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# Light and transmission electron microscopy on immature rabbit oocyte: A morphological study

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**ABSTRACT**

To succeed in *in vitro* maturation, *in vitro* fertilization, and to detect any molecular and morphological changes in the rabbit oocyte ultrastructure, the characteristics of immature rabbit oocytes must first be described. The present study aimed to define in detail the ultrastructure of the immature rabbit oocyte as a specific biological model. Oocytes were collected by scraping the ovarian follicles of rabbits. A total of 20 ovaries were used in this study, from which 850 oocytes were obtained. The excellent and good-quality cumulus-oocyte complexes (COCs) were prepared and analyzed by light and transmission electron microscopy (TEM). The zona pellucida (ZP) is completely located around the oocyte and many round cumulus cells surrounded it. The oocyte was determined by a nucleus in the germinal vesicle stage and the ooplasm containing subcellular organelles such as smooth and rough endoplasmic reticulum, mitochondria, Golgi complexes, vacuoles and lipid droplets. The ultrastructure of rabbit oocytes is morphologically similar to that of other mammalian oocytes; however, there are slight differences that exist between them.

**Keywords:** Rabbit, ovary, oocyte, transmission electron microscopy.

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## INTRODUCTION

To assay, ultrastructural changes of subcellular organelles in the cytoplasm and nucleus of oocytes occur in maturation, fertilization, and zygote formation, it is necessary to observe the structure of immature oocytes. TEM is the recommended valuable tool to evaluate the cell ultrastructure at an organelle and sub-organelle level of mammalian oocytes (Goldsmith and Miller, 2009; Sugawara et al., 2021). The TEM identifies and provides essential information of physiological and pathological significance that was not detected by other methods (Goldsmith and Miller, 2009). In TEM, a beam of electrons passes through ultra-thin sections (60 to 90 nm) made of the sample in the resin block, and the image is formed (Sugawara et al., 2021). Unfortunately, such efforts have limited significance because there are many issues with this technique. The tool is very expensive and laborious, and preparing an oocyte is a complicated process and requires high skill, time and concentration. All of the chemical materials required for fixation and embedding are toxic (Glauert and Lewis, 1998). In addition, one of the important challenges is the small size of the sample for manipulation and the loss and damage of so many oocytes during preparation (Tizro et al., 2019). The sample preparation process for TEM is very long and all stages must be done under a stereomicroscope and fume hood (Kovács, 2015). Ultramicrotome is a quite sensitive, precise, delicate and focused device. The oocytes were harmed in trimming and ultra-thin sectioning. It may tear and fold sections during cutting and some of the important parts of sections may fall on the mesh bars.

Despite all the issues mentioned several previous studies have described the ultrastructure of mammalian oocytes for instance humans (Trebichalská et al., 2021), mice (Merchant and Chang, 1971), sheep (Cran et al., 1980), Cattle (Hyttel et al., 1990) and goats (De Smedt et al., 1994). This study aimed to explain the ultrastructure of the nucleus and cytoplasm of the oocytes and cumulus

cells of immature rabbits, which have not been reported yet. The ovaries of immature rabbits (New Zealand) were punctured and COCs were recovered from Graafian follicles and analyzed by TEM.

## MATERIALS AND METHODS

This animal experimental study was done on 10 New Zealand adult white female rabbits, weighing 3 ± 0.3 kg and aged 4 to 4.5 months, and kept in standard conditions (12-h dark and 12-h light, 20 to 24°C and free access to food and water), and 40 to 60% humidity.

The ovaries were obtained from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Sciences. This study was approved by the ethics committee of Shiraz University of Medical Sciences (IR.SUMS.REC. 1396.S1013).

