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Maturation Competence of Swamp Buffalo Oocytes Obtained by Ovum Pick-Up and from Slaughterhouse Ovaries

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Contents

This study was designed with the final goal of improving *in vitro* embryo production in the Thai swamp buffalo (*Bubalus bubalis carabensis*). Oocytes were collected by ovum pick-up (OPU) from six non-lactating multiparous swamp buffalo twice per week for 10 consecutive sessions followed by once-weekly collection for 10 consecutive sessions without hormone stimulation. In addition, oocytes were collected from slaughterhouse ovaries that were classified as follows: ovaries from non-pregnant cows with a visible corpus luteum (NPCL); pregnant cows with a corpus luteum (P); and non-pregnant cows without a corpus luteum (NP). Follicles in each group of ovaries were categorized as small (2–4 mm), medium-sized (5–8 mm) or large follicles (≥9 mm). The quality of the oocytes was assessed by their capacity to undergo *in vitro* maturation. The total number of observed follicles per session (all sizes combined) was larger in the once-weekly OPU group compared with the twice-weekly OPU group. In particular, the numbers of small and large follicles were higher in the once-weekly OPU group (5.2 ± 0.7 and 0.9 ± 0.2, respectively) than in the twice-weekly OPU group (3.9 ± 0.5 and 0.5 ± 0.1). The number of medium-sized follicles did not differ between the groups. The percentages of oocytes with an abnormal spindle morphology were not different between oocytes from the twice-weekly (30.0%) and the once-weekly (28.6%) OPU groups. A higher percentage of oocytes obtained *in vitro* (49.5%) exhibited nuclear abnormalities compared with those obtained *in vivo* (≤34.8%) after *in vitro* maturation. In conclusion, oocytes can be successfully collected by OPU in the swamp buffalo, without hormonal pretreatment, and per week more good-quality oocytes can be collected by twice-weekly OPU. In addition, oocytes collected from slaughterhouse ovaries can be used with the reproductive status of the cow having no influence on the maturation competence of oocytes.

Introduction

The domestic Asian water buffalo (*Bubalus bubalis*) is a multipurpose livestock animal. It is of particular importance for meat and milk production in tropical and subtropical countries because of its ability to adapt to harsh environmental conditions (Das and Khan 2010). This buffalo species is responsible for over 95% of the total amount of milk produced for the dairy industry in Asia (Cockrill 1981; FAOSTAT, 2007). Considerable differences in reproductive traits among different breeds of buffaloes exist (Singh et al. 2000). River buffaloes (*Bubalus bubalis bubalis* chromosome number: 2n = 50) have a relatively high milk production, while swamp buffaloes (*Bubalus bubalis carabensis*; 2n = 48) are less productive (Nanda et al. 2003) and are also inefficient breeders because of their inherent susceptibility to environmental stress, which causes anoestrus and suboestrus (Singh et al. 2000; De Rensis and Scaramuzzi 2003). Although the world water buffalo population has increased significantly (FAOSTAT, 2007), there has been a considerable decline in the numbers of the swamp buffalo population in south-east Asia because of low calving rates and culling of females (Nanda and Nakao 2003). Hence, improvement in reproductive success and wider exploitation and dissemination of superior swamp buffalo genotypes is required and to be obtained through the implementation of assisted reproductive technology (ART).

ART including artificial insemination and embryo transplantation is common practice in cattle reproduction. Multiple ovulation and embryo transfer (MOET) has been attempted in the buffalo but typically resulted in a relatively low recovery of both unfertilized ova and embryos when compared with cattle (Madan 2005). However, the *in vitro* production of transferable embryos via ultrasound-guided transvaginal follicular puncture ovum pick-up (OPU) has been reported to be more efficient in this species than the *in vivo* production of embryos (Aboul-Ela 2000). Hence, OPU offers the opportunity to recover oocytes from genetically superior females. OPU for aspiration of relatively small and preovulatory follicles has been successfully employed in human (Kemeter and Feichtinger 1986), cattle (Pieterse et al. 1991; Bols et al. 1995), horse (Bruck et al. 1992), goat (Graff et al. 1999), llama (Brogliatti et al. 2000) and river buffalo (Neglia et al. 2003). This oocyte collection method has been used once and twice per week in dairy cows for 2- to 3-month periods. To yield as many oocytes as possible, pre-puncture hormone treatments have been applied to stimulate follicular growth in cattle (Gibbons et al. 1994; Bungartz et al. 1995; Goodhand et al. 1999; Bo et al. 2008; Scherzer et al. 2008) and in the river buffalo (Presicce et al. 2002). Hormone stimulation followed by OPU has also been applied in swamp buffaloes (Techakumphu et al. 2000), but it is time-consuming and a significant decrease in response has been observed following prolonged hormonal stimulation (Manik et al. 1998). Alternatives are therefore needed and observations in unstimulated cattle after several weeks of repeated follicle puncture showed this to be a most effective approach and in addition avoids expensive FSH treatments (Salamone et al. 1999). To improve *in vitro* embryo production of the swamp buffalo, adequate time schedules and oocyte collection procedures via the OPU technique need to be evaluated. In swamp buffaloes, no difference in the

number and quality of recovered oocytes by biweekly OPU was observed when control and hormonally stimulated buffaloes were compared (Promdireg et al. 2005). Manjunatha et al. (2008) reported the efficient recovery of oocytes for MOET by twice-weekly OPU in the river buffalo. Oocyte recovery has been reported to vary from 0.43 to between 2.4 and 3.3 oocytes per session in this subspecies (Totey et al. 1992; Boni et al. 1996; Gupta et al. 2006). Oocyte recovery and quality vary considerably depending on many factors including (sub)species and climate (Singh et al. 2009), and domestic buffaloes have a tendency to breed seasonally (Singh et al. 2000). Especially, the usability of OPU regarding oocyte quality and quantity in swamp buffalo under tropical conditions still remains to be established.

