

ULTRA-STRUCTURE STUDY OF MICROVILLI, MITOCHONDRIA AND LIPID DROPLETS OF EGYPTIAN BUFFALOES' OOCYTES WITH REFERENCE TO QUALITY

A. El-Sayed¹, Eitedal H. Elsayed², Dalia A. Ahmed¹, S.M. Salem¹ and A.H. Barkawi¹

1- Department of Animal Production, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt,

2- Animal Production Research Institute, Ministry of Agriculture, Dokki, Giza, Egypt

SUMMARY

The objective of this study was to describe and relate the ultra-structure of the micro-villi and mitochondria of buffalo oocytes as well as morphological aspects of lipid droplets with particular reference to oocytes' quality. Ovaries were collected from local slaughterhouse and transported to the laboratory within three to four h post-slaughter. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles of two to eight mm diameter using an 18-guage needle. A total number of 10 good and 10 denuded oocytes were selected and prepared to study the target structure using transmission electron microscopy.

Results indicated that good oocytes had large number ($P < 0.05$) of micro-villi, compared to that of denuded ones. In a few cases, micro-villi looked erected in good oocytes, while such cases were not clear in the denuded ones. The mitochondria were located at the periphery of the oocyte and a small number were found close to the center with round, hooded or oval shaped. The lipid droplets were found near to the mitochondria, they were small in size and number. The distribution of organelles in the cytoplasm of oocytes markedly differed between good and denuded oocytes.

Keywords: Buffalo, oocytes ultrastructure, mitochondria, microvilli, lipid droplets

INTRODUCTION

Buffaloes are considered one of the dual purpose domestic ruminants those have the potentiality for meat and milk production (Hinkovski, 1990). Applications of *in vitro* fertilization (IVF) technique was adopted during the last two decades to spread the distinguished genetic resources faster compared to the traditional breeding programs in farm animals (Camargo *et al.*, 2010). Hence, many trails were conducted to produce embryos from buffaloes (*Bubalus bubalis*) (Abbas, 1998, Abdoon *et al.*, 2001, Duran, 2000, Gasparrini, 2002 and Barkawi *et al.*, 2007) using *in vitro* technique. The main obstacles facing application of IVF are the low number of oocytes on their ovaries (Nandi *et al.*, 2002b) and unordinary follicular dynamics (Barkawi *et al.*, 2009), the quality of oocytes after harvesting (Rizos *et al.*, 2002 and 2003) and follicle size (Armstrong, 2001 and Nandi *et al.*, 2002a). After fertilization, ooplasm becomes the embryo cytoplasm, while the spermatozoon's participation in this process is minimal. For this reason, the quality of oocytes is a key factor in determination of the earliest steps of embryo development (Brevini-Gandolfi and Gandolfi, 2001) due to defects in oocyte maturation.

Selection of oocytes for *in vitro* culture based only on the morphological features is not enough to obtain better *in vitro* maturation

(IVM) and IVF (Blanco *et al.*, 2011). Factors affecting the 1st step of embryo development accumulated throughout the oogenesis period, but these factors are unknown.

Rare data are available to describe the cellular changes in bovine oocytes (Fleming and Saacke, 1972) and the fine structure of the bovine oocyte collected from the mature Graafian follicle during IVM process (Hyttel *et al.*, 1986).

Mondadori *et al.* (2010) described the ultrastructural changes in the nucleus and cytoplasmic organelles during IVM of buffalo cumulus-oocyte complexes. Also, Barakat *et al.* (2012) studied the changes in the cytoplasmic organelles of the buffalo oocytes before and after IVM.

As far as there are no reports discussing the competency of buffalo oocytes with particular reference to quality, the aim of this work was to compare the cellular changes (micro-villi and mitochondria in addition to lipid droplets) of good and denuded Egyptian buffaloes' oocytes during *in vitro* embryo production.

MATERIALS AND METHODS

Chemicals and media:

All chemicals, reagents and media, were purchased from Sigma-Aldrich Chemicals, Germany, unless otherwise mentioned. The final media for maturation were filtered (0.22 µm poor size, Durapure® membrane filter,

Ireland) and routinely equilibrated at 38.5°C with 5% CO₂ in humidified air for at least two h before using.

