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*Full Length Research Paper*

# Ultra-structural study of Egyptian Buffalo oocytes before and after *in vitro* maturation

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**The purpose of this study was to describe the changes occurring in the cytoplasmic organelles of the buffalo oocytes before and after *in vitro* maturation. The total number of oocytes used in this experiment was 250 oocytes; 50 *in vivo* matured oocytes, 100 immature oocytes, and the other 100 was *in vitro* matured oocytes cultured in TCM-199 + LH. The oocytes examined in this study showed normal ultra-structure of mitochondria, smooth endoplasmic reticulum (SER), zona pellucida (ZP), lipid droplets, vesicles and Golgi in the good type meanwhile, some differences and abnormalities in denuded oocytes were recorded. The most remarkable changes observed in the two different categories of oocytes (good and denuded) after maturation was the different complexes consisting endoplasmic reticulum, mitochondria (M), lipid droplets (L), vesicles (V) and ZP. Concerning the polar body (PB), group of *in vitro* matured oocytes showed a normal PB formation, vesicles, whereas mitochondria were dislocated towards the site of the PB. *In vitro* matured oocytes showed clusters of cortical granules which existed in aggregates throughout the peripheral ooplasm just beneath the oolemma. *In vitro* maturation of Egyptian buffalo oocytes could be elucidated by alterations that occurred in the cytoplasmic organelles of the oocytes as shown by transmission electron microscopy (TEM).**

**Key words:** Egyptian buffalo, oocytes, *in vitro* maturation, ultra-structure.

## INTRODUCTION

*In vitro* embryo production (IVEP) technology represents the best tool to improve maternal contribution to genetic progress in buffalo. Besides the progress obtained in the percentage of *in vitro* produced transferrrable embryos (Gasparrini et al., 2006, Manjunatha et al., 2009), the pregnancy rate achieved by transferring these structures remains poor (Gasparrini, 2002; Nandi et al., 2002a).

*In vitro* maturation (IVM) of oocytes from small antral follicles could reduce the need for exogenous gonadotrophin treatment and offer an alternative to hyperstimulation of ovulation during *in vitro* fertilization (IVF) (Yong-jie et al., 2009). Also, Yong-jie et al. (2009) showed that *in vitro* oocyte maturation is intended to stimulate the maturation of both the nucleus and the cytoplasm. Indices of the matured nucleus include breakdown of the nuclear envelop, segregation of chromosomes, and appearance of the first polar body. These changes are accompanied by a set of changes in the cytoplasm, such as nutrient accumulation and organelle redistribution. Maturation of the nucleus and cytoplasm synchronizes itself *in vivo,* whereas *in vitro* stimulation may only aid in the maturation of one or the other.

Oocytes maturation is the first and most critical step

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towards a successful *in vitro* production of embryos, but little information are available on *in vitro* maturation and fertilization of buffalo oocytes (Totey et al., 1991, 1992). Also, preliminary results obtained by various workers (Bacci et al., 1991; Chuangsoongneen and Kamonpatana, 1991; Lu and Hsu, 1990; Totey et al., 1992) on *in vitro* maturation and fertilization of buffalo oocytes reported poor results compared to cattle. During maturation, major changes took place in protein synthesis (Moor and Warnes, 1978; Crosby et al., 1981; Moor et al., 1981) and it has been proposed that such changes are essential for the continuation of cytoplasmic maturation (Thibault, 1977; Golbus and Stein, 1978; Moor et al., 1978). In addition to concomitant with nuclear maturation, cytoplasmic changes occur during oocyte maturation. At the ultra-structural level, cytoplasmic maturation encompasses morphological alterations in the distribution of organelles such as mitochondria, endoplasmic reticulum and cortical granules (Kruip et al., 1983; Blerkom and Bell, 1986; Loos et al., 1989). Ultra-structural changes in the oocyte during *in vitro* maturation have been studied in different mammalian species in detail [mouse (Merchant and Chang, 1971), human (Zamboni and Thomson, 1972) and cattle (Hyttel et al., 1997)]. Although, Ultra-structural studies on the oocyte during *in vitro* maturation in different mammalian species [mouse (Merchant and Chang, 1971), human (Zamboni and Thomson, 1972), cattle (Hyttel et al., 1997) and camel (Kafi et al., 2005)] have resulted in a better understanding of the biology of the oocyte and as a consequence, improvements in IVM and IVF. However, systematic studies on ultra-structure of buffalo oocytes during IVM have not been reported. Therefore, the objective of the present study was to describe the ultra-structure changes of Egyptian buffalo oocyte during *in vitro* oocyte maturation.

