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Ultrastructural analysis of human immature oocytes retrieved after two different protocols of controlled ovarian hyperstimulation

Dottoranda
Antonouli Sevastiani
Matr. 249249

Coordinatore del corso
Prof. ssa Maria Grazia Cifone

Tutore
Prof. Macchiarelli Guido
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Summary

The study presented in this PhD dissertation thesis was performed at the: 1) Dept. of Life, Health and Environmental Sciences of the University of L'Aquila and, 2) Dept. of Anatomy, Histology, Forensic Medicine and Orthopaedics, Sapienza University, Rome, Italy.

Introduction

Immature oocytes can be an alternative source of oocytes for Assisted Reproductive Technologies (ARTs). These oocytes can be retrieved by patients undergoing different controlled ovarian hyperstimulation protocols (COH), such as the conventional "***full***" (**fCOH**) or the less aggressive "***mild***" (**mCOH**) protocols. These, so-called "leftover" oocytes can increase the yield of total available oocytes, especially for poor responder patients or those having an unsynchronized cohort of follicles. The present PhD thesis aimed to evaluate the effects of two different COH protocols, a GnRH agonist (fCOH) stimulation and a GnRH antagonist (mCOH) stimulation, on the fine structure of fresh human immature GV-stage oocytes, to consider the ultrastructure of well-preserved organelles as indicator of GV quality preservation.

Methods

Fresh GVs were retrieved from consenting donor women undergoing ARTs due to tubal or male infertility factors. COH was achieved by: 1) a standard long protocol, with GnRH agonists (fCOH protocol) or 2) a mild protocol, with GnRH antagonists (mCOH protocol). After retrieval, oocytes were washed in phosphate buffered saline (PBS) solution, fixed in 2.5% glutaraldehyde/PBS and subjected to standard preparative for light (LM) and transmission electron microscopy (TEM). The following parameters were evaluated by LM and TEM and taken into consideration for qualitative assessment of the ultrastructural preservation of oocytes: general features (including oocyte shape and dimensions); morphology of the nucleus, nucleolus, chromatin and nuclear envelope

(including nuclear membrane integrity); microtopography, type and quality of the organelles; mitochondria shape, number and dimensions; presence and extent of ooplasmic vacuolization; position, number and dimensions of cortical granules; integrity of the oolemma and microvillus pattern; appearance of the perivitelline space (width, presence of fragments); zona pellucida texture.

Results

Most of fCOH and mCOH oocytes were well preserved and showed a roundish and centrally located nucleus. The nucleolus was reticulated and connected with dense heterochromatin spots in both oocyte groups. Mitochondria were usually located in a perinuclear position, sometimes associated to small vesicles; in the mCOH group, they showed a significant increase in the number, respect to fCOH. Numerous electron negative and membrane-bounded vacuoles were located close to the nucleus in both groups, but they appeared significantly larger in fCOH than in mCOH oocytes. Cortical granules were located both in the subplasmalemmal area and dispersed throughout the whole ooplasm, even if less abundant; the number of cortical granules was significantly higher in mCOH oocytes, compared to the fCOH ones. An intact and continuous zona pellucida were present in both groups; microvilli were uniformly distributed in both groups, with some areas of rarefactions in fCOH oocytes.

Conclusion

The data of the present study showed organelle-specific alterations in the oocytes observed, probably related to the applied COH protocol. In particular, the significantly lower number of mitochondria and the biggest size of vacuoles in fCOH oocytes TEM: transmission electron microscopy, respect to what observed in mCOH oocytes, may suggest that some microdomains of the GV-stage oocytes may be affected by the hormonal stimulation.

Publications

Published articles

Palmerini, M. G., Belli, M., Nottola, S. A., Miglietta, S., Bianchi, S., Bernardi, S., Antonouli, S., Cecconi, S., Familliari, G., Macchiarelli, G. (2018). Mancozeb impairs the ultrastructure of mouse granulosa cells in a dose-dependent manner. Journal of Reproduction and Development, 64(1), 75-82. doi: https://doi.org/10.1262/jrd.2017-143

Belli, M., Antonouli, S., Nottola, S.A. (2018). Mitochondria in mammalian oocytes and early embryos. A review on morphological and functional studies. EuroMediterranean Biomedical Journal, 13(26), 114-117. https://doi.org/10.3269/1970-5492.2018.13.26

Antonouli, S., Papatheodorou, A., Panagiotidis, Y., Petousis, S., Prapas, N., Nottola, S.A., Palmerini, M.G., Macchiarelli, G., Prapas, Y. (2019) The impact of sperm DNA fragmentation on ICSI outcome in cases of donated oocytes. Archives of Gynecology and Obstetrics, 300(1), 207–215. https://doi.org/10.1007/s00404-019-05133-9

Bianchi, S., Bernardi, S., Continenza, M.A., Vincenti, E., Antonouli, S., Torge, D., Macchiarelli, G. (2019) Scanning Electron Microscopy Approach for Evaluation of Hair Dyed with Lawsonia inermis Powder: in vitro Study. International Journal of Morphology, 38(1), 96-100.

Proceedings

Miglietta S, Agha-Rahimi A, Khalili MA, Moradi A, Antonouli S, Nottola SA. Morphology and viability of human spermatozoa vitrified with a new, cryoprotectant-free, artificial seminal fluid. Italian Journal Of Anatomy And Embryology, 1 supplement (121): 194. 70° Congresso nazionale SIAI, facoltà di Medicina e Chirurgia dell'Università Cattolica del Sacro Cuore, Roma, 15-17 Settembre 2016.

Belli M., Donfrancesco O., Palmerini MG., Miglietta S., Nottola SA., Cecconi S., Bianchi S., Antonouli S., Familiari G., Macchiarelli G. Ultrastructural analysis of in vitro matured granulosa cell under treatment with the fungicide Mancozeb. Book of abstract Ruđer Bošković Institute and Croatian Microscopy Society. 13th Multinational Congress on Microscopy September 24-29, 2017 in Rovinj, Croatia.

Antonouli S, Papatheodorou A, Panagiotidis Y, Petousis S, Nottola SA, Macchiarelli G, Palmerini MG, Bianchi S, Prapas Y. The impact of sperm DNA fragmentation on male fertility and ICSI outcome in cases of donated oocytes. Andrology, 2018; 6:103. 10th European Congress of Andrology (ECA), Budapest, Hungary, 11-13 October 2018.

Di Nisio V, Antonouli S, Rossi G, Tiboni GM, Budani MC, Macchiarelli G, Cecconi S. Effetti della stimolazione ovarica sull'espressione di proteine nelle tube di falloppio di topo. Congresso Nazionale S.I.F.E.S. e M.R., Riccione, 17-19 May 2018.

Antonouli S, Belli M, Palmerini MG, Bianchi S, Bernardi S, Cecconi S, Di Nisio V, Donfrancesco O, Familiari G, Nottola SA, Macchiarelli G. Ultrastructural analysis of human germinal vesicle-stage oocytes retrieved after conventional and mild ovarian stimulation. European Journal of Histochemistry, 2018; 62:19. 64° Convegno GEI – Società Italina di Biologia dello Sviluppo e della Cellula. L'Aquila, 11-14 June 2018.

Di Nisio V, Rossi G, Oddi S, Antonouli S, Macchiarelli G, Maccarrone M, Cecconi S. Major endocannabinoid-binding receptors are differentially modulated during oocyte meiotic maturation. European Journal of Histochemistry, 2018; 62:16. 64° Convegno GEI – Società Italina di Biologia dello Sviluppo e della Cellula. L'Aquila, 11-14 June 2018.

Bernardi S, Pinchi V, Di Angelo L, Antonouli S, Continenza MA, Bianchi S, Macchiarelli G. Uso della cheiloscopia nello studio dei pattern dei solchi labiali. 25° Convegno Pro.o.f. l'odontoiatra, l'odontologo e le sfide delle nuove norme. Complesso Didattico "Morgagni". Firenze, 28-30 June, 2018

Antonouli S, Belli M, Palmerini MG, Bianchi S, Bernardi S, Cecconi S, Donfrancesco O, Familiari G, Nottola SA, Macchiarelli G. Ultrastructural analysis of human germinal vesicle stage oocytes during the application of Assisted Reproductive Technologies.

ISMS book of abstract. XVII International Symposium on Morphological Sciences (ISMS), Motol University Hospital facilities, Prague, Czech Republic, 5-7 July 2018.

Bernardi S, Rastelli E, Bianchi S, Antonouli S, Palmerini MG, Belli M, Continenza MA, Macchiarelli G. Volumetric study of maxillary sinuses using Cone Beam Computed Tomography: An in vivo study. ISMS book of abstract. XVII International Symposium on Morphological Sciences (ISMS), Motol University Hospital facilities, Prague, Czech Republic, 5-7 July 2018.

Di Nisio V, Rossi G, Oddi S, Antonouli S, Macchiarelli G, Maccarrone M, Cecconi S. Major endocannabinoid-binding receptors are differentially modulated during oocyte meiotic maturation. Gordon Research Seminar and Conference on Mammalian Reproduction. Lucca, 28 July-3 August 2018.