In vitro maturation of oocytes:

Collection of ovaries:

Ovaries (n=192) were collected from local slaughterhouses (El-Monieb and El- Warak) within three to four h after slaughtering of the animal. Then, immediately, transported to the laboratory in a thermo flask containing warm physiological saline (35-37° C, 0.9% sodium chloride solution, w/v), and supplemented with 50 µg/ ml gentamicin. In the laboratory, ovaries were rinsed three times in saline at 35°C and attached tissues were removed by using sterile scissors. Thereafter, ovaries were washed with ethanol (70 %) to remove any contamination on the surface of the ovaries before being placed in glass gars containing warm physiological saline (37-38° C) and kept in a water bath adjusted to 37° C.

Oocytes retrieval:

Cumulus-oocyte complexes (COCs) were aspirated from 2-8 mm diameter antral follicles using an 18-guage needle connected to a 10 ml disposable syringe containing one ml of phosphate buffer saline (PBS) according to Hammam *et al.* (2010). The follicular fluid containing oocytes was pooled into a sterile 15 ml plastic falcon tubes and maintained in a water bath at 38 °C for 5 - 15 min for COCs sedimentation. The precipitate was taken into a sterile petri dish (nine cm) for evaluation of recovered oocytes under a stereomicroscopy and classified as grade-A (GA) (oocytes with compact cumulus mass ≥ 5 layers and homogeneous ooplasm), grade-B (GB): (oocytes with compact cumulus with 3-4 layers of cumulus cells and homogeneous ooplasm), grade-C (GC): (oocytes with less compact cumulus with 1-2 layers of cells and less homogenous ooplasm) and grade-D (GD): (oocyte without cumulus cells, (denuded) or with a shrunk cytoplasm). A total of 20 buffalo oocytes were selected to study the ultra-structure of oocytes as 10 good (grade A and B) and 10 denuded oocytes (grade D) according to the classification described by Faheem (2004).

Preparation of oocytes for transmission electron microscopy (TEM):

Selected oocyte were transferred to a separate vial and fixed in 2.5% glutaraldehyde supplemented with 0.1 mol/L sodium phosphate buffer (pH 7.4) for one h, followed by washing the samples in sodium phosphate buffer three times for 30 min each. Then, the buffer was pulled out and 1% of osmium-tetroxide (OsO₄) was added to the tube and

incubated for 1.5 h at 4°C. Oocytes were dehydrated in ascending ethanol series of 30%, 50%, 70%, 80% and 95%, for 15 min each before exposing to 100% two times for 15 min each (Fritschy, 2010). Dehydrated oocytes were infiltrate with Spurr's epoxy resin, then sectioned (90 µm thick) with ultra-microtome (Leica model EM-UC6, Japan), mounted on copper grids (400 mesh). Sections were stained with Uranyl Acetate and Lead Citrate, and then allowed to dry. Stained sections were examined by transmission electron microscopy (JEOL (JEM-1400 TEM, Japan).

Measurements:

The following traits were measured as

- *Micro- villi:* The number of micro-villi in one micron was carefully computed. Whereas, their length (µm) was measured as the distance from the vitelline membrane to the top of the micro-villi
- *Mitochondria:* The total number of mitochondria in µm² was concisely computed. At the same time, the number of each of peripheral, round, hooded, elongated and oval mitochondria in µm² was determined.
- *Lipid droplets:* Number of the lipid droplets which appeared as polygonal or round shape as shown in Plate (3) was derermind. Meanwhile, the diameter of the lipid droplets (µm) was calculated as the sum of the two diameters divide by 2 as shown in Plate (3).

Statistical analysis

The obtained data were statistically analyzed by T- test using XLSTAT (2012).

RESULTS AND DISCUSSION

Micro-villi

It is well known that, micro-villi are found on the surface of many cell types, including the mammalian oocyte, where they are thought to act in initial contact of sperm and oocyte plasma membranes. Images of sperm–oocyte fusion, from both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies, have implicated oocyte micro-villi in the fusion process (Runge *et al.*, 2007).

