***In Vitro* Fertilization of Follicular Oocytes from the Swamp Buffalo (*Bubalus bubalis*) and Kedah-Kelantan Cattle (*Bos indicus*) in Malaysia**

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Embryo transfer techniques (ET) have become a common tool for the improvement and breeding of *Bos taurus*. In eastern Asia, water buffalo (*Bubalus bubalis*) and zebu (*Bos indicus*) are the most important domestic ruminants as a source of milk, meat and draft power. However, embryo production in the water buffalo from gonadotropin-treated donors followed by nonsurgical uterine flushing has been disappointing [11]. The physiology and endocrinology of zebu are not identical to those of *Bos taurus* [5], and the results of ET in zebu are generally inconsistent with that in *Bos taurus* [22]. In particular, superovulatory response in zebu is poor as compared with that in *Bos taurus* [6]. The production of bovine embryos *in vitro* from oocytes recovered from the abattoir has developed, and commercial ventures for *in vitro* production of *Bos taurus* embryos have been established in Ireland, Scotland and Japan [9]. Thus, it seems that an alternate method for embryo production in the water buffalo and zebu is *in vitro* fertilization (IVF) of *in vitro* matured oocytes (IVM). IVF in the river buffalo was recently reported by several Indian groups [16, 18, 19, 21], but to our knowledge there has been no literature in the swamp buffalo and zebu.

The objective of this preliminary study was to evaluate the utilization of IVM-IVF procedure that is applied in *Bos taurus*, for the swamp buffalo and Kedah-Kelantan (K-K) cattle which are the most common indigenous species in Malaysia. In this study, we have used prepubertal calves of buffalo and K-K cattle as oocyte donors, because oocytes of gonadotropin-treated prepubertal *Bos taurus* has been successfully matured and fertilized *in vitro* [1, 12]. Prepubertal calves possess large number of growing and vesicular follicles which can be stimulated by gonadotropin-treatment [10], whereas ovarian follicular population in the swamp buffalo is only 20% of that of *Bos taurus* [15]. The ability of producing embryos from oocytes recovered from prepubertal calves also offers the potential for markedly reducing the generation interval, thereby substantially accelerating the rate of genetic gain that can be achieved through embryo transfer.

For oocyte donors, 4 adult (10–15 years old) and 3 prepubertal (2–3 months) swamp buffaloes and 3 prepubertal (2–3 months) K-K cattle were slaughtered. Both buffalo and K-K calves received an intramuscular injection of 1,500 IU PMSG 3 days before slaughter. Cumulus-

oocyte complexes (COCs) were recovered from antral follicles, 1–13 mm in diameter, by aspirating the follicular fluid using an 18-gauge needle attached to a 10 ml plastic syringe or by puncturing isolated follicles. *In vitro* maturation of oocytes was accomplished in HEPES-buffered TCM199 (Gibco Laboratory, Grand Island, N.Y., U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 0.02 U/ml FSH (from porcine pituitary, Sigma Chemical Co., St. Louis, MO, U.S.A.), 1 µg/ml estradiol-17β (Sigma), 0.2 mM sodium pyruvate, 100 U/ml penicillin G and 50 µg/ml streptomycin sulfate. Collected COCs were washed four times through the maturation medium, before placing them in a 100 µl-droplet of the same medium under paraffin oil. They were cultured for 24 hr at 39°C in humidified air with 5% CO₂.

