitttingham's modification of Dulbecco's phosphate buffered saline (PBS; Flow Laboratories, North Ryde, N.S.W. Australia) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, USA), and their stage of development was recorded and a photograph was taken.

RESULTS

A total of 19 (57.5%) normal and abnormal embryos was recovered in 33 attempts. The highest success rate (78%) was obtained by single embryo collection after natural estrus, while single embryo collection after induced estrus resulted in 46% recovery and post superovulation collection resulted in 55% (Table 1). Two of 33 recovery attempts failed because the cervix could not be passed. The quality of embryos and the percentage of normal embryos was the same for natural and induced estrus when single embryo collection was employed 71% (5/7) vs 83% (5/6). The lowest recovery rate of normal embryos was obtained in the superovulated group (38%,6/16;Table1). Retarded and degenerating (abnormal) embryos were also observed in the superovulated group (38%, 6/16). The percentage of unfertilized ova was approximately the same among the three collection methods. Embryos recovered on Day 5.5 were at the 16-cell stage; morulas or early blastocysts were found when the collection was made on Day 6.0, and blastocysts were obtained on Day 6.5. When collections were carried out on Days 7.0 and 7.5,hatched (collapsed or expanding) blastocysts were found. In addition, degenerating embryos and unfertilized ova were recovered. The morphology of normal embryos, degenerating embryos and unfertilized ova is shown in Figures 1 and 2.

DISCUSSION

The highest success rate of embryo recovery was 78% from the single egg collection after natural estrus compared to 46% after induced estrus and 54.5% superovulated animals. The reasons may partly be due to the fact that natural estrus does

not disturb normal reproductive endocrine function. In addition, estrus behavior in this species is a real problem as described earlier (12) appropriate time for mating will be critical especially after induction of estrus which resulted around 45% of conception rate (13, 14).

The quality of embryos obtained by single egg collection, either after natural or induced estrus, were better than those after superovulation. Poor quality of embryos might be due to inappropriate dose or method for ovarian stimulation. Few studies on the superovulatory treatment in buffalo have been reported generally the response was low and the unfertilized and abnormal eggs were obtained after stimulation (8, 3).

In this experiment, the embryo recovery was carried out from day 5.5 onwards as recent report in this species (Murrah breed) revealed that embryos age 4.5 days or more can be found in the uterus (3).

Our study showed that compaction of embryo occurred about day 6.0 and the transition from morula to early blastocyst occurred after day 6. This chronology of development to the early blastocyst stage is similar to that of cattle (15). A difference in rate of development between the swamp buffalo and cattle was observed from day 7.0 to 7.5 Hatching of embryos from the zona pellucida in the swamp buffalo seemed to occur earlier than in cattle. Hatched blastocysts were found on day 7.0 to 7.5 while they are found on day 8.5 to 10 in cattle. Our results were also different from the report of Karaivonov et al. (3) in Murrah buffalo as they reported that the hatched blastocyst was

found on day 5 after onset of estrus. In one attempt we could not find the embryo on day 4.5 after mating but repeat collection of the same animal was made on day 6.5 and the embryo was found. The difference of the result may be the fact that estrus behavior is difficult to observe in this species. In our experience the most practical way for heat detection in this species is use of a teaser bull, e.g. a bull with a deviated penis. Thus, the timing of ebryo development from the onset of estrus might be inaccurate time when compared to the time of standing heat. A comparative study of early embryonic development in Murrah and swamp buffalo should be conducted to explain this difference.

The present findings, indicate that the chronology of embryo development in swamp buffalo was different from that of cattle and of Murrah buffalo.

REFERENCES

1 Newcomb, P. 1976. Investigation of factors affecting superovulation, egg recovery, and transfer in cattle, M.Sc. Thesis, University of Cambridge, England. Cited by ADAMS, C.E. 1982. In : Mammalian egg transfer. CRC Press, Inc. Florida, p. 94.

2 Newcomb,R.,Rowson,L.E.A. and Trounson, A.O.1976. The entry of superovulated eggs into the uterus. In : Egg Transfer in Cattle L.E.A. (ed). Rowson. Commission of the European Communittees. Luxembourg. 1.

3 Karaivanov, C.,Vlahov,K., Petrov, M., Kacheva, D., Stojanova, M., Alexiev, A., Polihronov, O. and Danev, A. 1987. Studies on preimplantation development of buffalo embryo. Theriogenology. 28(5) 747-753.

4 Drost, M. and Elsden, R.P. 1985. Blastocyst development in the water buffalo (Bubalus bubalis). Theriogenology 23 (1) 191.

5 Lohachit, C. 1987. Anatomy and clinical examination on female reproductive organ of swamp buffalo. In : Swamp Buffalo Reproduction. Chantaraprateep, P., Virakul, P., Lohachit, C., and Kunavongkrit, A. (eds) 2nd edition Chulalongkorn University Press. pp. 93-115.

6 Bodhipaksha, P. 1987a. Physiology of female swamp buffalo reproduction. In:Swamp Buffalo Reproduction Chantaraprateep, P., Virakul, P., Lohachit, C., and Kunavongkrit, A., (eds) 2nd edition. Chulalongkorn University Press. pp. 117-124.

7 Chantaraprateep, P., Lohachit, C., Virakul, P., Kobayashi, G., Techakumphu, M., Kunavongkrit, A. and Prateep, P. 1988. Ovarian responses to gonadotrophin stimulation in swamp buffalo. Buffalo Bulletin. 7(4) 82-86.

8 Drost, M., Wright, J.M., Cripe, W.S. and Richter, A.R. 1983. Embryo Transfer in Water Buffalo (Bubalus Bubalis) Theriogenology. 20(5) 579-584.

9 Lohachit, C., Chantaraprateep,P., Virakul, P. Kunavongkrit, A. and Bodhipaksha, P. 1987. Technique of Embryo Transfer in Swamp Buffalo. In : In Vitro Fertilization and Embryo Transfer. M. Kamonpatana (ed) Chulalongkorn University Press. pp. 185-200. (in Thai).

10 Chantaraprateep,P., Lohachit, C., Virakul,P., Kunavongkrit,A., Prateep, P. and Bodhipaksha, P. 1987. Embryo Transfer in Swamp Buffalo. In : In Vitro Fertilization and Embryo Transfer. M. Kamonpatana (ed) Chulalongkorn University Press. pp. 321-330.

11 Bodhipaksha, P. 1987b. Embryo transfer attempts in the swamp buffalo. In : Swamp Buffalo Reproduction. Chantaraprateep, P. Virakul, P., Lohachit, C., and Kunavongkrit, A., (eds) 2nd edition. Chulalongkorn University Press. pp. 431-348.

12 Chantaraprateep, P. 1985. Oestrous signs in swamp buffalo its detection and improvement. The First World Buffalo Congress. Cairo. Egypt. 23 Dec - 1 Jan' 86 pp. 585-588.

13 Chantaraprateep, P. 1987. Control of estrous cycle of swamp buffaloes.In : Swamp Buffalo Reproduction. Chantaraprateep, P., Virakul, P., Lohachit, C., and Kunavongkrit, A., (eds) Chulalongkorn University Press. pp. 187-209.

14 Chantaraprateep, P.,Lohachit, C., Bodhipaksha, P.and Virakul, P. 1982. Prostaglandin F2 alpha for oestrus control of buffaloes. Proc. 2nd Internat. World Buffalo Production Congress. Casserta, Italy 29 Sept - 2 Oct. pp. 385-393.

15 Betteridge, K.J.1977. Superovulation. In : Embryo transfer in farm animals. A review of techniques and applications K.J. Betteridge (ed) Agriculture Canada. Monograph 16. p.3