## MATERIALS AND METHODS

### Oocytes collection and in vitro maturation

The ovaries were removed directly from abdominal cavity of Egyptian buffaloes after slaughtering and maintained in a thermo flask containing saline solution (0.9% NaCl) mixed with 50 µg/ml gentamycin sulphate at 30 to 38°C and were transported to laboratory within 2 h. At the laboratory, the ovaries were washed three times with pre-warmed isotonic saline solution supplemented with gentamycin sulfate to exclude adhering blood and other increment tissues. Immature oocytes were recovered by aspiration of the follicles on the ovaries using a 10 ml sterile syringe and an 18 G disposable needle. The oocytes were classified into three categories on basis of the presence of cumulus mass and homogeneity of cytoplasm (excellent, good and denuded oocytes) as described by Loos et al. (1989, 1991). The collected oocytes were washed three times with maturation medium (TCM-199 + 10% FCS + 0.02 IU FSH / ml + 0.023 IU LH / ml + 50 µg Gentamycin sulfate / ml + 1 µg Estradiol 17 β/ ml for 24 h at 38.5°C in 5% $CO_2$, and 95% humidity). Oocytes were cultured in groups in 4–well sterile plastic Petri dishes. The culture dishes were incubated for 22 to 24 h at 38.5°C in 5% $CO_2$, and 95% humidity.

### Experimental design

A total of 200 buffalo oocytes were selected to study the ultra-structure of oocyte before and after *in vitro* maturation; half of the oocytes (50 good and 50 denuded oocytes) were studied before maturation while the other half examined were studied after maturation. This is in addition to 50 *in vivo* matured oocytes which had expanded cumulus cells, which were randomly selected to investigate some fine structures of oocytes. Oocytes matured *in vitro* were cultured in maturation medium, in order to determine the ultra-structure changes of oocytes after *in vitro* maturation.

### Preparation of oocytes for transmission electron microscopy (TEM)

The procedure performed to study the ultrastructure of oocytes before and after *in vitro* maturation was that of Zaki (2000). This procedure was established and obtained from Electron Microscope Laboratory, Faculty of Science, Ain Shams University, Egypt.

### Ultra-structure evaluation

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

## RESULTS

### Ultra-structure of Egyptian buffalo oocytes matured *in vivo*

Mitochondria were observed in a normal number and distribution in the cytoplasm. The hooded (H), and round (R) mitochondria (M) were observed (Figure 1a) but no pleomorphic mitochondria were observed throughout the cytoplasm. The most abundant form of endoplasmic reticulum (ER) appeared as associated with the surfaces of hooded mitochondria (HM). Also, *in vivo* matured oocytes showed low number of microvilli (Figure 1a) and the vesicles were located in close proximity to mito-chondria. The cortical granules (CGs) were arranged just inside the oolemma. Golgi apparatus (G), and oval mitochondria were also detected (Figure 1b). A large number of small to medium size vesicles was also observed containing varying amounts of electron-dense material. The lipid droplets were randomly distributed with vesicles (Figure 1c).

### *In vitro* maturation of Egyptian buffalo oocytes

### Ultra-structure of oocytes before *in vitro* maturation

**Good quality oocytes:** The result of this group revealed that the immature oocytes were characterized by the presence of non-expanded cumulus cells. Cumulus cells foot process endings extended from the cumulus cells through the zona pellucida. The mitochondria were located at the peripheral of the oocytes with a small number close to the center. They were,generally rounded