In the present study, oocytes with good quality had a large number ($P < 0.05$) of micro-villi compared to that of denuded ones by about 1.7 folds (Table 1 and Plate 1). Microvilli in few cases of good quality oocytes were erected and a narrow perivitelline space was observed. Some of the denuded oocytes were degenerated and showed a large perivitelline space (Table 1 and Plate 1).

The present findings are in agreement with those obtained by Majerus *et al.* (2000), Salamone *et al.* (2001) and De Paz *et al.* (2001). They found that cow oocytes with more microvilli on their cell surface and more endocytic vesicles showed more blastocyst rate. This is confirmed by Runge *et al.* (2007) who reported that proper amount and positioning of microvilli on the oocyte surface is determinant for the initial contact and fusion of the fertilizing sperm with the oolemma.

Mitochondria:

Mitochondria are the energy supplying organelles, whose functional integrity is essential for cellular survival and development. Mitochondria in the oocyte can provide adenosine triphosphate (ATP) for fertilization and preimplantation embryo development and they can act as stores of intracellular calcium (Ca) and proapoptotic factors as well (Torner *et al.*, 2004)

Recent studies have shown that mitochondrial dysfunctions, such as the structural, spatial and genetic abnormalities in the oocyte, may influence normal embryo development, so mitochondrial characteristics and other mitochondrion-related changes can serve as signs of oocyte quality (Wang *et al.*, 2009).

The current results illustrated that in good buffaloes oocytes, mitochondria in some cases were located at the peripheral of the oocyte with a small number close to the center. This is in the contrast with the denuded oocytes, where mitochondria are concentrated in the middle of oocytes (Plate 2).

This is in agreement with that obtained by Stojkovic *et al.* (2001) who found that immature oocytes, staining with MitoTracker green revealed mitochondrial clumps in the periphery of the cytoplasm, with a strong homogenous signal in good oocytes. In addition, it has been stated that, high-polarized mitochondria are commonly seen in the pericortical cytoplasm of oocytes and early embryos, which may be associated with their functions in metabolic regulation including ATP production and calcium homeostasis (Van Blerkom *et al.*, 2003). Low-polarized mitochondria are associated with abnormal embryos (Wilding *et al.*, 2001). Moreover, Hyttel *et al.* (1997) reported that distribution of mitochondria throughout the cytoplasm in the periphery of oolema is characterized in the immature oocytes, which agree with what observed in the good oocytes in the present study.

The present result is in contrary with that obtained by Bavister and Squirrell (2000) in mice. They found that normal accumulations of mitochondria around the nuclei was indicator of competent oocytes compared to the abnormal mitochondrial distribution, in which mitochondria are randomly distributed and clumped. This observation suggests that there could be subtle differences between species in the localization of mitochondrial function and these differences might not be detected by observing the physical location of the mitochondria.

Data in Table (1) indicate that there were no significant difference between good and denuded oocytes regarding the total number and type of mitochondria (round, hooded, oval and elongated shapes). However, the values of good oocytes were higher relative to denuded ones (Table1). In addition, hooded mitochondria were present with a distinct finger-like appendage throughout the ooplasm in the denuded oocytes (Plate2).

Common feature of mitochondrial shapes observed in the present study is in agreement with the findings of Senger and Saacke (1970), Hyttel *et al.* (1987) and Assey *et al.* (1994) in bovine oocytes and Motta *et al.* (2000) in human oocytes.

Fleming and Saacke (1972) proposed that hooded mitochondria were a unique feature in ruminant's oocytes.

Lipid droplets:

Lipids (neutral lipids such as triacylglycerols or cholesteryl esters) are always present in the cytoplasm of mammalian oocytes where they serve as energy depots for the developing early embryo energy (Kikuchi *et al.*, 2002).

Lipid droplets in good oocytes looked smaller in size and less in number compared to denuded ones. In good oocytes, lipid droplets were observed near to the mitochondria, while those in denude oocytes were distributed in the cytoplasm. Number of lipid droplets / μ^2 were higher in denuded oocytes than good ones by 1.5 times, while the diameter of lipid droplets was longer by 1.4 times (Table 1 and Plate 3).