Bernardi S, Continenza MA, Bianchi S, Antonouli S, Macchiarelli G. Systematic review and meta-analysis of frequency and diameters of midline lingual foramina. Italian Journal of Anatomy and Embryology; 123 (supplement) 21. 72° Congresso Nazionale, Società Italiana di Anatomia e Istologia (SIAI), Parma, 20-22 September, 2018.

Di Nisio V, Antonouli S, Rossi G, Tiboni GM, Budani C, Macchiarelli G, Cecconi S. Repeated gonadotropin treatments and protein expression in mouse fallopian tubes. AIBG book of abstract. Comunicazione orale al XVIII Congresso Nazionale AIBG. Ferrara, 21-22 September 2018.

Di Nisio V, Di Luigi G, Antonouli S, Rossi G, Tiboni GM, Budani MC, Macchiarelli G, Cecconi S. L'invecchiamento riproduttivo e la gravidanza modulano l'espressione di VEGF/VEGFR2 e di proteine di controllo del ciclo cellulare. Congresso Nazionale S.I.F.E.S. e M.R. Riccione, 9-11 May 2019.

Di Luigi G, Di Nisio V, Rossi G, Antonouli S, Budani MC, Tiboni GM, Macchiarelli G, Cecconi S. Do reproductive ageing and parity modulate the expression of VEGF/VEGFR2 and cell cycle control proteins? Hum Reprod. 34(Suppl.1): i438-9. 35th Annual Meeting of European Society of Human Reproduction and Embryology (ESHRE). Vienna (Austria), 23-26 June 2019.

Antonouli S, Rossi G, Bianchi S, Palmerini MG, Donfrancesco O, Di Nisio V, Bernardi S, Nottola SA, Cecconi S, Macchiarelli G. Morphological changes of ampulla region from mouse oviducts after repeated cycles of hyperstimulation. 19th Congress of the International Federation of Associations of Anatomists Excel. London (United Kingdom), 9-11 August 2019.

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List of abbreviations

AL: annulate lamellae

AMH: anti-Müllerian hormone

ARTs: Assisted Reproductive Technologies

cAMP: cyclic AMP

CGs: cortical granules

COCs: cumulus oocyte complex

COH: controlled ovarian hyperstimulation

COX-2: cyclooxygenase

Cx: Connexin

DOR: diminished ovarian reserve

E2: estrogens

EGF: epidermal growth factor

fCOH: “full” controlled ovarian hyperstimulation protocol

GSH: glutathione

GVBD: germinal vesicle breakdown

hCG: human chorionic gonadotropin

ICSI: intracytoplasmatic sperm injection

ISMAAR: International Society for Mild Approaches in Assisted Reproduction

IVF: *in vitro* fertilization

IVF-ET: IVF and embryo-transfer

IVM: *in vitro* maturation

LM: light microscopy

MAPKs: mitogen activated protein kinases

mCOH: “mild” controlled ovarian hyperstimulation protocol

MI: meiosis I

MPF: maturation-promoting factor

MV: mitochondria-vesicle complex

NSN: non-surrounded nucleolus

OHSS: ovarian hyperstimulation syndrome

OMI: oocyte maturation inhibitor(s)

ORT: ovarian reserve test

PBI: polar body

PBS: phosphate buffered saline

PCM: Phase Contrast Microscopy

PCOS: polycystic ovary syndrome

PGC: primordial germ cells

PGS-2: prostaglandin endoperoxide synthase-2

POF: premature ovarian failure

POR: poor ovarian response

PR: progesterone receptors

PVS: perivitelline space

Rb: retinoblastoma

RER: rough endoplasmic reticulum

rFSH: recombinant FSH

SEM: scanning electron microscopy

SER: smooth endoplasmic reticulum

SN: surrounded nucleolus

TEM: transmission electron microscopy

TICs: theca interstitial cells

TZPs: transzonal projections

VEGF: vascular endothelium growth factor

ZP: zona pellucida

Part 1: Introduction

1.1 An *in vivo* approach of human oocytes enclosed in ovarian follicles: follicular and oocyte development, protein interactions, hormonal control and morphology of oocytes by Transmission Electron Microscopy

1.1.1 Ovarian physiology in regard to follicle and oocyte development

Human ovaries are enclosed in a fibrous and thick connective-tissue capsule, the tunica albuginea layer, consisting of the outer cortex and the inner medulla. All the follicles and the remains of ruptured follicles attached in vascular fibrous tissue are in the cortex. The inner medulla is highly vascular, including blood and lymphatic vessels and few follicles (Fig. 1B) (Johnson *et al.*, 2004). In humans, initial cells .transform into the primordial follicles in the embryonic development and initial cells called primordial germ cells (PGC) form the gametes by migrating from the yolk sac to the genital ridge/primordial gonad where they transform into oogonia (7^{th} week of gestation). The PGC proliferate during migration and continue to proliferate at the genital ridge (oogonia proliferation). In females are present an estimated number of 7×10^2 to 8×10^2 GC (5^{th} week of gestation). The transformation of oogonia into oocytes occurs once the division of oogonia stops and starts the meiosis (11-12^{th} week of gestation) (Baker, 1963). During meiosis, the oocytes go through different stages: leptotene, zygotene, pachytene, and diplotene (dictyate) (Franchi and Baker, 1973) characterized by the distinctive feature of the nucleus. Oocytes arrested at the dictyate stage of meiosis I, are called primary oocytes (Pan and Li, 2019). The total number of GC reaches a maximum of 6×10^6 - 7×10^6. The 2/3 are primary oocytes (20^{th} week of gestation). At 4^{th} month of fetal life initiates the formation of the follicles and between the 6^{th} and the 9^{th} month of gestation all the primordial follicles (oocytes) are formed. The duration and rate of oogonial proliferation and the extent of atresia are determinant for the number of oocytes in the ovaries at birth. The total number of GC at birth is around 1×10^6 to 2×10^6 and approximately 6×10^5 to 7×10^5 of them represent primordial follicles. Only about 3×10^5 to 4×10^5 follicles remain in puberty, and 95% of them represent the primordial follicle pool (Baker, 1963). For many decades, an equal number of follicles in both ovaries are in the primordial stage constituting the non-growing follicle pool, and once they activate, they start to grow (Fig. 1A) (Gougeon and Chainy, 1987). In humans, the process of activation, growth, and development of a primordial follicle containing an immature oocyte to a pre-ovulatory follicle including a mature oocyte has been described as folliculogenesis. The target of

folliculogenesis is to produce a single dominant follicle from a pool of growing follicles. During this process, are acquired the competence and the capacity of the oocyte enable to fertilize and to support a viable pregnancy (Sarma *et al.*, 2019). Human folliculogenesis is divided in two distinct phases: (i) gonadotropin-independent (preantral stages) and (ii) gonadotropin-dependent (antral stages). Distinct follicles represent each phase and local produced autocrine or paracrine factors as hypothalamus-pituitary hormones, and systemic growth factors are involved. The first phase lasts for about 300 days and is characterized by continuous recruitment and growth of non- growing follicles to primary follicles followed by progressive growth of primary follicles (initial follicle growth) into secondary (early follicle growth) and then large follicles (pre-antral follicle growth). The second phase occurs approximately 50 days and characterized by the growth of pre-antral follicles into early antral follicles evidenced by the appearance of fluid-filled cavities that aggregate to form the antrum. Further accumulation of fluid in the antrum along with follicle growth leads to the development of recruitable follicles from which is selected the dominant follicle for growth into the pre-ovulatory follicle (Rodgers and Irving-Rodgers, 2010). For ovulation, only one dominant follicle is chosen, and the rest of the recruited follicles undergo atresia. Noteworthy is the estimation that only about 4×10^2 of the primordial follicles ever develop into ovulation throughout the entire reproductive life of women. From the primordial to the pre-ovulatory stage, the follicle includes a diploid primary oocyte, and at the end of this stage, the oocyte resumes meiosis upon the ovulatory gonadotropin (LH) surge. In this process are acquired the expression and the accumulation of gene programs required for fertilization and early cleavage. The passage of a non-growing follicle growing into a pre-ovulatory follicle occurs as the result of the following successive events: (i) gonadotropin independent phase and (ii) gonadotropin-dependent phase (Fig. 1B and Table 1).

