IN VITRO PRODUCTION OF BUFFALO (*Bubalus bubalis*) EMBRYOS

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SUMMARY

Two experiments (Exp) were carried out to test the effect of different media on in vitro maturation (IVM) (Exp 1; n = 1142 ovaries with 2666 oocytes) and to study in vitro fertilization (IVF) of buffalo oocytes (Exp 2; n = 110 ovaries with 330 oocytes). In exp 1 the collected cumulus oocyte complex (COCs) was allocated to three maturational media; TCM-199 supplemented with hormones (M1), TCM-199 supplemented with epidermal growth factor (M2) and m-SOF (M3) overlaid with paraffin oil before incubating under 5 % CO_2 in air at 38.5^o C. A part of COCs was fixed to determine the stages of nuclear maturation after 24 hr. In exp 2 the matured oocytes were allotted with sperm suspension before incubating under 5 % CO_2 in air at 38.5^o C, for 22-24 hr. Presumptive embryos were placed, individually in 96 wells petri dish to determine at 12 hr intervals the kinetics of cleavage up to blastocyst stage. Average of collected COCs per ovary was 2.4 with 86 % of excellent and good graded COC's. M1 and M2 showed higher ($P<0.05$) maturation rate vs. M3. After culturing oocyte diameter and perivitalline space increased ($P<0.05$) relative to pre-culture diameters, while diameter of ooplasm and thickness of zona pellucida non significantly decreased . Fertilized ova and cleaved embryos (%) were 80.2 and 77.8 %, respectively. Early embryonic cleavage up to 32 cell stage lapsed took 84 hr, while corresponding periods to reach morula, and blastocyst stage were 101 and 134.5 hr, respectively. Out of the fertilized ova 31.5 % reached excellent and good graded blastocyst stage.

Keywords: buffalo, IVM, IVF, nuclear maturation, kinetics of embryonic development

INTRODUCTION

Buffaloes are the native bovine that is highly praised in Egypt. Its population is 3.4 million heads acting as the major source of milk (FAO, 2000). Efforts have been made in recent years to improve the reproduction potential of buffaloes using new techniques (Madan *et al.,* 1996 and Suzuki, 2001).

The application of the *in vitro* production of buffalo embryos will help in maintaining sound management of buffalo herds. Oocyte *in vitro* maturation (IVM) (Parrish *et al.*, 1992 and Tasripoo *et al.,* 2005) and culture system (Totey *et al.,* 1993) are integral parts of *in vitro* fertilization (IVF) technique. Several trials were conducted to process appropriate media for IVM and IVF through adding some hormonal supplements (Beker *et al*., 2002 and Mingoti *et al*., 2002) or growth

promoters (Chauhan *et al.,* 1999), and to find more parameters to define developmental competence of buffalo oocytes and nuclear maturation (Ganguli *et al.*, 1998 and Datta and Goswami, 1999) achieving successful IVF. Cell cycles during embryo cleavage showed noticeable variation concerning rate of cell cycle (Gondolfi *et al.,* 1989; and Totey *et al.,* 1996), since the oocyte and embryo show high sensitivity to environmental stress (Neglia *et al.,* 2003). Studies comparing the kinetic development of buffalo and cattle embryos showed faster cleavage rate for buffalo embryos (Totey *et al.,* 1996 and Tan-Shijian *et al.,* 1998).

In Egypt limited work (Abbas, 1998; Omaima *et al.,* 1999 and Abdoon *et al.,* 2001) has been carried out to improve the efficiency of IVF of buffaloes. The present work aimed at reaching to a protocol for buffalo IVF and to build up a database for IVF, nuclear maturation and kinetic development of embryos.

MATERIALS AND METHODS

Two experiments were carried out in the lab of animal physiology, Animal Production Department, Faculty of Agriculture, Cairo University. The objective of the first experiment was to test the effect of medium on the *in vitro* oocyte maturation (IVM) rate of buffalo oocytes, while the objective of the second was to verify the success rate of *in vitro* fertilization in buffalo oocytes.

Experiment I (IVM of oocyte)

Oocyte collection

A total of 1142 Egyptian buffalo (*Bubalus bubalis*) ovaries were collected from abattoirs throughout the period from November 2002 to May 2003. Within 1 hr post-slaughtering, ovaries were transported to the lab in a thermos containing warmed (35-38^{o}C) physiological sterile saline solution (0.9 % NaCl) supplemented with 50 µg/ml gentamycin sulphate. Prior to oocyte collection the ovaries were rinsed once in ethanol (70%) and twice by sterile normal saline. Follicles of 2 - 6 mm in diameter were aspirated using a 18-gauge needle connected to a 10 ml syringe to collect cumulus oocyte complex (COCs). To avoid COCs disruption the needle and syringe were primed with 0.25-0.50 ml of phosphate – buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA, Fraction V, Cat. No. A3311, Sigma, MO, USA). COCs and follicular fluids were slowly expelled into 10 ml glass tube and maintained in water bath (38 ^{o}C) for at least 5 min for COCs sedimentation. The precipitate was transferred into sterile petri dish for subsequent investigations.

