บทความปริทัศน์

Study on Embryonic Development and Embryo Freezing of Swamp Buffalo *(Bubalus bubalis)* in Thailand

การศึกษาการพัฒนาของตัวอ่อนและการแช่แข็งตัวอ่อนของกระบือปลัก (Bubalus bubalis) ในประเทศไทย

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บทคัดย่อ : **มงคล เตชะกำพุ. 2533. การศึกษาการพัฒนาของตัวอ่อนและการแช่แข็งตัวอ่อนของกระบือปลัก *(Bubalus bubalis)* ในประเทศไทย. วารสารวิจัยวิทยาศาสตร์การแพทย์ 4 (1) : 65-71**

การพัฒนาของตัวอ่อนและการแช่แข็งตัวอ่อนในกระบือปลัก ยังไม่มีผู้ใดศึกษามาก่อน จากการศึกษาของภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย พบว่า เวลาในการพัฒนาของตัวอ่อนของกระบือปลักแตกต่างจากรายงานที่พบในกระบือมูร่าห์และในโค นอกจากนี้ยังพบว่าตัวอ่อนกระบือปลักสามารถนำมาแช่เก็บไว้ได้ในไนโตรเจนเหลว เช่นเดียวกับสัตว์เลี้ยงลูกด้วยนมชนิดอื่น ๆ บทความนี้ได้กล่าวถึง วิธีการเก็บตัวอ่อน การตรวจหาและประเมินคุณภาพของตัวอ่อน การพัฒนาของตัวอ่อน และวิธีการแช่แข็งตัวอ่อนของกระบือปลัก

คำสำคัญ : *กระบือปลัก (Bubalus bubalis) ; ตัวอ่อน ; ทำให้เยือกแข็ง*

Abstract : **Mongkol Techakumphu. 1990. Study on Embryonic Development and Embryo Freezing of Swamp Buffalo *(Bubalus bubalis)* in Thailand. Thai J Hlth Resch 4 (1) : 65-71**

Development of swamp buffalo embryo and cryopreservation of embryo in this species have never been studied. From our work (OGR staff, Chulalongkorn University), we found that the chronology of embryonic development in swamp buffalo was different from the report in murrah buffalo and cattle. Other ways we found also that swamp buffalo embryo can be frozen and kept in liquid nitrogen as reported in other mammals. This article present the details of methods of embryo collection, searching and evaluation of embryo, chronology of embryonic development and freezing method for embryo in swamp buffalo.

Key words : *Buffalo, swamp (Bubalus bubalis) ; Embryo ; Freezing*

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Introduction

Development of swamp buffalo embryo in early stage prior to implantation occurs in fallopian tube and uterus like other mammals but its chronology remains unknown. Only few studies were carried out in Murrah type (Drost and Elsden, 1985; Karaivanov *et al.*, 1987). This type of buffalo is anatomically and physiologically different from Swamp type (Lohachit, 1987). The study on early embryo development in this species is prerequisite to application of biotechnology as of embryo transfer. In connection to embryo transfer development of deep freezing of embryo to -196° C will be useful for conserving high genetic potential animal, for the purpose of genetic improvement in this species.

Sources of embryos

Swamp buffalo embryo can be collected surgically or non surgically like the method described in cattle (Lohachit *et al.*, 1987). Briefly the donor is transquilized with xylazine HCl (20 mg/IM) and epidural anesthesized with 3 ml xylocaine. Embryos are collected with a two-way Foley catheter (French gauze 14 or 16) by flushing each uterine horn with 500 ml of isotonic phosphate buffer solution (PBS, Whittingham, 1971). The flushing medium is recovered into a 500 ml graduated cylinder.

The collection of embryo can be done by single egg collection after natural ovulation or by super-ovulated collection after gonadotropic stimulation during luteal phase. The method of superovulation was described recently by Chantaraprateep *et al.*, (1988a).