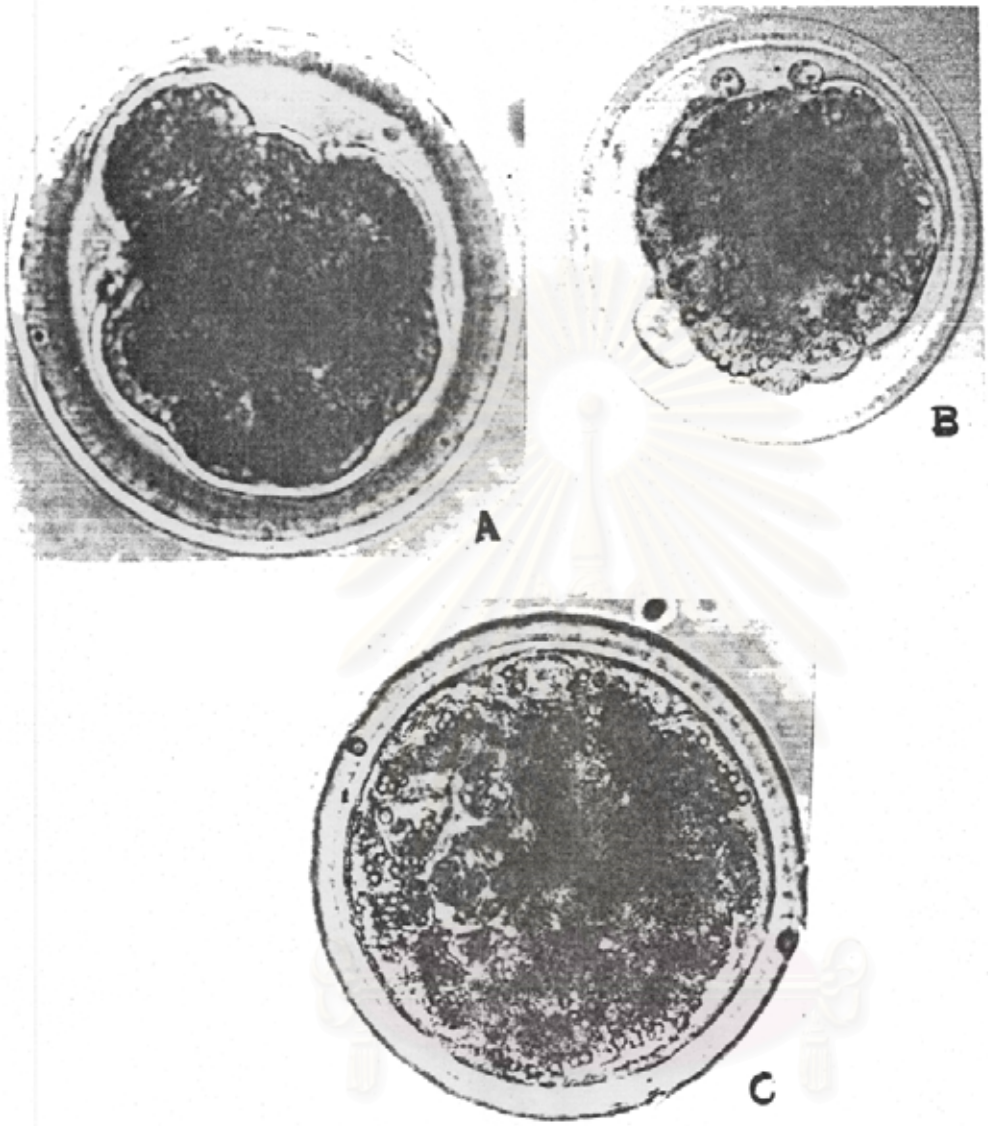

Figure 1 Various stages of swamp buffalo embryo development. A) A 5.5-day 16-cell embryo. The contour of each blastomere is evident, some blastomeres are beginning to fuse, the perivitelline space is present and the zona pellucida is thick. B) A 6-day embryo, blastomeres are in close apposition, resulting in a dense mass called the compact morula. Perivitelline space is still present. Some vacuoles may be observed on surface of blastomere. C) A 6.0-day embryo, early blastocyst stage with intact zona pellucida. Blastocoele occupies 25% of the volume of the embryo; undifferentiated blastomeres are still present, on perivitelline space.

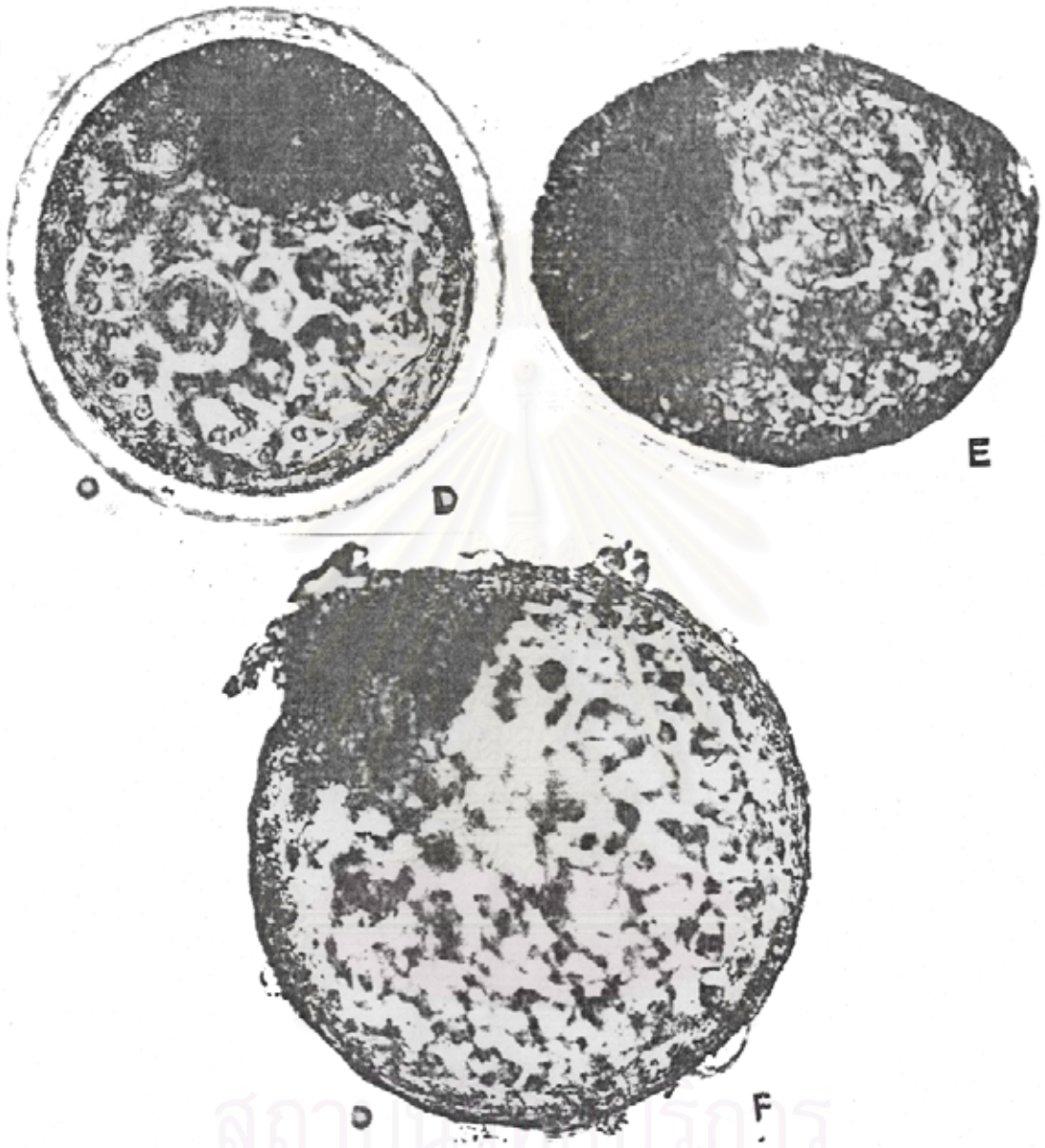

Figure 1 D) A 6.5-day embryo, expanding blastocyst stage. There is no perivitelline space. Trophoblastic cell line the zona pellucida; the inner cell mass is well differentiated; the blastocoele is about 75% of the volume of the embryo; the zona pellucida is considerably thinner, while the number of cells of the embryo has increased. E) A 7-day embryo, a partially collapsed hatched blastocyst; the zona pellucida is gone. Well-defined trophoblast cells and inner cell mass are present. F) A 7.5-day embryo, hatched expanding blastocyst (without a zona pellucida), increased diameter.

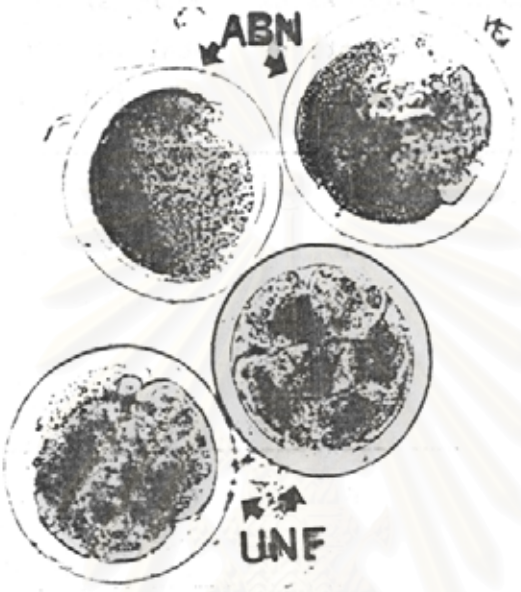

Figure 2 Morphology of unfertilized and abnormal embryos from a superovulated swamp buffalo recovered on Day 6 after mating. ABN = Abnormal embryo; UNF = Unfertilized ova. 200 x magnification.

Table 1 Results of embryo recovery from swamp buffalo after natural estrus, induced estrus and superovulation

Embryo collection	No. of collections	No. of successful collections (%)	Recovered embryos/ova Total	N (%)	ABN (%)	UNF (%)	Not found	Not able to collect
Single embryo collection (natural) estrus	9	7 (78)	7	5 (71)	-	2 (29)	1	1
Single embryo collection (induced estrus)	13	6 (45)	6	5 (80)	-	1 (20)	6	1
Embryo collection following superovualtion	11	6 (54.5)	16	6 (37.5)	6 (37.5)	4 (25)	5	-
TOTAL	33	19 (57.5)	29	16 (55)	6 (20.7)	7 (24.1)	12	2

N = normal embryo; ABN = abnormal embryo; UNF = unfertilized ova.