Determination of COCs quality

COCs were examined by stereomicroscope and graded according to cumulus investment and ooplasm homogeneity into five grades (Ravindranatha *et al.,* 2002). COCs graded as excellent (GA) and good (GB) were selected for the experimental work. They were washed twice by one of the two following media:

1- Tissue culture medium-199 (TCM-199) supplemented with 2% (v/v) inactivated Fetal Calf Serum (FCS, No. A11-043 PAA lab. Gmbh, Austria) (heated at 56^{o} C for 30 min) and 50 µg/ml gentamycin sulphate, for oocytes allotted for IVM in TCM-199 medium.
2- Modified phosphate-buffer saline (m-PBS) supplemented with 20% FCS, 0.3 mg/ml BSA and 50 µg/ml gentamycin sulphate for oocytes allotted for IVM in Synthetic Oviductal Fluid (SOF) medium.

Oocyte in vitro Maturation (IVM)

Three culture media were made-up fresh to be tested for buffalo oocytes IVM:

1- TCM-199 **(M1)**: 9 ml TCM-199 (Sigma) supplemented with 10 % FCS, 0.04 IU/ml FSH (no. F-2293; Sigma), 0.02 IU/ml LH (no. L-5269, Sigma), 1 µg/ml estradiol 17 β (E2) (Sigma) and 50 µg/ml gentamycin sulphate proposed by the authors.
2- TCM-199 **(M2)**: 9 ml TCM-199 (Sigma) supplemented with 10 % FCS, 20 ng/ ml epidermal growth factor (EGF, NO. E-1257, Sigma) and 50 µg/ml gentamycin sulphate as proposed by Chauhan *et al*. (1999).
3- m-SOF **(M3)**: 9.5 ml SOF supplemented with 0.3 mg/ml BSA, 0.1 IU FSH, 50 µg/ml gentamycin sulphate (Suzuki, 2001).

All media were adjusted at 7.2 pH by adding NaOH or Hcl (1M), filtered through 0.22 µm Millipore filter and were left for equilibrium at 38.5^{o} C in incubator under 5 % CO_2 in air for at least 2 h before using.

Culturing Procedure and Maturation Assessments

Aspirated COCs were washed twice in one of the washing media before being washed twice in the intended culturing medium. A number of 15-20 COCs was laid in a 100 µl drop of each culture medium in a small petri dish, then overlaid with liquid paraffin oil (No. M-8410; Sigma). The petri dishes were kept in an incubator under 5 % CO_2 in a humidified atmosphere at 38.5^{o} C for 24 hr. Expanded COC's were considered matured.

After 24 hr of *IVM*, oocytes were denuded from granulosa cells, fixed and stained as described by Moreira da Silva (2003). Nuclear configurations were classified according to Datta and Goswami (1999) as: Germinal Vesicle (GV), including either oocyte nucleus stage I or II, Metaphase I, Anaphase I, Telophase I and Metaphase II.

To assess the effect of maturation on oocyte morphological changes, a random sample (n= 159) of COCs was denuded by vortexing for 2 min immediately after recovery. Diameter of oocyte (OCD), diameter of ooplasm (OOD) and thickness of zona pellucida (ZP), were determined before and after treating with maturation medium, using inverted microscope equipped with the micrometer at X15 magnification. The perivitelline space (PVS= OCD - (OOD + ZP) was also determined.

Nuclear Development

To study the nuclear development, 69 denuded oocytes cultured for 24 hr in M1 were stained by aceto-orcein and examined microscopically for the stages of nuclear development in relation with the expansion response of the oocytes (Expanded, shrinked and not changed).

Experiment II (IVF)

Oocytes Collection and Maturation

330 COCs were collected from 110 ovaries and washed in TCM-199 HEPES buffered medium supplemented with 2% FCS, 0.3 mg/ml glutamine and 50µ g/ml gentamycin sulphate. Subsequently two washings were carried out in the maturation medium [TCM-199 HEPES supplemented with 10% FCS, 0.02 IU/ml FSH (Sigma), 1 µg/ml E_2 (Sigma) 0.15 mg/ml glutamine, 22 µg/ml Na-pyrovate (No. P-4562;

Sigma) and 50 µg/ml gentamycin sulphate]. Oocytes maturation was followed up as in experiment I.

Sperm Capacitation

Sperms were recovered from frozen semen by swim-up separation technique (Gasparrini, 2002) in tyroid albumin lactate pyrovate medium (TALP). The concentration of sperm was adjusted by adding IVF-TALP medium to reach 1 X 10^6 sperm / ml (counted by haemocytometer).

In Vitro Fertilization and Culturing Embryos

After 22 hr of IVM, the COCs were washed twice in HEPES-TALP and once in fertilization medium (IVF-TALP). COCs were arranged in groups of ten and placed into 50 µl droplets covered by parafin oil. Aliquots of sperm suspension (5-8 µl) were added to each droplet containing matured oocytes. A humidified gas atmosphere of 5 % CO_2 in air at 38.5^o C in 5 % CO_2 was used for 22-24 hr for IVF.