The ovaries, 30 min after being sacrificed, were transported to the laboratory in 0.9% of normal saline at 32 to 36°C using the "slicing" technique. The freshly COCs were recovered from Graafian follicles, with an insulin needle under the stereomicroscope. COCs were washed in warm normal saline then excellent and good quality COCs were selected. COCs were processed for TEM according to this procedure:

The oocytes fixation was performed in 2.5% glutaraldehyde for 2 h, rinsed 3 times (15 min) in sodium cacodylate buffer 0.1 M (pH 7.4), post-fixed in 1% osmium tetroxide in darkness (1 h), and rinsed again 4 times with the same buffer (15 min). Then dehydrated by ascending series of ethanol and finally three times 100% ethanol. Each oocyte was separately infiltrated with ethanol-resin (3:1, 1:1, 1:3, pure resin) by a commercial kit (AGAR, R1031), and embedded in epoxy resin. After trimming, sectioned by an ultra-microtome (OPTICAL RICHERT, OMU3). Semithin sections (1 µm thick) stained with toluidine blue (2%), and examined by light microscopy and photographed using a digital camera. After re-trim, ultra-thin sections (60 to 90 nm thick) were cut with a diamond knife, and mounted on 200 mesh grids. Then copper grids contrasted with uranyl acetate followed by lead citrate. The sections were observed and photographed by TEM (PHILIPS, CM10, and the Netherlands) operating at 80 kV.

To assess the morphology of the immature rabbit oocytes the following criteria were evaluated by LM and TEM: general features, type and quality of the organelles throughout the ooplasm, the compactness of cumulus cells, the integrity of the oolemma and ZP, and cumulus cell projections, and nucleus.

### Ethical consideration

The ethic committee of Shiraz University of Medical Sciences approved this study (IR.SUMS.REC 1396. S1013). All of the ethical guidelines for working with laboratory animals were covered.

## RESULTS AND DISCUSSION

The light microscopy and TEM allowed analysis of size, shape and organelle distribution and cumulus cells in immature rabbit oocytes.

### Light microscopy

Examination of semithin sections (1 µm) showed that most immature rabbit oocytes were spherical (90%) and others were oval. The oocytes were surrounded by multilayered compact cumulus cells that were in close contact with zona pellucida (ZP). The cumulus cells contain normal features pattern of cytoplasmic organelles and due to electron-dense heterochromatin, many of the nuclei were dark. The ZP was a distinct and uniform transparent layer in most oocytes and was located around them with a similar thickness. The perivitelline space (PVS) between ZP and the oolemma was often narrow. The ooplasm had a homogeneous appearance and was divided into a cortical part determined by many clusters of organelles, and a central part determined by a large number of lipid droplets and vesicles (Figure 1).

### Transmission electron microscopy

An eccentric spherical nucleus with a bilayer membrane in peripheral the ooplasm containing the number of nucleoli was clearly observed (Figure 2A).

The numbers of longitudinal, oblique and horizontal cumulus cells processes (CCP) were viewed across the ZP (Figure 2B). The cumulus cells communicate with each other and with the oocyte. The cumulus cells nuclei are irregular with indentations and there are round mitochondria, and vesicles in their ooplasm (Figure 2F).

The most prominent subcellular organelles aggregations were different sizes and shapes of vacuoles and lipid droplets. In addition, the lipid droplets were smaller and darker than the vacuoles. The vacuoles were generally empty, surrounded by a membrane often interrupted and associated with granules. Associated with vacuoles, mitochondria were observed. The mitochondria in different sizes and shapes, which were most spherical, both individually, and as groups distributed throughout the ooplasm. However, the cristae were not clearly visible (Figure 2B, C, D, E).

Some vesicular rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), Golgi complexes and a variable number of vesicles were observed. The

