

## 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) 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<sub>2</sub> in air at 38.5° 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<sub>2</sub> in air at 38.5° 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 perivitelline 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 buffalo. 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 was to test the effect of medium on the *in vitro* oocyte maturation (IVM) rate of buffalo oocytes and 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°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 °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° 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° C in incubator under 5 % CO<sub>2</sub> 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<sub>2</sub> in a humidified atmosphere at 38.5° C for 24 hr. Expanded COC's were considered matured.

After 24 hr of IVM, oocytes were denuded from granulose 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 examined the stages of nuclear development in relation with the expansion response of the oocytes (Expanded, shrunked 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<sub>2</sub> (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 1.

### ***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  $1 \times 10^6$  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 medical oil. Aliquots of sperm suspension (5-8 µl) were added to each droplet containing matured oocytes. A humidified gas atmosphere of 5 % CO<sub>2</sub> in air at 38.5° C in 5 % CO<sub>2</sub> 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-pyruvate, 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° C in humidified atmosphere of 5 % CO<sub>2</sub> 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,

$\mu$  = 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).

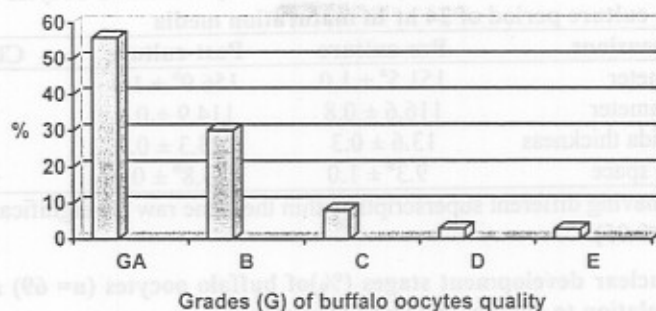


Figure 1. Quality grades of buffalo oocytes within COCs as collected from 2-6 mm ovarian follicles by aspiration method

#### 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  $\pm$  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 <sup>a</sup> $\pm$ 0.093 | 96.1 <sup>a</sup> $\pm$ 0.068 | 87.6 <sup>b</sup> $\pm$ 0.16 |

M1 (TCM-199 + 10 % FCS + 0.04 IU/ml FSH, + 0.02 IU/ml LH + 1  $\mu$ g/ml E2 + 50  $\mu$ g/ml gentamycin.

M2 (TCM-199 + 10% FCS, 20 ng/ml EGF + 50  $\mu$ g/ml gentamycin.

M3: SOF + 0.3 mg/ml BSA + 0.1 IU FSH + 50  $\mu$ g/ml gentamycin.

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

#### Maturation morphometry

After 24 hr of IVM, the oocyte diameter and perivitelline 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 58.3% and 50.0% for shrunk 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 ( $\mu\text{m}$ , Mean  $\pm$  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 <sup>a</sup> $\pm$ 1.0 | 156.9 <sup>b</sup> $\pm$ 1.0 | + 3.7    |
| Ooplasm diameter         | 116.6 $\pm$ 0.8              | 114.9 $\pm$ 0.8              | - 1.5    |
| Zona pellucida thickness | 13.6 $\pm$ 0.3               | 13.3 $\pm$ 0.3               | - 2.2    |
| Previtelline space       | 9.3 <sup>a</sup> $\pm$ 1.0   | 15.8 <sup>b</sup> $\pm$ 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 |
| Shrunked    | 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