After the IVF, the presumptive embryos were denuded from granulose cells by pipetting. The final washing was done in culture medium consisting of TCM-199 supplemented with 3 mg/ml BSA, 22 µg/ml Na-pyurvate, 10 µl/ml non-essential amino acids (100 X), 20µl/ ml essential amino acids (50 X) and 50 µg / ml gentamycin sulphate. Presumptive embryos were placed, individually in 96 wells petri dish in culture medium, covered with paraffin oil and incubated at 38.5^o C in humidified atmosphere of 5 % CO_2 in air. Half of medium was changed every 48 hr and checked for monitoring kinetic cleavage every 12 hr by inverted microscope up to blastocyst stage. In the case of changing the embryonic stage between the two successive checks 6 hr (half of the interval between two successive checks) were subtracted from the time at which the new stage was observed. The obtained embryos were classified as excellent, good, fair, poor and degenerate according to Kennedy *et al. (*1983).

Statistical Analysis

Data were subjected to analysis of variance as repeated measurements (split plot in time) according to SAS (1998), while differences among means were tested using Duncan test (1955). The following model was used for data analysis to find out the effect of medium on maturation rate of oocytes:

$Y_{ij} = \mu + M_i + e_{ij}$

Where:

Y_{ij} = the measured trait,
μ = the overall mean
M_i = effect of media (M= 1,2,3)
e_{ij} = a random error

RESULTS

Experiment I

Oocytes Recovery Rate

Out of 1142 buffalo ovaries, 2666 COCs were collected with an average of 2.4 per ovary and about 86 % of the collected oocytes were graded as A and B (Figure 1).

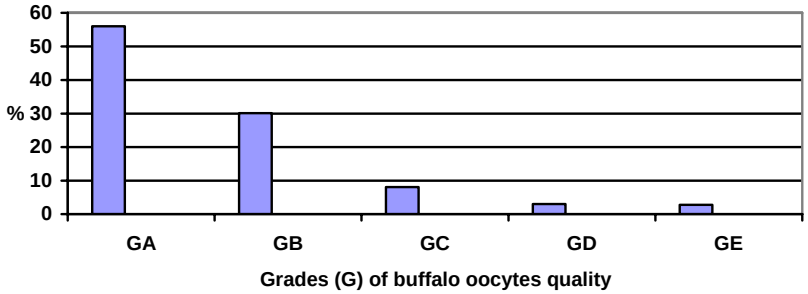

Figure 1. Quality grades of buffalo oocytes within COCs as collected from 2-6 mm ovarian follicles by aspiration method

Effect of type of medium on IVM

Oocytes matured in TCM-199 medium supplemented either by hormones (M1) or epidermal growth factor (M2) showed higher (P< 0.05) expansion *vs.* SOF medium (M3) (93.9, 96.1 and 87.6%, respectively) (Table 1).

Table 1. Effect of type of medium on *in vitro* maturation (Mean ± SE) of buffalo oocytes as measured by COCs expansion

Trait	Type of medium		
	M1	M2	M3
No of collected COCs	765	990	911
No of accepted COCs	637	881	777
No of matured oocytes	603	850	687
Maturation rate (%)	93.9^{a} ±0.093	96.1^{a} ± 0.068	87.6^{b} ± 0.16

M1 (TCM-199 + 10 % FCS + 0.04 IU/ml FSH, + 0.02 IU/ml LH + 1 µg/ml E2 + 50 µg/ml gentamycin.
M2 (TCM-199 +10% FCS, 20 ng/ ml EGF + 50 µg/ml gentamycin.
M3: SOF + 0.3 mg/ml BSA + 0.1 IU FSH + 50 µg/ml gentamycin.
a,b; values having different superscripts within the same row are significantly different (P<0.05)

Maturation morphometery

After 24 hr of IVM, the oocyte diameter and perivitalline space increased (P<0.05) in comparison with their pre-culture measurements. In contrast, the diameter of ooplasm and thickness of zona pellucida decreased, however the differences were not significantly (Table 2).

Nuclear development

Nuclear development results showed that about 70% of oocytes with expanded COCs reached MII (fully matured oocytes) compared to 50.0% and 58.3% for shrinked and non-expanded oocytes (Table 3), respectively. The stages of nuclear maturation of Egyptian buffalo oocytes are shown in Plates 1 a, b, c, d, e, and f.

Table 2. Changes in dimensions (µm, Mean ± SE) of buffalo oocytes (n= 159) in response to culture period of 24 hr in maturation media

Oocyte dimensions	Pre-culture	Post-culture	Change %
Oocyte diameter	$151.5^{a} \pm 1.0$	$156.9^{b} \pm 1.0$	+ 3.7
Ooplasm diameter	116.6 ± 0.8	114.9 ± 0.8	- 1.5
Zona pellucida thickness	13.6 ± 0.3	13.3 ± 0.3	- 2.2
Preivitelline space	$9.3^{a} \pm 1.0$	$15.8^{b} \pm 0.9$	+ 61.4

a, b; values having different superscripts within the same raw are significantly different (P<0.05)

Table 3. Nuclear development stages (%)of buffalo oocytes (n= 69) after 24 hr culture in relation to change in oocyte diameter