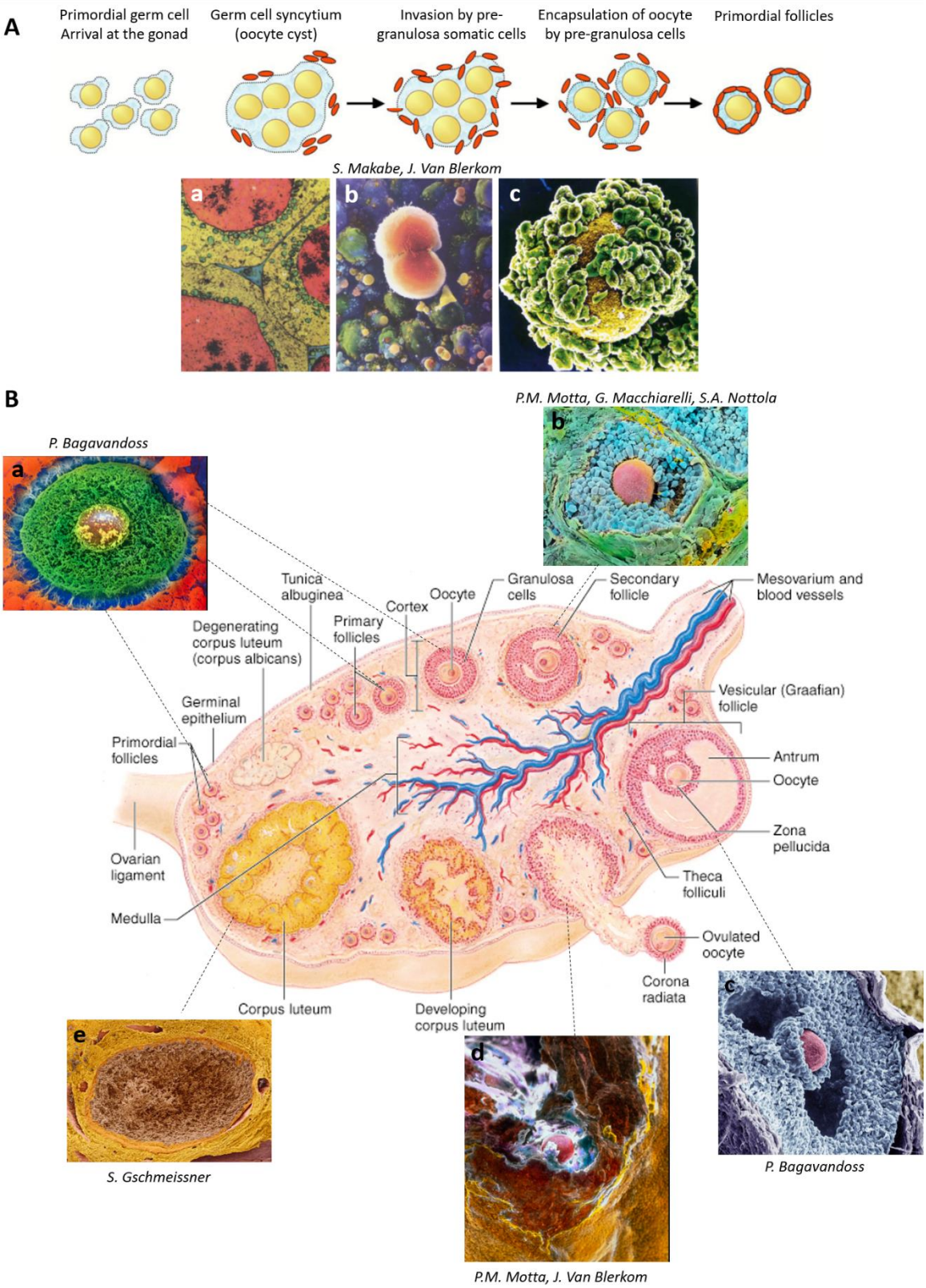


Figure 1. ***A) Division of germ cells and the formation of follicles.*** Representative Scanning Electron Microscopy (SEM) micrographs of germ cells (a, b) and of an isolated oocyte surrounded by granulosa cells (c). (Modified by Bristol-Gould *et al.*, 2006). ***B) Follicular development in the mammalian ovary.*** (Modified by Marieb, 2001)**.** Representative SEM micrographs (a, b, c, d, e) show the ultrastructure of follicles in each developmental stage.

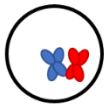
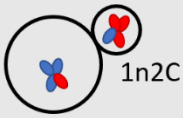
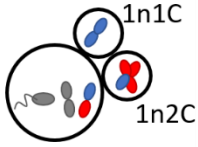
		Germ cell and follicle stages	Time span or period of existence	Cell cycle phase of germ cells	DNA status
Oogenesis	Oocytogenesis	Oogonia	Fetus	Mitosis	2n2C 
				Interphase: G1, S (DNA replication), G2 Meiosis I: Prophase I: leptotene, zygotene, pachytene, diplotene	2n4C 
		Primordial follicle (primary oocyte)	Fetus, newborn to adult	G2/Prophase I* Arrest at diplotene (GV stage oocyte)	
	Ootidogenesis/Folliculogenesis	Primary follicle (primary oocyte)	Fetus, newborn to adult	G2/Prophase I Diplotene (GV stage oocyte)	
		Secondary follicle (primary oocyte)	Fetus, newborn to adult	G2/Prophase I Diplotene (GV stage oocyte)	
		Tertiary follicle-early antral (primary oocyte)	Fetus, newborn to adult	G2/Prophase I Diplotene (GV stage oocyte)	
		Tertiary follicle-late antral (Graafian) (primary oocyte)	Puberty (menarche) to adult	G2/Prophase I Diplotene (GV stage oocyte)	
		Graafian follicle (pre-ovulatory oocyte)	Puberty to adult	G2/Prophase I (GV stage oocyte)	
		→ LH Surge			
		Graafi an follicle (pre-ovulatory oocyte)	Puberty to adult	Diakinesis (fully chromosome condensation) (GV→GVBD→MI stage oocyte)	2n4C 
		Ovulatory follicle (secondary oocyte)	Puberty to adult	Extrusion of the fi rst polar body and oocyte arrest at metaphase of meiosis II (MII)	1n2C 
				After fertilization, meiosis II in oocytes ends with the extrusion of the second polar body	1n1C 

Table 1. ***Folliculogenesis and oogenesis in humans.*** (Modified by Sadeu and Nogueira, 2011).

1.1.2 Folliculogenesis: Preovulatory stages of follicular growth in humans

1.1.2.1 Gonadotropin independent phase

1.1.2.1.1 Primordial follicle growth

The entry of primordial follicles in the growing phase is known as initial recruitment, and it occurs continuously throughout reproductive life from fetal development until menopause. This process is characterized by the reduction in the number of primordial and/or primary follicles due to their entry from the non-growing pool to the growing phase, and it is regulated by many stimulatory and inhibitory hormones and/or growth factors of local and/or systemic origins. Based on the morphological characteristics, there are three types of non-growing follicles (Westergaard *et al.*, 2007): (i) primordial, (ii) intermediary; and (iii) small primary follicles. All the non- growing follicles differ on the proportion of flattened and cuboidal granulosa cells and in their diameter due to different numbers and sizes of granulosa cells, but they do not differ in oocyte and its mean nuclear diameters (Gougeon and Chainy, 1987). Even if morphometric studies suggested that the differentiation of flattened to cuboidal granulosa cells could be the marker- mechanism of the follicle growth initiation, the factors that trigger the follicle growth activation are still not fully investigated. Due to the above-mentioned morphological features, the transition from primordial to small primary follicles has been considered as a slow maturation process (Gougeon, 1996), but the small primary follicles are considered as growing follicles (Hirshfield, 1989). Morphologically, primordial follicles (~35 μm in diameter) are small primary oocytes (~32 μm in diameter), surrounded by a monolayer of flattened or squamous (pre)granulosa cells and a basal lamina without blood vessels (Reynolds, 1950) and high transcriptional or translational activity (Fig. 1B). By virtue of the basal lamina, the (pre)granulosa cells and the oocyte are present in a microenvironment in which contact with other cells is not evident. The primordial follicles do not have a proper blood supply (Reynolds, 1950), and it seems that they have limited access to the endocrine system. Intermediary follicles (~38 μm in diameter) includes an oocyte (~32 μm in diameter) surrounded by a mixture of flattened and cuboidal granulosa cells and small primary follicles (~46 μm in diameter) show an oocyte

(~33 μm in diameter) surrounded by a monolayer of cuboidal granulosa cells (Gougeon and Chainy, 1987).

In the past, discrepancies have been developed regarding the involvement of gonadotropins in primordial follicle recruitment with some studies to suggest that gonadotropins may play a role (Wang and Greenwald, 1993; Ataya *et al.*, 1995; Flaws *et al.*, 1997) and others to demonstrate that primordial follicle recruitment is gonadotropin independent (Kumar *et al.*, 1997; Diedrich and Felberbaum, 1998) and that the primordial follicle does not have functional gonadotropin (FSH and LH) receptors (Oktay *et al.*, 1997; O'Shaughnessy *et al.*, 1997). However, it is considered as gonadotropin-independent with indirect facilitation to follicles by FSH or pituitary factors (Williams and Erickson, 2000). It has been suggested that the role of the oocyte during this follicle growth phase is passive since the mean diameter of the oocyte does not seem to increase prior to primary follicle development (Tsafriri, 1997). However, there are several shreds of evidence derived from animal models suggesting that autocrine and/or paracrine signalling involving growth factors from the (pre)granulosa cells, the interstitial stromal cells and the oocyte regulate the primordial follicle recruitment (Lee *et al.*, 2004; Ding *et al.*, 2013). In these factors are included the retinoblastoma (Rb) protein (Bukovský *et al.*, 1995) and Myc oncogene (Li *et al.*, 1994) that are expressed in oocytes and links to the inhibition of granulosa cell proliferation (Picton *et al.*, 1998) in non-growing and growing follicles. Another crucial regulator of primordial follicle recruitment is the anti-Müllerian hormone (AMH) that inhibits primordial follicle recruitment towards the growing phase of early folliculogenesis and is secreted by granulosa cells of growing follicles (Durlinger *et al.*, 1999). AMH is found to modulate the sensitivity of growing follicle to FSH (Durlinger *et al.*, 2001) and the expression of AMH mRNA is appeared to be regulated by the oocyte (Salmon *et al.*, 2004). Other factors shown to be important for primordial follicle growth are the Forkhead box L2, fibroblast growth factor 2 and 7, leukaemia inhibitory factor, epidermal growth factor, insulin-like growth factor, transforming growth factor (Palma *et al.*, 2012), as well as factors that stimulate cyclic adenosine 3',5'-monophosphate production (Buffet and Bouchard, 2001). As for the rate of non-growing follicles stimulation to grow is found to be directly proportional to the number of non-growing follicles (Gougeon, 1996).