Morphology and developmental competence of collected cumulus–oocyte complexes (COCs) is influenced by a number of factors including method of collection (*in vivo* vs *in vitro*), stage of the ovarian cycle and follicle size. In addition, the degree of cumulus development around the oocyte at recovery influences oocyte developmental capacity (Hinrichs and Schmidt 2000). Adequate presence of cumulus cells is necessary for cytoplasmic and nuclear maturation in cattle (Zhang et al. 1995). An integral aspect of *in vitro* embryo production is successful oocyte maturation. Complete nuclear maturation encompasses the progression of the oocyte from the dictate stage in meiosis I to metaphase in meiosis II and extrusion of the first polar body, requiring proper meiotic spindle formation.

This study was designed with the final goal of improving *in vitro* embryo production in the swamp buffalo, a first step being efficient oocyte collection from superior donors. However, as both the frequency, suitability of timing and the quantity and quality of the COCs to be collected after unstimulated OPU is currently unknown in Thai swamp buffaloes, the purposes of this study were to determine (i) the average number of oocytes that can be collected from the swamp buffalo after once- and twice-weekly OPU without hormone stimulation and from slaughterhouse ovaries and (ii) the quality of collected oocytes with respect to their nuclear developmental stage after *in vitro* maturation.

Materials and Methods

Oocytes collected *in vivo*

Six non-lactating multiparous Thai swamp buffaloes (*Bubalus bubalis carabensis*), 6–12 years of age, weighing 445–518 kg were used in this experiment. The animals belonged to Chulalongkorn University, Nakorn-Patom campus. They were fed 3 kg/day/head commercial concentrate, and corn leaf, dry grass and water was offered *ad libitum*. The animals had continuous access to a paddock. The experiment was carried out between August 2005 and January 2006 (end of the rainy season and the subsequent winter season). Transvaginal ultrasound-guided follicle ablation was carried out as a method of synchronizing follicular wave emergence starting at a random stage of the oestrous cycle without the requirement of exogenous hormone stimulation. Follicles were subsequently punctured twice a week for 10 consecutive sessions immediately followed by follicular puncture once a week for 10 consecutive sessions. The buffalo cows were sedated using XylazineHCl (1 mg/100 kg body weight; Bayer Health Care, Monheim, Germany) and received epidural anaesthesia by 1.5–2.5 ml of 2% Lidocaine HCL (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). Faeces were removed and the vaginal region cleaned. A real-time B-mode linear array ultrasound scanner (Aloka SSD-500; Tokyo, Japan) equipped with a 7.5-MHz ultrasound transducer was placed in the vagina. Follicles were categorized into three classes according to their diameter size: small (2–4 mm), medium (5–8 mm) and large (≥9 mm). For oocyte collection, one hand was placed into the rectum to position the ovary against the vaginal wall adjacent to the face of the transducer. A 17-G, 60-mm-long needle was inserted into the needle guide and advanced to the vaginal fornix to puncture the follicles. The contents of follicles ≥5 mm were aspirated using a vacuum pump set at a suction pressure of 70 mmHg. Subsequent curettage of the follicular wall was performed by slowly rotating the needle inside the follicle during collection. The follicular aspirates were collected in 15-ml centrifuge tubes (Corning Inc., New York, NY, USA), and the contents were allowed to settle for 10–15 min.

Oocytes collected *in vitro*

Swamp buffalo ovaries were collected at an abattoir, immediately put into a thermos flask (0.9% saline at 37°C) and transported within 5 h to the laboratory. The ovaries were classified as follows: (i) ovaries from non-pregnant cows with a visible corpus luteum (NPCL); (ii) ovaries from pregnant cows with a corpus luteum (P); and (iii) ovaries from non-pregnant cows without a corpus luteum (NP). Excess of tissue was removed, and the ovaries were rinsed once with water (37°C). Follicles in each group of ovaries were categorized to one of three groups: small (2–4 mm), medium-sized (5–8 mm) and large follicles (≥9 mm) and were aspirated with an 18-gauge needle attached to a 5-ml disposable syringe. Follicular fluid with oocytes was collected in 15-ml centrifuge tubes and allowed to settle for 10–15 min.

The sediments containing the oocytes from both sources were placed in 100-mm Petri dishes for COC isolation using a stereomicroscope with 20 × magnification. Cumulus–oocyte complexes were classified and evaluated by stereomicroscopy with 50 × magnification and classified into one of the following five groups based on the presence and morphology of their surrounding cumulus cells: (i) ≥4 compact layers of cumulus cells: COC-I; (ii) 1–3 compact layers: COC-II; (iii) partial layer of cumulus cells: P; (iv) no cumulus cells: D; or (v) expanded cumulus cells: EXP.