Our results are in agreement with those of Hyttel *et al.* (1997) and Sturmey *et al.* (2006) who found that distribution of small lipid droplets in the periphery of the cytoplasm being characteristic of immature oocytes. Abe *et al.* (2002) and Leroy *et al.* (2008) stated that excessive lipid accumulation in oocytes reduces their quality, developmental competence and cryotolerance. Moreover, accumulation of cytoplasmic lipid deposits (as

lipid droplets, membrane-bounded vesicles or membrane-bound vacuoles) leads to misdistribution affecting oocyte maturation and quality (Adamiak *et al.*, 2006 and Leroy *et al.*, 2008), as it had been seen in heifers of the Swedish Red breed.

In addition, the relative occupancy of osmophilic lipid droplets in the cytoplasm was higher in oocytes of bad quality compared with good ones (Awasthi *et al.*, 2010). Such accumulation of lipid droplets may imply increase sensitivity to oxidative stress, hinder cytoplasmic maturation and lead to sub fertility. In contrary with these results, Jeong *et al.* (2009) stated that, lipid rich oocytes were shown to possess greater developmental competence in bovine.

GENERAL DISCUSSION

Describing the localization of mitochondria and lipid droplets, our results showed that, in good oocytes lipid droplets were observed near to the mitochondria, while in denuded oocytes they were distributed in cytoplasm. This is in agreement with the result recorded by Homa *et al.* (1986) who found that the majority of oocytes lipids were found in the form of triglyceride (TG) which are metabolized by β -oxidation and the tricarboxylic acid (TCA) cycle within the mitochondrial matrix. For this to occur, mitochondria and lipid droplets, the primary source of TG, should ideally reside in close proximity. This has been observed in oocytes of cattle (Kruip *et al.*, 1983 and Hyttel *et al.*, 1986) and pig (Sun *et al.*, 2001), where mitochondria and lipid droplets were seen to associate, forming 'metabolic units' which tend to accumulate at the edge of the oocyte, thus ensuring a steady and readily accessible supply of O_2 for oxidative processes.

Collectively, changes in mitochondrial morphology and distribution as well as lipid droplets mean a shift in oocyte metabolism from cumulus cells dependency to a dependence on stored energy sources and nutrients (Fleming and Saacke, 1972).

Present results have added more insights into the ultra-structure of immature oocytes in buffalo. The results confirm the rare studies which have described previously in buffaloes' (Mondadori *et al.*, 2010 and Barakat *et al.*, 2012).

However, further studies are needed to find out the relation between cellular components and molecular characteristics of oocytes with different quality based on phenotype 0

In conclusion, results indicated that, cellular components (number and distribution of micro-villi, mitochondria and lipid droplets)

have a close relation to oocyte quality in the Egyptian buffaloes.