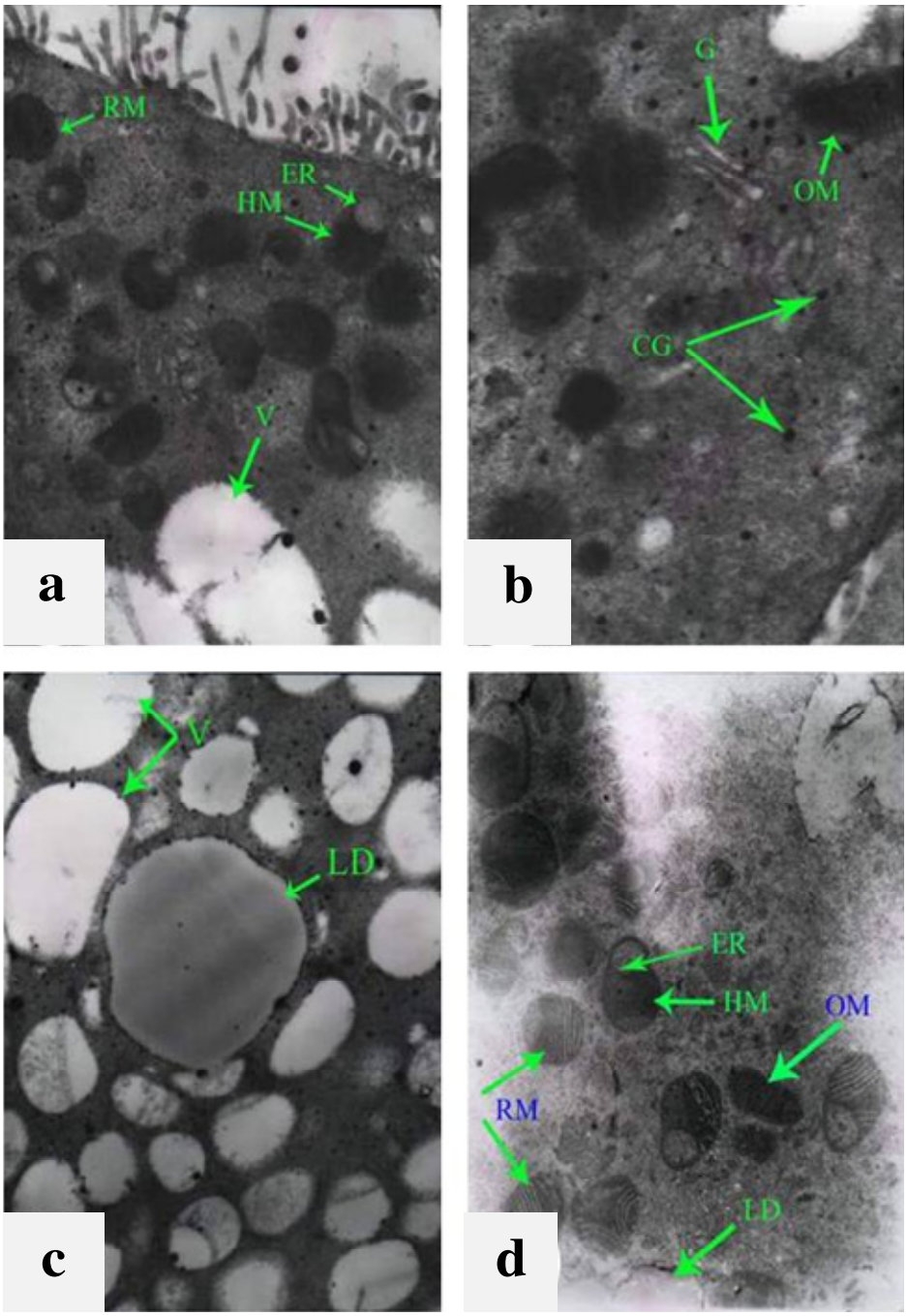


**Figure 1.** The electron micrograph (TEM) of *in vivo* matured Egyptian buffalo oocytes showing; a) Hooded mitochondria (HM), round mitochondria (RM), endoplasmic reticulum (ER) and vesicles (V); b) oval mitochondria (OM), Golgi apparatus (G) and cortical granules (CGs); c) lipid droplets (LD) and vesicles (V) and d) hooded mitochondria (HM), oval mitochondria (OM), round mitochondria (RM), lipid droplets (LD), and endoplasmic reticulum (ER).

hooded, or oval shaped. Pleomorphic mitochondria were not found in this group (Figure 1d). The lipid droplets were found mainly near the mito-chondria; they were small in size and number (Figure 1d). Endoplasmic reticulum (ER) were detected both in association with mitochondria and distributed throughout the ooplasm (Figure 1d). A large number of vesicles were distributed all over the oocyte except at the extreme periphery and large numbers of microvilli were found extended from the plasma membrane through foot