Recovered COCs were placed into TCM-199 with Hepes (Gibco BRL, Paisley, UK) (289 mOsm, pH 7.35) and were washed twice in TCM-199 with Hepes without prior selection. Maturation medium consisted of bicarbonate-buffered TCM-199 supplemented with 10 μl/ml E2 (Organon, Welwyn Garden City, UK), 10 μl/ml FSH (Organon) and 10% FCS (Gibco BRL), pH was

adjusted to 7.2, and osmolarity was 276 mOsm. The oocytes were cultured in 4-well plates in maturation medium that was equilibrated with 5% CO_2 for at least 2 h prior to use. Samples were incubated for 24 h at 38.5°C with 5% CO_2 in a humidified atmosphere. After 24 h, oocytes were fixed with 3% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS and stored at 4°C.

Staining was performed as described by Tremoleda et al. (2001). Briefly, denuded oocytes were washed two times in PBS and incubated for 5 min in PBS supplemented with 2% (v/v) goat serum and monoclonal mouse-anti-tubulin (1 : 100 in PBS-serum; Molecular Probes Inc., Burlington ON, Canada) for 1 h. The oocytes were washed 3 times in PBS with 0.1% (v/v) Tween, washed for 5 min in PBS serum and incubated with goat-anti-mouse Alexa633-labelled antibodies for 1 hr (1 : 100 in PBS-serum, GAMA633, Molecular Probes Inc.). Then, oocytes were washed 3 times in PBS-Tween, and the microfilaments were stained for 30 min with Alexa Fluor 568-labelled phalloidin (A-12379, Molecular probes Inc.). The oocytes were washed two times with PBS and stained for 5 min with sytox-green (1 : 100, S-7020, Molecular Probes Inc.). The oocytes were loaded with PBS-Vectashield (1 : 1) (Vector-Labs, Burlingame, CA, USA) and mounted in pure Vectashield. The slides were examined using confocal laser scanning microscopy (Leica TSC MP, Heidelberg, Germany).

The oocytes were classified into four categories: (i) germinal vesicle stage (GV), oocytes with diffuse or slightly condensed chromatin; (ii) metaphase I (MI), oocytes aligned chromosomes between two spindle poles, no polar body; (iii) metaphase II (MII), oocytes with either a polar body or two chromatin spots; and (iv) degenerated (Deg), oocytes with e.g. dispersed chromatin and disorganized spindle elements (Fig. 1; Table 1).

Oocytes were evaluated using a modification of the classification described by Saunders and Parks (1999) (Table 1). Briefly, the meiotic spindle was classified as normal if it assumed the classical, symmetrical barrel-shape with two anastral poles and two equal sets of chromosomes aligned at its centre (Tremoleda et al. 2001). Chromatin quality was classified as normal if it aligned at the centre, and clumping or dispersal of chromatin was not found. A normal polar body was identified by its spherical shape and condensed chromatin, while a polar body was classified as abnormal when its chromatin was dispersed.

The significance of difference between means was evaluated by a chi-square test and ANOVA. Differences were considered significant at the 5% level.

Results

In vivo collection of oocytes

The total number of observed follicles per session (all sizes combined) was larger ($p < 0.05$) in the once-weekly OPU group compared with the twice-weekly OPU group (Table 2). In particular, the numbers of small (2–4 mm) and large (≥9 mm) follicles were higher in the once-weekly OPU group (5.2 ± 0.7 and 0.9 ± 0.2) than in the twice-weekly OPU group (3.9 ± 0.5 and 0.5 ± 0.1; $p < 0.05$), but the number of medium-sized follicles (5–8 mm) did not differ between groups (Table 2). Per week, more oocytes were therefore collected in the twice-weekly group compared with the once-weekly group.