SUCCESS OF EMBRYOS TRANSFER IN THAI SWAMP BUFFALO (Bubalus bubalis)

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ABSTRACT

Nonsurgical embryo transfer was carried out in swamp buffalo cows using 3 donors and 4 recipients . Two types of embryo collection were used, single embryo recovery and recovery after superovulation. Single embryo recovery was performed in one donor on 7.5 day after estrus and the embryo found was a hatched blastocyst which was transferred to a recipient. Pregnancy diagnosis performed 2 months later showed the recipent to be nonpregnant. A hatched blastocyst and a degenerated embryo were recovered from a superovulated donor on day 7.0, and two morulae were recovered from a second superovulated donor on day 6.5. The hatched blastocyst and the 2 morulae were transferred to 3 recipients and the 2 recipients which received the morulae, were subsequently diagnosed pregnant, and calved 3 bull calves.

This is the first report of successful nonsurgical embryo transfer in Thai swamp buffalo.

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INTRODUCTION

Attempts had been made to carry out embryo transfer in swamp buffalo since 1981 as recently described (Chantaraprateep et al., 1987). Although embryos were recovered from donors and transfers were made to recipients non resulted in pregnancy. The first successful nonsurgical embryo transfer resulted in the birth of a bull calf but it was performed in water buffalo of Jafarabadi breed by Drost et al.(1983). A second report came from Bulgaria where Drost et al. (1988) produced five pregnancies in Mediterranean buffalo, which resulted in the birth of four live calves. However, attempts had also been made in Malaysia by Sharifuddin and Jainudeen (1984) in swamp buffalo but no ova were recovered from the flushings. Failure may be caused partly by lack of knowledge of embryo development and technique used as described by different authors. Our recent report on different rate of embryo development in this species with a total of 33 collections were performed(Chantaraprateep et al.,1989) suggested a more appropriate time for recovery of embryo for transfer in this species. The purpose of the present study was to perform embryo transfer and evaluate the technique used in swamp buffalo.

MATERIALS AND METHODS

Animals

Seven female swamp buffaloes (1 heifer and 6 cows) between 4 to 12 years old weighing between 357 to 482 kg were used in this experiment. All animals were raised under similar management conditions at the Research and Training Centre, Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom province. Two teaser bulls, one white and another polled, were used for estrus detection. Both bulls had been operated for deviation of penis more than 1 year earlier. A fertile bull was used to breed the donors.

Products

Prostaglandin F 2 alpha (PGF 2 alpha) (Prosovin^{R}, Intervet, Holland), 1 ml contains 7.5 mg.

Follicle stimulating hormone (FSH-P), (Burns-Biotec Lab, USA). Each vial contains FSH-P for injection equivalent of 50 mg Armour Standard.

Pregnant Mares' Serum Gonadotropin (PMSG) (Intervet) contains 5000 I.U./ 25 ml.

Gonadotropin releasing hormone (GnRH) (Receptal^{R} Hoechst, West Germany) 1 ml contains 0.4 mg Busereline.

Flushing medium : Isotonic Ringers Solution or Whittingham's Modification of Dulbecco's phosphate buffered saline (Flow Lab., N.S.W. Australia) which was fortified with 2 percent heat treated bovine serum and sodium penicillin G 100,000 I.U. per 1 liter of solution.

METHODS

Three attempts were made at embryo recovery, one single egg collection and two from superovulated donors. When animals were found to have a corpus luteum (CL) by rectal examination, they were treated with 2 ml PGF 2 alpha and estrus was confirmed through the observation of the behavior of the teaser bulls. Estrus generally occurred mostly within 3 to 4 days after the treatment. For the single egg collection first natural mating was performed at the onset of standing heat and the second mating took place 12 h later. Two regimens were used for superovulation PMSG 2700 I.U. (N=1) or FSH 32 mg (N=1). PMSG was given on day 10 of estrous cycle, two days later 15 mg PGF 2 alpha was injected intramuscularly in the morning and evening. FSH was given twice daily, 6-6, 5-5, 3-3 and 2-2 mg (32 mg) with the first injection on day 8 of the estrous cycle 15 mg PGF 2 alpha was given morning and evening on the last day of the FSH treatment and 1 mg GnRH on

the day of mating. Estrus in the recipients was synchronized with that of the donors and they received 15 mg PGF 2 alpha intramuscular treatment 12 h prior to the donors. Selection of the experimental animals and pregnancy diagnosis were performed by rectal examination of the reproductive tract. The techniques used for nonsurgical embryo collection and transfer were similar to those described by Kobayashi (1980) and Kobayashi et al.(1981) with some modifications according to species as metioned elsewhere (Drost et al., 1983; Bodhipaksha, 1987; Chantaraprateep et al., 1987; Lohachit et al., 1987; Chantaraprateep et al., 1989). Briefly described, tranquilized the donor with xylazine 15 mg IM and posterior epidural anaesthesized using xylocaine 3 ml. Response to superovulation was determined by examination per rectum of the ovaries and counting number of corpora lutea. The perineal region and vulva were thoroughly washed and disinfected with diluted iodine solution and the tail was tied. After empty content in the rectum, cervical dilator was inserted through vaginal canal into the cervix. A 2 way gauge Foley catheter with 30 ml inflatable balloon was used. A sterile stylet was inserted the full length of the catheter and fixed with artery forceps in order to render it sufficiently rigid to allow introduction into the uterus. The lips of the vulva were parted and the Foley catheter was inserted into the vagina and cervix. The inflatable balloon was placed first at the point beyond the middle of the ipsilateral horn to corpora lutea and it was slowly inflated with 15-20 ml of air. Subsequently, the stylet was retracted and a 20 ml catheter tip syringe was attached to the catheter. The flushing medium in 1 liter bottle was gently introduced into the uterus and allow to flow back into the 500 ml graduate cylinder. The process was repeated until the medium was used up the effluent was then placed in room temperature protected from sunlight, the embryos were allowed to settle to the bottle of the cylinder for 30 min. The supernatant fluid was rinsed, the

remaining 100 ml was poured into a petri dish and examined for embryo under a dissecting stereo-microscope at 10 x to 40 x magnification. The same procedure was repeated in the remaining horn using a separate sterile equipment.

Fresh embryo transfer was carried out similar to AI technique, by loading embryo into a French mini-straw and inserted into inseminating gun. Embryo was deposited into anterior portion of the ipsitaleral horn to corpora lutea.

The investigation was carried out during January 1988 to March 1989.

RESULTS

Synchronization of estrus in swamp buffalo by using 15 mg PGF 2 alpha was successful in 50 to 70% of the treated animals. Detection of estrus by using teaser bulls rendered the best solution in this species as recently reported (Chantaraprateep, 1988). Responses of ovaries and embryos collected as well as results of embryos transfer to recipients are shown in table 1.

Ovarian responses to the two regimens of superovulation by FSH-P and PMSG in this study were similar based on the number of corpora lutea (CL) on ovaries and recovery rate of embryos 44.4% (4/9). One hatched blastocyst (HB) and 1 degenerated embryo were recovered from cow No. 57 on day 7.0 and one hatched blastocyst from cow No. 8 on day 7.5. Two morulae were recovered from cow No. 8 on day 6.5. The blastocysts and the morulae were transferred to four recipients and 2 months later, the two recipients received the hatched blastocysts were not pregnant. The two recipients which received the two morulae on 23 April, 1988 (one recepient was mated naturally once on the day of estrus) were diagnosed pregnant.

The recipient which has previously mated prior to embryo transfer calved male twin calves weighed 15 and 25 kg. (12 March 1989) and the second cow calved a male calf weighed 33 kg. Their gestation lengths were 329 (12 March 1989) and 332 days (15 March 1989) respectively.

Table 1 Ovarian responses of swamp buffalo cows to superovulation and results of embryo recovery and transfer.