Oocyte size	No of oocytes	Stages of nuclear development					
		GV	GVBD	MI	AI	TI	MII
Expanded	37	0	18.9	2.7	2.7	5.4	70.3
Shrinked	20	7.7	20.0	10.0	0	15.0	50.0
No change	12	0	8.3	16.7	0	16.7	58.3
Total	69	1.4	17.4	7.4	1.4	10.1	62.3

GV = Germinal vesicle GVBD= Germinal vesicle break down MI= Metaphase I
AI= Anaphase I MII= Metaphase II TI= Telophase I

Experiment II

In vitro fertilization

Out of collected oocytes (GA & GB) 94.7 % reached maturity as evidenced by expanded COCs. Percentage of obtained zygote and cleaved embryos was 80.2 and 77.8 %, respectively (Figure 2). Following stages of embryonic development are shown in Plates 2 a, b. c, d, e, and f.

Cleavage rate showed wide variation concerning the period between the successive stages of embryonic cleavage (Table 4). The lapsed time from zygote stage to 32 cell stage averaged 84 hr, while it extended to 101 and 134.5 hr to reach morula and blastocyst, respectively.

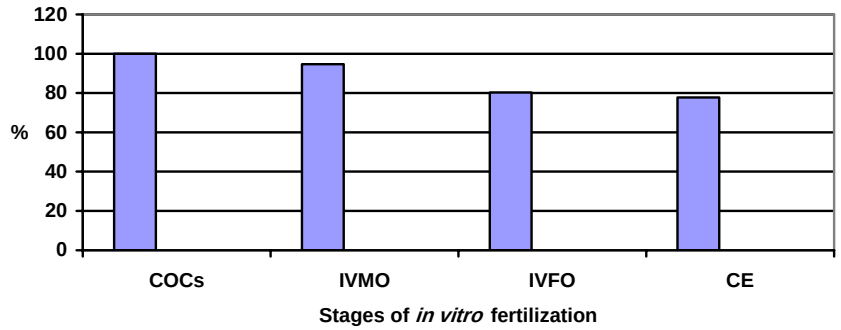

Figure 2. Rates (%) of successive stages towards *in vitro* fertilization of buffalo oocytes (COCs = collected; IVMO = *in vitro* matured oocytes,; IVFO= *in vitro* fertilized ova and CE= cleaved embryos)

Table 4. Kinetics of embryonic cleavage (Mean ± SE) of buffalo embryos (time interval)

Stages of embryonic development		Between stages (h)	Till a particular stage (h)	Days
Cells	2	14.5 ± 2.0	14.5	
	4	12.0 ±2.0	26.5	1
	8	20.3 ± 2.9	46.8	2
	16	15.8 ± 1.6	62.6	2 – 3
	< 32	21.2 ± 3.5	83.8	3 – 4
Morula	Early	17.5 ± 1.3	101.3	4 – 5
	Late	16.9 ± 1.1	118.2	5
Blastocyst	Early	16.3 ± 1.0	134.5	6
	Proper	28.9 ± 2.2	163.4	7
	Expanded	26.9 ± 1.2	190.3	8

Out of the fertilized ova 45.5 % reached the blastocyst stage. This may be because 33.5% of the embryos were blocked at the 8 cell stage and 6.7 % blocked at the 16 cell stage. Regarding the quality of embryos about, 69.3 % showed GA & GB, while the rest had low quality (Figure 3).

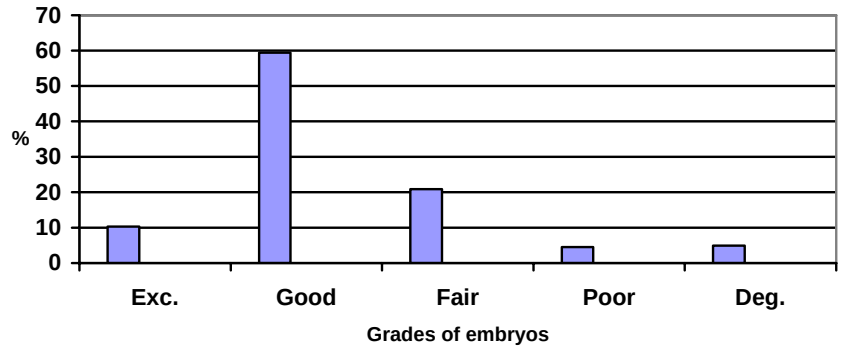

Figure 3. Grades (%) of embryos during cleavage process (Exc. = Excellent) (Deg = Degenerated)

DISCUSSION

The present results indicated that the recovery rate of oocytes per ovary (about 2. 5) is close to that reported for buffaloes (Kumar *et al*., 1997 and Abbas , 1998; 2.7) but less than that reported for cattle (Gandolfi *et al*., 1997; >10). This is most probably attributed to the low number of recruited follicles within the ovarian follicular waves (Barkawi *et al*., 2007) due to the low number of oocytes that existed in buffalo ovaries compared to cattle (Palta and Chauhan, 1998). The high percentage

of GA and GB (86 %, Figure 1) is about double of that reported in buffaloes (47.2 %, Samad and Raza, 1999), while less than that of cattle (91.2 % ; Wit *et al.,* 2000).