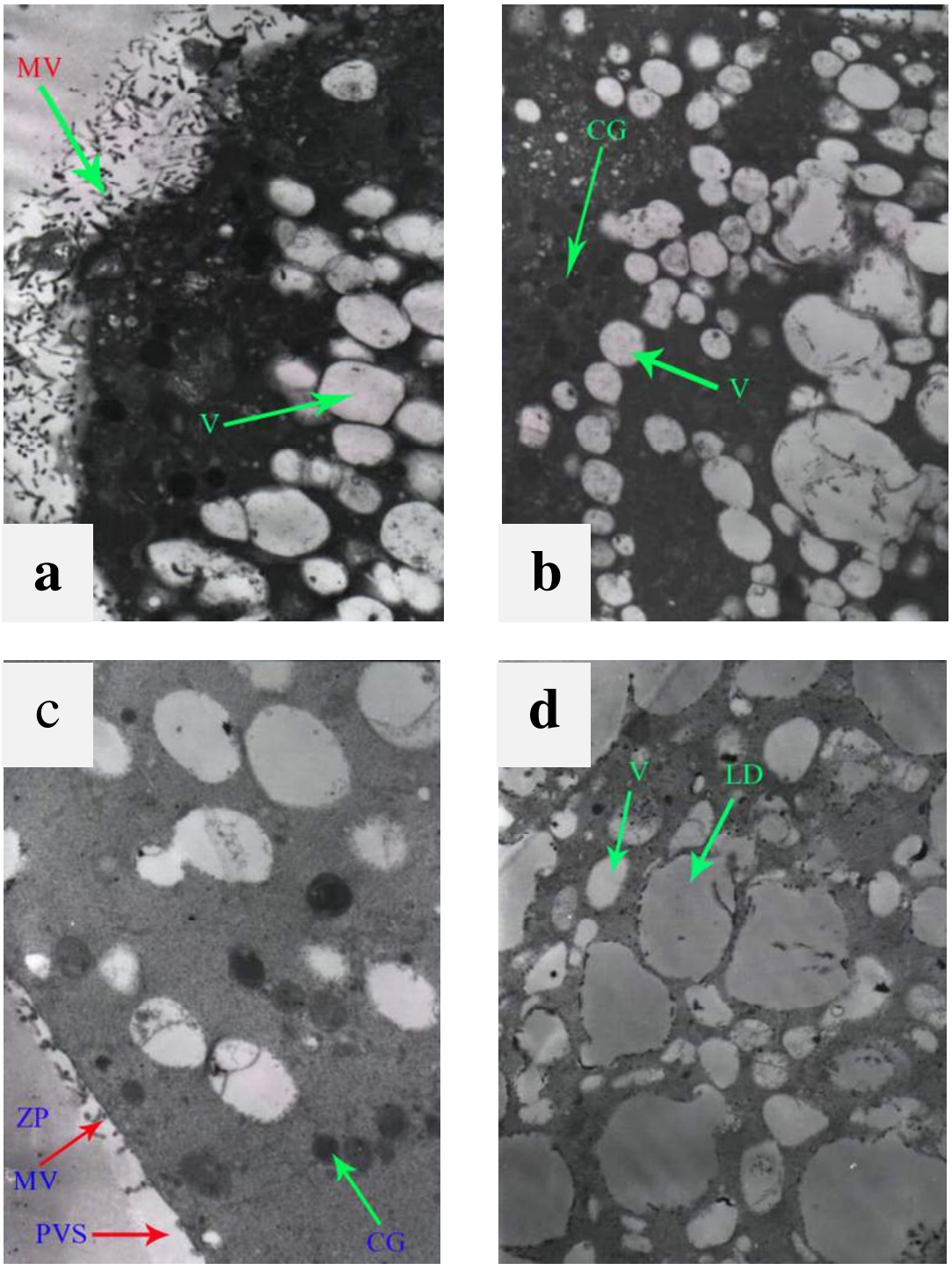


**Figure 2.** The electron micrograph of *in vivo* matured Egyptian buffalo oocytes showing; a) vesicles (V) and microvilli (MV); b) a large number of vesicles (V) and cluster of cortical granules (CG); c) microvilli (MV), cortical granules (CGs), perivitelline space (PVS) and Zona pellucida (ZP) and d) vesicles (V) and lipid droplets (LDs).

processes into zona pellucida (Figure 2a). Cluster of cortical granules were observed in the deep cortex of ooplasm (Figure 2b). Moreover, immature good oocytes had a very large number of vesicles as shown in Figure (2b).

**Denuded oocytes:** Ultra-structural observation of denuded immature oocytes showed an enlarged perivitelline space (PVS), less number of microvilli and some of them were degenerated and detachment of foot

processes. The populations of cortical granules were generally arranged individually or in small clusters of the oocytes. ZP showed irregularity fractures (Figure 2c). Large numbers and sizes of lipid droplets and empty vesicles were found and some of them contained electron dense materials. Also, the numbers of vesicles were very low (Figure 2d). While the hooded mitochondria were rarely observed, the rounded mitochondria were mainly seen in this group. Also, Golgi apparatus was seen in denuded oocytes (Figure 3a).

### Ultra-structure of oocytes after *in vitro* maturation

**Good quality oocytes:** In good matured oocyte observed in this study, the electron micrographs showed the ER entering and closely related to the hooded mitochondria (Figure 3b) as well as associating with the outer surface of other mitochondrial and cytoplasmic inclusions. Vesicles, and mitochondria observed in the ooplasm were related to well developed cisternae of ER (Figure 3c). Ultra-structurally, in mature good oocytes, the zona pellucida was composed of a moderately staining homogenous material (Figure 3d). Cumulus cell processes and microvilli arising from the oocyte were observed within the zona pellucida. The perivitelline space appeared as a lighter staining band between the zona pellucida and vitelline membrane, this band appeared as a flocculent layer easily differentiated from zona pellucida. The cumulus cells had numerous projections and a structure similar to that of the zona pellucida were observed between these projections and the oolemma. A perivitelline space was small with a limited number of microvilli lying with their long axis; the cortical granules were dispersed to solitary positions forming a closure to the oolemma (Figure 4a). The lipid droplets were peripherally located in the good oocytes and bound layer of endoplasmic reticulum, but randomly distributed in more mature oocyte (Figure 4b). The Golgli apparatus was clearly observed in aggregating groups more than that in the *in vivo* matured oocytes and oocytes before maturation (Figure 4c). Concerning the polar body, the group of *in vitro* matured oocytes showed a normal polar body formation, vesicles, and the mitochondria was dislocated towards the site of polar body (Figure 4d). *In vitro* matured oocytes showed clusters of cortical granules found in aggregates throughout the peripheral ooplasm just beneath the oolemma (Figure 5a).

**Denuded oocytes:** On the other hand, the denuded oocytes showed some abnormal mitochondria, extensive vacuolization, disappearance of cristae, dilated and pleomorphic shaped of mitochondria membrane, however, other mitochondria showed normal membrane and cristae which were clearly delineated (Figures 5b and c). Also, electron micrograph of denuded oocytes showed the vacuolated mitochondria which was not found in the other groups (Figure 4b). Moreover, denuded oocytes had low number of vesicles, microvilli and lipid droplets (Figures 4b and c). Cortical granules were absent in this group. In denuded oocytes, Golgi apparatuses were distributed in cytoplasm of the oocytes (Figure 5d). Abnormal polar body formation (lightly stained and considered to be degenerated was found in *in vitro* matured denuded oocytes with no appearance of mitochondria or vesicles (Figure 6).