Donor No.	treatment	Ovarian responses				Embryos recovered	Transfer yes/no	Pregnancy diagnosis
		FL	CL	FR	CR			
57	FSH 32 mg	0	2	0	2	HB	yes	Nonpregnant
						DEG	no	
8	PMSG 2700 I.U.	0	2	0	3	2 morulae	yes	Pregnant

Legend : FL Follicle on left ovary, FR Follicle on right ovary.
CL Corpus luteum on left ovary, CR Corpus luteum on right ovary.
DEG Degenerated egg HB Hatched blastocyst
T Transfer

DISCUSSION

Responses of swamp buffalo cow to the treatment of PGF 2 alpha in term of estrus was variable between 50 to 70% and some animals may not response to such treatment as previously reported (Chantaraprateep et al., 1982; Chantaraprateep, 1987). Signs of estrus in this species are very difficult to detect and teaser bulls are necessary for estrus detection. Bulls with surgically deviated penis were very useful. It was easy to observe the bulls follow the estrous animal and try to mount as well as exhibit other sexual behaviors as previously described (Chantaraprateep, 1985; Chantaraprateep, 1988). Our earlier experiences and the present results indicated that embryos from day 7 onwards were hatched blastocysts (Chantaraprateep et al., 1987; Chantaraprateep et al, 1989). Transfer at this stage probably is not suitable as we did not get result of pregnancy possibly due to the fact that the embryo without its zona pellucida is prone to damage by several factors including diseases as shown by Wrathall (1987) and Singh (1980). However, the first sucessful embryo transfer in water buffalo was that of a hatched 7-day old embryo (Drost et al., 1983). Embryos around day 6(morula) in this species seemed to be suitable stage for transfer as its zona pellucida is still intact and serves as an important barrier which protects the embryo. We used mainly isotonic Ringer solution for flushing embryo except the two morulae recovered with Whittingham medium for cow No.8 resulted in two pregnancies.

The technique used for recovery of embryo in this species may need to be adjusted due to smaller reproductive tract when compared to those of cattle. Sharifuddin and Jainudeen failed to recover embryos from 10 cows superovulated with PMSG plus GnRH or PMSG. They suggested that it was due to difficulty

of manipulation of the reproductive genital tract and leakage of flushing medium into the abdomen.

For one of the two pregnant recipients the onset of estrus was synchronized with that of the donor while for the other the onset occurred about 12 h after that of the donor. The 2 embryos transferred successfully were day 6.5 morulae with intact zonae pellucidae which has also proved to be the optimal time for the transfer with highest conception rate in cows (Newcomb et al., 1982). Both recipients were reconfirmed pregnant 4 months after the transfers and they calved an average of the gestation length of swamp buffalo (Chantaraprateep et al., 1982). This is the first successful embryo transfer reported in swamp buffalo.

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REFFERENCES

Bodhipaksha, P. 1987. Embryo transfer attempts in the swamp buffalo. In: Swamp Buffalo Reproduction. P. Chantaraprateep, P. Virakul, C. Lohachit and A. Kunavongkrit (eds) 2nd edition. Chulalongkorn University Press. pp. 341-348.

Chantaraprateep, P. 1988. Estrous Symptoms and AI Techniques in Water Buffalo. Proc. FFTC Seminar. Manila, Bangkok and Taipei, 4-17 September. 13 pages.

Chantaraprateep, P. 1987. Control of estrous cycle of swamp buffalo. In: Swamp Buffalo Reproduction. P. Chantaraprateep, P. Virakul, C. Lohachit and A. Kunavongkrit. (eds). Chulalongkorn University Press. pp. 187-209.

Chantaraprateep, P., Lohachit, C., Bodhipaksha, P. and Virakul, P. 1982. Prostaglandin F2 alpha for oestrus control of buffaloes. Proc. 2nd Internat World Buffalo Production Cong. Casserta, Italy. 29 Sept - 2 Oct. pp. 385-393.

Chantaraprateep, P., Lohachit, C., Virakul, P., Kunavongkrit, A., Prateep, P. and Bodhipaksha, P. 1987. Embryo transfer in swamp buffalo. In: In Vitro Fertilization and Embryo Transfer. M. Kamonpatana. (ed). Chulalongkorn University. Press. pp. 321-330.

Chantaraprateep, P., Lohachit, C., Kobayashi, J., Virakul, P., Kunavongkrit, A., Techakumphu, M., Prateep, P. and limskul, A. 1989. Early Embryonic Development in Thai Swamp Buffalo. Theriogenology. 31 (6) : 1131-1139.

Drost, M., Wright, J.M., Cripe, W.S. and Richter, A.R. 1983. Embryo Transfer in Water Buffalo (Bubalus Bubalis). Theriogenology. 20(5) : 579-584.

Drost, M., Vlahov, K., Alexiev, A., Cripe, W.S., Karaivanov, Ch., Leonards, A.P., Kacheva, D., Polihronov, O., Nicolov, N., Petrov, M. and Dragoev, A. 1988. Successful Nonsurgical Embryo Transfer in Buffaloes (Bubalus bubalis) in Bulgaria. Theriogenology, 30(4): 659-668.

Karaivanov, C., Vlahov, K., Petrov, M., Kacheva, D., Stojanova, M., Alexiev, A., Polihronov, O. and Danev, A. 1987. Studies on preimplantation development of buffalo embryo. Theriogenology. 28(5): 747-753.

Lohachit, C., Chantaraprateep, P., Virakul, P., Kunavongkrit, A. and Buffalo. Technique of Embryo Transfer in Swamp Buffalo. In: Vitro Fertilization and Embryo Transfer. M. Kamonpatana. (ed). Chulalongkorn University, Press. pp. 185-200 (in Thai).

Newcomb. R. 1982. Egg Recovery and Transfer in Cattle. In: Mammalian Egg Transfer. Cyril E. Adams. (ed). CRC Press, Inc, Florida. pp. 81-118.

Sharifuddin, W. and Jainudeen, M.R. 1984. Superovulation and non-surgical collection of ova in the water buffalo (Bubalus bubalis). Proc. 10 th Internat. Cong. Anim. Reprod. and AI. University of Illinois II: 240.

Singh, L.E. 1988. Determining the disease transmission potential of embryos and seman. Proc. 3rd World Congress on sheep and Beef Cattle Breeding. 19-23 June, Paris, Volume 1. pp. 659-672.

Wrathall, A.E. 1987. Disease Transmission by Semen and Embryo Transfer Techniques and Implications for International Trade. State Vet. J. 41(118): 19-30.

Induction of Twin Calving by Transcervical Transfer of an Extra Embryo in Swamp Buffalo

Brief communication :

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INTRODUCTION

The first successful nonsurgical embryo transfer in water buffalo of Jafarabadi breed in U.S.A. was achieved by Drost et al.(1983) and resulted in the birth of a bull calf and a second report came from Bulgaria where Drost et al. (1988) produced four calves in Mediterranean buffalo. Our recent reports on production of embryos using gonadotrophin stimulation (Chantaraprateep et al., 1988) and early embryonic development in Thai swamp buffalo (Chantaraprateep et al., 1989) revealed a good evidence for embryo recovery and transfer in this species. In cattle, embryo transfer does provide a very real alternative in the production of twins (Mapletoff, 1986). Kobayashi et al. (1982) have demonstrated the possibility of induction of twinning in cattle by supplementation of extra embryo after artificial insemination. Furthermore, our previous reports on estrus synchronization in swamp buffalo using prostaglandin F 2 alpha (Chantaraprateep et al., 1982) and norgestomet plus PMSG (Virakul et al., 1988) resulted in twinning of 8.3% (2/14) and 11.8%(3/26)

respectively. The present objective is to achieve twinning by supplementation of extra embryo transfer in mated swamp buffalo cow.

MATERIAL AND METHOD

A swamp buffalo cow aged 7 years old, weighed 482 kg. served as a recipient. When she was in heat, was mated once with a polled bull, 6.5 days afterward she received one embryo of morula stage (figure 1) from a heifer donor collected on the same day. The embryo was transferred nonsurgically into the right horn which was ipsilateral to the corpus luteum. The techniques of embryo collection and transfered in this species have been described in details elsewhere (Kobayashi, 1980; Kobayashi et al., 1981; Chantaraprateep et al., 1987; Chantaraprateep et al., 1989).

RESULTS

The recipient was diagnosed pregnant at 2 mo. after mating and calved a twin bull calves with a gestation length of 329 days. Birth weight of the first and second bull calves were 15 and 25 kg. respectively and the interval of the 2 births was 25 min. Both 2 bull calves resulted from the present trial are healthy and phenotypically different as shown in figures 2 and 3. At the age of 3.5 mo. old they weighed 62 and 71 kg. respectively for the first and second born calves. The bull calves on the right and left were born from natural mating and ET respectively.

DISCUSSION

This study indicated the possibility of production of twinning in swamp buffalo when transferred fresh embryo to the ipsilateral horn of previously mated cow. The bull calf born from the natural mating (right side of figures 2 and 3) is a polled which is corresponded to the mated sire. Both calves were pregnant on the same right horn and the different body size of the sires (680 kg and 400 kg for the ET and natural sires respectively) may influence partly to the birth weight of the 2 calves. So far report on twinning in swamp buffalo has not been documented yet. However, in cattle Rowson et al. (1971) who acknowledged significant differences in twinning rates between ipsilateral and bilateral embryo transfer techniques (50 and 75% respectively). In addition, Anderson et al. (1978) obtained good results of bilateral surgical embryo transfer while Diskin et al. (1987) obtained a twinning rate of 47% when they transferred fresh embryos to the ipsilateral horn. Recently, Suzuki et al. (1989) conducted similar studies in cattle but using two whole or demi-embryos and transferred into ipsilateral horn using transcervical method,their results of twinning rates of 23 to 50%. Jones and Rouse (1920) cited by Johansson et al.(1974) reported results of an analysis of data from beef breeds of the Hereford and Aberdeen Angus herdbooks in the USA. The average twinning incidence was found to be 0.45% for 527,000 calvings in the Hereford and 0.41% for 219,200 calvings in the Aberdeen Angus breed. Genetic selection for twinning in beef cattle has been largely unsuccessful. Mechling and Carter (1964) reported on the twinning frequency in an Aberdeen-Angus herd by selection. Only twin bulls were used as sires in the herd, and the females used for breeding were born as twins or were daughters of twinning

cows. After some 15-20 years average twinning frequency in the herd was 1.71% in 585 calvings. However, it has been estimated that beef production can be increased by 60% through twinning in intensively managed herds.