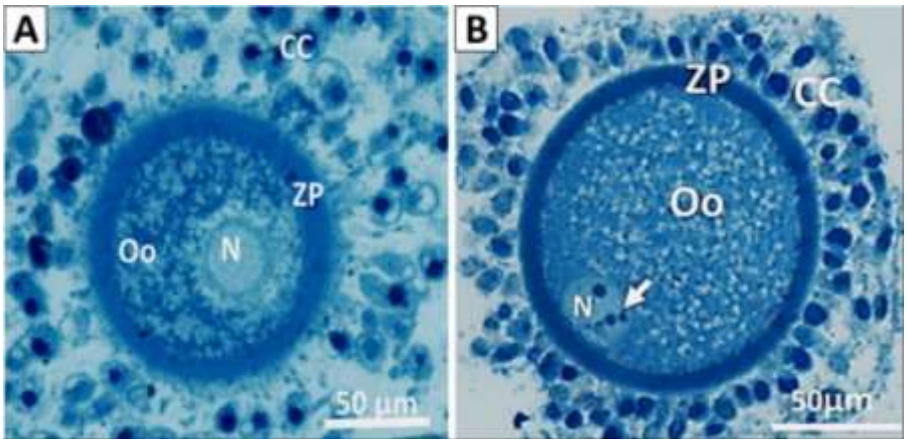


**Figure 1**. Light micrograph of a semithin section of immature rabbit oocyte stained with toluidine blue, showing cumulus cells (CC), zona pellucida (ZP), ooplasm (Oo), nucleus (N), and nucleolus (arrows).

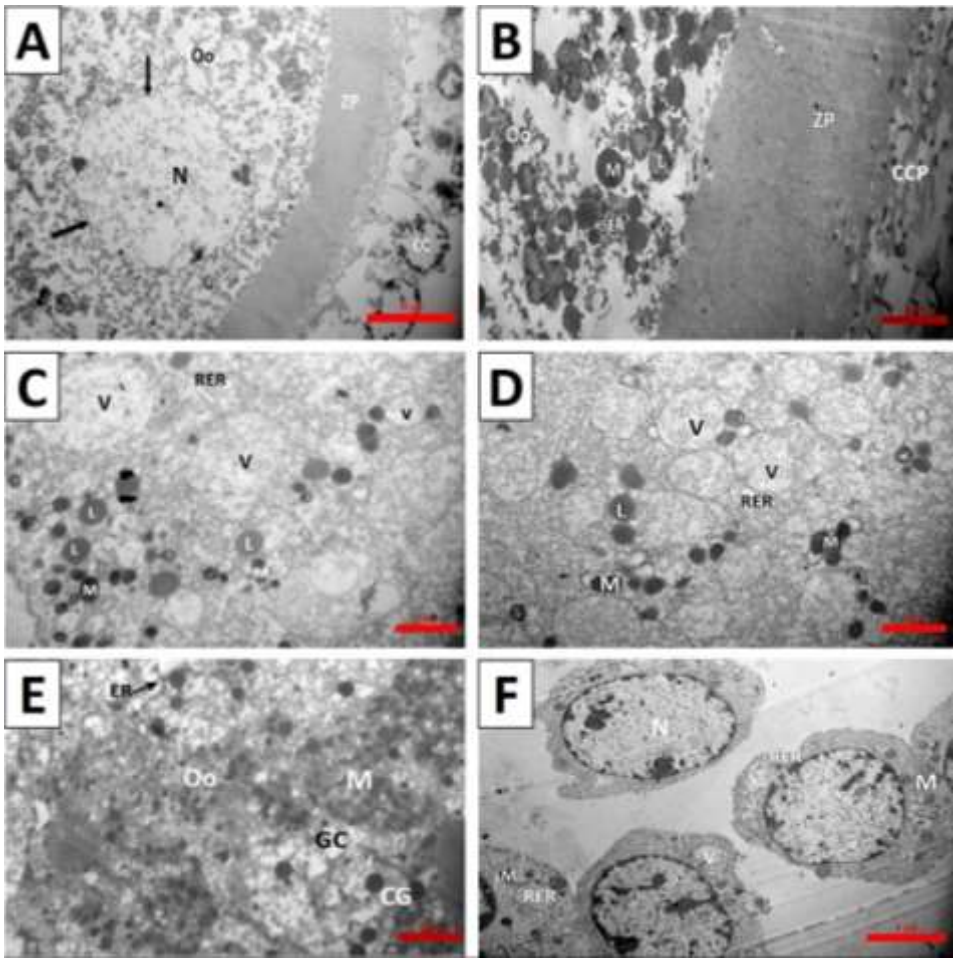


**Figure 2.** Transmission electron micrographs of immature rabbit oocytes showing: nucleus (N), nuclear envelope (arrow), zona pellucida (ZP), cumulus cell projections (CCP), cortical granule (CG), vacuole (V), lipid droplet (L), ooplasm (Oo), endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), Golgi complexes (GC) and the cluster of round mitochondria (M). Figure F show cumulus cells (scale bar = 10, 2 and 4 µm).

cortical granules were electron-dense and round, and scattered over all the ooplasm or were located near the oolemma (Figure 2B, C, D, E).