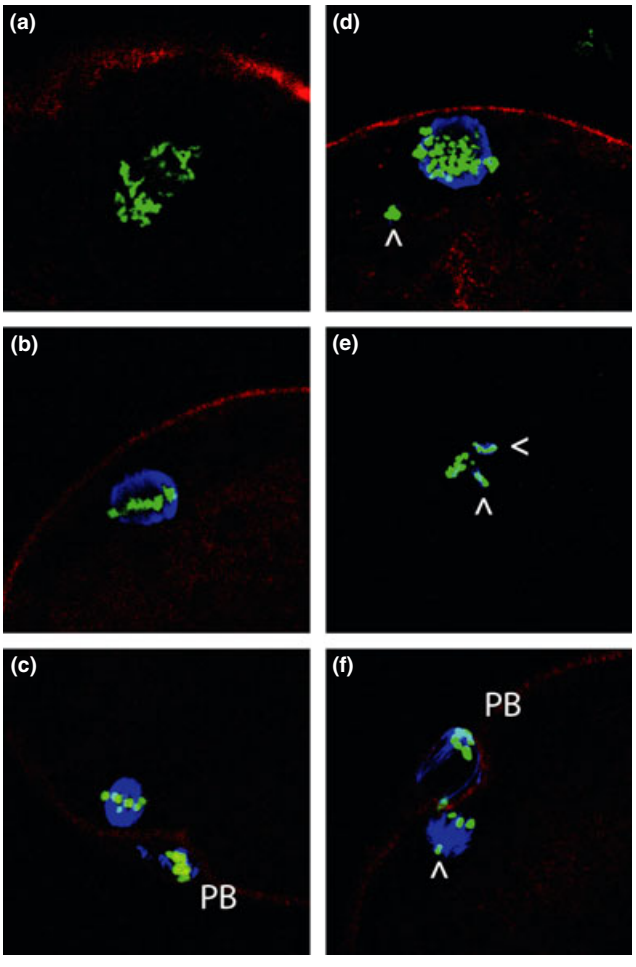

Fig. 1. Confocal laser scanning photomicrographs of normal (a–c) and abnormal (d–f) oocytes collected *in vivo*. Blue: tubulin; green: chromatin; red: microfilaments. (a) Germinal vesicle stage, (b) Metaphase I stage, a barrel-shaped spindle with chromosomes aligned on a mid-plate; (c) Metaphase II stage, a barrel-shaped spindle with chromosomes aligned on mid-plate with polar body (PB) formation; (d) Aberrant DNA, the chromatin is not aligned (arrow) (e) Dispersed spindle, spindle fragmentation (arrow) without chromatin alignment (f) Aberrant MII, arrow indicates dispersed DNA, and the polar body (PB) appears normal

There was no difference regarding the location of the ovary punctured either twice weekly (right: 0.8 ± 0.2, 50.5%; left: 0.8 ± 0.2, 49.5%) or once weekly (right: 0.9 ± 0.1, 48.7%; left: 0.9 ± 0.11, 51.3%). Also the number of oocytes recovered per session did not differ between one and two times OPU per week (0.7 ± 0.2 vs 1.0 ± 0.1, respectively). No difference was observed in the morphology of the oocytes recovered from OPU performed once or twice weekly regarding absence (denuded or partly denuded oocytes) or presence (1–3 or ≥4 layers) of cumulus cells. However, the percentage of expanded cumulus oocytes in the once-weekly group was 24.5% and was lower (4.8%) in the twice-weekly OPU group (Table 3).

Table 1. Morphological classification of oocytes at the MII stage by spindle morphology and chromatin formation (adapted from Saunders and Parks 1999)

Cell structure	Normal	Abnormal	Absent
Meiotic spindle	Symmetrical, barrel-shaped spindle with two anastral poles	Disorganized, clumped or dispersed spindle elements, or multiple spindle-like structure	No visible spindle, despite 2 sets of chromatin being present (MII plate)
Chromatin	Two sets of chromatin aligned at the centre of the barrel-shaped spindle	Aberrations of chromatin arrangement; clumping, or dispersal from the centre of the spindle	Not applicable
Polar body	Spherical shape, clumped chromatin	Fragmented shape, not spherical, dispersed chromatin	Not applicable

Table 2. Number of observed follicles (mean/buffalo/session ± SEM) and relative distribution among follicle size classes in six Thai swamp buffaloes

Puncture interval	No. of Sessions	Total observed follicles/session, mean ± SEM	Average number of follicles, mean ± SEM (%)		
			Small (2–4 mm)	Medium (5–8 mm)	Large (≥9 mm)
Twice weekly	60	5.8 ± 0.2[a]	3.9 ± 0.5 (67.5)[a]	1.7 ± 0.3 (29.0)	0.5 ± 0.1 (3.4)[a]
Once weekly	60	7.7 ± 0.3[b]	5.2 ± 0.7 (67.7)[b]	1.6 ± 0.3 (21.0)	0.9 ± 0.2 (12.0)[b]

Values with a different superscript (within column) are significantly different at the 0.05 level using ANOVA.

Table 3. Cumulus investment morphology of oocytes recovered in six Thai swamp buffaloes. COC-I (≥4 layers cumulus cells/oocyte); COC-II (1–3 layers cumulus cells/oocyte); P, partial cumulus complex; D, denuded oocyte; EXP, expanded cumulus complex (non-significant difference between COC-I, COC-II and P in twice and once ovum pick-up, $P = 0.104$)

Puncture interval	Total no. of punctured follicles (mean ± SEM/session)	Total no. of recovered oocytes (mean ± SEM/session)	Recovery rate (%)	No. of oocytes (%)/cumulus investment category				
				COC-I	COC-II	P	D	EXP
Twice weekly	113 (1.8 ± 0.2)	42 (0.7 ± 0.1)	37.2	4 (9.5) 35 (83.3)	17 (40.5)	14 (33.3)	5 (11.9)	2 (4.8)
Once weekly	151 (2.5 ± 0.2)	53 (1.0 ± 0.1)	35.1	5 (9.4) 35 (66.0)	18 (34.0)	12 (22.6)	5 (9.4)	13 (24.5)
Total	264	95		9 (9.5)	35 (36.8)	26 (27.4)	10 (10.5)	15 (15.8)

The immature oocytes with cumulus obtained via aspiration (twice/week n = 35, 83.3% or once/week n = 35, 66.0%) were matured for 24 h. The percentages of oocytes from the twice-weekly and once-weekly OPU that reached metaphase II were 65.7% and 51.4%, respectively, which shows a statistical trend (p = 0.065) (Table 4).

MI and MII stage oocytes obtained after once (n = 30)- and twice (n = 30)-weekly collection were assessed for spindle morphology after a 24-h maturation period. No obvious differences in morphology were observed between the two collection groups. The number of abnormalities in the twice-weekly OPU group was 30.0% and similar to the 28.6% found in the once-weekly OPU group. Abnormal spindle formation and aberrant DNA distribution were more frequently observed than abnormalities of polar body formation (Table 5, Fig. 1a–c) but no differences between the groups were detected.