The present result indicates the possibility of application of biotechnological technique for improving swamp production and enhancing reproduction of superior genetic potential animals. To avoid the constraint of freemartinism due to twinning for pure breeding purposes and with the advent of biotechnological method production of twinning is more appropriate following sexing of embryos.

As the cost of buffalo herds is mostly associated with the maintenance of animals. Thus, buffalo cows production twin calves would be cheaper to maintain than cows producing single calves.

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REFERENCES

Anderson, P., Cupps, T., Drost, M., Horton, M.B. and Wright, R.W. 1987. Induction of twinning in beef heifers by bilateral embryo transfer. J. Anim. Sci. 46 : 449-452.

Chantaraprateep, P., Lohachit, C., Bodhipaksha, P. and Virakul, P. 1982. Prostaglandin F2 alpha for oestrus control of buffaloes. Proc 2nd Internat. World Buffalo Production Congress. Casserta Italy - 29 Sept - 2 Oct. pp. 385-395.

Chantaraprateep, P., Lohachit, C., Virakul, P., Kunavongkrit, A., Prateep, P. and Bodhipaksha, P. 1987. Embryo Transfer in Swamp buffalo. In : In Vitro Fertilization and Embryo Transfer M. Kamonpatana (ed).Chulalongkorn University Press. pp.321-330

Chantaraprateep, P., Kobayashi, G., Techakumphu, M., Virakul, P., Kunavongkrit, A. and Prateep, P. 1988. Ovarian Responses to Gonadotrophin Stimulation in Swamp Buffalo. Buffalo Bulletin. 7(4) : 82-86.

Chantaraprateep, P., Lohachit, C., Kobayashi, G., Virakul, P., Kunavongkrit, A., Techakumphu, M. and Limskul, A. 1989. Early Embryonic Development in Thai Swamp Buffalo. Theriogenology. 31(6) : 1131-1139.

Diskin, M.G., Mc Donagh, T. and Sreeran, J.M. 1987. The experimental induction of twin calving in beef cows by embryo transfer. Theriogenology. 22 : 224 (abstr.)

Drost, M., Wright, J.M., Cripe, W.S. and Richter, A.R. 1983. Embryo Transfer in Water Buffalo (Bubalus Bubalis). Theriogenology. 20(5) : 579-584.

Drost, M., Vlahor, K., Alexiev, A., Crippe, W.S., Karaivanov, C., Leonards, A,P., Kacheva, D., Polihronov, O., Nicolov, N., Petrov, M. and Dragoev, A. 1988. Successful Nonsurgical Embryo Transfer in Buffaloes in Bulgaria. Theriogenology. 30(4) : 659-668.

Jones, S.V.H. and Rouse, J.E. 1920. Cited by Johansson, I., Lindhe, B. and Pirchner, F. 1974. Causes of variation in the frequency of monozygous and dizygous twinning in various breeds of cattle. Hereditas. 78 : 201-234.

Kobayashi, G.1980. A successful cervical bovine embryo transfer. J. Res. Anim. Husb. (Chikusan-No-Kenkyu) 34(10) : 1229-1230.

Kobayashi, G., Sakamoto, K. and King, S.J. 1981. Results of non-surgical cattle embryo recovery. J. Res. Anim. Husb. (Chikusan-No-Kenkyu). 35(6) : 798-800.

Kobayashi, G. Baker, A.A. and Oda, Y. 1982. Supplementation of bovine embryo transfer after artificial insemination. Japanese Society of Anim. Reprod. In Tottori.

Mapletoff, R.J. 1986. Embryo Transfer and Genetic Engineering. In : Current Therapy in Theriogenology. D.A. Morrow (ed). W.B. Saunders Company. Philadelphia. pp. 51-53.

Mechling, E.A. and Carter, R.C. 1964. Selection for twinning in a grade Aberdeen Angus herd. J. Hered. 55 : 73-75.

Rowson, L.E.A., Lawson, R.A.S. and Moor, R.M. 1971. Production of twins in cattle by egg transfer. J. Reprod. Fertil. 25 : 261-268.

Suzuki, T., Sakai, Y., Ishida, T., Matsuda, S., Miura, H. and Itoh, K. 1989. Induction of Twinning in Crossbred Heifers by Ipsilateral Frozen Embryo Transfer. Theriogenology. 31(4) : 917-926.

Virakul, P., Chantaraprateep, P., Lohachit, C., Prateep, P. and Demakan,T. 1988. Synchronization of oestrus in swamp buffalo by using Norgestomet and Norgestomet plus PMSG. Buffalo J. 1 : 95-98.

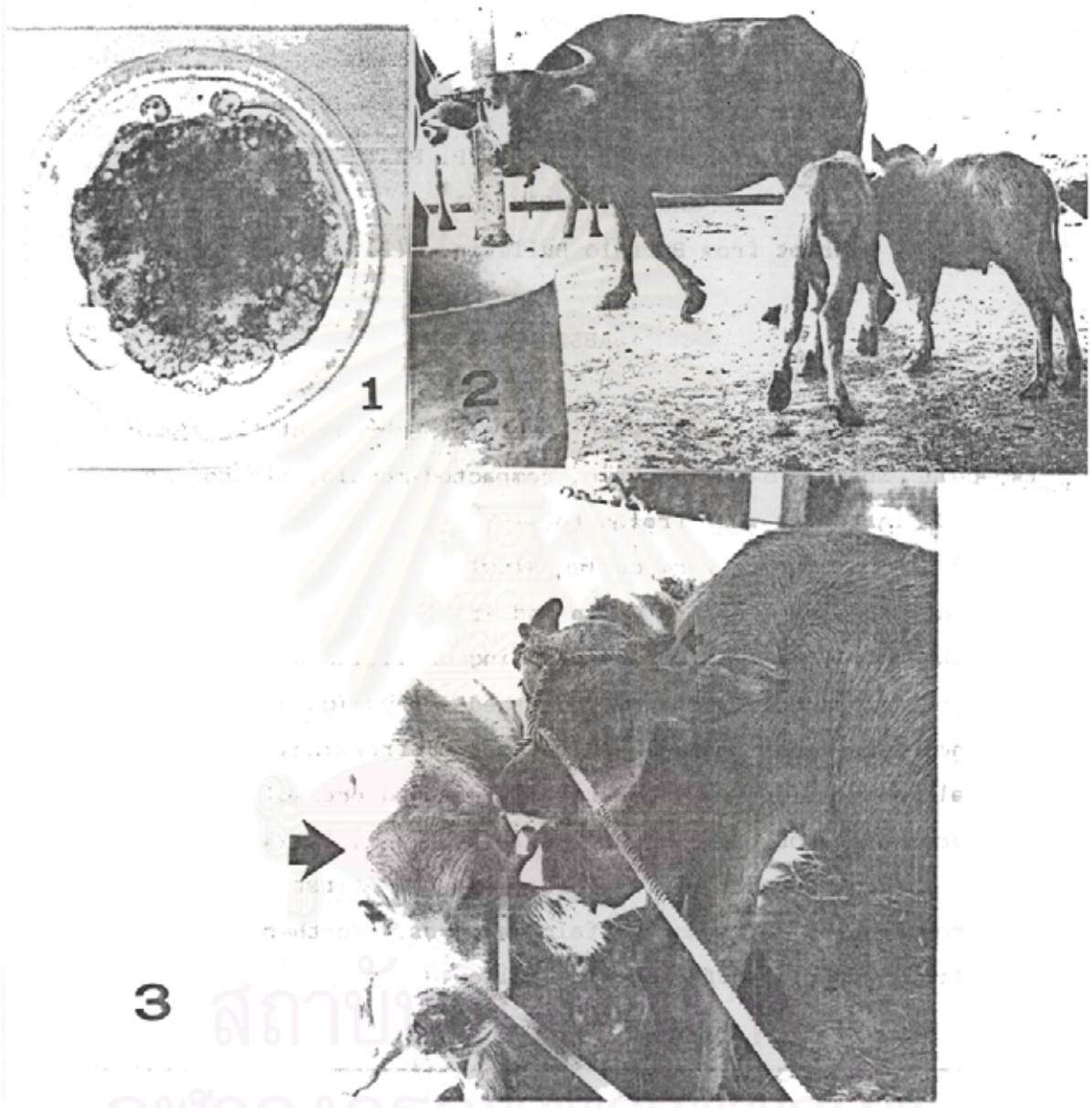

Figure 1 Embryo at morula stage, collected at day 6.5

Figure 2 Two bull calves age 5 days with their dam

Figure 3 Two bull calves aged 1.5 months with different phenotype, one with horn bud (left) and another one (right) with horn.