Fresh ejaculated semen was collected from one K-K and one swamp buffalo bull by an artificial vagina. The semen was diluted 10-fold with an isotonic defined medium (DM) [2] supplemented with 5 mg/ml fatty acid-free bovine serum albumin (BSA, Sigma), and washed three times by centrifugation at 500 × g for 5 min. A 50 µl of fertilization droplet was prepared by adding a 25 µl aliquot of sperm suspension to 25 µl of the DM containing 10–50 µg/ml heparin (Sigma) and 2.5–5 mM theophylline (Sigma) to give a final sperm concentration of 2.5–5 × 10⁶ cells/ml. After washing once with DM, the COCs were put into the fertilization droplets (4–5 COCs per droplet) and incubated for about 24 hr at 39°C in humidified air with 5% CO₂. At the end of co-incubation with sperm, the COCs were transferred to a 10 ml conical tube, and vortexed for 2 min with 2 ml DM to remove their cumulus cells. Immediately after the removal of cumulus cells, the oocytes were fixed for 1 day with a mixture of ethanol and acetic acid (3:1) except trial 2. They were then stained with 1% aceto-orcein, and examined under phase-contrast microscopy to evaluate their fertilization. Oocytes were defined as penetrated when they had a swollen sperm head(s) or male pronucleus(ei) with corresponding sperm tail(s). The oocytes with a pair of pronuclei or cleaved were determined as normally fertilized oocytes. Unfertilized oocytes reaching metaphase II were considered as mature.

In trial 2, oocytes from adult buffaloes were transferred to a 50 µl of oviductal cell suspension 24 hr after insemination, and they were co-cultured with buffalo oviductal cells. Preparation of oviductal epithelial cells

Table 1. *In vitro* maturation and fertilization of follicular oocytes from the swamp buffalo (*Bubalus bubalis*) and Kedah-Kelantan (K-K) cattle (*Bos indicus*)

Trial No.	1	2	3	4
Breed of oocyte donor	Swamp buffalo			K-K cattle
Adult/prepuberal No. of donors	Adult 2	Adult 2	Prepuberal 3	Prepuberal 3
No. of oocytes inseminated	20	9	29	22
No. of degenerated	3	2	4	0
No. of unfertilized				
Metaphase-telophase I	9	3	10	0
Metaphase II	4	2	7	2
No. of penetrated				
With swollen sperm	0	0	2	9 ^{a)}
Normally fertilized	4	2 ^{b)}	6	11
Maturation rate (%)	8/20 (40)	4/9 (44)	15/29 (52)	22/22 (100)
Normal fertilization rate (%)	4/20 (20)	2/9 (22)	6/29 (21)	11/22 (50)
	12/29(41) 6/29(21)			

a) Including two polyspermic oocytes.

b) Both oocytes cleaved and developed into the morula stage.

was done as described by Eyestone and First [8] with slight modification. Briefly, oviducts from one of the oocyte donor buffaloes were trimmed free of connective tissue. After scraping the epithelium with a slide glass, extruded mucosal cells were transferred to a 12 ml plastic conical tube with 10 ml of TCM199+FCS. The oviductal cells were washed in four changes of TCM199+FCS, and then resuspended in TCM199+FCS to a ratio of 1:50 (V/V). Five ml of the cell-suspension was placed in a 50 ml plastic flask and cultured for 2 days at 39°C in a CO₂ incubator. In this trial, embryonic cleavage was checked after 1 day of co-culture (2 days after insemination *in vitro*), and non-cleaved oocytes were fixed and stained as described above. Cleaved oocytes were allowed to develop *in vitro* by co-culturing another 4 days. At the end of co-culture period (6 days after *in vitro* insemination), their cell numbers were determined by an air-dry procedure [20].

In vitro fertilization results for the swamp buffalo and the K-K calves are shown in Table 1. The proportion of degenerated oocytes in the present swamp buffalo was relatively high (16%, 9/58), although all recovered oocytes were cultured and inseminated without grading their quality because of a small sample size. The frequency of follicular atresia in the buffalo was reported to be higher than that for *Bos taurus* [4]. Suzuki *et al.* [19] also demonstrated that more than 50% of oocytes (n=536) aspirated from antral follicles of the river buffalo ovaries were degenerated, and that only 9.5% and 17% of recovered oocytes were found to be good and fair qualities, respectively.