## DISCUSSION

Available information about the ultra-structural changes in Egyptian buffalo oocytes after *in vitro* maturation is considered to be very limited. So, the results obtained in this study were compared to other related species. The low number of COCs collected probably might be due to the result of some peculiarities inherent to buffaloes, such as the reduced number of antral and preantral follicles, approximately ten times lower than in cattle (Drost, 2007; Mondadori et al., 2008). The results reveal that the mitochondria observed were similar to those of other domestic species, containing few cristae and frequently opposed vesicular of cisternal endoplasmic reticulum (Cran et al., 1980). Senger and Saacke (1970) had previously described the mitochondria in a study of bovine oocytes from tertiary follicles. It was apparent from their study that the membranes were actually continuous with the cisternae of the endoplasmic reticulum; ribosomes were occasionally associated with these cisternae. The close association of the cisternae with the outer surface of mitochondria appeared to be very similar to that reported by Fleming and Saacke (1972) in cattle oocytes. Also, Kruip et al. (1983) suggested that existence of mitochondrial clusters and vesicles were rearranged ~ 15 h after LH peak. Similar organelle rearrangements were reported in un-stimulated cattle as taking place more than 19 h after the LH peak (Kruip *et al.,* 1983). Hence, in un-stimulated cattle and stimulated cattle, these major organelle rearrangements of the oocyte occur with an increase in the progesterone dominance in the follicular fluid.

Immature COCs showed typical structure previously described for buffalo (Boni et al., 1992; Mondadori et al., 2008), as well as for bovine (Kacinskis et al., 2005; Nagano et al., 2006), ovine (O'Brien et al., 2005) and camel (Kafi et al., 2005) oocytes. Confirming previous observations (Mondadori et al., 2008), the most important difference observed between the species is the larger number of lipid droplets in buffalo ooplasm. The same sort of GC–oocyte junctions previously described for buffalo (Mondadori et al., 2008) and bovines (Fair and Hyttel, 1997) was also observed in some immature oocytes. It is well known that these junctions play an important role during oogenesis (Mondadori et al., 2007) and IVM in different species (Suzuki et al., 2000).