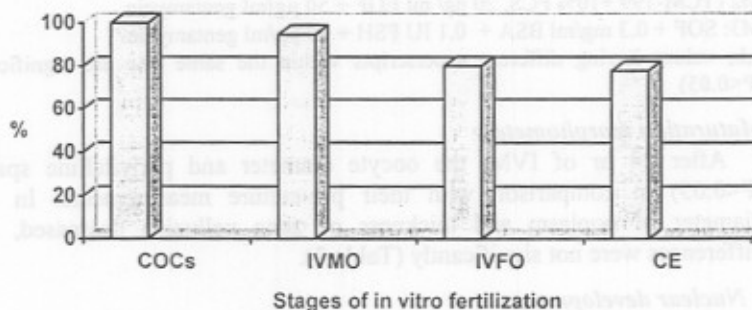
TI= Telophase I

## 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  $\pm$  SE) of buffalo embryos (time interval)

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

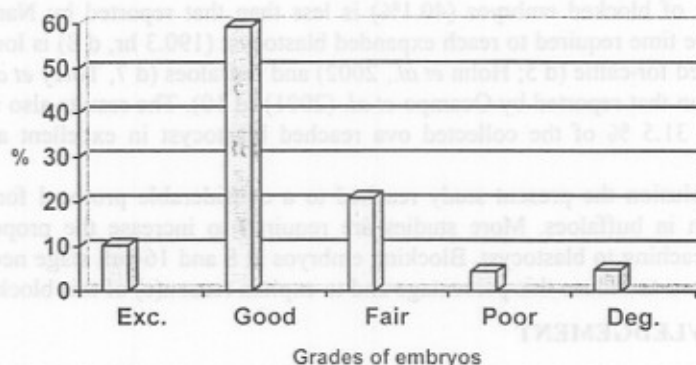


Figure 3. Grades (%) of embryos during cleavage process (Exc. = Excellent) (Deg = Degenerated)

## 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.*, 2006) 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  $\mu\text{m}$ , Table II) is less than that reported by Dobson and Kamonpatana (1986) in Swamp buffaloes (169  $\mu\text{m}$ ). Gupta *et al.* (2000) reported wide variation in buffalo oocytes diameter (45 – 270  $\mu\text{m}$ ), however 55 % of the oocytes had diameter of 151 – 200  $\mu\text{m}$ . Results obtained by Wit and Kruip (2001) showed similar diameter (151  $\mu\text{m}$  ) 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 III) 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





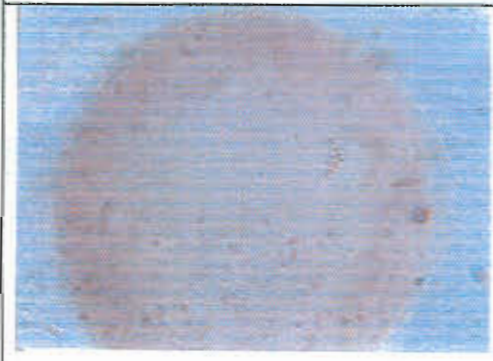

The obtained fertilization rate (Table 3) is less than that reported by Omaima *et al.* (1999) and Chohan and Hunter (2003) (84.4 %). On the other hand, the percentage of blocked embryos (40.1%) is less than that reported by Nandi *et al.* (1998). The time required to reach expanded blastocyst (190.3 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 collected 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  |  |