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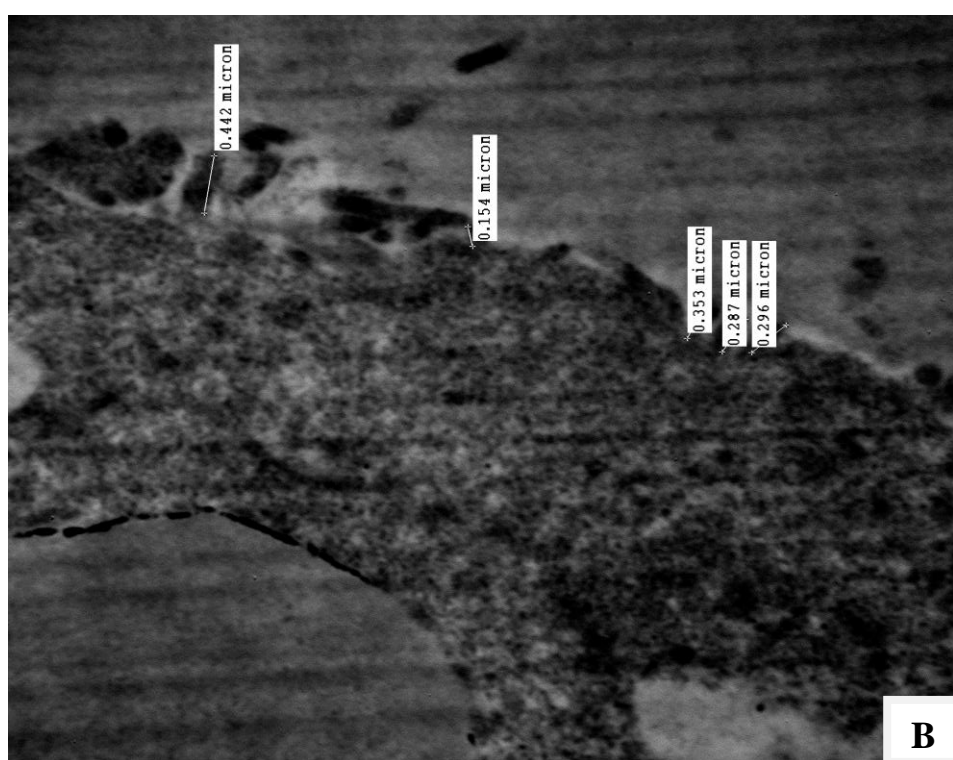
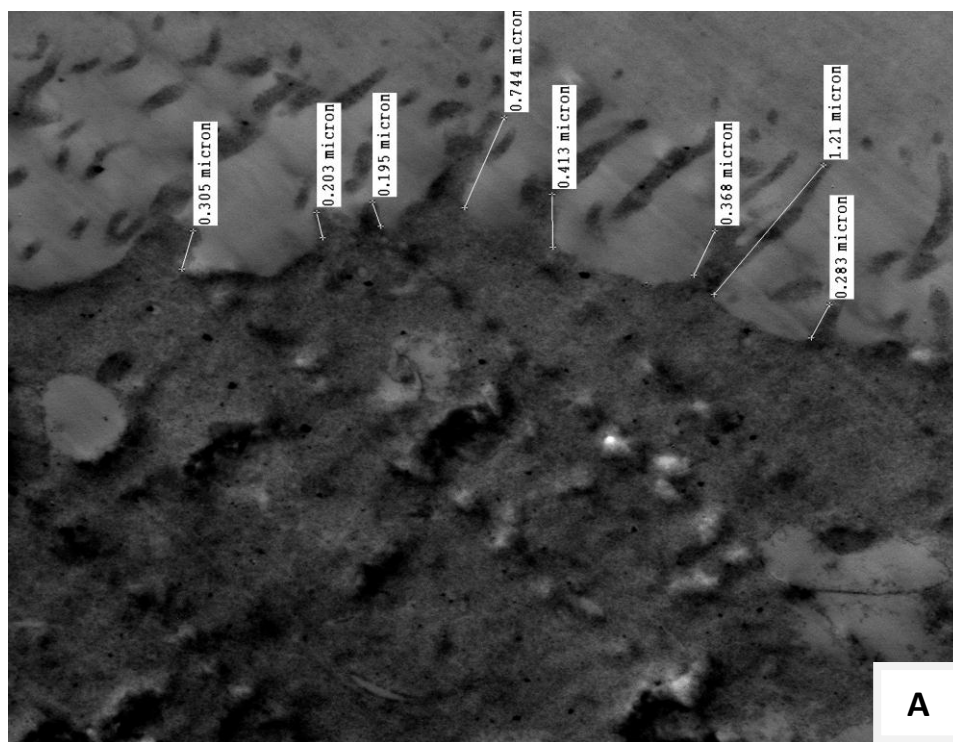


Plate1. Electron micrograph of micro-villi (MV) in good Egyptian buffalo oocytes (A) and denuded one (B), (X 15000)