1.1.2.1.2 Primary follicle growth

A primary follicle (~46 μm) consists of one or more cuboidal granulosa cells that are arranged in a single layer surrounding the oocyte. The development of the follicle is characterized by the differentiation of elongated somatic (pre-granulosa) cells of primordial follicles (~35 μm) into cuboidal granulosa cells (Fig. 1B). The cuboidal-shaped granulosa cells express the FSH receptor (Oktay *et al.*, 1997). Even if the granulosa cells express the FSH receptors, the normal levels of plasma FSH during regular menstrual cycle do not influence granulosa responses due to the lack of an independent vascular system in primary follicles (Nakamura *et al.*, 1993). However, high levels of plasma FSH showed an acceleration in primary follicle development (Fortune *et al.*, 2000), but knockout mouse models for GnRH or FSH revealed that the follicles are capable of growing until pre-antral follicle (Cheon, 2012). Based on these pieces of evidence, FSH seems to take part in the development of primary follicles without being necessary to the growth of the follicle, demonstrating a gonadotropin-independent grow until the early antral follicle. In the oocyte, it is noticed a progressive increase in the level of RNA synthesis. Ribosomal mRNAs are synthesized and accumulate for the production of proteins necessary for later stages of oocyte maturation. At this phase, several important oocyte genes are transcribed and translated. The oocyte synthesizes the glycoprotein of zona pellucida (ZP) that consists of a layer around the oocyte. Genes encoding the ZP proteins (i.e. ZP-1, ZP-2, and ZP-3) translated, thus contributing to oocyte growth and maturation (Wassarman *et al.*, 1996). The importance of the zona pellucida is due to the fact that the carbohydrate moiety of ZP-3 is the species-specific sperm-binding molecule (Gong *et al.*, 1995) indispensable for initiating the acrosome reaction in capacitated sperm during oocyte fertilization (Wassarman, 1988). Important molecular factors that take part in normal primary follicle development is the growth differentiation factor-9 (GDF-9) and bone morphogenic protein-15 (BMP-15) that they found to be expressed in oocytes at early follicle development in humans (Aaltonen *et al.*, 1999).

During primary follicle growth, the granulosa cells send processes through the zona layer, where they establish specialized cell contact systems, the gap or adherent junctions with the oocyte cell membrane, or oolemma (Gougeon, 1996). Adherent junctions are associations of filamentous proteins on the intercellular sides of the plasmalemma. They are essential in order to maintain communication within the avascular granulosa cell

layer. Gap junctions are composed of proteins called connexins. There are 13 members of the connexin family. They may directly couple adjacent cells to allow the diffusion of ions, metabolites, and other low-molecular-weight signalling molecules such as cyclic AMP (cAMP) and calcium (Beyer, 1993; Kumar and Gilula, 1996) with the Connexin 37 (Cx37) to be an oocyte-derived connexin that forms gap junctions between the oocyte and surrounding granulosa cells (Simon *et al.*, 1997). Cx43 is also a major gap junction protein expressed in the granulosa cells (Beyer *et al.*, 1989). The impact of gap junctions on the primary follicle is that it is a metabolically and electrically unit and the intercommunication of oocyte with granulosa cells continues throughout follicle growth.

1.1.2.1.3 Secondary follicle growth

The transition from a primary to a secondary follicle (0.12-0.2 mm) is characterized by the composition of at least two layers of granulosa cells and the acquisition of the theca (ovarian stroma cells) layer, forming parallel sequence next to the basal lamina (Fig. 1B). Oocyte-derived factors (GDF-9, BMP-15, and Cx43 molecules) found to be essential for the formation of the secondary follicle (Galloway *et al.*, 2000). Like primary follicle, also in secondary follicle growth, gap or adherent junctions have a predominant role with Cx43, Cx57 to be the most significant connexins involved in oocyte- granulosa cells communication (Kidder and Mhawi, 2002). The granulosa cells of this phase express FSH, estrogen, androgen, and IGF-1 receptors (Gougeon, 1996; Oktay *et al.*, 1997). As the secondary follicle increases in diameter, the stromal tissue differentiated into the theca layers: the inner theca and the outer theca. The second one is composed of undifferentiated theca cells (smooth muscle cells). The fibroblast-precursor cells of the inner layer in secondary follicles contain LH receptors, IGF-1 receptors and blood vessels that form one or two arterioles connecting the layer with the capillaries of granulosa cells basal lamina (Channing and Kammerman, 1973; Tajima *et al.*, 2007). The capillary vessels transfer nutrients and gonadotropins and collect functional or not factors from/to the growing follicle. The fibroblast-precursor cells subsequently differentiate into steroidogenic cells called theca interstitial cells (TICs), due to the response of the plasma LH delivered by the theca vascular system (Erickson *et al.*, 1985). In the secondary follicle, all the granulosa cells express FSH receptors probably caused by the diffusion of plasma FSH into the follicle, provoking FSH-dependent granulosa responses (Yamoto *et al.*, 1992). When the pre-antral follicle growth is complete (Fig. 1B), the follicle includes

a fully-grown oocyte surrounded by ZP layer, granulosa cell layers (approximately nine) that express FSH, estrogen and androgen receptors, a basal lamina, a theca interna expressing LH receptors, a theca externa, and blood as well as lymphatic circulatory vessels. The LH receptor produces estradiol through the theory of two gonadotropin-two cells (Raju *et al.*, 2013). The activate LH receptors produce androstenedione that passes through the basal lamina in granulosa cells that activate the FSH path leading to the production of P450 aromatase, a protein that metabolizes androstenedione to estrogens (E_2).

1.1.2.2 Gonadotropin-dependent phase

1.1.2.2.1 Graafian or antral follicle growth

The development of antral follicle is defined structurally by the formation of a fluid-filled cavity or antrum and due to this characteristic structural unit is called antral follicle, synonym to Graafian follicle that the name given after the death of the anatomist Regnier de Graaf who was the first who described this stage follicle development (Fig. 1B) (Jay, 2000). Antral development includes a phase of early antral follicle growth, follicle (cycle-gonadotropin control) recruitment, dominant follicle (pre-ovulatory) selection, and maturation. The antral follicle includes six major components: the theca externa and interna, the basal lamina, granulosa cells, the fully-grown oocyte, and the fluid-filled cavity. In the early antral follicle, small antral cavities (0.04 mm in diameter) growth to then coalesce in a single and large antral cavity. In humans, the pre-antral (0.12-0.2 mm in diameter) follicle development into early antral follicles (0.2-0.4 mm in diameter) lasts around 25 days. The role of the antral cavity is to maintain follicular growth and development by providing a functional intrafollicular microenvironment that derives from blood to follicular cells nutrients and growth regulators, e.g. gonadotropins, proteoglycans, etc. In humans, there is a lack of knowledge in molecular pathways involved in antrum formation. However, FSH, LH, activin, and KL in rodents (Jones and Shikanov, 2019), as well EGF in bovines (Gutierrez *et al.*, 2000) seem to be the principal regulators. The theca externa consists of smooth muscle cells innervated by autonomic nerves, and it may be involved in atresia and ovulation (Motta and Familiari, 1981; Erickson *et al.*, 1985). The theca interna consists of differentiated TICs located within a matrix of loose connective tissue and blood vessels. LH is the key hormone for TIC

function by regulating TIC androgen production, specifically during the early stages of antral follicle development the TICs express their differentiated androgen, androstenedione-producing cells (Erickson *et al.*, 1985). The theca interna is highly vascularized and delivers required components for the growth and differentiation of the oocyte and granulosa cells, e.g. FSH, LH, nutrient, vitamins, etc. The differentiation of granulosa cells into the four distinct layers takes part in the formation process of the antral cavity. The granulosa cell layers are the membrana granulosa (or mural granulosa) that is the outermost layer, the periantral that is the innermost layer, the cumulus oophorus that is the intermediate layer, and the corona radiata layer that is juxtaposed to the ZP of the oocyte. The membrana layer is composed of a pseudostratified epithelium of tall columnar granulosa cells placed in the basal lamina. Each of these cell types responds differently to FSH actions and the growth, as well as the development of the antral follicle, is strictly associated with granulosa cell and theca cell differentiation. The increase in the size of the antral follicle is, at first, the result of antral cavity formation and around two menstrual cycles (70 days) the diameter increases and varies from 0.12-0.2 mm (pre-antral follicle) to 2-5 mm (antral follicle).