The obtained initial diameter of buffalo oocytes (151.5 µm, Table 2) is less than that reported by Dobson and Kamonpatana (1986) in Swamp buffaloes (169 µm). Gupta *et al.* (2000) reported wide variation in buffalo oocytes diameter (45 – 270 µm), however 55 % of the oocytes had diameter of 151 – 200 µm. Results obtained by Wit and Kruip (2001) showed similar diameter (151 µm) to that obtained in the present study.

The increase in oocyte diameter after 24 hr of culturing agrees with the findings of Arlotto *et al.* (1996) and Osaki *et al.* (1997), which may be due to the synthesis of RNA during oocyte growth (Lucas *et al.*, 2002). The expulsion of the first polar body during IVM process may be the cause of increasing the perivitelline space (Ramesha *et al.*, 2000) and shrinking of ooplasm (Hyttel *et al.,* 1986).

The total percentage of oocytes which reached MII after 24 hr of maturation process (62.3 %, Table 3) is lower than that reported by Datta and Goswami (1999) (92.1 %), Ocampo *et al.* (2001) (78.7 %) and Chohan and Hunter (2003). Meanwhile, the positive relation of COCs expansion with percentage of oocytes that reached MII stage agrees with the findings of Otoi *et al.* (1997). This finding supports the assumption of expansion of COCs during maturation process as a good evidence for oocyte maturation

The obtained fertilization rate (Table 3) is less than that reported by Omaima *et al.* (1999) and Chohan and Hunter (2003) (84.4 %). On the other hand, the percentage of blocked embryos (40.1%) is less than that reported by Nandi *et al.* (1998). The time required to reach expanded blastocyst (190.3 hr, d 8) is longer than that reported for cattle (d 5; Holm *et al.,* 2002) and buffaloes (d 7, Totey *et al.*, 1996) but less than that reported by Ocampo *et al*. (2001) (d 10). The results also indicated that about 31.5 % of the fertilized ova reached blastocyst in excellent and good stages.

In conclusion the present study reached to a considerable protocol for *in vitro* fertilization in buffaloes. More studies are required to increase the proportion of embryos reaching to blastocyst. Blocking embryos at 8 and 16-cell stage needs more investigations to reduce this percentage and to explain reason(s) of this blocking.

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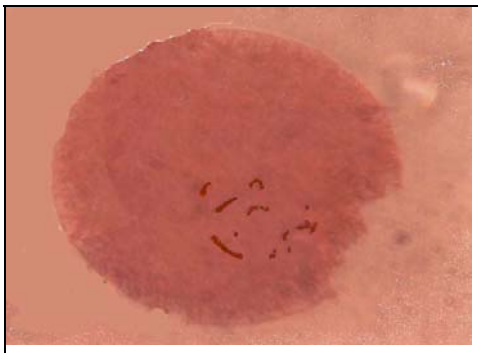

Plate 1a: Nucleus at germinal vesicle breakdown (GVBD) X200

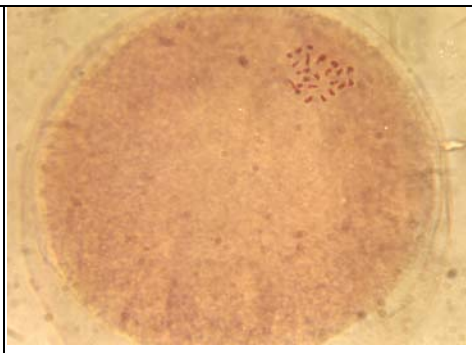

Plate 1b: Nucleus at Metaphase I (M I) X400

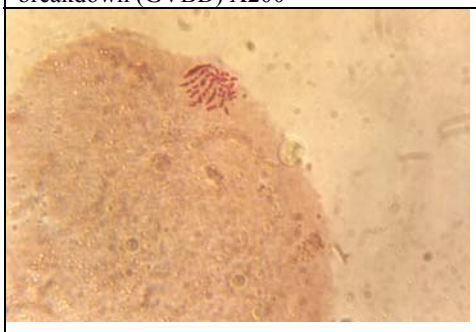

Plate 1C: Nucleus at Anaphase I (A I) X400

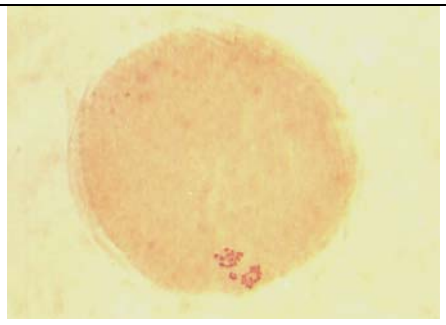

Plate 1d: Nucleus at Late Telophase I (T I) (X200)

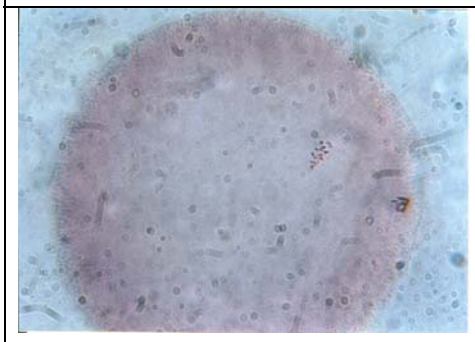

Plate 1e: Nucleus at Metaphase II (X400)

