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Full Length Research Paper

Fine structure of Egyptian buffalo oocytes (*Bubalus bubalis*) during different *in vitro* maturation periods using transmission electron microscopy (TEM)

I. A. H. Barakat[1,2*] and A. R. Alhimaidi[1]

[1]Zoology Department, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia.
[2]Cell Biology Department, National Research Center, Giza, Egypt.

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This study was carried out to illustrate the ultrastructure of Egyptian buffalo oocytes through *in vitro* maturation (IVM) period (24 h). Selected cumulus oocytes complexes (COCs) were divided into three groups in relation to the time of maturation (8, 16 and 24 h >). After different maturation intervals, the matured oocytes were examined by light and transmission electron microscope (TEM). Results showed that the cumulus cells were close to each other and zona plucida (ZP) in the first group than the second and third group, lipid droplets (LD) appeared normal and nearly from plasma membrane in the group of oocytes matured *in vitro* for 8 h than oocytes matured *in vitro* for 24 h, microvilli (Mv) appeared with more number, higher thickness and penetrating the ZP and cytoplasm in the first group than other two groups, and mitochondria (M) in the first group were located near the plasma membrane, while in the third group it appeared as individual and scattered through the oocyte cell. Finally, the first polar body was observed in the third group only. This study showed the ultra-structural concepts of Egyptian buffalo oocytes during different IVM periods, and provided insights about the changes that occurred in oocytes during maturation process in the bovine species.

Key words: Egyptian buffalo, oocytes, *in vitro* maturation (IVM), ultra-structural.

INTRODUCTION

The buffalo (*Bubalus bubalis*) population in the world is actually about 168 million herd: 161 million in Asia (95.83%); 3.717 million in Africa, almost entirely in Egypt (2.24%); 3.3 million (1.96%) in South America, 40,000 in Australia (0.02%) and 500,000 in Europe (0.30%) (Antonio, 2005). However, Santos et al. (2011) showed that there are 174 million herd of buffalo (*B. bubalis*) in the world. In Brazil, this has over three million herds alone, the population increased by 9.1% in the last 10 years. Buffalo milk production already corresponds to 12.4% of the world production, with an increase of 70.6% in the last 10 years (Santos et al., 2011). Productivities of buffalo are affected mainly by performance of reproductive female status. Buffaloes have a longer gestation period (Madan, 1988), a later puberty (Drost, 2007a) and short-day seasonal breeders (Baruselli et al., 1993) when compared with cattle. It is also known that super-ovulation is even less predictable in buffalo than in cattle (Drost, 2007b). Additionally, buffalo female has shorter oestrous cycles, a longer gestation period, and late puberty, compared to the cattle female (Madan et al., 1996). Also, buffalo has a smaller number of primordial follicles than bovine species (10,000 to 19,000 versus 150,000, respectively) (Danell, 1987), higher incidence of atresia, and smaller antral follicles (82 to 92%) (Kumar et al., 1997; Palta and Chauhan, 1998). These problems could be solved by using assisted reproductive technologies such as super-ovulation, artificial insemination (AI),

*Corresponding author: E-mail: ibrahimahb@yahoo.com.

Abbreviations: IVM, *In vitro* maturation; **IVF, *in vitro* fertilization; GV,** germinal vesicle; **GVBD,** germinal vesicle breakdown; **MI,** metaphase I; **AI,** anaphase I; **MII** metaphase II; **IVEP,** *in vitro* embryo production; **FSH,** follicle stimulating hormone; **LH,** luteinizing hormone; **FBS,** fetal bovine serum.

embryo transfer (ET), and *in vitro* fertilization (IVF), to reduce the generation intervals in buffalo, and to increase the number of offspring from selected females. Laboratory production of embryos technology is the best way to provide an excellent and inexpensive source of embryos for carrying out basic researches in farm animal breeding, developmental physiology, and for commercial application of the emerging bio-techniques like nuclear transfer and transgenesis, embryo sexing, intracyto-plasmic sperm injection (ICSI) and cloning (Baldassarre et al., 2002; O'Brien et al., 2004; Wang et al., 2002).

Considerable advancements have been made during the past two decades as a result of continuous efforts of scientists. However, various previous reviews (Palta and Chauhan, 1998; Gasparrini, 2002; Nandi et al., 2006) and recent studies suggest that the rate of transferable embryo yield remains decreased (Jamil et al., 2007; Anand et al., 2008; Manjunatha et al., 2007, 2009). Some modifications were made to improve the process of *in vitro* embryo production (IVEP) in buffalos which based on the bovine model (Gasparrini, 2002; Presicce, 2007). In general, embryo production in buffaloes is only ranged between 15 and 30% as reported by different research groups (Presicce, 2007; Liang et al., 2008; Manjunatha et al., 2008). The mammalian ovary contains a pool of inactive primordial follicles. Each follicle is composed of a small inactive oocyte and one layer of granulosa cells. A great number of molecular and ultrastructure changes occur during oocyte development and affect the competence of oocytes for fertilization and development (Hyttel et al., 1997).

Transmission electron microscopy (TEM) is a technique which allows in depth investigation of folliculogenesis (Mondadori et al., 2007, 2010a) and cytoplasmic maturation in a variety of livestock species. This technique is also used by various groups to elucidate the details of immature oocytes (Cran et al., 1980; Lopes et al., 2010) and mature (Hyttel et al., 1986a) oocyte morphology, besides supporting the evaluation of oocytes from super-ovulated females (Hyttel et al., 1986a) and dominant versus subordinate follicles in bovines (Assey et al., 1994). Moreover, ultra-structural studies on the oocyte during *in vitro* maturation in different mammalian species such as mouse (Merchant and Chang, 1971), human (Zamboni and Thomson, 1972), cattle (Hyttel et al., 1997), camel (Kafi et al., 2005), pigs (Cran, 1985), equines (Alvarenga and Alvarenga, 2006) and buffaloes (*B. bubalis*) (Mondadori et al., 2010b) have resulted in a better understanding of the biology of the oocyte and, as a consequence, improving *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) rates. However, systematic studies on ultrastructure of buffalo oocytes during IVM have not been extensively reported.