Similar mitochondrial migrations have been reported in

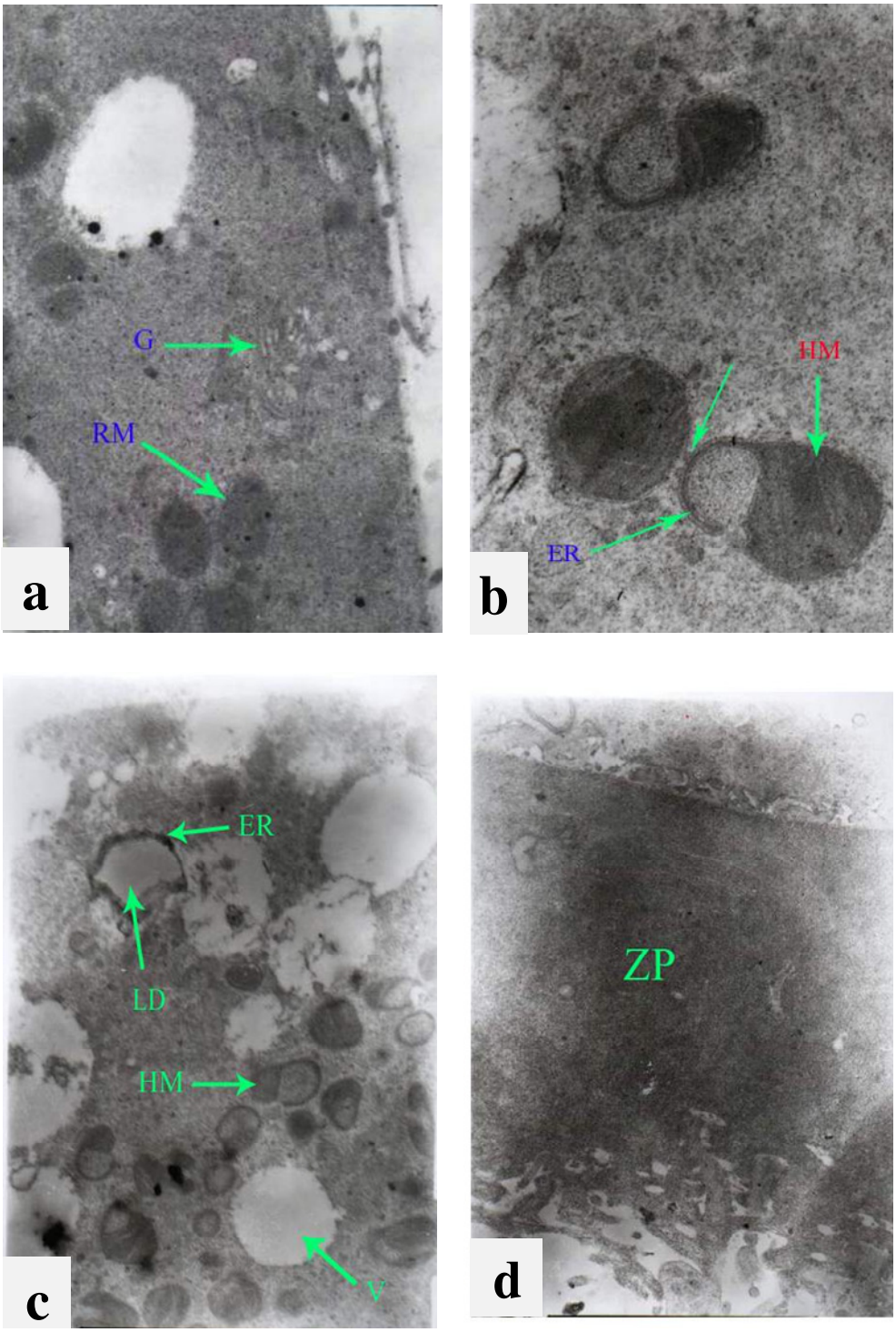


**Figure 3.** The electron micrograph of *in vivo* matured Egyptian buffalo oocytes showing; a) rounded mitochondria (RM) and Golgi apparatus (G); b) Endoplasmic reticulum (ER) entering and closely related to the hooded mitochondrial (HM) (indicated by arrow) as well as associating with outer surface of other mitochondrial and cytoplasmic inclusions; c) the mitochondria (M), clusters and spatial distribution of vesicles (V) and endoplasmic reticulum (ER) and d) the zona pellucida (ZP).

mice by Van Blerkom, and Runner (1984) who claimed this rearrangement as being essential for the pre-ovulatory maturation and suggested then to be necessary for elevated concentrations of adenosine triphosphate for