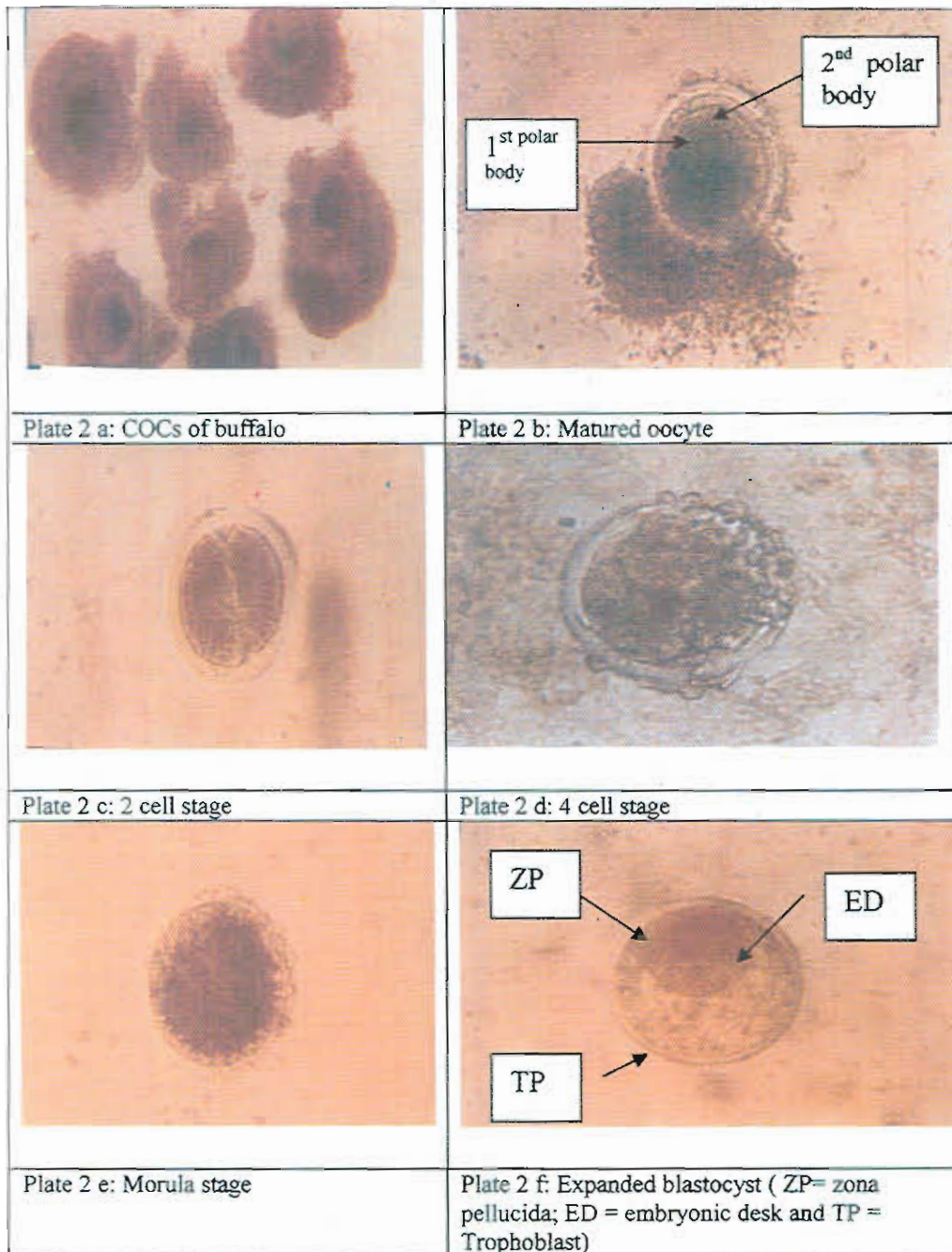


Plate 2. Embryonic cleavage stages of buffaloes as studied by *in vitro* fertilization

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## الإنتاج المجهري لأجنة الجاموس

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شملت هذه الدراسة إجراء تجربتين علي بويضات الجاموس المصري ، كان الهدف من التجربة الأولى اختيار البيئات المختلفة للإخصاب البيضي خارج الجسم واستخدم فيها 2666 بويضة ، قسمت إلي ثلاثة مجموعات طبقا للبيئة المستخدمة في الإخصاب ، المجموعة الأولى واستخدم فيها بيئة TCM-199 مضافا إليها الهرمونات، وفي المجموعة الثانية تم استخدام نفس البيئة مضافا إليها عامل نمو الجلد ( EGF ) ، أما في الثالثة فتم إخصاب البويضات باستخدام بيئة m-SOF . تم تغذية البويضات البويضات المتحصل عليها بزيت البرافين وتم تحضينها علي درجة 38.5 °م 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 °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 الأولى 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 الزيغوت 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 البويضات المخصبة التي قيمت بحالة جيدة إلي 31.5 %.