There are many variations between laboratories concerning embryo production rate, and there is a need to analyze the protocols, conditions, and factors that affect the successful rate of embryo production in buffaloes. Thus, information about the fine ultra-structural changes occurring during IVM in the oocyte of different species could facilitate the understanding of oocyte biology and allows the improvement of species-specific systems for IVEP. In the light of this, the objectives of this study were described as the ultra-structural changes and nuclear division evaluation occurring during *in vitro* maturation of Egyptian buffalo oocytes, using light and transmission electron microscopy.

MATERIALS AND METHODS

Chemicals

All Chemicals and media used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of media

Handling medium

The collection medium used for harvesting oocytes is modified Dulbecco's phosphate buffer saline (mD-PBS) supplemented with 0.03 g/ml bovine serum albumin (BSA) and 50 µg/ml gentamycin sulfate (Chauhan et al., 1997).

Oocytes maturation medium

The basic maturation medium is TCM-199 supplemented with follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol 17β, fetal bovine serum (FBS) and gentamycin sulfate (Chauhan et al., 1997).

In vitro maturation (IVM)

Collection of ovaries

Ovaries of female Egyptian buffaloes were collected (n = 550) from slaughter houses in Giza region and transported to the laboratory, within 2 to 4 h, in warmed saline solution (30 to 35°C) supplemented with 50 µg/ml gentamycin sulfate. Upon arrival, the ovaries were cleaned of the extraneous tissues and washed three times using sterile normal saline. Then, they were placed into a beaker containing warmed sterile normal saline and kept in a water bath at 37°C during oocytes collection.

Recovery of oocytes

Oocytes from all visible antral follicles (2 to 8 mm) on the surface of the ovaries were aspirated with a 20 g needle attached with a 10 ml disposable syringe containing 0.5 ml from handling medium**.** The medium, together with the aspirated follicular fluid, were expelled into a sterile 15 ml conical tube in a water bath at 37°C. Oocytes were recovered from the settled sediment after 15 min, almost upon completion of the whole process of oocytes collection, using low power of a stereomicroscope. The quality of the oocytes is categorized according to their combined morphology of the cumulus investment and cytoplasm into four grades. Briefly, grade A (Excellent oocytes): corresponds to immature COCs which are completely invested with several layers of dense cumulus cells and

homogenous cytoplasm; grade B (good oocytes): COCs which have fewer layers of compact cumulus investment or are partially denuded oocytes with homogenous cytoplasm; grade C (denuded oocytes): oocytes which have no cumulus cell layers but they have homogenous cytoplasm and grade D (degenerated oocytes): defined as those with one or more of the following characteristics; very expanded cumulus, fragmented nucleus, misshaped, partially absent or vacuolated cytoplasm, empty zona pellucida (O'Brien et al., 1997; Wang et al., 1998). Once graded, grades A, B and C are chosen for *in vitro* maturation, while grade D was discarded.

In vitro maturation of buffalo oocytes

The selected oocytes were washed one time with handling medium and twice with the IVM medium. Groups of 10 to 15 oocytes were cultured in 50 µl droplets of maturation medium for three different times: 8, 16 and 24 h, respectively in the incubator at 39°C, 5% CO_2 in air and 95% relative humidity.

Assessment of nuclear maturation

At the end of the assigned *in vitro* culture period, 1200 oocytes (400 oocytes for each group) were first evaluated depending on expansion of cumulus cells, then transferred into 400 µl of PBS solution and stripped from their cumulus cells by mechanical displacement by gentle mouth pipetting, using a small-bore glass pipette. Matured oocytes were fixed with acetic alcohol (methanol and acetic acid, 3:1, v/v) for 24 to 48 h and stained with 1% aceto-orcein in 45% (v/v) acetic acid. The stages of oocytes were classified according to Santos et al. (2006) as follows, intact germinal vesicle (GV; unidentifiable nucleolus and very fine filaments of chromatin); germinal vesicle breakdown (GVBD; presence of different patterns of chromatin condensation, chromosomes coiled up and no visible individual chromosomes); metaphase I (MI), anaphase I (AI) and metaphase II (MII), once the formation of bivalents were completed and appeared on 1 or 2 sets of chromosomes.

Ultrastructure study

Preparation of oocytes for transmission electron microscope examination

The procedure to study the matured oocytes was performed according to John (2007) with some modifications. Briefly, *in vitro* matured oocytes (n = 450; 150 oocytes for each culture period) were fixed in buffered aldehyde (2.5% glutaraldhyde) for 3 h at room temperature, and washed with buffered solution, oocytes were fixed again in osmium tetroxide for 1 h, washed with buffered solution, and were dehydrated in a graded ethanol series 25, 50, 75, and two 100% each for 10 min. Then, oocytes were placed in acetone using two times, 10 min each, embedded out in 1:2 Epon 812 acetone mixture for half an hour, then in 1:1 Epon 812 acetone for further 3 h, with slight shaking. Oocytes were then transferred to a third mixture of 2:1 Epon 812 acetone where they were kept for 6 h at room temperature. Finally, the oocytes were transferred to pure Epon 812 and left overnight. The oocytes were then embedded in rubber boats filled with fresh Epon 812. Blocks were polymerized at 60°C for 24 h. Semi-thin sections of 0.7 µm thickness were cut with glass knives by ultramicrotome. They were mounted on glass slides and stained with 0.25% toluidine blue. For the electron microscope preparations, thin sections were cut from a pre-selected area of the block provided by viewing a semi-thin section with a light microscope. Silver thin sections of 60 to 90 nm, were prepared by diamond knife and collected on copper grids. These sections were stained with 5% uranyl acetate for 20 min, and then stained in lead citrate for other 20 min. The sections were examined and photographed by a JEOL 1200 EXII transmission electron microscope (Japan).