Searching of embryo

After collection, the cylinder will be placed for 30 min at room temperature. All but the bottom 100 ml is siphoned off drop to drop into a 500 ml glass bottle. The 100 ml is then swirled around and poured into a sterile petri dish. The embryo is subsequently searched under stereomicroscope at 10×to 40 × magnification and isolated into a small petri dish with 1 ml of holding medium. The medium should be the same type as used for flushing or the culture medium which is gazed with 5% CO_2. Usually the holding medium is a phosphate buffer saline supplemented with 10 to 20% of fetal bovine serum. The medium should be fresh and sterilized by filtration through a 22 μm or 45 μm milipore filter. The collected embryos are washed twice in the holding medium and stored for further manipulation.

The storage of embryo during recovery from the donor and transfer to recipients is important for further development. Several factors which can affect the viability of the embryo such as : time of searching, variation of temperature, high or low temperature, sunlight or UV light, pH, osmolality, sterility, kinds of medium, toxicity of medium etc.

The suitable condition for embryo storage is conserving in an enriched nutrient solution with pH 7.2 to 7.6 and its osmolality of 270 to 310 m.osm. at 37° C. But it is better to keep embryos at room temperature in order to avoid the variation of temperature in case that the transfer or other manipulations will be done in the same day.

Evaluation of embryos

The swamp embryos are evaluated basing on various criterias : shape of embryo, presence and form of zona pellucida, size of embryo, number of cells (blastomeres), equality and colors of cells and knowledge of the age of the embryo.

The embryos will be graded in four categories :

Grade A (good) embryo : referred to embryo with spherical form, zona pellucida and blastomeres are regular with homogenic colors. In case of blastocyst the presence of inner cell mass, trophoblast and blastocoele is necessary.

Grade B (fair) embryo : referred to embryo with spherical form, regular zona and irregular blastomeres, some vesicles or dead cells may present on the surface of blastomeres.

Grade C (poor) embryo : referred to embryo with non-spherical form such as oval or flat as a deform embryo. Some embryos have a small opening to a crack zona pellucida. The blastomeres are divided irregularly, some scattered. In case of blastocyst, the area of inner cell mass, and trophoblast is not evident.

Grade D (very poor) embryo : referred to unfertilized ova and degenerated embryo.

For the quality of swamp buffalo embryos, the study of Chantaraprateep *et al.* (1988b) shows that higher percentage of normal embryo (grade A + grade B) could be obtained after single egg collection than superovulated stimulation, 77% vs 37.5%. Table 1. Abnormal eggs (grade D) was 37.5% in superovulated group compared to 0% in single egg collection group.

Table 1 *Quality of swamp buffalo embryo after single egg collection and superovulation.*

Method	No. of collection	No. recovered eggs	Quality of embryo		
			Normal	Abnormal	Unfertilized
Single egg collection	22	13	10 (77%)	—	3 (23%)
Superovulation	11	16	6 (37.5%)	6 (37.5%)	4 (25%)

(Modified from Chantaraprateep ***et al.****, 1989)*

The percentage of unfertilized eggs is rather equal in the two groups 23% and 25% respectively. From this study, it is suggested that the superovulation in swamp buffalo might influence on the development of embryo due to the alteration of the *in vivo* level of sex homones.

Chronology of embryonic development

From a total of 33 collections as presented in Table 1 at Day (D) 5.5, 6.0, 6.5, 7.0, 7.5 after onset of estrus. The finding shows that the differnt stages of embryonic development on these days are 16-cell stage, compact morula, blastocyst, hatched blastocyst and hatched expanding blastocyst respectively (Chantaraprateep *et al.*, 1989). Morphologically swamp buffalo embryos are similar to bovine embryo in terms of size and color.