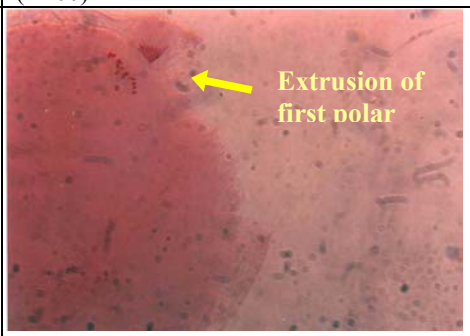

Plate 1f: Nucleus at Metaphase II (M II) X400

Plate I: Nuclear maturation phases

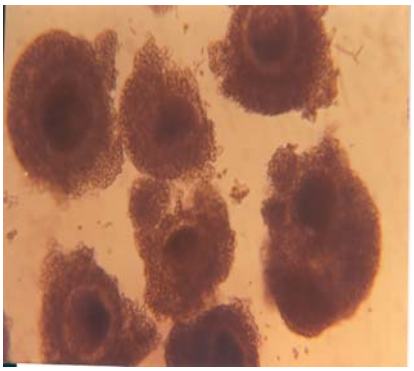

Plate 2 a: COCs of buffalo

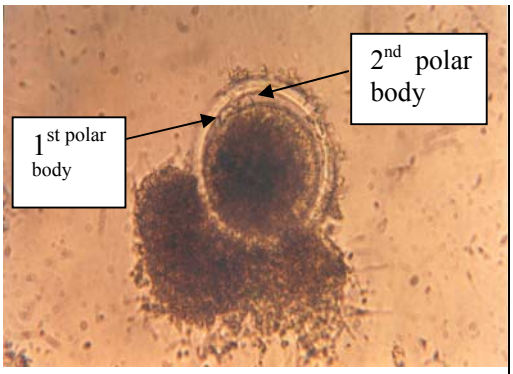

Plate 2 b: Fertilized ovum

Plate 2 c: 2 cell stage

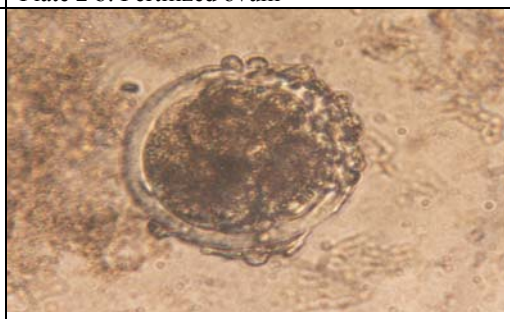

Plate 2 d: 4 cell stage

Plate 2 e: Morula stage

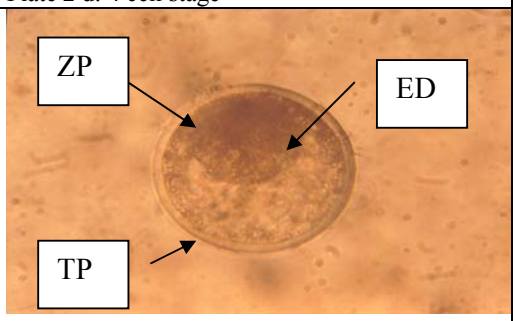

Plate 2 f: Expanded blastocyst (ZP= zona pellucida; ED = embryonic disk and TP = Trophoblast)

Plate 2. Embryonic cleavage stages of buffaloes as studied by *in vitro* fertilization

REFERENCES

Abbas, H.E., 1998. Investigation on *in vitro* fertilization in buffaloes. Ph.D. Thesis, Faculty of Veterinary Medical, Zagazig Univ., Egypt.

Abdoon, A.S.S., Kandil, M. Omaima., T. Otoi, and T. Suzuki, 2001. Influence of oocyte quality, culture media and gonadotropins on cleavage rate and development of *in vitro* fertilized buffalo embryos. Animal Reproduction Science 65:215-223.

Arlotto, T., J. L. Schwartz, N.L. First and M. L Leibfried-Rutledge, 1996. Aspects of follicle and oocyte stage that affect *in vitro* maturation and development of bovine oocytes. Theriogenology 95:943-956.

Barkawi, A.H., Y. Hafez, Amal K. El-Asheeri, S.A. Ibrahim, and N. G. Othman, 2007. Characteristics of ovarian follicular dynamics throughout the estrous cycle of Egyptian buffaloes. Animal Reproduction Science (Under publication).

Beker, A.R., C.L. Colenbrander, and M. M. Bevers, 2002. Effect of 17 β-estradiol on the *in vitro* maturation of bovine oocytes. Theriogenology 58: 1663-1673.

Chauhan, M.S., S.K., Singla, P. Palta, R. S. Manik and M.L. Madan, 1999. Effect of epidermal growth factor on the cumulus expansion, meiotic maturation and development of buffalo oocyte *in vitro*. Veterinary Record. 144:266-267.