Ultrastructure evaluation

Ultrastructural analysis were examined, interpreted and assessed according to the scheme based on previous works by Barakat et al. (2012), Barakat (2005), Fuku et al. (1995a, 1995b) and Kanwal (1999).

Statistical analysis

Data of the effect maturation time on *in vitro* maturation of buffalo oocyte complexes were analyzed using one way analysis of variance (ANOVA). Differences were considered significant at the 0.05 level by using Duncan's multiple range test procedure (Duncan, 1955).

RESULTS

Cytoplasmic maturation of the oocytes was assessed by electron microscopy. Immature oocytes were randomly collected immediately after slaughter of female buffaloes in slaughterhouse, and ovaries were collected. The distribution of cytoplasm organelles such as cortical granules, vesicles, lipid droplets, mitochondria, and the occurrence of gap junctions between cumulus cells and the oocyte was used to estimate oocyte maturity.

Egyptian buffalo oocytes after 8 h of culture

In vitro matured Egyptian buffalo oocytes after 8 h of culture were analyzed by TEM and characterized by near of cumulus cells to each other and to the zona pellucidaa, the mean diameter of these cells reached to 8.6 µm (7.95 to 9.20 µm) at magnification power (MP) 3000×. Lipid droplets appeared in more number, normal shape and close to the plasma membrane. The average diameter of lipid droplets ranged between 3.02 to 4.80 µm, with mean as 3.9 µm. Moreover, the mitochondria appeared in groups and nearly to the plasma membrane (Figure 1a). The Microvilli in oocytes matured *in vitro* were characterized by thick, dense and penetrating to the zona, pellucida and cytoplasm. Also, they appeared with more vesicles which were located nearly from the plasma membrane. The width of the perivitelline space was 1.1 µm almost at 15000× (MP) (Figure 1b). In addition, the distance between the cumulus cells and zona pellucida ranged between 3.1 to 4.4 µm at 5000× (MP) (Figure 1c) and the shape of most mitochondria in matured oocytes was either circular or oval as shown in Figure 1d.