The finding from this study indicates that the compaction of swamp buffalo embryo occurs about D 6.0 (Fig 1) and the transition from morula to early blastocyst occurs after D 6.0 (Fig 2). This chronology of development to the early blastocyst stage is similar to that of cattle. A difference in rate of embryo development between the swamp buffalo and cattle is observed from D 7.0 to D 7.5. Hatching of embryos from the zona pellucida in swamp buffalo seems to occur earlier than in cattle. Hatched blastocysts are found on D 7.0 to 7.5 (Fig 3) while they are found on D 8.5 to 10 in cattle. The comparative chronology of development between swamp buffalo and cattle is resumed in Table 2.

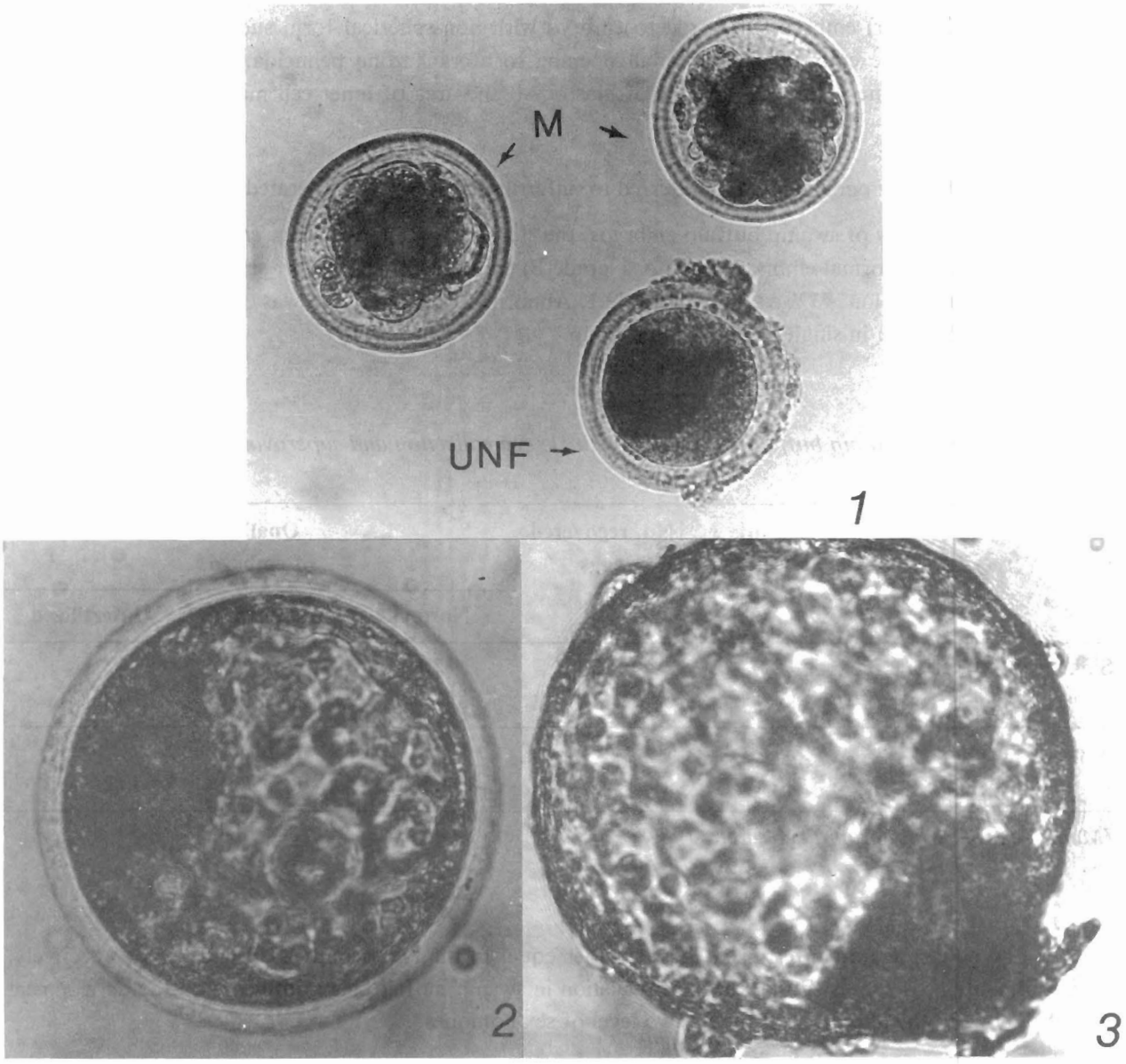

Fig. 1 : Embryo at morula (M) stage and unfertilized egg (UNF) collected at Day 6.0 after superovulation (X 200).