1.1.2.2.1.1 Cyclic recruitment in antral follicles-gonadotropin control

Once the diameter of the antral follicles reaches the size of 5 mm, the follicles remain dependent on cyclic FSH changes, following growth and development into ovulation (Fig. 2) (Gougeon, 1996). During antral follicle growth, the cyclic gonadotropin control and the selection of the dominant follicle followed by the maturation and ovulation of a pre-ovulatory follicle are important events. After increases in circulating FSH during the perimenstrual period, a small cohort of antral follicles (2-5 mm) is rescued from atresia due to the survival action of FSH. It is suggested that in the ovaries of healthy young women are included four-to-six healthy antral follicles, 0.9-5 mm in diameter and that in order to prevent the atresia of approximately 15 follicles, measuring 2-5 mm in diameter, an increase of FSH from the physiological levels up to 10-30% is required (Brown, 1978). The lack of FSH is shown to block the follicle recruitment and selection (Kumar and Sait, 2011), but increased levels of the gonadotropin is found to contribute to the following procedures: formation of follicular fluid, proliferation of granulosa cell, synthesis of E_2, induction of the expression of FSH and LH receptors in granulosa cells, and of non-steroidal growth factors such as IGF-1, FGF, TGF-β, activin, and the aromatase enzyme

complex (Gougeon, 1996). However, once the follicle reaches the pre-ovulatory stage is noticeable the induction of high levels of LH receptor expressed in granulosa cells, and when the follicle exceeds the size of 10 mm in diameter the aromatase enzyme complex is completely functional (Gougeon, 1996). At the initial stages of the phase, the largest follicles with the highest number of FSH receptors are present, providing to the follicle a low FSH threshold (Kumar and Sait, 2011). Thus, only the largest follicles can survive and respond to lower FSH levels and then grow. The follicle of which the granulosa cells can respond better to lower FSH levels is the selected follicle to grow into a pre-ovulatory follicle, the dominant follicle. The selection process occurs at the beginning of the phase (day 5-7 of the menstrual cycle). The largest healthy antral follicle is selected among follicles sized in diameter between 5.5 and 8.2 (Gougeon and Lefèvre, 1983) and the rest, non-selected follicles undergo atresia.

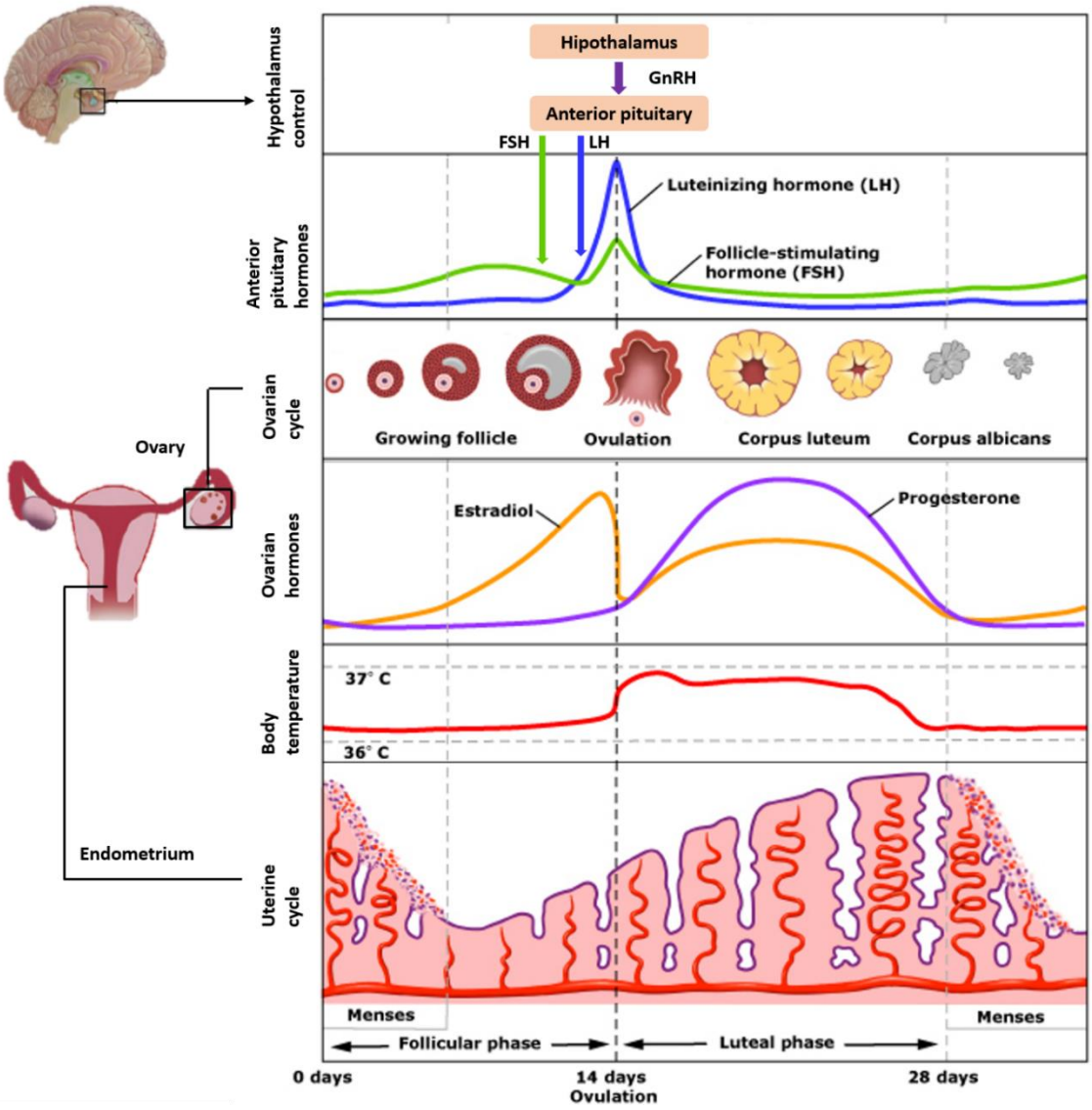


Figure 2. ***Hormonal fluctuations over a physiological menstrual cycle in women.*** (Modified by free available online archives. No authorized copyright)**.**

1.1.2.2.1.2 Selected dominant follicle

The larger diameter, the presence of FSH and LH in the follicular fluid, the continuous proliferation of granulosa cells, the increased production of E_2 and inhibin are the characteristics of the dominant follicle (Fig. 2) (Gougeon and Lefèvre, 1983). The selection of a single dominant follicle to participate in single ovulation is suggested to be caused by the induction of aromatase by FSH, which results in the rise in peripheral levels of E_2. This, along with inhibin, suppress FSH secretion such that plasma concentrations of FSH fall below the threshold. The falling of FSH under the threshold is essential for stimulating the development of less mature follicles. In addition, the simultaneous induction of LH receptors on granulosa cells by FSH can be considered as extra gonadotropic support to the dominant follicle, enabling continuous growth under the presence of FSH concentrations. At the same moment, these levels of FSH concentration are insufficient to support the development of the other follicles (Zeleznik, 2004). Thus, the dominant follicle is the one that grows at a quicker rate, contains a detectable FSH level and differs substantially in the estradiol concentration at follicular fluid than the other follicles (McNatty, 1982).

1.1.2.2.1.3 Ovulation - Pre-ovulatory follicle

In the final phase of the follicle growth, the dominant follicle becomes a pre-ovulatory follicle (20 mm) by continuing granulosa cell proliferation, theca cell growth (new vascularization and hypertrophy), and further accumulation of follicular fluid in the antral cavity until the ovulatory LH surge. In the pre-ovulatory follicle, it increases the production of LH together with the expression of LH receptors in mural granulosa cells. Approximately 10 days after selection, the size of the dominant follicle had considerably increased from 8 to 20 mm in diameter. During the maturation of the pre-ovulatory follicle, the synthesis of E_2 increased, correlating with increased aromatase expression in granulosa cells. Therefore, the onset of the LH surge induces terminal differentiation of granulosa cells and their expression of luteal-specific genes (Hunzicker-Dunn and Mayo, 2015), and causes a significant decline in gap junctions (Amsterdam and Rotmensch, 1987) leading to dissociation of mural granulosa cells and expansion of the cumulus-oocyte complex. Decreased levels of intrafollicular E_2 and androstenedione with

augmented levels of progesterone and 17-α-hydroxyprogesterone are present (Fig. 2) (Zeleznik and Hillier, 1984). The matured pre-ovulatory follicle 20 mm in diameter can be triggered to ovulate after LH surge stimulation. In the case of LH-surge failure follows the degeneration of the follicle. Regarding the fully-grown oocyte maturation, the LH surge suppresses the responsible inhibitors for oocyte maturation, allowing the diploid primary oocyte arrested in the dictyate stage of prophase I to resume meiosis and proceed to metaphase II stage (Hodgen, 1989). For completing oocyte maturation, an LH threshold must be reached and maintained for 14 to 27 h (Zelinski-Wooten *et al.*, 1992). At the time of ovulation, the haploid cell arrested in metaphase II is in the size of 120 μm in diameter. In humans, the process of ovulation lasts approximately 36-38 h, and negative feedback of progesterone and 17β-hydroxyprogesterone on the hypothalamus-pituitary-ovarian axis is needed to terminate the LH surge (Gougeon, 1996). Some hours before ovulation and before the LH-surge the granulosa cell wall which was avascular before the mid-menstrual cycle LH surge appears to be bloody after blood vessel invasion from theca cells. At the onset of LH surge, except from the induction of granulosa cells mitosis suppression that stimulates their luteinization, is noticed an induction in the expression of prostaglandin endoperoxide synthase-2 (PGS-2) or cyclooxygenase (COX-2) and progesterone receptors (PR) in the granulosa cells and a decrease in estrogen and androgen levels in follicular fluid. The molecular mechanisms included in the degradation of the extracellular matrix are the dissociation by the PR and PGS-2 of fibroblasts and collagen of the theca externa and tunica albuginea on the apex of the follicle, plus the production of interleukin-1 and chemokines. Significant factors are also the responsible factor for the initiation of angiogenesis, the vascular endothelium growth factor (VEGF) and the ERK1 and ERK2 that are identified to play a key role in ovulation signalling (Fan *et al.*, 2009).