Egyptian buffalo oocytes after 16 h of culture

Examination of *in vitro* matured Egyptian buffalo oocytes

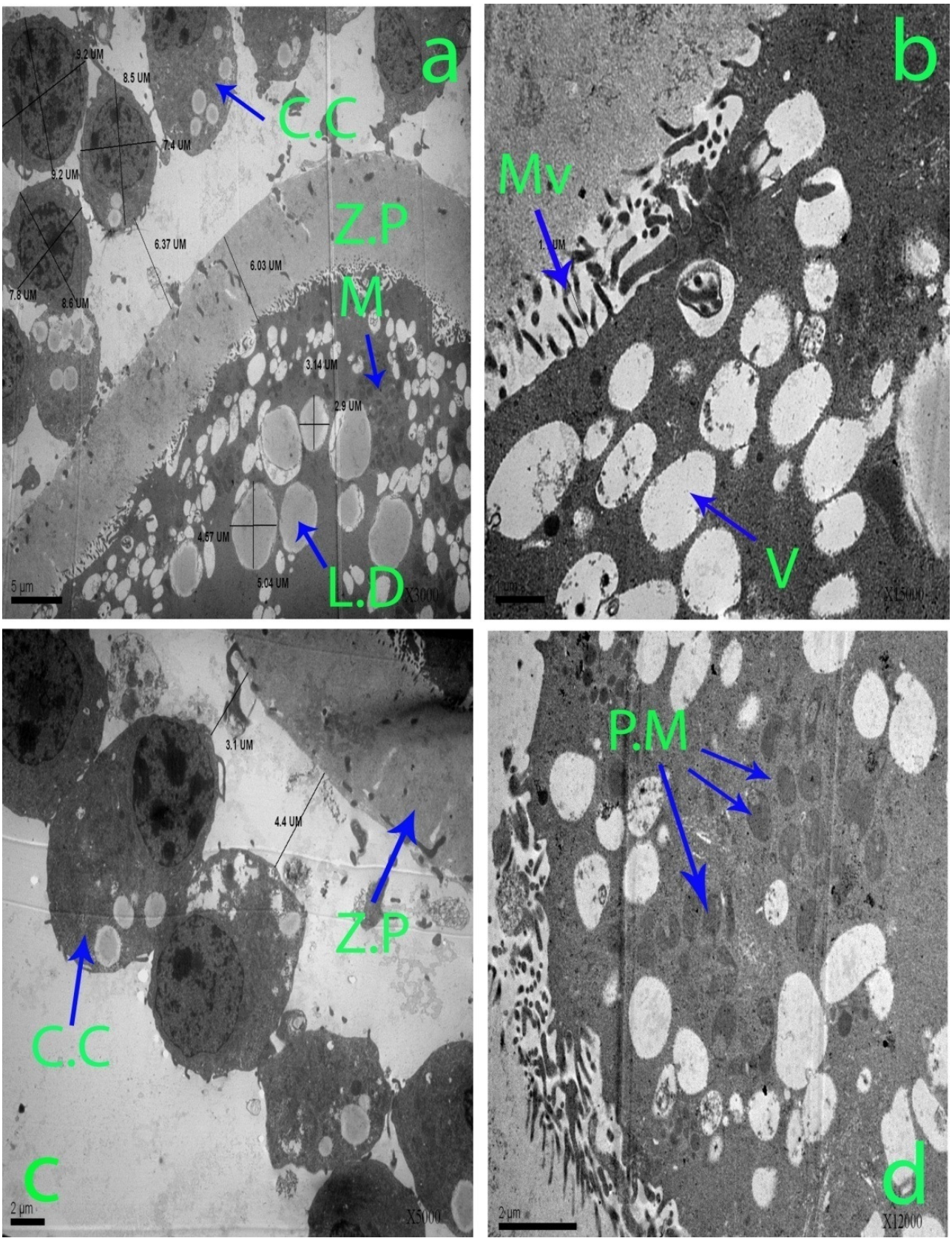

Figure 1. The electron micrograph (TEM) of *in vitro* matured Egyptian buffalo oocytes (8 h) showing; (a) Cumulus Cells (CC), zona pellucida (ZP), lipid droplets (LD) and mitochondria (M); (b) Microvilli (Mv) and vesicles (V); (c) Cumulus cells (CC) and zona pellucida (ZP) and (d) polymorphic mitochondria (PM).

by TEM showed some different characteristics; the distance between cumulus cells from each other and zona pellucida was larger than in oocytes cultured for 8 h only, vesicles were present in large number and far away from the plasma membrane, lipid droplets are also found far from plasma membrane than oocytes which cultured for 8 h, in large number and normal shape (Figure 2a). Also in the same matured oocytes, mitochondria were found scattered and far from the plasma membrane (Figure 2b). In addition to the above, microvilli had less thickness and fewer numbers and did not penetrate for the zona pellucida and cytoplasm than oocytes, cultured for 8 h, as well as, the diameter of zonal pellucida reached about 6.6 µm at 8000× (MP) (Figure 2c). In terms of microvilli, vesicles and mitochondria in this group, Figure 2d shows low number of microvilli in the perivitelline space, greater number of vesicles and it was located far from the plasma membrane, and mitochondria were found also far from plasma membrane and existed in group but in few number and scattered. Also, the same figure showed low number of cortical granules scattered in the cytoplasm of the oocyte. Figure 3 shows increased numbers of mitochondria with normal shape (hooded shape) in the oocytes matured *in vitro* for 16 h.

Egyptian buffalo oocytes after 24 h of culture

Study of Egyptian buffalo oocytes at the end of *in vitro* maturation period (24 h) with TEM revealed some differences between this group and the other two groups (8 and 16 h). With respect to the mitochondria, vesicles, and cortical granules (C.G) status, Figure 4a shows that the vesicles were found in low number, an increase in size for some vesicles and was located nearly from the plasma membrane, while for mitochondria; the same figure shows that the mitochondria were individual and scattered in the cytoplasm of the cell, as well as, CGs observed in oocytes groups matured *in vitro* for 24 h. Also, the diameter of zona pellucida reached 8.6 µm at 1300× (MP). Examination of the same oocytes showed that the lipid droplets were found intensively but with irregular shape than lipid droplets in oocytes which matured for 8 and 16 h, and mitochondria appeared with individual and scattered image throughout all oocytes (Figure 4b). *In vitro* maturation of buffalo oocytes for 24 h led to spacing of cumulus cells from zona pellucida with more transparency than the other groups and the distance between them ranged from 7.5 to 25.8 µm as shown in Figure 4. Also, in the same figure, the polar body appeared in the perivitelline space and the diameter of zona pellucida reached to 10.8 µm at 1200× (MP). In addition, Figure 4d shows a decrease of the microvilli in the perivitelline space and did not penetrate the zona pellucida and cytoplasm, and also the emergence of the first polar body in perivitellinee space. Finally, mitochondria appeared with different shapes (circular, hooded, etc.) as indicated in Figure 5.

Nuclear maturation during maturation period

Nucleus of immature oocytes passed with different stages during the maturation process, until they reach the metaphase stage of Meiosis II division, then this stage, was considered as evidence of the maturity of the oocytes. Table 1 shows that the culture of Egyptian buffalo oocytes *in vitro* for 24 h resulted in significant results when compared with the two other groups (8 and 16 h) where the nuclei of these oocytes were arrested in M II at 8, 16 and 24 h of culture (0.0 ± 0.0, 12.6 ± 1.03 and 27.4 ± 1.17), respectively. On the same approach, a greater percentage of oocytes were arrested in the GV stage (52.7%) after culture for 8 h only, while higher percentage of oocytes were reached to M II stage (45.7%) after cultured for 24 h (Figures 6 and 7).

DISCUSSION

To our knowledge, this might be the first trial to studying the changes that occurs during *in vitro* maturation of Egyptian buffalo oocytes. The follicle and oocyte structures of some domesticated species such as porcines (Greenwald and Moor, 1989), cattle (Schmidt et al., 1995; Beckers et al., 1996; Fair et al., 1997), canines (Durrant et al., 1998), caprines (Lucci et al., 2001) and felines (Jewgenow and Go¨ ritz, 1995) have already been described. Folliculogenesis has been studied in buffalo foetuses (El-Ghannam and El-Naggar, 1974, 1975) and adults (Danell, 1987; Smith et al., 1991; Le Van et al., 1989; Kumar et al., 1997). However, only Mondadori et al. (2007) evaluated the morphometric and ultrastructural characteristics of preantral follicles in adult animals.