Preliminary report on cryopreservation of Thai swamp buffalo embryos : manual and automatic methods

By

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ABSTRACT

A total of nine swamp buffalo embryos at the stage of two-cell embryo, 16-cell embryo, compacted morula, blastocyst and hatched blastocyst were frozen to -196°C by manual and automatic methods. The embryos were cooled slowly from room temperature to -7°C at the rate of 1.0°C/min and from -7°C to -15°C or -30°C with the rate 0.3°C/min before plunging in liquid nitrogen. The post thawed morphology showed undamaged embryo (grade-A) could be obtained after both manual and automatic freezing, and also for partial damaged (grade-B) and totally damaged embryos (grade-C). The percentage of each group was 22.2% (2/9), 22.2% (2/9) and 55.6% (5/9) respectively. This was the first report on cryopreservation of swamp buffalo embryos. Further studies on viability after transfer should follow.

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INTRODUCTION

To date, since the first birth of mouse from a frozen embryo (Whittingham,1971), different species of animals,including human beings have been successfully frozen and normal young have been born (Renard, 1982; Mohr et al., 1985). However, no report of cryopreservation of a water buffalo embryo has been published yet. In Thailand,the swamp buffalo was a major population of ruminant which was around 6million heads. The development of some biotechnologies such as embryo transfer has been started since 1981 (Thungtanawatet al.,1981) and recently, the success of two pregnancies was reported by Chantaraprateep et al. (1988a). Associated with embryo transfer, in the form of "embryo bank" will be useful for genetic improvement and for basic reproductive research in this species. This study, therefore, aimed at investigating the freezability of swamp buffalo embryo by manual and automatic methods.

Materials and method

Embryos : A total of 9 embryos at different stages, 1 retarded two-cell embryo (two cells), one 16-cell embryo (16 cells) three compacted morulae (M), 3 blastocysts (B) and one hatched blastocyst (HB) were recovered non surgically by single egg collection (n = 2) or superovulated collection (n = 3). These embryos were collected during day 6 to 7 after natural mating. The methods of collection and of superovulation were described previously by Kobayashi et al. (1981); Lohachit et al.(1986) and Chantaraprateep et al.(1988b). The collected embryos were washed twice in 2 ml of Whittingham's modifications of Dulbecco's buffered saline (PBS, Flow Laboratories, Australia) supplemented with 20 % of fetal bovine serum (FBS, Flow Laboratories,

Australia) and were conserved in this medium before freezing. The interval between collection and freezing was about three hours.

Freezing procedures

Addition of cryoprotectant : Glycerol was used as cryoprotectant. The final concentration was 1.5 molar or 10% of glycerol in the solution of PBS +20% FBS (volume/volume). At room temperature (27°C), the cryoprotectant was added in four steps, starting from 0.25 M, 0.50 M, 1.0 M and 1.5 M for 5, 5, 10 and 10 minutes respectively. The embryo was loaded into the middle part of 0.25 ml French mini-straw in a small volume of freezing medium (1.5 M of glycerol in PBS +20% FBS) separated by two air bubbles. The loading of embryo was similar to that described for cattle (Massip et al., 1978).

Freezing : The freezing was performed by two methods, manual and automatic. A total of nine embryos were frozen. Four embryos (1-2 cells, 1-M, 1-B and 1-HB) and five embryos (1-16 cells, 2M, 2B) were frozen by manual and automatic respectively.

The manual freezing was carried out by using the RPE Embryo Freezer (Elsden & Associated, Inc, Fort Collins, U.S.A.). The loaded straw was put into the freezer apparatus which was placed vertically into the neck of a liquid nitrogen tank. The vapor of liquid nitrogen will cool the freezer progressively. The adjustment of distance between the freezer apparatus and the level of liquid nitrogen helped to monitor the cooling rate. The cooling rate was controlled regularly every two minutes by means of a probe attached to a digital readout thermocouple thermometer. This probe was placed in a reference straw filled with 1.5 molar of freezing medium. Automatic freezing was done using a programmable freezer (Planer, Biomed Kryo series U.S.A.).

In both manual and automatic methods, the cooling rate was around 1.0°C/min decreasing from room temperature (27°) to -7°C. At -7°C crystallization was induced by touching the surface of straw with metallic forceps precooled in liquid nitrogen. The embryos were then slowly frozen from -7°C to -15°C or -30°C at the rate of 0.3°C/min before plunging into liquid nitrogen. This freezing curve followed that described for bovine embryos (Renard et al., 1982). The terminal temperature for manual freezing was -30°C with no equilibration time before freezing. In automatic freezing, the two terminal temperatures were -15°C and -30°C followed by a 10 minute equilibration thime before freezing.

Thawing and dilution of cryoprotectant : The embryos were thawed by removing the straw from liquid nitrogen and directly plunging it into a water bath at 37°C. The cryoprotectant was later removed by four steps in reverse of addition which was 1.5 M, 1.0 M, 0.5 M and 0.25 M for 10 min each. After complete dilution the embryos were washed five times in small drops of PBS +20 % FBS and kept in this medium for further investigation. One morula from manual freezing was cultivated for 24 hours in Ham F 10 medium in order to allow it to restore its structure or to develop into a blastocyst, and thus demonstrate its viability in vitro.

Viability of embryo : The viability of frozen-thawed embryos was done determinated by embryonic investigation under stereomicroscope at 10 to40 magnifications. The embryos were classified into three grades :

Grade A : embryo classified as "undamaged" which had intact zona pellucida and intact blastomeres. For hatched blastocyst the presence of inner cell mass and trophoblast was judged as undamaged.

Grade B : embryo classified as "partial damage" were intact or had small damage in zona pellucida and with or without damage in the blastomeres.

Grade C : embryo clasified as "totally damageed" had discarded blastomeres or zona pellucida or other embryonic cells of blastocyst.

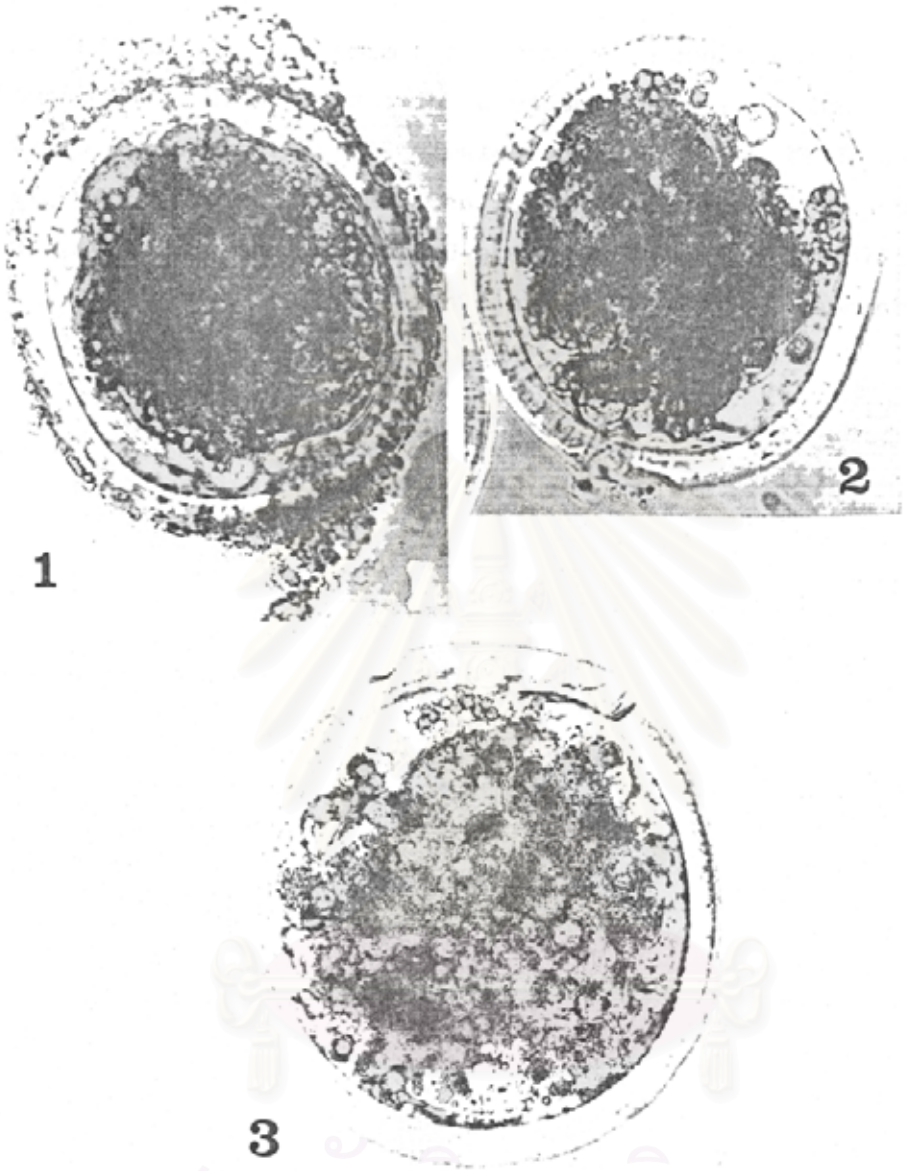
RESULT

The details of the morphology of the post thawed swamp buffalo embryos are presented in Table 1. With manual freezing, one normal hatched blastocyst with intact inner cell mass and trophoblast and one partial damaged morula with small cracking of zona pellucida were obtained. The blastocyst was judged a grade-A embryo. The morula, whose blastomeres were partially destroyed during freezing was graded a grade-B embryo. The morphology compared with an unfrozen embryo is shown in Fig. 1 and 2. The confinement of blastomeres was clearer after culture for 24 hours (Fig. 3). The other two embryos, two-cell stage and blastocyst were severely damaged. The two-cell embryo had an intact zona pellucida with total damage of blastomeres while the blastocyst was totally destroyed.