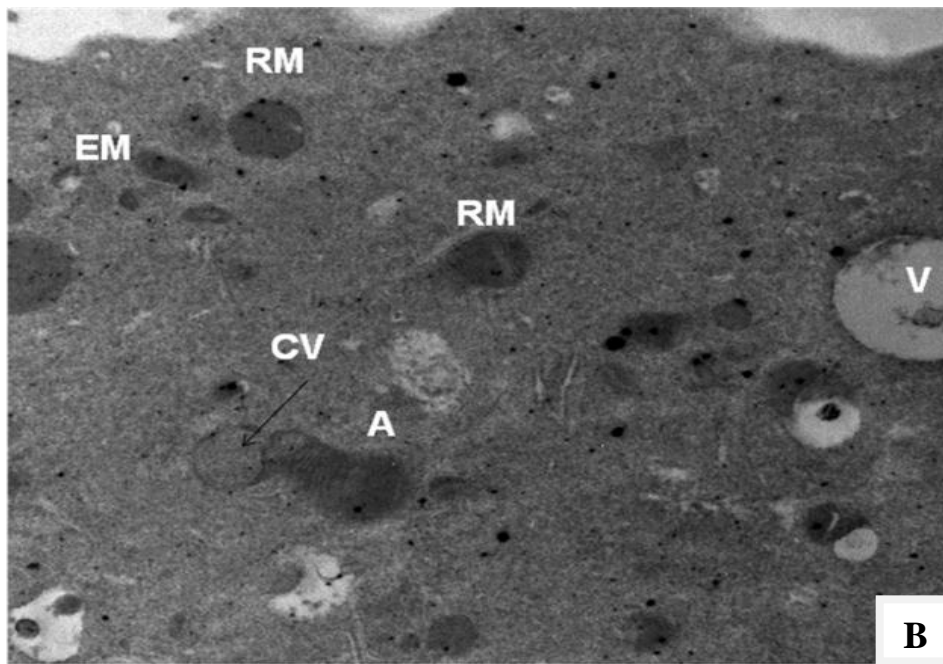
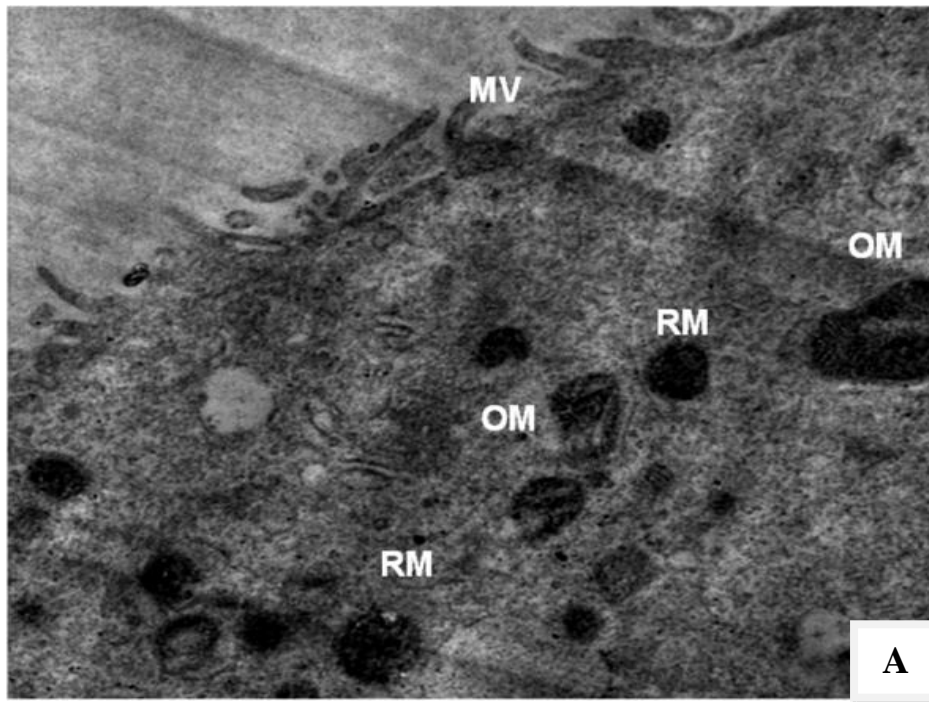