To work on the success of *in vitro* production of embryos, increase the rates of blastocyst production and embryo transfer success, detection and selection of excellent oocytes must be performed, which will be used in that, as well as, studying all the circumstances relating to the success and increased rates of *in vitro* maturation such as study of buffalo oocytes at the cellular level by TEM. The current study showed that in the beginning of *in vitro* maturation (8 h), the cumulus cells appeared close to each other and to ZP area as observed in immature oocytes by Cran et al. (1980) for sheep and other species, like horses (Alvarenga and Alvarenga, 2006); buffaloes (Mondadori et al., 2007); pigs (Cran, 1985) and cattle (Hyttel et al., 1986b). This is perhaps one of the reasons for entering the eggs in the process of maturation, as the granulosa cells are thought to have an important role at the beginning of the process of maturation (Shirazi et al., 2007), it is also believed that these cells do not play an important role in the process of maturation during the rest of IVM peroid. The separation

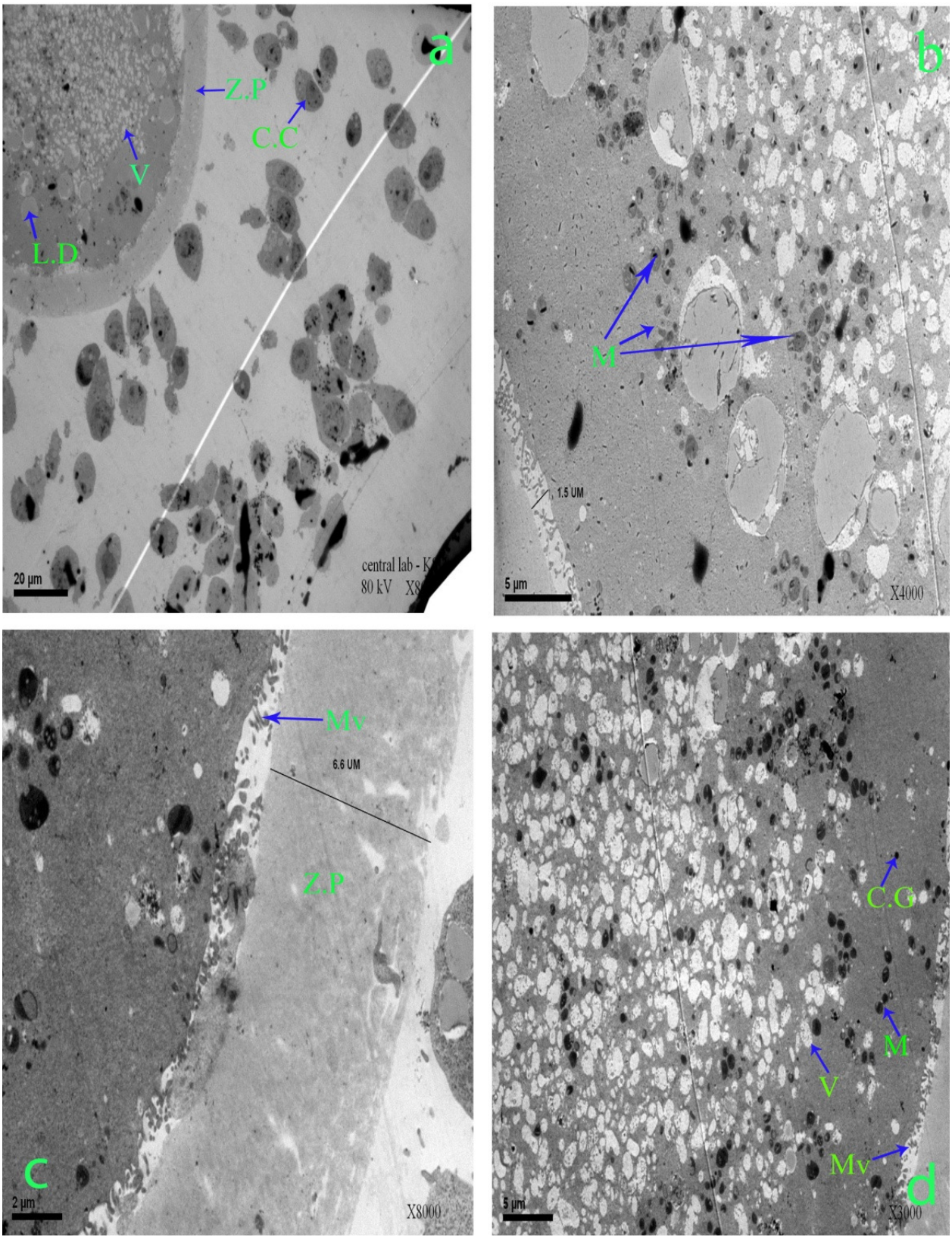

Figure 2. The electron micrograph (TEM) of *in vitro* matured Egyptian buffalo oocytes (16 h) showing; (a) Cumulus cells (CC), zona pellucida (ZP), vesicles (V), and lipid droplets (LD); (b) Mitochondria (M) distribution; (c) Microvilli (Mv) had less thickness and number and diameter of zonal pellucida (ZP) and (d) Microvilli (Mv), vesicles (V), mitochondria (M) and cortical granules (CG) status.