1.1.3 Ootidogenesis: Oocyte growth and stages of maturation in humans

1.1.3.1 Primary oocyte growth (GV-stage)

During oocyte growth, human GV-stage oocytes size from 35 μm up to fully grown oocyte of 110-120 μm in diameter and it's the largest cell in the female body. Throughout primordial follicle growth, the enclosed primary oocytes remain in an abeyant growth phase with the arrested nucleus at the dictyate-prophase stage of meiosis I until the peri-

ovulatory antral stage (Table 1). A few decades can possibly last the continuous nuclear and ooplasmic alterations for primary oocytes surviving via the renewal of organelles and molecules, the uptaking of ribonucleotides and amino acids and the regulation of RNA polymerase activity. In humans, the oocyte obtains the maximum final diameter in the antral stage, although it grows mainly in the pre-antral stage (Gougeon, 1996). During growth, the mammalian oocytes are able to undergo meiosis, a species and size-dependent ability that is not necessarily connected to the maximum size of the oocyte, e.g. the proper oocyte diameter in mouse is 70-80 μm (Wassarman and Josefowicz, 1978), in bovine is 100-110 μm (Fair *et al.*, 1995) and in humans is 90-120 μm (Durinzi *et al.*, 1995). Both a significant increase in oocyte volume and intense ooplasmic modifications are noticed at the phase of pre-ovulatory antral development. The growth and the maturation of the oocyte, as well as the early embryo development, are strictly dependent on the gradual synthesis of proteins, RNA and other components occurring during oocyte growing. In non-growing or fully-grown oocytes, the rate of RNA synthesis is two to three times less in comparison with growing oocyte whereon a rate is also a simultaneousness event along with the increase in the volume (Schultz *et al.*, 1988; Bachvarova, 1985). At primary oocyte development occurs an increased proliferation in the number of ooplasmic organelles which afterwards translocated by microtubule network (Albertini, 1992). The nucleolus has highly transcriptive activity and includes well-defined fibrillary and granular structures (non-surrounded nucleolus, NSN) (Parfenov *et al.*, 1989). In the oocytes of secondary follicles, the ZP formation requires the above-mentioned organelle contribution. In GV-stage, gap junctions develop between the oocyte and granulosa cells and primary clusters of cortical granules produced by Golgi complexes are present in the cytoplasm. In order to ensure the viability of an oocyte enclosed in a non-atretic pre-ovulatory follicle is mandatory the differentiation of granulosa cells, the interactions between oocyte and granulosa cells, and the influence of gonadotropin regulation on follicular development. Along with the increase in the follicular size, the oocyte undergoes cellular proceedings, essential for its later maturation and developmental success. The buildup and storage of transcripts, the synthesis and accumulation of proteins, and the post-translational modifications as phosphorylation are some of the ooplasmic procedures (Wickramasinghe and Albertini, 1993; Wassarman and Florman, 2011). The maturation-promoting factor (MPF) and protein kinase mos are crucial proteins that participate in meiotic progression and cell cycle control (Wickramasinghe and Albertini, 1993). Other ooplasmic changes include the microtubular and

microfilament rearrangements that initially involve in the migration of organelles during oocyte development, then in the formation and migration of the metaphase spindle at maturation and play a key role for the accurate division of the embryonic cells. Ultrastructural modifications as the dispersion of elongated mitochondria and the migration of cortical granules to the periphery of the oocyte occur before the LH surge (Hyttel *et al.*, 1997). The increase of glutathione (GSH) responsible for the protection of the oocyte against oxidative stress is a biochemical change that occurs before ovulation (de Matos *et al.*, 1996). A peculiar nuclear process is the progressive chromatin condensation resulting in the surrounded nucleolus (SN) from the NSN nucleolus (Bogolyubova and Bogolyubov, 2013). Although at the final stages of the oocyte growth, there is a remarkable decrease in the rate of RNA synthesis, this synthesis will continue to stop at the germinal vesicle breakdown (GVBD).

1.1.3.2 Acquisition of nuclear competence (GV-stage)

In human oocytes, the competence of the nucleus is obtained gradually during follicular growth. One crucial aspect of the coordination of oogenesis and folliculogenesis receiving widespread support is to consider the oocyte–granulosa cell communication as a dominant force in sustaining the protracted phase of oocyte growth in slowly developing primary and secondary follicles (Albertini *et al.*, 2001). A variety of mechanisms associated with the enclosed oocyte in each follicular developmental stage sustains the meiotic arrest. During folliculogenesis and oogenesis, GVBD-incompetent is the oocytes arrested in prophase I that grow inside follicles from primary to the early antral stage (Table 1). Oocytes unable to undergo GVBD are these inside the follicles that they have not reached the species-specific minimal size yet. Regarding this along with meiotic proceeding, the oocytes are not able yet to synthesize the substantial quantities of regulators and/or the position of regulators in the oocytes is still incorrect (Albertini and Carabatsos, 1998). GVBD-competent oocytes are the arrested in prophase I oocytes enclosed in antral follicles their spontaneous meiotic resumption is inhibited by many factors originating in ovarian somatic cells and follicle fluid. One of the most important inhibitory factors that seem to have a key role in follicle-enclosed oocytes to sustain the immaturity until stimulation is the oocyte maturation inhibitor(s) (OMI); others are the sex steroids, c-kit ligand molecule, nucleotides and the purine hypoxanthine (Wassarman and Florman, 2011). The presence of the last one and its interaction with the somatic cells

maintains and sustains the correct levels of cAMP inside the oocyte enclosed in the antral pre-ovulatory follicle when the interaction of the oocyte with the surrounding follicle fluid occurs (Norris *et al.*, 2009).

1.1.3.3 Transition from Primary to Secondary oocyte (GV-stage → GVBD → MII stage): Nuclear and ooplasmic maturation

The maturation of the human oocyte nucleus occurs at the time that the oocyte resumes meiosis to metaphase II and requires nuclear modifications. In humans, GV-arrest can last decades and is only broken postnatally by gonadotropin cues arising from puberty onwards (Homer, 2011). After the LH surge, this huge intact nucleus in the immature GV-stage oocyte arrested at prophase I undergo GVBD or a rapid disappearance of the nucleolus followed shortly thereafter by the dissolution of the nuclear membrane and the condensation of chromatin in bivalents that align at the equator of the first meiotic spindle. The signalling cascade initiated by the LH and triggered by mitogen-activated protein kinases (MAPKs), then induces a decrease of the ooplasmic levels of cAMP followed by the destabilization of the after MPF, with the consequent meiotic cell cycle progression (Tiwari *et al.*, 2018). GVBD is, indeed, the marker of meiosis I resumption. Oocytes then discriminate recombined homologous chromosomes before first polar body (PBI) extrusion after telophase I. PBI constitutes the morphological marker to exit from meiosis I (MI) (Homer, 2011). Oocytes, then, enter meiosis II and progress to metaphase II (MII), where they arrest for a second time. Oocytes at the time of ovulation are arrested at the second meiotic division (one chromosome, 1n containing two sets of DNA, 2C) (Table 1). The MII in females is not completed until after fertilization. In fact, the oocyte becomes a "truly haploid cell" (1n1C) after fertilization, since the second DNA set is extruded PBII when the sperm cell enters the egg introducing another DNA set (1n1C) and forms a 2n2C cell (i.e., zygote). Ooplasmic maturation of the oocyte is determined as all the acquired modifications occurring in the cytoplasm and is essential for the success of fertilization and pre-implantation embryo development. The acquisition of ooplasmic competence or the capability maturity ooplasmic-model integration, including the accumulation of proteins and short poly-A tails mRNAs occurs before the oocyte resumes meiosis (Eichenlaub-Ritter and Peschke, 2002). In paragraph 1.1.3.5, it is described the organelle distribution of GV and/or MII stage oocytes by TEM.