Plate 2.Electron micrograph of Egyptian buffalo oocytes. good oocytes (A) round mitochondria (RM), oval mitochondria (OM), micro villi (MV), denuded oocytes(B), A mitochondrion presents with a distinct finger-like appendage (A); a cytoplasmic vesicle (CV) lies within the cavity formed by the hood, elongated mitochondria (EM), vesicles (V) ,(X 15000)

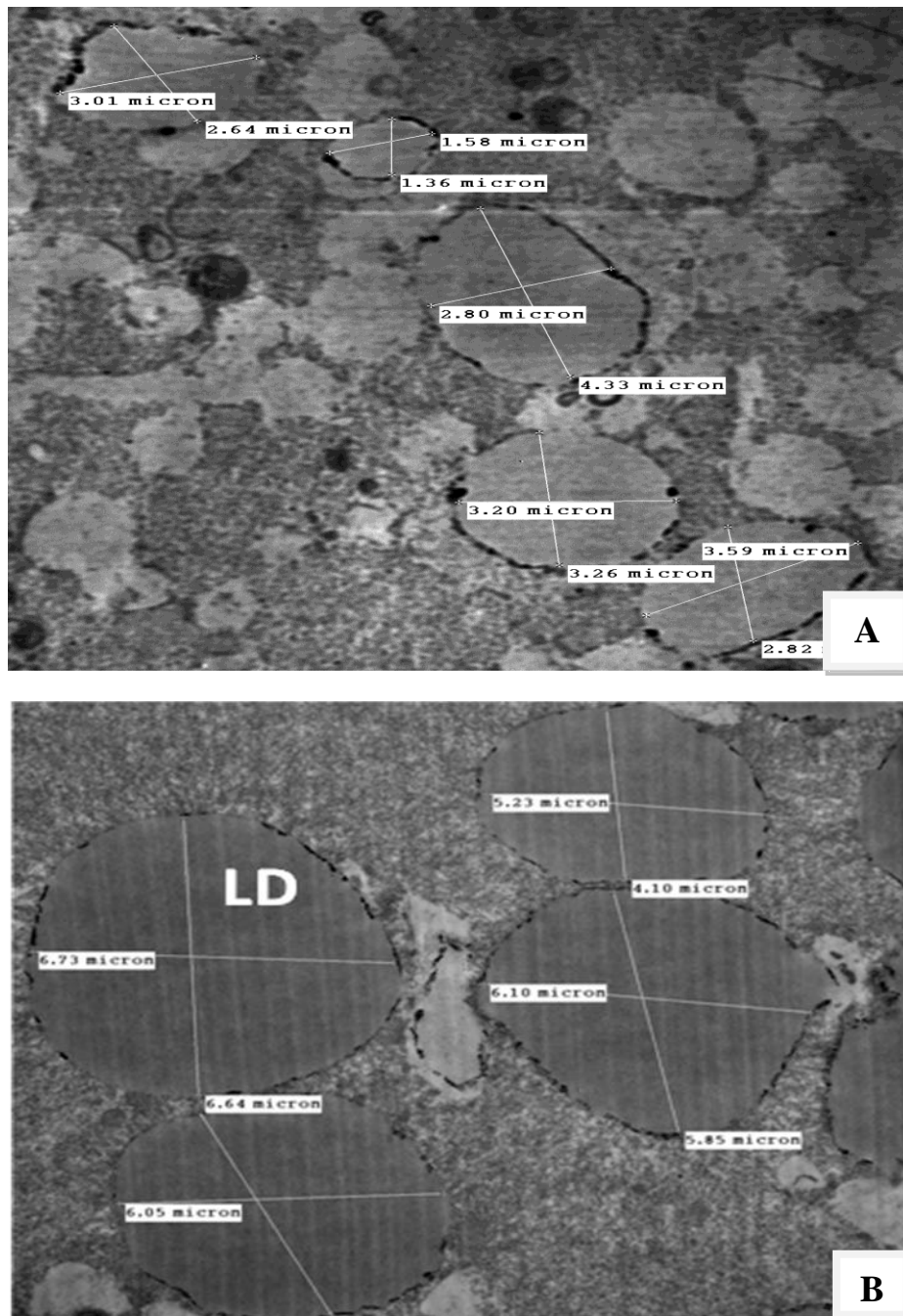


Plate 3. Electron micrograph of Egyptian buffalo oocytes showing lipid droplets (LD) in good oocytes (A) and in denuded oocytes (B), (X 3000)