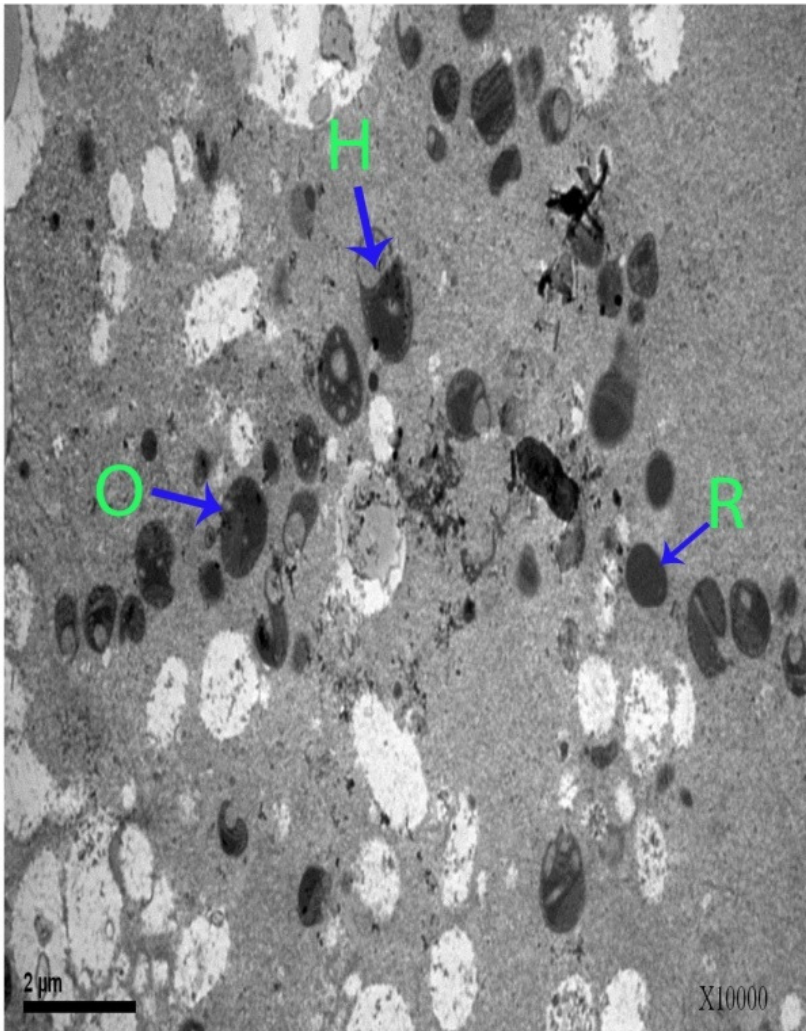

Figure 3. The electron micrograph (TEM) of *in vitro* matured Egyptian buffalo oocytes (16 h) showing polymorphic mitochondria [hooded (H); oval (O), round (R)].

of cumulus cells probably due to the CC hyaluronic acid production is induced by the presence of gonadotrophins (Chen et al., 1990). Also, lipid droplets and groups of mitochondria were found near to the plasma membrane as in oocytes matured for 6 h (Máximo et al., 2011). This redistribution of organelles is related to the beginning of cytoplasmic maturation, necessary to allowing the oocyte to reach the metaphase II stage as described by Máximo et al. (2011) and Mondadori et al. (2010b). After 8 h of *in vitro* culture, mitochondria exhibited polymorphic appearance and many of them had a hooded shape as agreed with Máximo et al. (2011). This form is a small excess appendix on the mitochondria to form a curve or arc. This curve or arc increases the area of mitochondria and may be related to an increase of metabolic activity of mitochondria during the process of maturation, which includes the cytoplasmic and nuclear maturation. Lipid droplets appeared irregular or without consistent shape after 24 h of culture than in the beginning of maturation, this change in lipid droplets may be due to its utilization as a source of energy in mitochondria metabolism from start of maturation until 18 h of culture as reported by Máximo et al. (2011). Cortical granules in this study were observed after 16 h of culture, but observed after 12 h of maturation time by Máximo et al. (2011) and 18 h of maturation by Mondadori et al. (2010).

Ultrastructure study of Egyptian buffalo oocytes revealed that the polar body was extruded after 24 h of culture as reported by earlier studies (Yadav et al., 1997; Santos et al., 2002; Nandi et al., 2002; Gasparrini et al., 2008). On the contrary, Mondadori et al. (2010) found that the first polar body was observed after 12 h of culture. This difference for first polar body extrusion time is probably due to the difference of buffalo species. Contact between cumulus cells and oocytes is an important factor for oocyte maturation and this is shown