Table 4. Nuclear meiotic stage of oocytes, collected by ovum pick-up in 6 Thai swamp buffaloes, after culture for 24 h

Puncture frequency	Oocytes, n	Developmental stages (%)			
		GV	MI	MII	Deg
Twice weekly	35	1 (2.9)	7 (20.0)	23 (65.7)	4 (11.4)
Once weekly	35	2 (5.7)	12 (34.5)	18 (51.4)	3 (8.6)

In vitro collection of oocytes

A total of 746 oocytes were collected from follicles, and these oocytes were categorized in five groups based on the presence and morphology of their surrounding

Table 5. Abnormalities of *in vitro* matured oocytes in six Thai swamp buffaloes according to classification by spindle morphology and chromatin formation

Puncture interval	Stage	No. of abnormal oocytes (%)	No. of abnormalities		
			Abnormal DNA	Abnormal PB	Abnormal spindle
Twice weekly (n = 30)	MI (n = 7)	1 (14.3)	1	0	0
	MII (n = 23)	8* (34.8)	4	2	4
		9 (30.0)	5	2	4
Once weekly (n = 28)	MI (n = 10)	3* (30.0)	2	0	2
	MII (n = 18)	5 (27.8)	2	0	3
		8 (28.6)	4	0	5

MI = metaphase I, MII = metaphase II.
*Oocytes with more than 1 abnormality.

cumulus cells. Most oocytes were obtained from non-pregnant donors with presence of a corpus luteum in the ovary (Table 6). Also most oocytes were harvested from small follicles (n = 398), while less could be harvested from medium-sized (n = 299) and large (n = 49) follicles. Non-expanded cumulus cell layers were associated with 85–90% of the oocytes irrespective of category (pregnant vs non-pregnant; small vs medium sized). Only few COCs with expanded cumulus were observed, mainly originating from large follicles, but the total number of COCs from large follicles was low. Pregnancy did not negatively influence oocyte morphology.

The 574 immature oocytes with cumulus cell layers (COC-I: ≥4 compact layers of cumulus cells; COC-II: 1–3 compact layers) were matured for 24 h. These oocytes were subsequently assessed for nuclear and cytoplasmic maturation. The percentages of oocytes that reached metaphase I and metaphase II were 21.9% (126/574) and 59.6% (342/574), respectively (Table 7). Pregnancy did not markedly affect maturation rate. A significantly higher percentage of oocytes from medium-sized follicles (64.9%; 150/231) reached the metaphase II stage compared to that of small follicles (54.6%; 175/320).

The percentage of oocytes with nuclear abnormalities following *in vitro* maturation of MII oocytes was 49.4% (169/342) and was not significantly different between oocytes that originated from small follicles (51.4%; 90/175) compared to those obtained from medium-sized follicles (46.0%; 69/150). As in oocytes obtained *in vivo*, abnormal spindle formation and aberrant DNA distribution were more frequently observed than abnormalities of polar body formation (Table 8). A higher percentage of oocytes obtained *in vitro* exhibited nuclear abnormalities (49.4%) compared with those obtained *in vivo* (< 35.0%) when assessed after *in vitro* maturation.

Discussion

This study demonstrates that ultrasound-guided OPU can be successfully performed once and twice weekly in swamp buffaloes similar to what has been described for river buffaloes (Liang et al. 2008; Manjunatha et al. 2008). Because the average dimensions of buffalo ovaries are small (Drost 2007), OPU in swamp buffaloes is a relative difficult procedure compared to OPU in cattle.

The majority of follicles were of small size (≤4 mm), independent of the frequency of OPU, similar to what has been described for bovine (Goodhand et al. 1999). However, when OPU was performed once weekly, follicle size had significantly shifted from medium sized (5–8 mm) to large (≥9 mm). Consequently, the weekly harvest of oocytes was almost twice as high in the twice-weekly protocol compared to once-weekly sessions. The oocyte recovery (0.8/session) was somewhat lower than that described by Manjunatha et al. (2008) in river buffaloes (1.2/session) in twice-weekly sessions. A discrepancy in genetic background and climate conditions influencing ovarian activity could cause such difference in oocyte production. The yield was similar to that described by Promdireg et al. (2005) (0.7–1.4) obtained in biweekly sessions. Seasonal differences (mainly winter season vs mainly rainy season) might underlie this variation as season has been described to influence oocyte recovery rate, being highest in the breeding season (Manjunatha et al. 2008).

Quality of the COCs obtained by OPU is of great significance for the embryo production *in vitro*. Cumulus investment is a significant criterion in this respect. Disruption of cumulus cell layers or partly denudation will reduce developmental competence in cattle (Merton et al. 2003). Collection frequency did not influence cumulus investment morphology of oocytes, apart from a slight increase in COCs with an expanded cumulus in the once-weekly group. Indeed, such COCs are more matured and are more apt to be successfully fertilized for embryo production (Sirard and Lambert 1985).