Chohan, R. and G. Hunter, 2003. *In vitro* maturation and fertilization of water buffalo oocytes. Buffalo Journal 1:91-101.

Datta, T. K. and S. L Goswami, 1999. Time dynamics and chronology of meiotic progression of buffalo *(Bubalus bubalis)* oocytes during *in vitro* maturation. Buffalo Journal 1:53-60.

Dobson, H. and M. Kamonapatana, 1986. A review of female cattle reproduction with special reference to a comparison between buffaloes, cows and zebu. Journal of Reproduction and Fertility 77:1-36.

Duncan, B. D., 1955. Multiple range and Multiple F test. Biometrics, 11: 1-42.

FAO, 2000. Production Year Book of Food and Agricultural Organization of United Nations, Rome.

Gandolfi, F., T.A.L., Brevini, L. Richardson, C. R., Brown, and R. M. Moor, 1989. Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Reproduction and Fertility. Development 106:302-312.

Gandolfi, F., A. M., Luciano, S. Modina, A. Ponzini, P. Pocar, D. T.Armstrong and A. Lauria, 1997. The *in vitro* developmental competence of bovine oocytes can be related to the morphology of the ovary. Theriogenology 48:1153-1160.

Ganguli, G., A. Indra, and P. Gupta, 1998. Suitability of the follicular oocytes obtained from slaughtered buffalo ovaries and assessment of their nuclear maturation. Buffalo Journal 2:217-227.

Gasparrini, B., 2002. In vitro embryo production in buffalo species : state of the art. Theriogenology 57: 237 – 256.

Gupta, P.S.P., S. Nair, and P. V. Sarma, 2000. Cytometry of oocytes in buffaloes. Buffalo Journal 1:111-114.

Holm, P., P. J. Booth, and H. Collesen, 2002. Kinetics of early *in vitro* development of bovine *in vivo*- and *in vitro*-derived zygotes produced and/or cultured in

chemically defined or serum containing media. Reproduction Cambridge 123:553-565.

Hyttel, P., H. Callesen, and T. Greve, 1986. Ultrastrucutral features of preovulatory oocyte maturation in superovulated cattle. Journal of Reproduction and Fertility 76:645-656.

Kennedy, L.G., M. P. Boland, and I. Gordon, 1983. The effect of embryo quality at freezing on subsequent development of thawed cow embryos. Theriogenology 19:823-832.

Kumar, A. V.; S. Salanki, S. K.Jindal, V. N. Tripathi, and G. C. Jain, 1997. Oocyte retrieval and histological studies of follicular population in buffalo ovaries. Animal Reproduction Science, 47: 189-195.

Lucas, X., E.A., Martinez, J. Roca, J. M. Vazquez, M. A. Gil, L. M. Pastor, and J. L. Alabart, 2002. Relationship between antral follicle size, oocyte diameters and nuclear maturation of immature oocytes in pigs. Theriogenology 58:871-885.

Madan, M.L., S. K. Das, and P. Palta, 1996. Application of reproductive technology to buffaloes. Animal Reproduction Science 42:299-306.

Mingoti, G.Z., J. M. Garcia, and A. A. M. Rosa, 2002. Steroidogenesis in cumulus cells of bovine cumulus-oocyte-complexes matured *in vitro* with BSA and different concentrations of steroids. Animal Reproduction Science 69:175-186.

Moreira da Silva, F., 2003. (Personal communication), Universidade dos Açores Departamento de Ciências Agrárias, Reprodução Animal, Portugal.

Nandi, S., M. S. Chauhan, and P. Palta, 1998. Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo *(Bubalus bubalis)* oocytes matured and fertilized *in vitro*. Theriogenology 50:1251- 1262.

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

Ocampo, L.C., F. V. Mamuad, T. Mori, H. Shimizu, and M. B. Ocampo, 2001. *In vitro* production of pre-implantation buffalo embryos. Buffalo Journal 1:145-154

Omaima, M. Kandil, A.S.S. Abdoon, M. Murakami, T. Otoi, and T. Suzuki, 1999. New technique, using a portable CO_2 incubator, for the production of *in vitro* fertilized Egyptian buffalo embryos. Journal Reproduction Development 45:315-320.

Osaki, S., K. Matsumura, K.Yamamoto, T. Miyano, M. Miyake and S. Kato, 1997. Fertilization of bovine oocytes grown *in vitro.* Reproduction and Fertility. Development 9:781-787.

Otoi, T., K. Yamamoto, N. Koyama, S. Tachikamawa and T. Suzuki, 1997. Bovine oocyte diameter in relation to developmental competence. Theriogenology 48: 769-772.

Palta, P. and M. S. Chauhan, 1998. Laboratory production of buffalo (*Bubalus bublis*) embryos. Reproduction and Fertility Development 10:379 - 391.

Parrish, J. J., C. I. Kim, and I. H. Bae, 1992. Current concepts of cell cycle regulation and its relationship to oocyte maturation, fertilization and embryo development. Theriogenology 38: 277- 296.