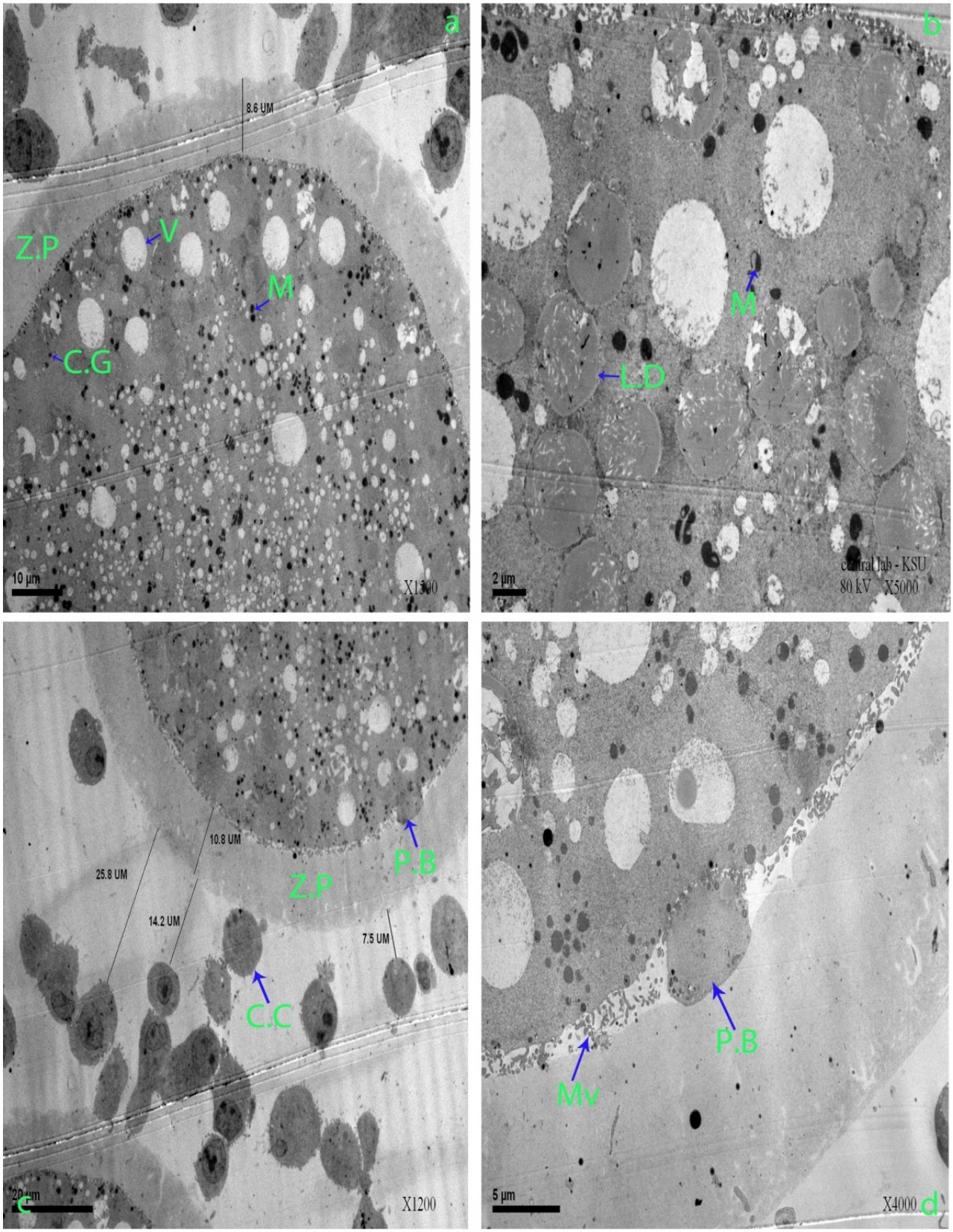

Figure 4. The electron micrograph (TEM) of *in vitro* matured Egyptian buffalo oocytes (24 h) showing; (a) vesicles (V), mitochondira (M), cortical granulse (CG) and zona pellucida (ZP) diameter; (b) lipid droplets (LD) and mitochondria (M); (c) cumulus cells (CC), polar body (PB), and zona pellucida (ZP) and (d) microvilli (Mv) and first polar body (PB).

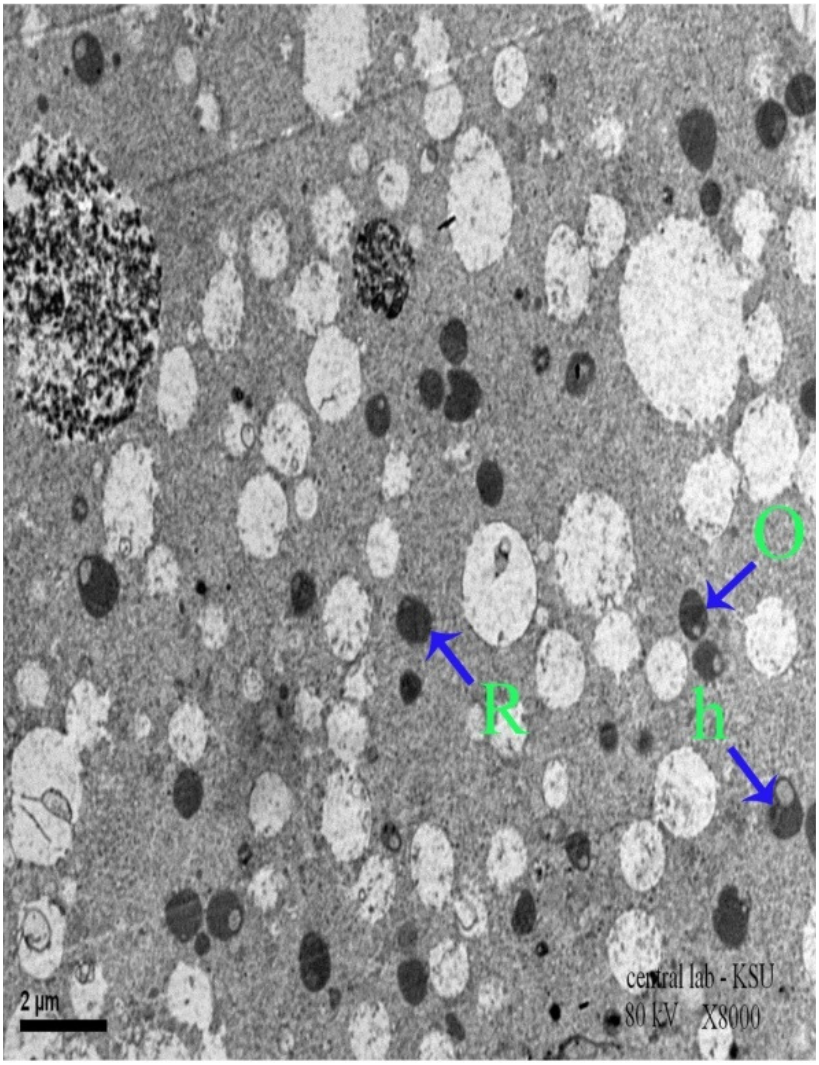

Figure 5. The electron micrograph (TEM) of *in vitro* matured Egyptian buffalo oocytes (24 h) showing polymorphic mitochondria [hooded (H), oval (O), round (R)].

Table 1. Nuclear maturation stages following different *in vitro* maturation periods.