With automatic freezing, one grade-A and one grade-B morulae were obtained when freezing was terminated at -30^{o}C before plunging in liquid nitrogen. The morphology of frozen-thawed grade-A embryo was similar to an unfrozen one (Fig.4, 6 and 7). The grade-B embryo had cracked zona pellucida and 20% of destroyed blastomeres when compared to unfrozen ones (Fig.5 and 6).

The freezing to -15^{o}C gave one grade-B blastocyst with 50% of blastomeres were destroyed. Two other embryos, one 16-cell stage and one blastocyst frozen to -15^{o}C were mostly

damaged. No appearance of inner cell mass and trophoblast was found in blastocyst. The embryos prior to freezing post thawed were shown in Fig.7 and 8. Conclusively 22%(2/9) of frozen embryos presented morphologically normal as grade-A embryo, 33% (3/9) were partially damaged as grade-B embryo and 45% (4/9) were totally damaged as grade-C embryo (Table 2).

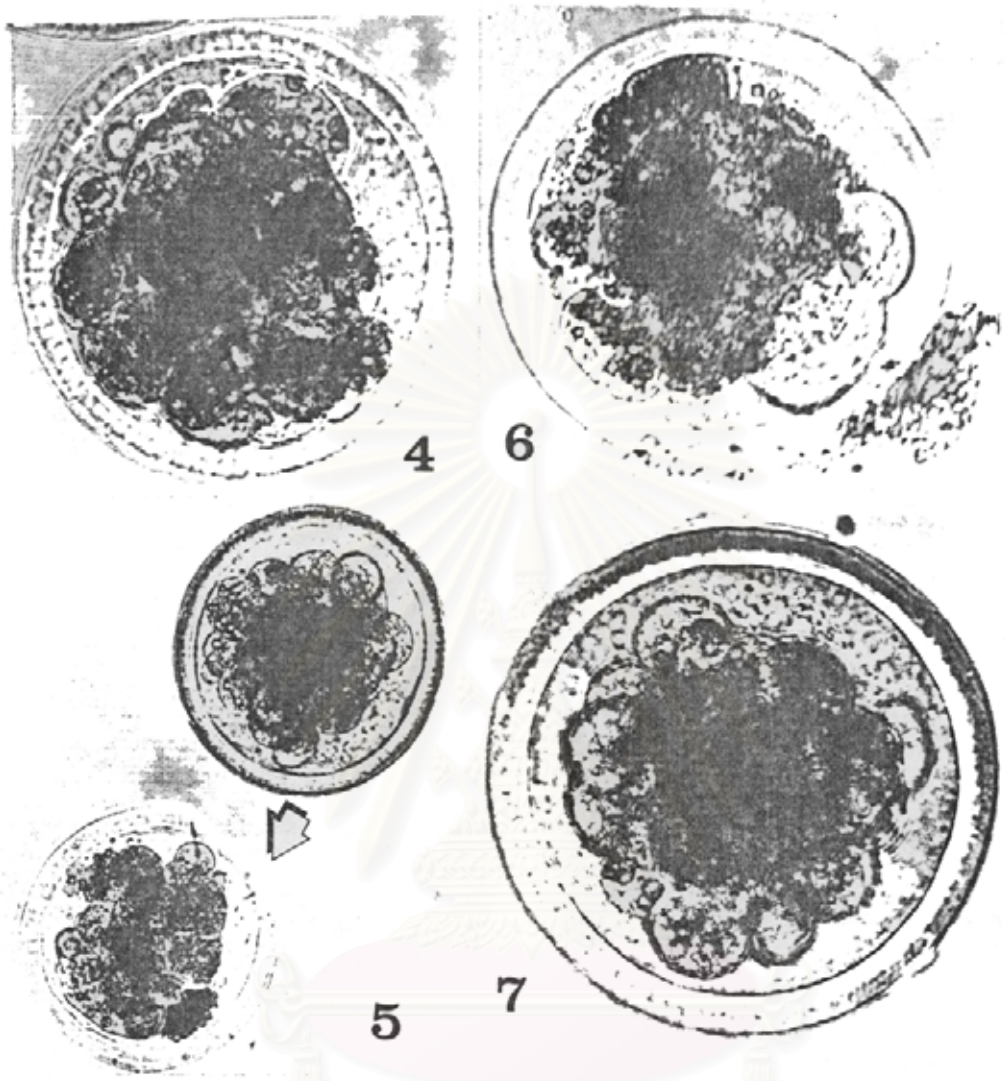


Figures 1, 2 and 3 Embryos treated by manual freezing

Fig 1 Embryo at stage of compacted morula before freezing (x 400)

Fig 2 Grade-B morula with small opening of zona pellucida and some destroyed blastomeres (x 400)

Fig 3 Frozen-thawed embryo (fig 2) after culture for 24 hrs. the confinement of blastomeres was evident and some degenerated cells were on the contour of embryo (x 430)



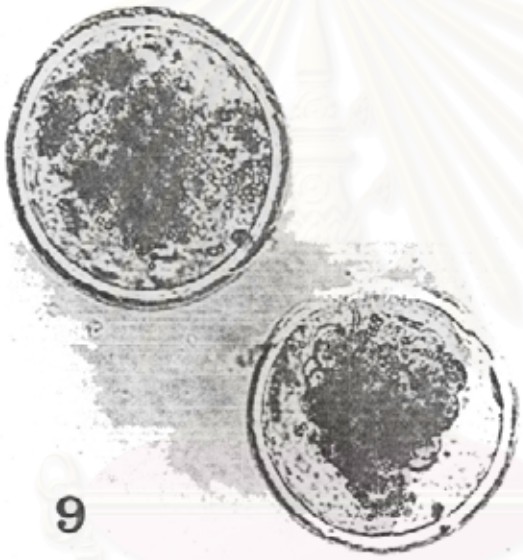
Figures 4, 5, 6 and 7 Embryos treated by automatic freezing to -30°C before plunging into liquid nitrogen

Fig 4 Embryos at morula stage before freezing (x 400)

Fig 5 Embryos at morula stage before freezing (x 400)

Fig 6 Grade-A embryo (upper from fig 4) with intact zona pellucida and intact blastomeres and Grade-B embryo (lower) with cracked zona pellucida (arrow) and about 20% destryoed blastomers. (x 200)

Fig 7 Grade-A morula (fig 4) after freezing (x 400)



Figures 8 and 9 Embryos treated by automatic freezing to -15°C before plunging into liquid nitrogen

Fig 8 Two blastocysts before freezing (x 200)

Fig 9 One Grade-C embryo (left) with intact zona pellucida and total damaged cells and one Grade-C embryo (right) with cracked zona pellucida and 50% degenerated blastomeres (x 200)

Table 1 Morphology of swamp buffalo embryos at different stages after freezing by manual and automatic methods.

Stage	Morphology after freezing-thawing
Manual	
two-cell embryo	cracked zona pellucida with destroyed blastomeres Grade C
Morula	intact zona pellucida with some blastomeres destroyed Grade B
Blastocyst	embryonic cells totally destroyed, no zona pellucida recovered Grade C
Hatched blastocyst	intact inner cell mass and trophoblast embryo was contracted during dilution of cryoprotectant Grade A
Automatic	
Morula*	normal embryo with intact zona pellucida and blastomeres Grade A
Morula*	partial damage embryo with cracked zona pellucida and some blastomeres (=20%) destroyed Grade B
16-cell embryo	totally destroyed Grade C
Blastocyst**	intact zona pellucida but the embryonic cells totally destroyed Grade C
Blastocyst**	cracked zona pellucida with about 50% of blastomeres destroyed Grade B

* = temperature before plunging in liquid nitrogen was -30°C.

** = temperature before plunging in liquid nitrogen was -15°C.

Table 2 Classification of swamp buffalo embryos after freezing by manual and automatic methods.