The round shape of the immature rabbit oocytes is similar to that of other mammals. Ultrastructure observed in the ZP is similar to Zamboni (1966) who reported the ZP in rabbits at some stages is filamentous. The majority of the cumulus cells in this study were cuboidal and round similar to another report (Zamboni and Mastroianni Jr, 1966). The cumulus cells are compactly close together and adherence junctions are common between them and the oocyte, which is properties of immature oocytes in a variety of mammalian species (De Loos et al., 1989). The cumulus cell projections are infiltrating into the ZP where gap junctions are among oocyte and cumulus cell membranes (Kacinskis et al., 2005; Fair et al., 1997). The rabbit oocytes in this study similar to previous reports showed no perivitelline space (Lu et al., 2010).

The ooplasm of oocytes of all mammals contains lipid droplets or inclusion bodies with no membranes in different sizes (0.1 to 1.6 µm) (Belák et al., 1990) as an energy source (Brown, 2001). During the preparation process with osmium tetroxide, they are reduced and are seen as dark round in the images (Belák et al., 1990). The content varies between species in terms of plenty and specifications. In pigs, lipid droplets as small dark round structures are abundant in the ooplasm (Silva et al., 2011). We were able to see in the rabbit ooplasm of oocytes the rough, dark granules of the ooplasm similar to another study (Cai et al., 2005).

The endoplasmic reticulum in the electron micrograph has a granular appearance and includes sacs and tubules. The main activity of the ER is protein degradation, lipid metabolism, calcium gradient, and lattice construction (Ferreira et al., 2009). In some species like the rabbit in the present study, often the ER, mitochondria and lipid droplets are visible side by side (Fair et al., 1997; Sheng et al., 2022). Because lipid synthesis requires enzymatic activity associated with the RER and mitochondria (Raturi and Simmen, 2013). In goats, buffaloes and sheep, many vesicles as the metabolic unit are scattered across the cytoplasm containing proteins or mucopolysaccharides (Mondadori et al., 2007; Lucci et al., 2001; Reader, 2007; Guraya et al., 1998). Activation of metabolic pathways through protein synthesis and phosphorylation is essential for cytoplasmic maturation. Mitochondria play a very important role in this process because they are the main organs of energy metabolism. At oocyte maturation, the mitochondria produce ATP to synthesize essential proteins during embryonic development (Krisher and Bavister, 1998).

In immature rabbit oocytes similar to other mammals, the cortical granules are clustered throughout the ooplasm (Hosoe, 1997; Pratt, 2021). They are specific organs of oocytes and are composed of proteins, structural molecules, GAGs, and enzymes. These structures prevent polyspermy (Landim-Alvarenga and Maziero, 2018).

The mammalian oocyte remains arrested at the diplotene of prophase I or germinal vesicle (GV) from the fetus stage to puberty (Hunt and Hassold, 2008; Ghanem et al., 2021). As shown in the results of the present study, GV with a spherical nucleus, intact nuclear envelope, filamentous chromatin and several nucleoli are identified. The presence of dense cumulus cells, GV nuclei, and uniform ooplasm in immature oocytes indicates the ability to resume meiotic division and readiness for puberty (Dell'Aquila et al., 1996; Ridlo et al., 2021; Santiago-Rodriguez et al., 2021).

Collectively, our observations of light microscopy and TEM revealed that most but not all features of the ultrastructure of immature rabbit oocytes are similar to other mammals.

It must be noted that TEM is an intensive and rigorous technique in that we have to use a large number of samples in a study.

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## Conflicts of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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