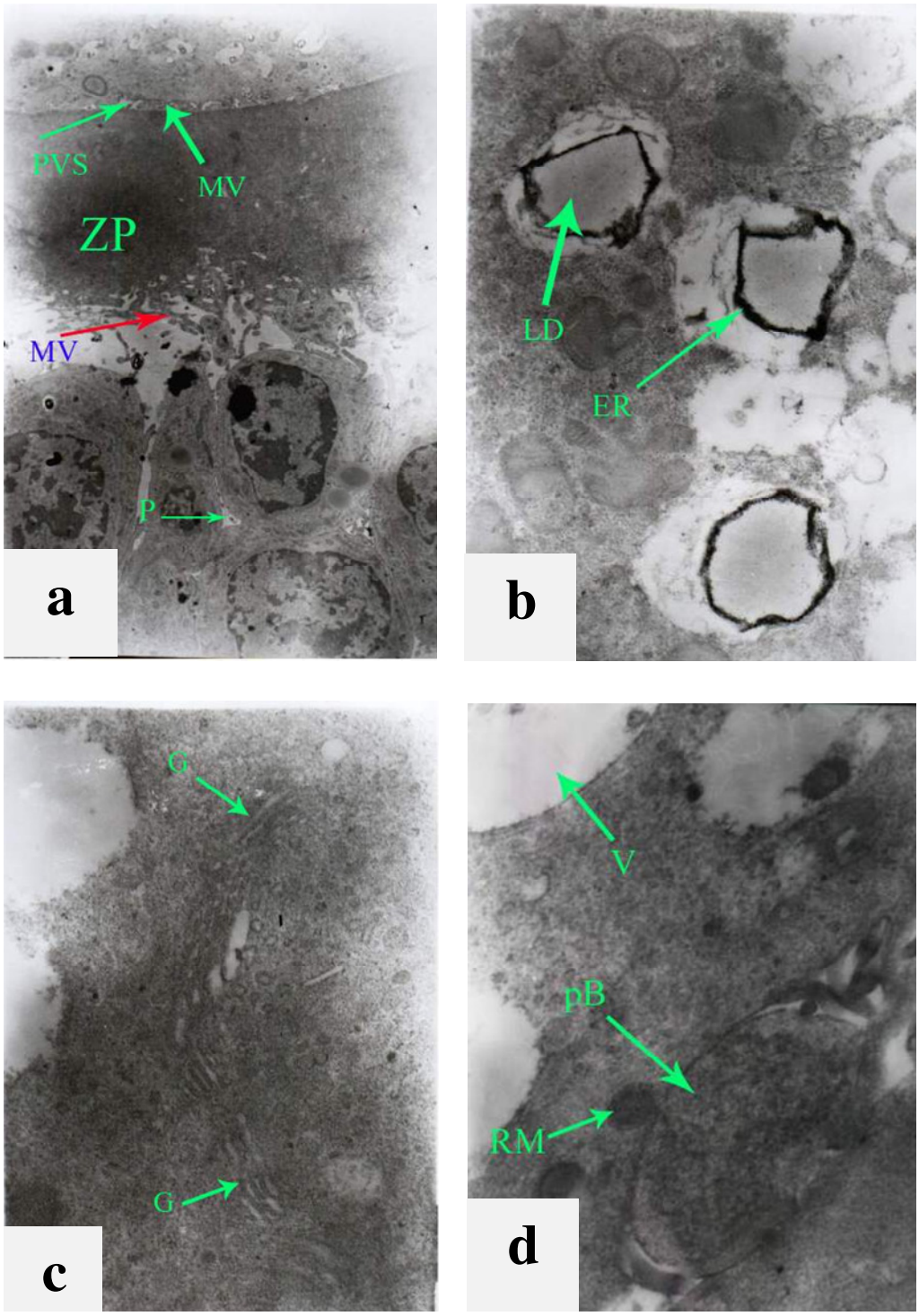


**Figure 4.** Electron micrograph of matured *in vitro* good oocyte of Egyptian buffaloes showing; a) granulosa cell processes associated with the surface of the ooplasm appear to be degenerating due to their very granular appearance. Pinocytotic vesicles (P) are apparent between cumulus cells. Numerous microvilli are seen; b) a lipid droplet (LD) and endoplasmic reticulum (ER); c) groups of Golgi apparatuses in cytoplasm and d) polar body (PB), vesicles (V) and mitochondria (M) which are dislocated towards the site of normal polar body formation.

localized activities in the ooplasm. The close association of the smooth endoplasmic reticulum to the inner surface of the hood also suggests that the hood may provide a specific microenvironment, facilitating the exchange of

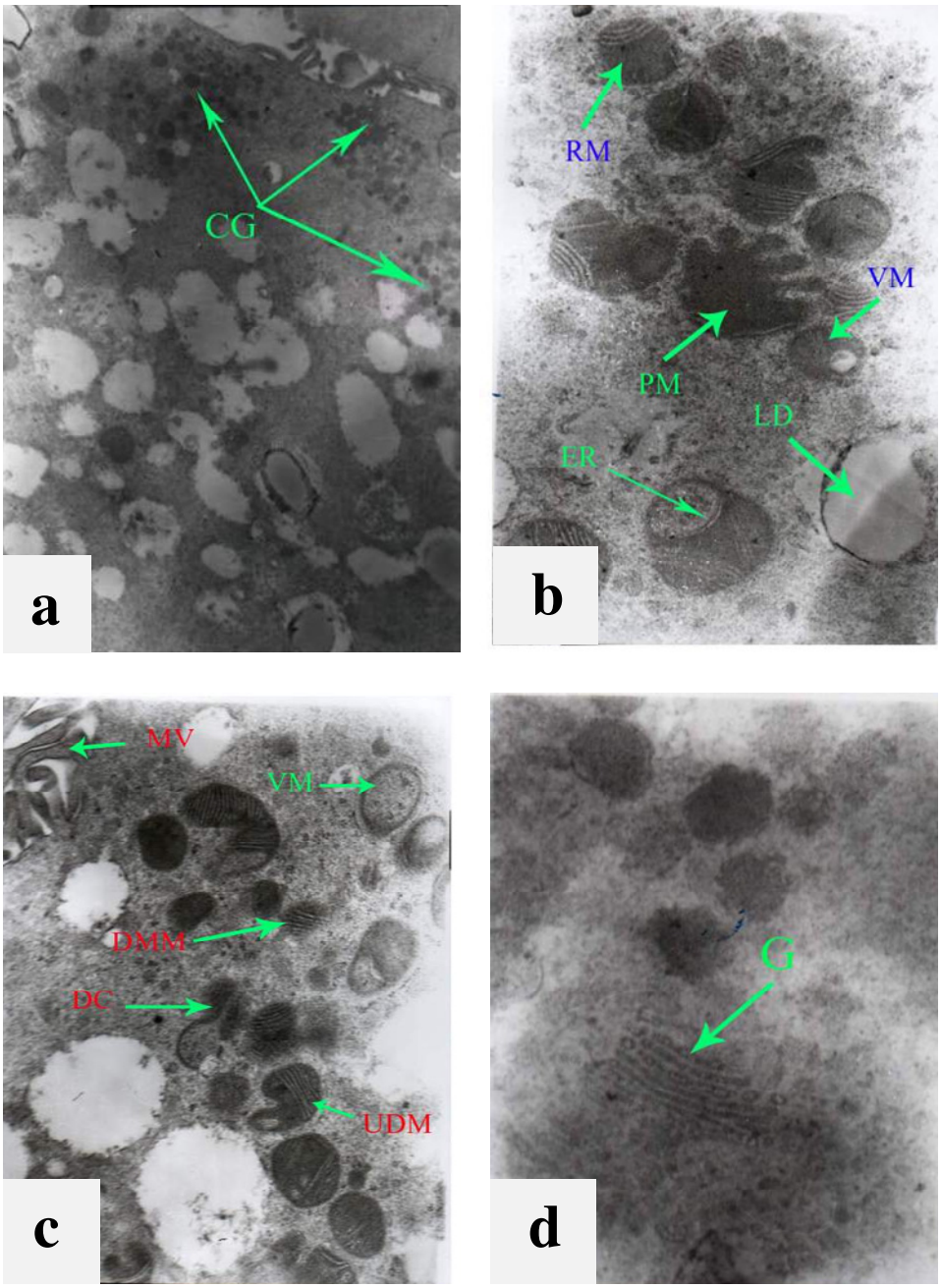


**Figure 5.** Electron micrograph of *in vitro* matured good oocyte of Egyptian buffalo showing; a) cluster of cortical granules (CG); b) abnormal mitochondria exhibit extensive vacuolization, disappearance of cristae and dilated and pleomorphic mitochondrial (PM) membrane and vacuolated mitochondria (VM) other mitochondria showing normal membrane are continuous and cristae are clearly existed; c) undulating mitochondrial membranes and deformed cristae (DC), distorted mitochondrial membrane (DMM), and extensive vacuolization (EV) disappearance of most of cristae and damage of the mitochondrial membrane and microvilli (MV) and d) groups of Golgi apparatus (G).