The percentage of immature oocytes of the twice-weekly OPU group was higher than that of the once-weekly OPU group (95.2% vs 75.5%). The percentage of matured oocytes, with an expanded cumulus complex, in the once-weekly group (24.5%) was similar to results obtained by Techakumphu et al. (2000) in hormonally treated prepubertal swamp buffaloes. The

Table 6. Classification and morphology of buffalo oocytes obtained from ovaries at different reproductive stages (RS) and from different sizes of follicles (NPCL: ovaries with corpus luteum from non-pregnant donor, P: ovaries from pregnant donor, NP: ovaries without corpus luteum from non-pregnant donor, S: small, 2–4 mm; M: medium, 5–8 mm; L: Large, ≥9 mm)

Ovary RS follicle size	Number of oocytes, n	Stage				
		COC-I, n (%)	COC-II, n (%)	P, n (%)	D, n (%)	EXP, n (%)
NPCL-S	221	68 (30.8)	105 (47.5)	23 (10.4)	14 (6.3)	11 (5.0)
NPCL-M	178	52 (29.2)	93 (52.2)	16 (9.0)	5 (2.8)	12 (6.8)
NPCL-L	31	11 (35.5)	3 (9.7)	2 (6.4)	6 (19.4)	9 (29.0)
P-S	55	19 (34.5)	31 (56.4)	4 (7.3)	0 (0.0)	1 (1.8)
P-M	40	18 (45.0)	14 (35.0)	3 (7.5)	1 (2.5)	4 (10.0)
P-L	4	1 (25.0)	1 (25.0)	0 (0.0)	1 (25.0)	1 (25.0)
NP-S	122	38 (31.2)	59 (48.4)	12 (9.8)	6 (4.9)	7 (5.7)
NP-M	81	21 (25.9)	33(40.7)	14 (17.3)	5 (6.2)	8 (9.9)
NP-L	14	3 (21.4)	4 (28.6)	2 (14.3)	2 (14.3)	3 (21.4)
Total	746	231 (31.0)	343 (46.0)	76 (10.2)	40 (5.3)	56 (7.5)

Oocytes were classified into one of the following five groups based on the presence and morphology of their surrounding cumulus cell: COC-I, >4 layers compact layers of cumulus cell; COC-II, 1–3 compact layer; P, partial layer of cumulus cell; D, no cumulus cells; EXP, expanded cumulus cells.
COC, cumulus–oocyte complex.

Table 7. Nuclear meiotic stage of *in vitro* matured buffalo oocytes obtained from ovaries at different reproductive stages (RS) and from different sizes of follicles (NPCL: ovaries with corpus luteum from non-pregnant donor; P: ovaries from pregnant donor; NP: ovaries without corpus luteum from non-pregnant donor; S: small; 2–4 mm, M: medium, 5–8 mm, L: Large, ≥9 mm)

Ovary RS follicle size	Number of oocytes	No. of stages			
		GV (%)	MI (%)	MII (%)	Deg (%)
NPCL-S	173	6 (3.5)	34 (19.7)	103 (59.5)	30 (17.3)
NPCL-M	145	2 (1.4)	23 (15.8)	94 (64.8)	26 (17.9)
NPCL-L	14	0 (0.0)	1 (7.1)	11 (78.6)	2 (14.2)
P-S	50	1 (2.0)	19 (38.0)	26 (52.0)	5 (8.0)
P-M	32	0 (0.0)	6 (18.8)	24 (75.0)	2 (6.2)
P-L	2	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)
NP-S	97	7 (7.2)	26 (26.7)	46 (47.4)	8 (8.2)
NP-M	54	0 (0.0)	15 (27.7)	32 (59.3)	7 (13.0)
NP-L	7	0 (0.0)	2 (28.6)	4 (57.1)	1 (14.3)

percentage of oocytes from the once-weekly OPU that reached metaphase II was 51.4%. In contrast, the percentage of oocytes that reached the metaphase II stage was higher when oocytes were collected twice weekly (65.7%), indicating improved quality of oocytes in this group. However, statistically, this would be referred to as a trend ($p = 0.065$), probably because of the low numbers. In cattle, the morphological quality and developmental competence of oocytes expressed as blastocyst rate is also higher when OPU is performed twice weekly compared with one-weekly OPU (Lopes et al. 2006). This finding is in agreement with the hypothesis that a dominant follicle emerging approximately 3 days after OPU will exert a negative effect on developmental competence of oocytes of subordinate follicles and thus on the oocytes collected during weekly sampling (Merton et al. 2003).

An effect of the frequency of oocyte collection on oocyte nuclear morphology was not observed. Abnormalities in spindle formation and improper chromosome alignment can frequently be observed following *in vitro* maturation of oocytes and are incompatible with normal embryonic development. These could be attributed to the intrinsic oocyte quality but also induced during *in vitro* manipulation (Christopikou et al. 2010). There was no difference regarding the side of the punctured ovary either twice or once weekly and right or left side of ovaries, similar to results obtained by Techakumphu et al. (2000).

When oocytes were collected via follicle puncture of slaughterhouse ovaries, most follicles were small (≤4 mm). Consequently, 53.3% of oocytes originated from this category, while 40.1% was obtained from medium-sized follicles. As in river buffaloes, morphological appearance of oocytes did, however, not differ significantly between follicle size classes (Raghu et al. 2002). Developmental competence of oocytes from medium-sized follicles as evaluated by *in vitro* maturation rate (reaching MII stage) was, however, superior to that of oocytes from small-sized follicles. Also in the river buffalo (Raghu et al. 2002) and in cattle (Blondin and Sirard 1995; Lonergan et al. 1999), oocytes from small follicles following fertilization have lower cleavage rates and embryo yields than those from medium-sized and larger follicles. Oocytes of small-sized follicles (> 3 mm) in cattle have a similar developmental competence as medium-sized follicles (< 8 mm) both requiring *in vitro* maturation (Hendriksen et al. 2000). However, variation in oocytes from medium-sized follicles can also be expected because of presence of a dominant follicle of which negative effects are more obvious on medium-sized than on smaller follicles (Hendriksen et al. 2000).