Treatment group (h)	Nuclear stage				
	GV	GVBD	M I	Anaphase	M II
8	31.6±0.8^{c}	24.8±0.8^{c}	2.2±0.4^{a}	1.4±0.2^{a}	0.0±0.0^{a}
16	22.8±1.2^{b}	16.6±1.0^{b}	3.8±0.4^{b}	3.6±0.6^{b}	12.6±1.0^{b}
24	12.0±0.6^{a}	8.6±0.6^{a}	5.8±0.4^{c}	6.2±0.7^{c}	27.4±1.2^{c}

Means with different superscript (a, b) within the same column are significantly different (P ≤ 0.05).

by an increase of cumulus cell number contacted with the ZP of oocyte at the beginning of maturation period, and this contact was lost with extension of maturation period. This fact is in agreement with our results and previous studies (Browder, 1984; Alvarenga and Alvarenga, 2006; Hadek, 1965; Hyttel et al., 1989).

In conclusion, the present study may be the first trial in this field to describe the ultra-structural changes of cytoplasmic maturation using TEM and nuclear maturation using light microscope during IVM in Egyptian

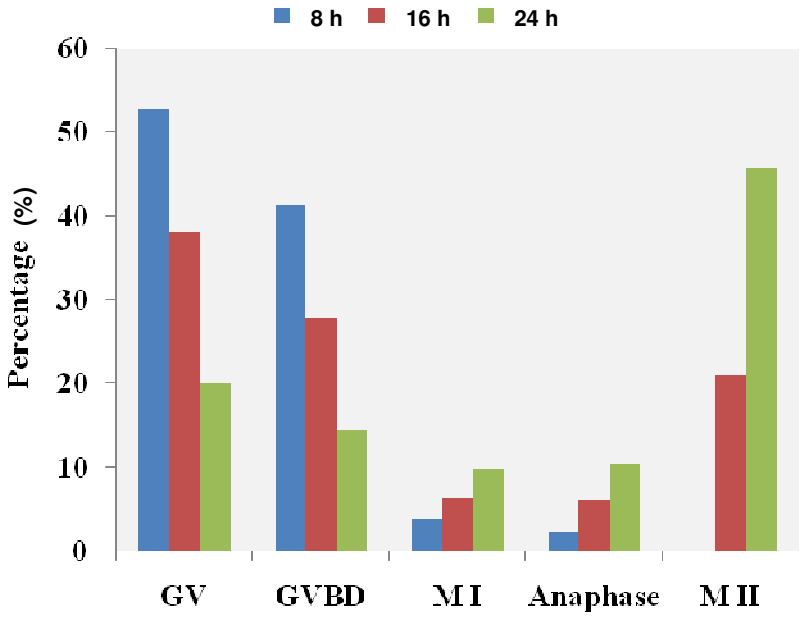

Figure 6. Percentage of nuclear stages at different times of *in vitro* maturation of Egyptian buffalo oocyte.

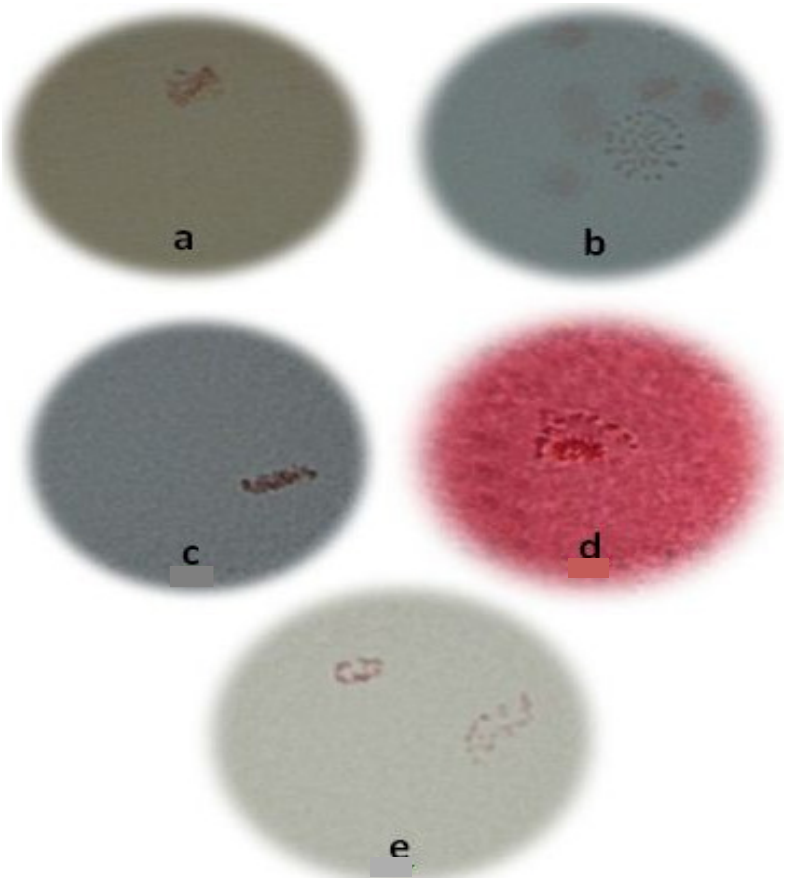

Figure 7. Different stages of nuclear maturation of Egyptian buffalo oocytes. (a) Germinal vesicle (GV); (b) germinal vesicle break down (GVBD); (c) metaphase I (M I); (d) anaphase I (Anaph I) and (e) metaphase II (M II).

buffalo oocytes. The cytoplasmic rearrangements which were observed by TEM enabled the elucidation of the changes to occur during the *in vitro* maturation period of buffalo oocytes which could be used as a basis for future studies.

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