metabolic intermediates between mitochondria and endoplasmic reticulum. The highly closure of endoplasmic reticulum and mitochondria to the surface of lipid droplet suggests that the oocyte may be utilizing lipid store and could be important in providing nutrients for the final maturation of the oocyte (Kruip et al., 1983 and Fair et al., 2001), which can explain the success of good type in our results to reach maturation. On the other hand, degeneration or fusion and the observed dilation of the mitochondrial envelope may be an indication that some

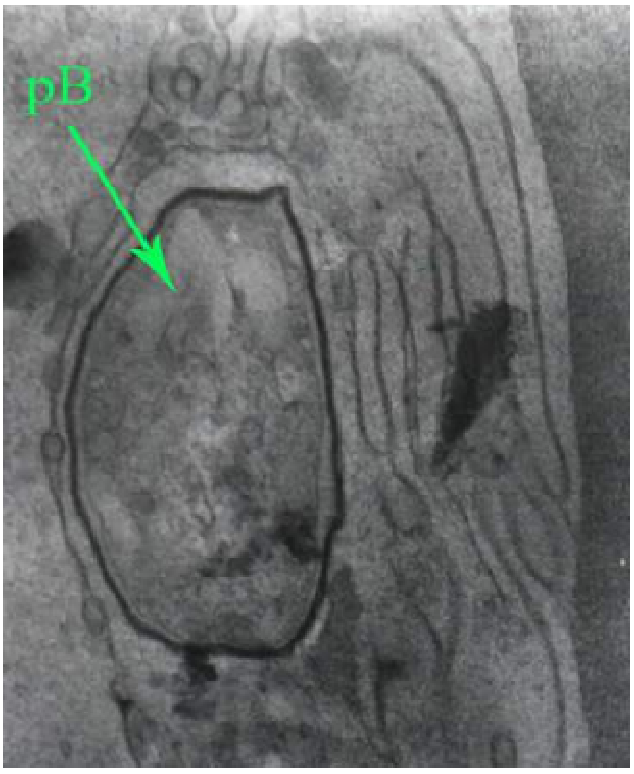


**Figure 6:** Electron micrograph of *in vitro* matured denuded oocyte of Egyptian buffalo showing abnormal polar body formation with no appearance of mitochondria or vesicles.

loss of mitochondria by degeneration was taking place, few markedly degenerate organelles was observed. It seems unlikely therefore that the reduction in number may be accounted for by this means (Kruip et al., 1983). Common feature of mitochondrial shape were seen in this study as showed by many authors such as pleomorphic mitochondria, hooded, and other shapes including dumbbell and cloverleaf shapes were noted, also vacuolated mitochondria described in compact bovine morulae (Crosier et al., 2000) were observed. Hooded mitochondria have been described previously as common in bovine oocytes (Senger and Saacke, 1970; Fleming and Saacke, 1972; Hyttel et al., 1987; Assey et al., 1994). Fleming and Saacke (1972) proposed that the hood served to increase surface area and facilitated transport from the endoplasmic reticulum to hooded mitochondria. They proposed that hooded mitochondria were a unique feature of ruminant oocytes as they were found also in oocytes from goats and sheep. Changes in mitochondrial morphology were observed in this study and at the same time the distribution of mitochondria became more clustered often in association with lipid droplets. These changes may represent a shift in oocyte metabolism from a dependence on the cumulus cells to a dependence on internal stores of energy sources and nutrients. Further studies focusing on oocyte mitochondrial function may yield interesting information about oocyte metabolism (Robert, 1999). Large aggregates of smooth endoplasmic reticulum (SER) surrounded by mitochondria are considered to be typical of *in vivo* matured oocytes 24 h after the LH peak (Gosing and Jonas, 1998). *In vitro* matured oocytes started to form such clusters at about 18 h of maturation and the aggregation was pronounced more than 24 h of *in vitro* maturation (Hyttel et al., 1986b). At that time it was considered to be sign of certain metabolic activities maintained throughout the culture period of cattle oocytes (Kruip et al., 1983). Generally, the main differences observed in follicles were cytoplasmic vesicles quantity, mitochondria shape and inner content, ZP deposition and granulosa cells–oocyte junctions (Mondadori et al., 2007). These morphological differences described could be responsible for some functional differences observed in *Bubalus bubalis in vitro* embryo production and follicular dynamics (Manik et al., 2002; Neglia et al., 2003; Mondadori et al., 2007). From the start of IVM, as a result of the resumption of meiosis, nucleus morphology changes and PVS grow, preparing to receive the polar body.

In most oocytes studied, metaphase stage II was achieved after 24 h of IVM period as shown by earlier reports (Nandi et al., 2002b; Gasparrini et al., 2008) but in contrast to Rafael et al. (2010).

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