Method	No. of frozen embryos	Grade		
		A	B	C
Manual	4	1	-	3
Automatic	5	1	2	2
Total	9	2	2	5
		22.2%	22.2%	55.6%

DISCUSSION

The present findings of post thawed morphology of swamp buffalo embryos revealed that they could resist being frozen to -196°C by the two techniques used. Both manual and automatic freezing gave normal embryos with intact zona pellucida and intact embryonic cells. The advantage of manual freezing is that it is simple, inexpensive and portable. Frank et al. (1986) reported no difference in embryo survival between simplified embryo freezing equipment and programmable freezer. The percentage of post thawed embryos and Their development in culture medium for 12 and 24 hours were comparable between the two methods. However, in spite of its simplicity, the standardized protocol of freezing as described for bovine embryo (Renard et al., 1982) were not strictly followed. No equilibration time at -7°C can be allowed due to difficulty in controlling the temperature because of the

distance between the freezing apparatus and the level of liquid nitrogen in the tank. Neither was an equilibration time at -30°C allowed before plunging in liquid nitrogen for the same reason. Leibo (1981) suggested that the equilibration time influenced the survival rate. This equilibration time would allow time for the embryos to undergo extensive dehydration. Normally the embryo should be equilibrated for 5 to 10 minutes at seeding temperature and about 10 minutes at the last temperature before liquid nitrogen. However, without the equilibration time at these two temperatures, one undamaged grade A hatched blastocyst and one partial damaged morula could be obtained after manual freezing including intact zona pellucida of two-cell embryo which used for testing the efficiency of manual freezing.

In automatic freezing, the post-thawed morphology of embryos frozen at -30°C seemed to be better than those frozen at -15°C. Only one embryo with 50 % of undamaged blastomeres was obtained when the slow cooling was terminated to -15°C while one grade-A and one grade-B embryos were obtained after termination at -30°C. This observation could be explained by insufficient dehydration at -15°C as compared with -30°C. The amount of water still in the embryo would lead to the formation of intracellular ice, which was one of the major causes of cell destruction during thawing (Leibo, 1977). Massip et al. (1987) showed that in the presence of glycerol alone, slow cooling to -35°C was necessary before abrupt cooling in liquid nitrogen. A higher pregnancy rate was obtained with cow embryos when the last temperature before liquid nitrogen was -35°C than when it was -25°C. So, as with cow embryos, it would be reasonable to cool the swamp buffalo embryos to -30°C before plunging in liquid nitrogen.

The limited number of frozen embryos for this study was due to low response to superovulation treatment reported earlier in Thai swamp buffalo by Chantaraprateep et al. (1988b) and to the low rate of successful recovery, which was around 56 % (Chantaraprateep et al., 1989). Nevertheless, this preliminary study gives some information on the feasibility of cryopreservation of swamp buffalo embryos. Further study on viability by transfer to a suitable recipient would help confirming the final viability.

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REFERENCES

Chantaraprateep, P., Kobayashi, G., Lohachit, C., Virakul, P., Kunavongkrit, A., Techakumphu, M. and Prateep, P. 1988a. Embryos Transfer in Thai Swamp Buffalo (Bubalus bubalis). 15th Annual Veterinary Science Conferences, Ambassador Hotel, Bangkok. 2-4 November. (Abstracts). 42.

Chantaraprateep, P., Lohachit, C., Virakul, P., Kobayashi, G., Techakumphu, M., Kunavongkrit, A. and Prateep, P. 1988b. Ovarian responses to gonadotrophin stimulation in swamp buffalo. Buffalo Bulletin. 7(4) : 82-86.

Chantaraprateep, P., Lohachit, C., Techakumphu, M., Kobayashi, G., Virakul, P., Kunavongkrit, A., Prateep, P. and Limskul, A. 1989. Early embryonic development in Thai swamp buffalo (Bubalus bubalis). Theriogenology. 31 (6) : 1131-1139.

Frank, G.C., Coley, S.L., Betterbed, B. and Page, R.D. 1986. The effect of freezer type, cryoprotectant, and processing methods on viability of frozen embryos. Theriogenology. 26 : 135-144.

Kobayashi, G., Sakamoto, K. and King, S.J. 1981. Results of non-surgical cattle embryo recovery. J. Res. Ani. Hus. 35 : 799-800.

Leibo, S.P. 1977. Fundamental cryobiology of mouse ova and embryos. In : The freezing of mammalian embryos. Cifa Foundation Symposim (new series). Elsevier Experta Medica. North Holland, Amsterdam. pp. 69-92.

Leibo, S.P. 1981. Introduction to embryo freezing. In : Frozen storage of laboratory animals. G.H. Zeilmaker. (ed). New York. pp. 1-19.

Lohachit, C., Chantaraprateep, P., Virakul, P., Kunavongkrit, A. and Bodhipaksha, P. 1988. Technic of embryo transfer in swamp buffalo. In : In Vitro Fertilization and Embryo Transfer. M. Kamonpatana. (ed). Chulalongkorn University Press. pp. 135-200. (in Thai).

Massip, A., Jacguelot, B., Ectors, F., De Coster, R., D'ieteren, G., Hanzen, C. and Derivaux, J. 1978. Congelation d'embryons suivie de transfert chez les bovins : premiere utilisation des paillettes. Ann. Med. Vet. 122 : 515-526.

Massip, A., Van Der Zwalmen, P. and Ectors, F. 1987. Recent progress in cryopreservation of cattle embryos. Theriogenology. 27 : 69-79.

Mohr, L.R., Trounson, A. and Freemann, L. 1985. Deep-freezing and transfer of human embryos. J.in vitro fertil embryo transf. 2 : 1-10.

Renard, J.P. 1982. La conservation des embryos. chez les mammiferes. In : Transfert d'embryons chez les mammiferes. 2 eme Congres. International Annecy. Collection Foundation MARCEL MERIEUX, Lyon, France. p. 285-306.

Renard, J.P., Heyman, Y. and Ozil, J.P. 1982. Congelation de l'embryon bovin : une nouvelle methode de decongelation pour le transfert cervical d'embryons conditionnes une seule fois en paillettes. Ann. Med. Vet. 126 : 23-32.

Thungtanawat, P., Aroonprasert, D., Kietsommat, S., Lohachit, C., Chantaraprateep, P. and Bodhipaksha, P. 1981. Induction of superovulation in swamp buffaloes. Senior Project. Fac. of Vet. Sci., Chulalongkorn University. 16 pages (in Thai).

Whittingham, D.G. 1971. Survival of mouse embryos after freezing and thawing. Nature (Lond) 233 : 125-126.

DISCUSSION

Resposes of swamp buffalo ovaries to stimulation by exogenous gonadotrophin both FSH and PMSG were low as indicated by low rate of embryo recovery (average embryos). These findings were in accordance with those previous reports (Nittayawattana et al., 1982 : Drost et al., 1983; Sharifuddin and Jainudeen, 1984). The reasons of such poor responses due partly to genetic make up, nutrition and state of the estrous cycle in connection to the regimen of gonadotrophin applied. Different stages of early embryonic development on Days. 5.5, 6.0, 6.5, 7.0 and 7.5 were identified as the 16-cell stage, compact morula, blastocyst, hatched blastocyst and hatched expanding blastocyst, respectively. These findings serve as a milestone for further application of biotechnological techniques particularly embryo transfer in this species. It was also shown that a higher recovery rate and normal embryos were obtained with single embryo collection after natural estrus than after induced estrus or superovulation. Three attempts of non surgical fresh embryo transfer were performed, the first 2 were on Days 7.5 and 7.0 and the embryos were hatched blastocept, it was found nonpregnant. The last attempt was carried out on Day 6.5 from superovulated donor 2 morulae were recovered and transfered to 2 recepients and they were found pregnant and calved a twin bull calves and a bull calf in March 1989 with gestation length of 329 and 332 days respectively. The success due to information gathered from the studies of early embryonic development. In view of more acceptable for further impliment of biotechnology in this species freezing of embryos is compulsory. Our preliminary investigation on cryopreservation of swamp buffalo embryos using manual compared to automatic methods. A total of 9 embryos at different stages of development ; 2 cell embryo,

16 - cell, compacted morula, blastocyst and hatched blastocyst. They were frozen at -196^{o}C using manual and automatic methods. The post thawed morphology of embryos were similar for both methods. The normal grade-A embryo, partial damaged grade-B and total damaged grade-C embryos were 22.2 % (2/9), 22.2 % (2/9) and 55.6 % (5/9) respectively.

In conclusion, the findings from these 4 studies indicated the possibilities of production of swamp buffalo embryos through stimulation of ovarian responses by gonadotrophin either FSH or PMSG. However low ovarian responses would be expected. Such phenomenon can be overcome by genetic selection together with proper management in terms of feed and feeding and estrus cycle. Embryo transfer in swamp buffalo can be carried out more sucessfully if it's performed with intact zona pallucida embryo or prior to Day 7 embryo. Freezing of embryo should be encouraged for preservation of genetic resource and future investigation.