Fig. 2 : Embryo at blastocyst stage collected at Day 6.5 (X 400).

Fig. 3 : Embryo at hatched blastocyst collected at Day 7.5 (X 400).

Table 2 *Comparative chronology of early embryonic development between swamp buffalo and cattle*

Days after onset of estrus	Swamp buffalo[1]	Cattle[2]
5.5	8-16 cells	16-32 cells
6.0	Morula Early blastocyst	Morula
6.5	Blastocyst	—
7.0	Hatched blastocyst	Early blastocyst
7.5	Hatched expanding blastocyst	—
8-9	—	Blastocyst
10	—	Hatched blastocyst
11	—	Hatched expanding blastocyst

1) Chantaraprateep ***et al.,*** *1989.*
2) INRA, 1980.

Our observation is in accordance with Drost and Elsden (1985) that the buffalo embryo developped about 24 to 36 hr. faster than cattle on corresponding days. However, our results are different from the report of Karaivanov *et al.* (1988) in Murrah buffalo as they reported that the hatched blastocyst was found on D 5.0. A comparative study of embryonic development between two species, Murrah and Swamp type should be conducted to explain this difference.

The outcome of this study is useful for embryo collection in this species which is an important key of success in embryo transfer in swamp buffalo which was firstly reported by our team (Chantaraprateep *et al.*, 1988b)

Freezing of embryos

The development of embryo freezing techniques was reported around 20 years ago in differents species including human. The usefulness of this techniques are :-

1) To conserve high genetic valuable animal.
2) To associate with embryo transfer (ET) for local breeding improvement.
3) To transport embryos.
4) To economize the expenses in preparing a large number of recipients in ET program due to variation of superovulation responses.
5) To export or to import lines or breeds with the minimum of risk of introducing diseases.
6) To facilitate the preservation and multiplication of endangered species.

Preliminary study was conducted in swamp buffalo in 1989 by Techakumphu *et al.* The method of freezing is followed as described by Renard *et al.* (1982). The embryo was frozen in 0.25 ml french straw with a slow rate of freezing, 0.1-0.3°C/min to -30 to -35°C and plunging directly in liquid nitrogen (-196°C). The induction of crystallization is done at -6°C to -8°C. The thawing is carried out by putting the embryo from -196°C to +37°C in water bath for 1/2 to 1 min. The concentration of morphology of embryo is observed immediatly after thawing. The glycerol at 1.5 M is used as cryoprotectant by stepwise addition and dilution. We compared the two methods of freezing : automatic programmable and manual

methods (Techakumphu *et al.*, 1989). The first results showed that the swamp buffalo could be frozen as others species. The post-thawed embryos gave a normal embryo either by automatic or manual methods (Fig 4A, 4B). The results are resumed in Table 3.