1.1.3.4 Oocyte-somatic cell interactions

During follicle growth and development, crucial is the communication between follicle-enclosed oocytes with their surrounded cells for the simultaneousness of developmental success. Upon follicle growth, the oocyte-somatic cell conjugation renders increasingly intense. The cells (granulosa and theca) provide substantial support to the insufficient-oocyte such as hormones, nutrients and growth factors (Eppig, 2001). A recent animal study showed that paracrine and juxtacrine signals derived from the oocyte regulate the rate of follicle growth (Eppig, 2001). The contribution of the oocytes to the follicular growth is the synthesis and the release of essential factors for granulosa cell proliferation, function, survival and differentiation, the recruitment of theca cells, and the secretion of extracellular matrix components (Gilchrist *et al.*, 2008). The oocytes play an important role at the initial stage of primordial follicle development and promote the transition of the follicles: primary to secondary (Eppig, 2001) and pre-antral to antral (Latham *et al.*, 2004). Prior to LH surge, the oocytes control the proliferation and differentiation of granulosa cells (Gilchrist *et al.*, 2008), the glycolysis and cholesterol biosynthesis of cumulus cells (Su *et al.*, 2008), the expansion of cumulus, and the ovulation after the LH surge along with their GDF-9 and BMP-15 regulators (Dragovic *et al.*, 2007).

1.1.3.5 Ultrastructure of immature and mature oocytes by TEM

In precise, the term GV-stage naturally defines a stage of oocyte maturation inside the antral follicle when the nuclear maturation is not yet complete (immature oocyte), and the nucleus is arrested at the prophase I stage of the first meiotic division (Yang *et al.*, 2009). The fully grown immature GV-stage oocyte was described in 19th century as a unique cell owning to its size (nearly 0.1 mm or 100 μm diameter), enclosed by a clear 'glass-like shell' or zona pellucida provided with a large nucleus or germinal vesicle, and containing at least one prominent spherical nucleolus in association with dispersed chromatin and often with dense spherical bodies, the nuclear bodies (Fig. 3A) (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006; Nottola *et al.*, 2014). The nucleoli that may be found inside a GV nucleus show four different components: a fibrillar component; a granular component; a fibrillar center, which is round-shaped and dense structure completely encircled by fibrillar components; and small vacuoles or interstices (El Shafie *et al.*, 2000). The nucleus is located generally in an eccentric

position (just before GVBD the nucleus approaches the oocyte periphery) surrounded by a nuclear membrane or envelope rich in pores, and a plethora of chromatin dispersed in the adjacent nucleoplasm. The nuclear membrane seems to promote the large molecular exchanges between the ooplasm and the nucleoplasm within the immature oocyte (Fig. 3A). Even if immature, the oocyte is enclosed by the ZP, a translucent acellular shell, as referred above, containing ooplasmic projections called as transzonal projections (TZPs), and more externally by a wall of follicular cells, called mural granulosa cells, responsible for the production of steroids, among which estrogens are the most abundant before ovulation. The innermost domain of the wall, the corona radiata is located just above the zona pellucida and communicates directly with the oocyte through the TZPs; and above the corona radiata is located the cumulus oophorus, an important domain for the acrosomal reaction of the spermatozoon in the act of fertilization (Sathananthan *et al.*, 1985, Conti and Chang, 2016). Cellular ultrastructure of both domains may show irregular nucleus with reticulated nucleoli, microvilli, mitochondria, rough endoplasmic reticulum cisternae, Golgi complexes, digestive vacuoles and secondary lysosomes containing phagocytosed material (El Shafie *et al.*, 2000). Detail of the ooplasmic context can show spherical and in abundance mitochondria (about 150,000) with very dense cristae, which however are not developed enough due to the presence of few ridges present in the mitochondrial matrix (Figs. 4A-B) (Motta *et al.*, 2000); a variety of lysosomal inclusions; isolated small vesicles of smooth endoplasmic reticulum (SER), and usually an increased vacuolization in close proximity to the nucleus (Figs. 3A, 4B). The vesicles of SER are small, having lighter or denser content and appearing free or associated with mitochondria (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006; El Shafie *et al.*, 2000). The vesicles in close association with mitochondria called MV complexes and small of them have been detected in GV oocytes (Fig. 4A inset). The lysosomes are very abundant in the cortical or subcortical cytoplasm of a GV, and are named differently according to their content: if they contain mainly vesicles, they are called multivesicular bodies; if they contain many small lipid droplets they are called lipofuscin bodies; if they contain membrane portions they are called multilamellar bodies. These secondary lysosomes result from the fusion of primary lysosomes, containing acidic digestive hydrolases, and vesicles including phagocytosed material, or cellular structures and organelles that required recycling (El Shafie *et al.*, 2000; Khalili *et al.*, 2012). Unlike the mature oocyte, in the GV-stage, there are still few and small Golgi complexes (Fig. 4A). In addition, rarely cortical granules are present, arranged in clusters

scattered in the cytoplasm or organized as a single discontinuous layer just below the oolemma (Fig. 4A) (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006). Often, in the subcortical area of a GV, annulate lamellae (AL) may also be present, cytomembranes deriving from the nuclear envelope that will be transformed into SER cisternae and dense vesicles of unknown nature that can also be found in a mature oocyte (El Shafie *et al.,* 2000). At the time of regaining maturation, the nuclear membrane of a GV-stage oocyte becomes highly folded and subsequently breaks down, in the so-called GVBD phenomenon as referred before. The chromatin, therefore, condenses to form chromosomes that later on will separate, and the one or more nucleoli disappear (Nottola *et al.*, 2014). Then, the oocyte blocks in MI-stage where the barrel-shaped mitotic spindle is present, positioned beneath the oocyte surface without the presence of centrioles and the chromosomes are in pairs, positioned at the equator of the spindle (Fig. 3B). At this stage, the polar body is not yet present, while below the oolemma there is a single or a double layer of cortical granules (Sathananthan *et al.*, 1985).

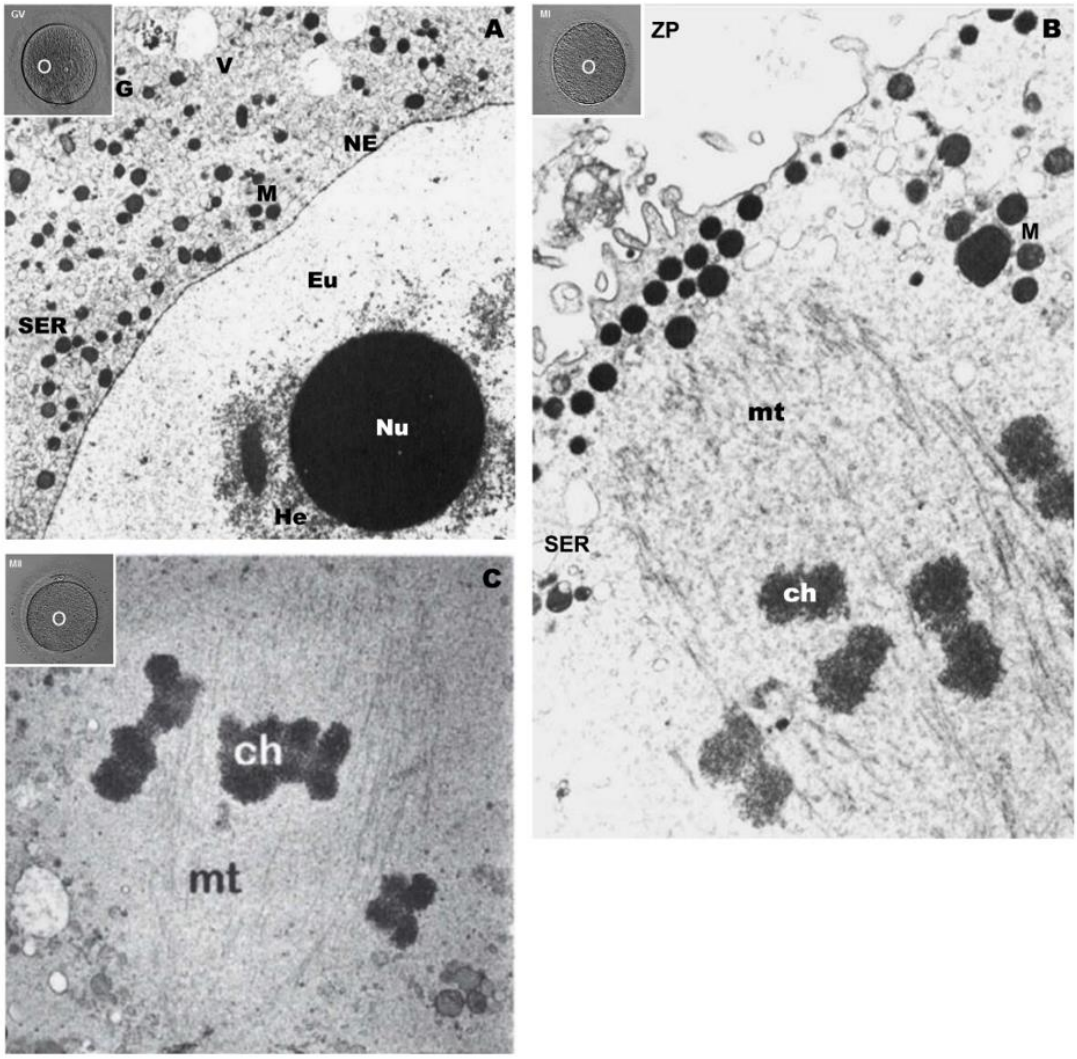


Figure 3. ***Representative TEM micrographs of the three maturation stages of the human oocyte, A) GV, B) MI, C) MII.*** A) A nucleus of GV-stage oocyte presented by a dense nucleolus (Nu) associated with dense heterochromatin (He) and abundant euchromatin (Eu), surrounded by a nuclear envelope (NE). Note also the presence of smooth endoplasmic reticulum (SER), a Golgi complex (G), vacuoles (V) and mitochondria (M) in close proximity to the nucleus. B and C) The presence of meiotic spindle in MI and in MII stages oocyte, respectively, composed of microtubules (mt) and associated chromosomes (ch) in the ooplasm. Close to the meiotic spindle in B) SER and M are present. The inset in A) indicates a GV-stage, in B) an MI-stage and in C) an MII-stage human oocyte, by phase-contrast microscopy. (Modified by Santhananthan *et al.*, 1985 and Nottola *et al.*, 2008).