Table 1. Ultra-structure measurements (Mean±SE) of microvilli, mitochondria and lipid droplets of good and denuded Egyptian buffaloes' oocytes (n=10 /group)

Trait	Good oocytes	Denuded oocytes
Zona pelucida		
Thickness (μm)	3.05*±0.061	5.85±0.23
Previtalline space (μm)	-	15±3.11
Micro-villi		
Length (μm)	0.42 ±0.04	0.36± 0.04
Number/ micron	1.18* ±0.29	0.7±0.11
Mitochondria		
Number of Mitochondria / μm ²	9.75± 1.19	7.50 ± 1.59
Number of peripheral mitochondria/ μm ²	5.12± 2.20	3.0 ± 1.90
Number of round mitochondria/ μm ²	5.87± 1.14	4.50 ± 0.90
Number of hood mitochondria/ μm ²	1.50 ±0.60	1.3±0.46
Number of elongated/ μm ²	0.62±0.26	0.62±0.33
Number of oval mitochondria/ μm ²	1.75±0.41	1±0.5
Lipid droplets		
Number of lipid droplets / μm ²	3.28±0.71	4.89±0.26
Diameter of lipid droplets	3.34 *±0.18	4.76±0.22

دراسة التركيب الدقيق للخمالات والميتوكوندريا وحببيبات الدهن وعلاقتها بجودة البويضات في الجاموس المصري باستخدام الميكروسكوب الإلكتروني

أشرف عبد الحليم السيد¹، إعتدال حسن السيد²، داليا عبد الرحمن أحمد¹، سالم محمد سالم¹، أشرف هشام برقأوي¹

1- قسم الإنتاج الحيواني، كلية الزراعة، جامعة القاهرة، 12613، جيزة، مصر، 2- وزارة الزراعة، معهد بحوث الإنتاج الحيواني، الدقي، جيزة، مصر

الهدف من هذه الدراسة هو وصف الخمالات والميتوكوندريا وحببيبات الدهن تبعا لجودة البويضات باستخدام الميكروسكوب الإلكتروني .

تم جمع المبايض من مجزر المنيب والوراق ونقلها الي المعمل خلال 3- 4 ساعات بعد الذبح حيث تم استخلاص البويضات من حويصلات مبيضية قطرها 2- 8 مم باستخدام سرنجة 10 مم.

في هذه الدراسة تم مقارنة 10 بويضات جيدة التقييم من حيث السيتوبلازم والخلايا الحبيبية المحيطة بها مقابل 10 بويضات أخرى غير جيدة من حيث التقييم للدراسة وذلك باستخدام الميكروسكوب الإلكتروني النافذ.

اشارت نتائج الدراسة الى وجود فروق معنوية في اعداد وتوزيع العضيات المختلفة داخل البويضة طبقا لجودتها. حيث زاد عدد الخمالات وطولها في البويضات الجيدة عن البويضات الغير جيدة.

بالرغم من وجود اختلافات في اعداد الميتوكوندريا بين البويضات الجيدة والغير جيدة إلا أن هذه الفروق لم تكن معنوية في حين ان توزيع الميتوكوندريا تركز في الاجزاء الطرفية في البويضات الجيدة. كما اظهرت النتائج زيادة قطر حببيبات الدهن في البويضات الغير جيدة في حين ان توزيع هذه الحبيبات في البويضات الجيدة تركز حول الميتوكوندريا وباعداد قليلة وصغيرة الحجم.

من هذه الدراسة يمكن إستخلاص ان المكونات الخلوية للبويضة (الخمالات والميتوكوندريا وحببيبات الدهن) من ناحية العدد والتوزيع يرتبط ارتباطا وثيقا بجودة البويضات في الجاموس.