Reproductive state has no major influence on oocyte quality. Indeed, in cattle, OPU is successfully performed during the cycle but also during pregnancy in commercial breeding programmes (Merton et al. 2009). Morphological abnormalities are more frequently encountered in oocytes obtained from *in vitro* than in those collected *in vivo*. This is not surprising as animals to be slaughtered encompass a higher percentage of subfertile and aged individuals, characteristic for a bias to reduced oocyte quality. Moreover, post-mortem deterioration could affect oocyte quality when adequate preservation conditions cannot be met under these tropical conditions.

In conclusion, oocytes can be successfully collected by OPU in the swamp buffalo, without hormonal pretreatment, and per week more good-quality oocytes can be collected by twice-weekly OPU compared with once-weekly OPU. In addition, oocytes collected from

Table 8. Abnormalities of *in vitro* matured MII buffalo oocytes obtained from ovaries at different reproductive stages (RS) and from different sizes of follicles (NPCL: ovaries with corpus luteum from non-pregnant donor; P: ovaries from pregnant donor; NP: ovaries without corpus luteum from non-pregnant donor; S: small; 2–4 mm; M: medium, 5–8 mm; L: Large, ≥9 mm)

Ovary RS follicle size	No. of oocytes	No. of MII oocytes (%)	No. of abnormalities		
			Abnormal DNA (%)	Abnormal PB (%)	Abnormal spindle (%)
NPCL-S	103	53 (51.5)	28 (27.2)	13 (12.6)	12 (11.7)
NPCL-M	94	45 (47.9)	18 (19.1)	3 (3.2)	24 (25.5)
NPCL-L	11	6 (54.5)	2 (33.3)	1 (9.1)	3 (27.3)
P-S	26	16 (61.5)	7 (26.9)	3 (11.5)	6 (23.1)
P-M	24	10 (41.7)	5 (20.1)	2 (8.3)	3 (12.5)
P-L	2	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)
NP-S	46	21 (45.7)	10 (21.7)	2 (4.3)	9 (19.6)
NP-M	32	14 (43.8)	7 (15.2)	2 (6.25)	5 (15.6)
NP-L	4	3 (75.0)	2 (50.0)	0 (0.0)	1 (25.0)
	342	169 (49.5)	80 (23.4)	26 (7.7)	63 (18.4)

slaughterhouse ovaries can be used, and the reproductive status has no influence on oocyte quality.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

The experiments were designed by MY and BC. MY conducted the experiments, together with MT, CL, SS and AN. MY, BAJR and BC analysed the results and wrote the article.

References

Aboul-Ela M, 2000: Superovulation in the buffaloes: constraints and manipulation. Buffalo J **16,** 1–20.

Blondin P, Sirard MA, 1995: Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev **41,** 54–62.

Bo GA, Guerrero DC, Adams GP, 2008: Alternative approaches to setting up donor cows for superstimulation. Theriogenology **69,** 81–87.

Bols PE, Vandenheede JM, Van Soom A, de Kruif A, 1995: Transvaginal ovum pick-up (OPU) in the cow: a new disposable needle guidance system. Theriogenology **43,** 677–687.

Boni R, Roviello S, Zicarelli L, 1996: Repeated ovum pick-up in Italian Mediterranean buffalo cows. Theriogenology **46,** 899–909.

Brogliatti GM, Palasz AT, Rodriguez-Martinez H, Mapletoft RJ, Adams GP, 2000: Transvaginal collection and ultrastructure of llama (Lama glama) oocytes. Theriogenology **54,** 1269–1279.

Bruck I, Raun K, Synnestvedt B, Greve T, 1992: Follicle aspiration in the mare using a transvaginal ultrasound-guided technique. Equine Vet J **24,** 58–59.

Bungartz L, Lucas-Hahn A, Rath D, Niemann H, 1995: Collection of oocytes from cattle via follicular aspiration aided by ultrasound with or without gonadotropin pretreatment and in different reproductive stages. Theriogenology **43,** 667–675.

Christopikou D, Karamalegos C, Doriza S, Argyrou M, Sisi P, Davies S, Mastrominas M, 2010: Spindle and chromosome configurations of human oocytes matured in vitro in two different culture media. Reprod Biomed Online **20,** 639–648.

Cockrill WR, 1981: The water buffalo: a review. Br Vet J **137,** 8–16.

Das GK, Khan FA, 2010: Summer anoestrus in buffalo – a review. Reprod Domest Anim **45,** e483–e494.

De Rensis F, Scaramuzzi RJ, 2003: Heat stress and seasonal effects on reproduction in the dairy cow – a review. Theriogenology **60,** 1139–1151.

Drost M, 2007: Bubaline versus bovine reproduction. Theriogenology **68,** 447–449.

FAOSTAT, 2007: FAO (Food and Agriculture Organization of the United Nations). http://faostat.fao.org/site/573/default.aspx#ancor.

Gibbons JR, Beal WE, Krisher RL, Faber EG, Pearson RE, Gwazdauskas FC, 1994: Effects of once- versus twice-weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. Theriogenology **42,** 405–419.

Goodhand KL, Watt RG, Staines ME, Hutchinson JS, Broadbent PJ, 1999: In vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. Theriogenology **51,** 951–961.

Graff KJ, Meintjes M, Dyer VW, Paul JB, Denniston RS, Ziomek C, Godke RA, 1999: Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology **51,** 1099–1119.