In the adult buffalo, 6 out of 18 unfertilized oocytes

reached to metaphase II, and 6 penetrated oocytes were normally fertilized. In the prepuberal buffalo, 7 out of 17 unfertilized oocytes were at metaphase-II stage, and 8 penetrated oocytes were mature at anaphase-telophase II or pronuclear stage. Therefore, the maturation rates for adult and prepuberal buffaloes were 41% (12/29) and 52% (15/29), respectively. These maturation rates in the present swamp buffalo were comparable to that for the previous report in the river buffalo which showed 40% of oocyte maturation after incubation with TCM199 supplemented with hormones and FCS [21]. An improved oocyte maturation rate (about 65%) was reported in the previous river buffalo study when the oocytes were cultured in Ham's F-10 with FCS and hormones instead of TCM199 [21]. However, those values were still lower than that for the present K-K calves (100%) and other data in *Bos taurus* (more than 80%) under a similar maturation condition [14]. Thus, there must be difference in the requirement for oocyte maturation such as nutrients and hormones among buffalo, zebu and *Bos taurus*.

In the river buffalo, *in vitro* fertilization rate and cleavage rates after insemination with fresh ejaculated sperm in the presence of heparin and caffeine were reported to be 28% and 10%, respectively [21]. Another Indian group [19] also used heparin and caffeine for the *in vitro* insemination, and they have shown that 25% of inseminated oocytes cleaved. The present result of 21% (6/29) normal fertilization for both the adult and prepuberal swamp buffalo is very similar to those reported for the river buffalo. For K-K calves, an IVF result was better with 50% (11/22) normal fertilization rate. This value was

similar to those for the previous reports in *Bos taurus* in which heparin and caffeine or theophylline were added in an insemination medium (DM) resulting in 68% of fertilization rate [17] and 48% of cleavage rate [12]. However, no direct comparison between the present results of swamp buffalo and K-K cattle can be made, since numbers of oocytes and trials were limited, and the conditions for IVF, such as dose of heparin and concentration of sperm in the insemination medium were different among the trials. Moreover, it is known that there is a great variation in fertilization rates among individual bulls even if the doses of chemicals (heparin and caffeine or theophylline) and concentration of sperm in the insemination medium were fixed [12, 17].

One pregnancy has been reported in the river buffalo after the transfer of morulae derived from IVF of ovarian oocytes, although only 5.8% (14/240) of IVF embryos developed into the late morula stage, even 6 days after *in vitro* insemination following co-culture of embryos with cumulus cells [19]. In the present trial 2, two out of 9 inseminated oocytes cleaved and developed into the morula stage within 5 days after *in vitro* insemination. At the end of co-culture, 6 days after *in vitro* insemination, these two embryos possessed 40 cells (36 live and 4 pyknotic cells) and 29 cells (26 live and 3 pyknotic cells), respectively, but did not form blastocyst. Embryonic development in the water buffalo is faster than that in *Bos taurus*. Embryos collected from gonadotropin-treated river and swamp buffaloes were reported to develop into the blastocyst stage 6 days after the estrus [3, 7, 13]. It is likely that the water buffalo embryos might require more complex nutrients or growth promoting factors for their development and blastocyst formation. The present co-culture system might be insufficient to support the *in vitro* development of swamp buffalo embryos. Otherwise, the present conditions for IVM and IVF might be not adequate so that the embryonic development was retarded at the morula stage.

In conclusion, we have demonstrated for the first time that ovarian oocytes from prepuberal swamp buffalo and Kedah-Kelantan (Asian zebu) calves can mature and be fertilized normally *in vitro* using an IVM-IVF procedure generally applied in *Bos taurus*. It is, however, suggested that some modifications in IVM-IVF procedure might be required, especially for IVM in the swamp buffalo because of their lower oocyte maturation rate. Further studies for IVM, IVF and culturing of fertilized ova *in vitro* are in progress to establish a reliable system for embryo production in the water buffalo and zebu.

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