Ramesha, K.P., M. Balakrishnan, L. K. Murthy and C. R. Balakrishnan, 2000. Additive effect of pentoxifylline and heparin on buffalo sperm motility and fertilization of oocytes. Buffalo Journal 1:63-71.

Ravindranatha, B. M., S. Nandi, P. S. P. Gupta, and P. V. Sarma, 2002. *In vitro* effects of different levels of commercially available PMSG oocyte maturation. Buffalo Journal 1:101 -107 .

Samad, H.A. and A. Raza, 1999. Factors affecting recovery of buffalo follicular oocytes. Pakistan Veterinary Journal 19:56-59.

SAS, 1998. SAS user′s guide for personal computers, SAS Institute Inc., Cary, NC., USA.

Suzuki, T., 2001. Personal communication, School of Veterinary Sciences, Lab. of Animal Reproduction and Biotechnology, Yamaguchi University, Japan.

Tan-Shijian, Yang-Nian Sheng, Shi-Deshun and Lu-Kehuan, 1998. Application of bovine *in vitro* fertilization procedures to buffalo. Journal-of-Guangxi-Agricultural-University. 17: 312-317.

Tasripoo, K., K. Srisakwattana, W. Suthikrai, S. Chethasing and M. Kamonpatana, 2005. Potential uses of buffalo oocytes from ovaries with CL and without CL for *in vitro* maturation and fertilization. Buffalo Journal, 21 (3): 221-228.

Totey, S.M., M., Daliri, K.B.C. Appa Rao, C. H. Pawshe, M. Taneja and R. S Chillar, 1996. Differential cleavage and developmental rates and their correlation with cell numbers and sex ratios in buffalo embryos generated *in vitro*. Theriogenology 45: 521-533.

Totey, S.M., C. H. Pawshe, and G. P. Singh, 1993. *In vitro* maturation and fertilization of buffalo *(Bubalus bubalis)*: Effects of media, hormones and sera. Theriogenology 39: 1153 – 1171.

Wit, A. A. C. and T. A. M. Kruip, 2001. Bovine cumulus-oocyte-complex-quality is reflected in sensitivity for α-amanitin, oocyte-diameter and developmental capacity. Animal Reproduction Science 65:51-65.

Wit, A. A. C., Y. A. Wurth, and T.A.M. Kruip, 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. Journal of Animal Science 78:1277-1283.

الإنتاج المجهري لأجنة الجاموس

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شملت هذه الدراسة إجراء تجربتين علي بويضات الجاموس المصري ، كان الهدف من التجربة الأولي اختبار البيئات المختلفة للإنضاج البيضي خارج الجسم واستخدم فيها 2666 بويضة ، قسمت إلي ثلاثة مجموعات طبقا للبيئة المستخدمة في الإنضاج ، المجموعة الأولي واستخدام فيها بيئة TCM-199 مضافا إليها الهرمونات، وفي المجموعة الثانية تم استخدام نفس البيئة مضافا إليها عامل نمو الجلد (EGF) ، أما في الثالثة فتم إنضاج البويضات باستخدام بيئة m-SOF . تم تغطية البويضات البويضات المتحصل عليها بزيت البرافين وتم تحضينها علي درجة 38.5 ºم تحت هواء جوي يحتوي علي 5 % ثاني أكسي الكربون لمدة 22-24 ساعة. تم أخذ عينة من البويضات الناضجة وتم تثبيتها لتقدير معدل النضج النووي. أما التجربة الثانية فكانت بهدف اختبار كفاءة الإخصاب المجهري وأجريت علي 330 بويضة تم أنضجها في بيئة TCM-199 مضافا إليها الهرمونات. تم اخذت البويضات الناضجة ووضعت مع سائل منوي للجاموس قبل تحضينها علي درجة 38.5 º م تحت هواء جوي يحتوي علي 5% من ثاني أكسي الكربون لمدة 22-24 ساعة . بعد ذلك تم وضع الأجنة منفردة لمراقبة معدل الإنقسام الخلوي (التطور الجنيني) كل 12 ساعة حتي الوصول إلي مرحلة البلاستوسست.

كان متوسط البويضات المتحصل عليها 2.4 بويضة / مبيض منها 86 % في حالة جيدة. كان معدل النضج البيضي في المجموعة الأولي والثانية أعلي ($P<0.05$) منها في المجموعة الثالثة. كما زاد قطر البويضة والمسافة من سطح البويضة إلي الطبقة الشفافة ($P<0.05$) بعد النضج (الزراعة في البيئة) مقارنة بما كان قبل النضج (الزراعة في البيئة). وصلت نسبة البويضات المخصبة والأجنة المتطورة إلي 80.2 و 77.8 % علي التوالي. استغرق تطور الأجنة من مرحلة الزيجوت إلي مرحلة الأجنة ذات 32 خلية حوالي 84 ساعة ، بينما استغرقت الفترة للوصول إلي مرحلة الموريولا والبلاستوسست حوالي 101 و 134.5 ساعة علي التوالي. كما بلغت نسبة الأجنة التى وصلت إلى مرحلة البلاستوسست وبحالة جيدة إلي 31.5 % من البويضات المخصبة.