Gupta V, Manik RS, Chauhan MS, Singla SK, Akshey YS, Palta P, 2006: Repeated ultrasound-guided transvaginal oocyte retrieval from cyclic Murrah buffaloes (Bubalus bubalis): oocyte recovery and quality. Anim Reprod Sci **91,** 89–96.

Hendriksen PJ, Vos PL, Steenweg WN, Bevers MM, Dieleman SJ, 2000: Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology **53,** 11–20.

Hinrichs K, Schmidt AL, 2000: Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. Biol Reprod **62,** 1402–1408.

Kemeter P, Feichtinger W, 1986: Transvaginal oocyte retrieval using a transvaginal sector scan probe combined with an automated puncture device. Hum Reprod **1,** 21–24.

Liang XW, Lu YQ, Chen MT, Zhang XF, Lu SS, Zhang M, Pang CY, Huang FX, Lu KH, 2008: In vitro embryo production in buffalo (Bubalus bubalis) using sexed sperm and oocytes from ovum pick up. Theriogenology **69,** 822–826.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP, 1999: Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J Reprod Fertil **117,** 159–167.

Lopes AS, Martinussen T, Greve T, Callesen H, 2006: Effect of days post-partum, breed and ovum pick-up scheme on bovine oocyte recovery and embryo development. Reprod Domest Anim **41,** 196–203.

Madan ML, 2005: Animal biotechnology: applications and economic implications in developing countries. Rev Sci Tech **24,** 127–139.

Manik RS, Singla SK, Palta P, Madan ML, 1998: Effect of presence of a dominant follicle on the superovulatory response in buffalo (Bubalus bubalis). Theriogenology **50,** 841–852.

Manjunatha BM, Ravindra JP, Gupta PS, Devaraj M, Nandi S, 2008: Oocyte recovery by ovum pick up and embryo production in river buffaloes (Bubalus bubalis). Reprod Domest Anim **43,** 477–480.

Merton JS, de Roos AP, Mullaart E, de RL, Kaal L, Vos PL, Dieleman SJ, 2003: Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology **59,** 651–674.

Merton JS, Ask B, Onkundi DC, Mullaart E, Colenbrander B, Nielen M, 2009: Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. Theriogenology **72,** 885–893.

Nanda AS, Nakao T, 2003: Role of buffalo in the socioeconomic development of rural Asia: current status and future prospectus. Anim Sci J **74,** 443–445.

Nanda AS, Brar PS, Prabhakar S, 2003: Enhancing reproductive performance in dairy buffalo: major constraints and achievements. Reprod Suppl **61,** 27–36.

Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Campanile G, Antonio Presicce G, Zicarelli L, 2003: Bovine and buffalo in vitro embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology **59,** 1123–1130.

Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, Taverne MA, 1991: Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology **35,** 857–862.

Presicce GA, Senatore EM, De SG, Stecco R, Terzano GM, Borghese A, De Mauro GJ, 2002: Hormonal stimulation and oocyte maturational competence in prepuberal Mediterranean Italian buffaloes (Bubalus bubalis). Theriogenology **57,** 1877–1884.

Promdireg A, Adulyanubap W, Singlor J, Na-Chiengmai A, Techakumphu M, 2005: Ovum pick-up in cycling and lactating postpartum swamp buffaloes (Bubalis bubalis). Reprod Domest Anim **40,** 145–149.

Raghu HM, Nandi S, Reddy SM, 2002: Follicle size and oocyte diameter in relation to developmental competence of buffalo oocytes in vitro. Reprod Fertil Dev **14,** 55–61.

Salamone DF, Adams GP, Mapletoft RJ, 1999: Changes in the cumulus-oocyte

complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology **52,** 549–561.
Saunders KM, Parks JE, 1999: Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. Biol Reprod **61,** 178–187.
Scherzer J, Fayrer-Hosken RA, Ray L, Hurley DJ, Heusner GL, 2008: Advancements in large animal embryo transfer and related biotechnologies. Reprod Domest Anim **43,** 371–376.
Singh J, Nanda AS, Adams GP, 2000: The reproductive pattern and efficiency of female buffaloes. Anim Reprod Sci **60-61,** 593–604.
Singh B, Chauhan MS, Singla SK, Gautam SK, Verma V, Manik RS, Singh AK, Sodhi M, Mukesh M, 2009: Reproductive biotechniques in buffaloes (Bubalus bubalis): status, prospects and challenges. Reprod Fertil Dev **21,** 499–510.
Sirard MA, Lambert RD, 1985: In vitro fertilization of bovine follicular oocytes obtained by laparoscopy. Biol Reprod **33,** 487–494.
Techakumphu M, Lohachit C, Tantasuparak W, Intaramongkol C, Intaramongkol S, 2000: Ovarian responses and oocyte recovery in prepubertal swamp buffalo (Bubalus bubalis) calves after FSH or PMSG treatment. Theriogenology **54,** 305–312.
Totey SM, Singh G, Taneja M, Pawshe CH, Talwar GP, 1992: In vitro maturation, fertilization and development of follicular oocytes from buffalo (Bubalus bubalis). J Reprod Fertil **95,** 597–607.
Tremoleda JL, Schoevers EJ, Stout TA, Colenbrander B, Bevers MM, 2001: Organisation of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev **60,** 260–269.
Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA, 1995: Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development in vitro. Mol Reprod Dev **40,** 338–344.

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