Table 3 *Post-thawed morphology of swamp buffalo embryos after freezing to -196°C by automatic or manual methods.*

Method	No. of frozen embryo	Post-thawed embryo		
		Normal	Partial damage	Total damage
Manual	4	1	1	2
Automatic	5	1	2	2
Total	9	2 (22%)	3 (33%)	4 (45%)

From Techakumphu ***et al.****, 1989.*

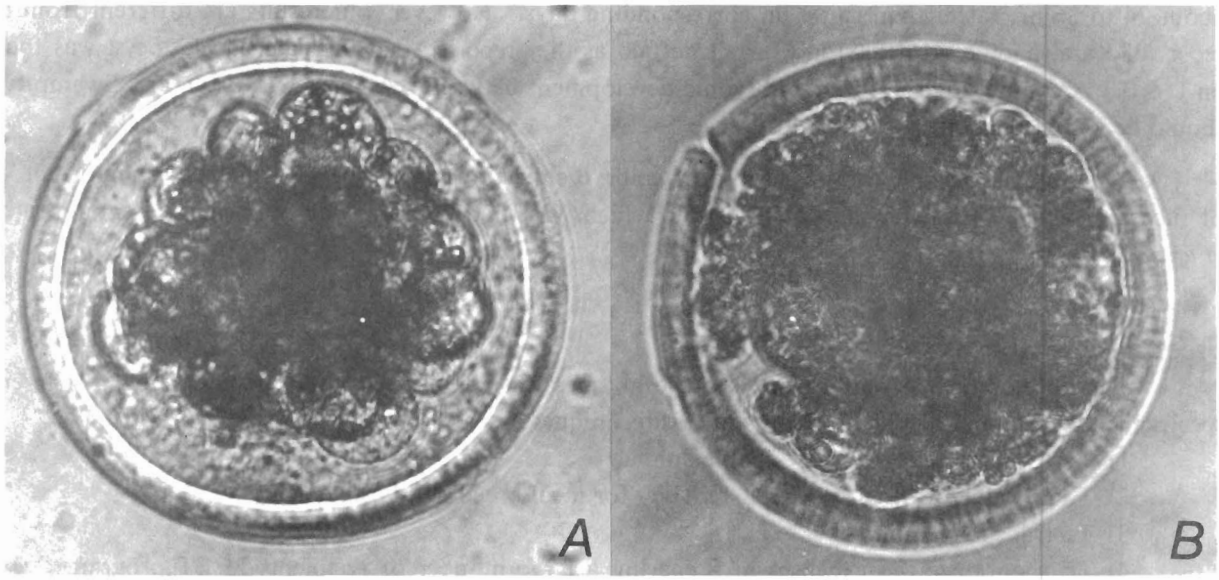

Figure 4 **Post-thawed morphology of swamp buffalo embryo after automatic freezing (A) and after manual freezing (B) (X 400)**

Due to low response of superovulation in this species (Chantaraprateep *et al.*, 1988a) only 9 embryos could be used for freezing study. Further investigation with a large number of embryos should be carried out in order to acquire the appropriate techniques of freezing such as cooling and thawing rates, type and concentration of cryoprotectants etc. Furthermore, the viability of frozen-thawed embryos should be confirmed by transfer to appropriate recipients.

REFERENCES

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

Chantaraprateep P, Kobayashi G, Lohachit C, Virakul P, Kunavongkrit A, Techakumphu M, and Prateep P. 1988b. Embryos transfer in Thai swamp buffalo *(Bubalus bubalis)*. 15th Annual Veterinary Science Conferences, 2-4 November. Indra Hotel, Bangkok (Abstracts) P 42.

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

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

INRA. 1980. Blastographie du développement précoce de l' embryon bovin superovulé. Paris, France.

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. 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.

Lohachit C, Chantaraprateep P, Virakul P, Kunavongkrit A, and Bodhipaksa 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).

Renard J P, Heyman Y, and Ozil J P. 1982. Congélation de l' embryon bovin une nouvelle method de décongélation pour le transfert cervical d'embryons conditonnés une seule fois en paillettes. Ann Med Vet 126 : 23-22.

Techakumphu M, Lohachit C, Chantaraprateep P, Prateep P, and Kobayashi G. 1989. Preliminary report on cryopreservation of Thai swamp buffalo embryos : manual and automatic methods. Buffalo Bulletin 8(2) : 29-36.

Whittingham D G. 1971. Culture of mouse ova. J Reprod Fert (Suppl) 14 : 7-21.