The mature, MII-stage oocyte consists of similar cellular organelles with similar functions to all the other human cell types; however, its ultrastructural organization is immensely peculiar. Initially, the MII oocyte has terminated its nuclear and cytoplasmic maturation and is a single cell isolated from other cells of the same type. In fact, it is a large and spherical cell (90-100 μm in diameter), surrounded by cellular and acellular layers that contribute to maintaining the architecture and physiology of the egg cell. Similarly too immature, the mature oocytes surrounded by the ZP composed of fibrils and granules immersed in an amorphous matrix and plays a crucial role in the binding of the

spermatozoon during fertilization. Between the oocyte surface and the ZP is the perivitelline space that includes the first polar body, extracted from the first meiotic division that contains isolated chromosomes in association with microtubules arising from the mitotic spindle, a few and spheroidal mitochondria, vesicles, endoplasmic reticulum and dense cortical granules arranged in a single row beneath the plasma membrane (Fig. 4C) (Zamboni *et al.*, 1966; Bianchi *et al.*, 2014; Coticchio *et al.*, 2016; Nottola *et al.*, 2014). Around ZP, there is a layer of cells, the corona radiata, that extend long cytoplasmic processes through ZP, until reaching the oocyte membrane and forming junctional complexes. In junctional complexes, communicating desmosomes and communicating junctions have been identified, while cellular processes contain microfilaments, microtubules, and secretory granules. TZPs represent a means by which bidirectional communication occurs between the oocyte and the follicular cells assisted by many small vesicles rich in molecules, even though in the mature oocyte the corona radiata is retracted for isolating the oocyte. Morphologically, the cells of corona radiata present lipids, nucleoli, SER and ribosomes; and some of them exhibit protrusions, called "blebs", typically found in cells that export proteins or other molecules in small cytoplasmic compartments. In the oolemma the second meiotic spindle is barrel-shaped, is anastral and devoid of centrioles, composing by numerous microtubules, whilst the chromosomes are arranged in pairs at the equator (Fig. 3C), although it has been suggested that the maternal centrosomes are located at each pole (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006). Protrusions of microvilli to perivitelline space (PVS) are found normally in MII oocytes, presenting a core of microfilaments presumably composed of actin (Fig. 4D) (Nottola *et al.*, 2014). Spherical or ovoid-shaped mitochondria are the most abundant organelles dispersed in the ooplasm of the mature human oocyte and contain a dense matrix and a few peripheral, arched or transversal, cristae (Figs. 4D-F). Sometimes, clear vacuoles within the mitochondria matrix have been detected. Often, they are characteristically associated with SER membranes, resulting in the formation of peculiar structures mainly located in the cortical ooplasm. These structures are composed of voluminous aggregates of anastomosing tubules or appear as small vesicles containing a flocculent, slightly electron-dense material. Both tubules and vesicles appear surrounded by mitochondria and are named M-SER aggregates and MV complexes, respectively (Fig. 4E). In fact, SER can be found as tubular aggregates or as isolated vesicles, however, in mature oocytes, the rough endoplasmic reticulum (RER) is not present (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006; Palmerini *et al.*,

2014a; Motta *et al.*, 2000; Coticchio *et al.*, 2016; Khalili *et al.*, 2013). M-SER aggregates are spatially separated, but form a network in the ooplasm that responds to stimuli in a coordinated manner, regulating, for example, the mitochondrial production of ATP, given that calcium is a mediator of oxidative phosphorylation (Makabe and Van Blerkom, 2006; Bianchi *et al.*, 2014; Motta *et al.*, 2000; Coticchio *et al.*, 2016). Golgi complexes and free ribosomes are rarely detected, while SER membranes of rough endoplasmic reticulum and vacuoles are mostly absent. However, Golgi complexes are abundant in oocytes at the GV-stage and in fertilized oocytes, where they develop a secretory activity allowing the formation of both lysosomes and cortical granules. Cortical granules (with a diameter of 300-400 nm) appeared as membrane-bounded organelles with a very dense and homogenous matrix, positioned right below the surface and form a continuous layer (Fig. 4D) (Sathananthan *et al.*, 1985; Makabe and Van Blerkom, 2006; Palmerini *et al.*, 2014a; Bianchi *et al.*, 2014).

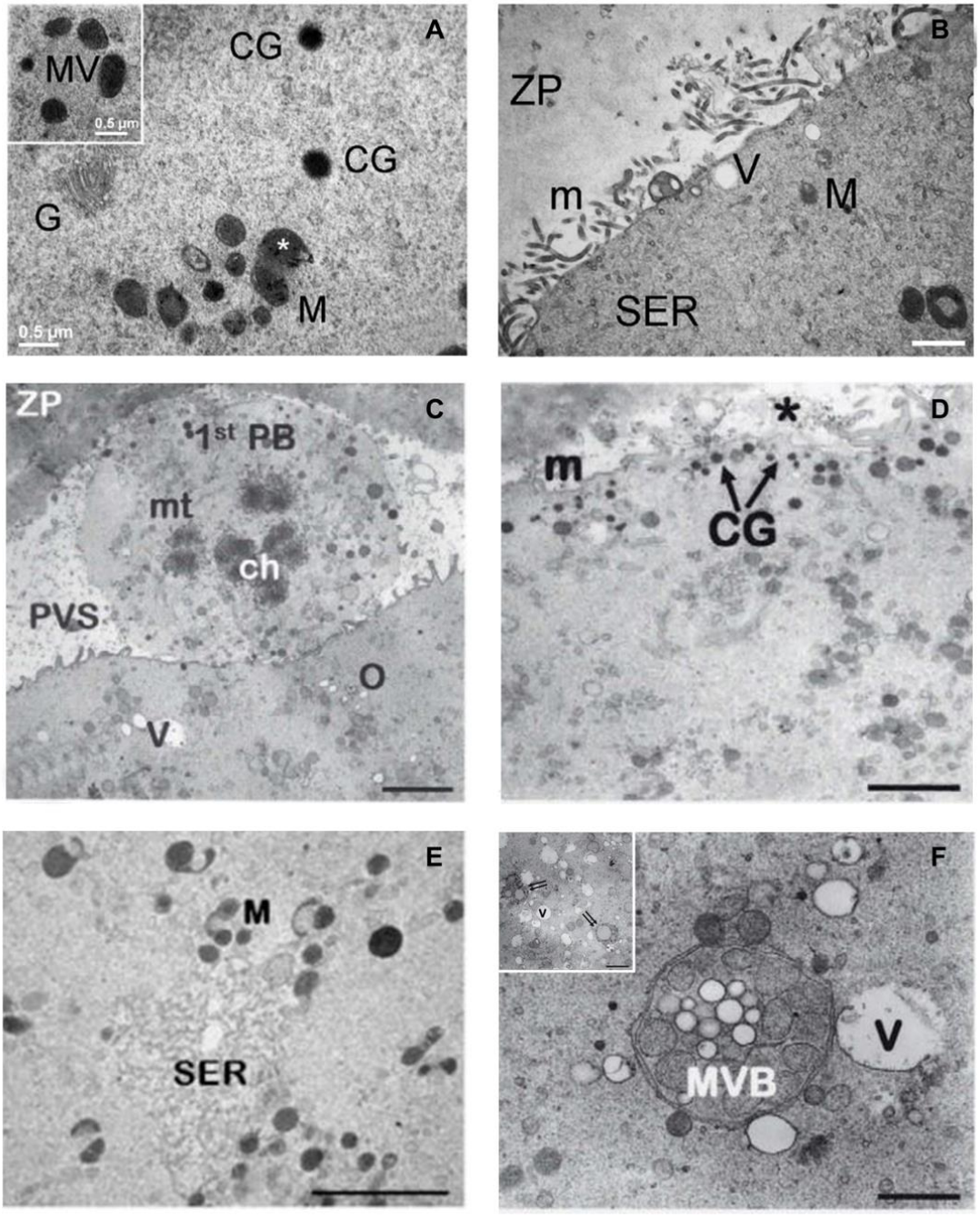


Figure 4. ***Representative TEM micrographs of cortical and subcortical regions of human oocytes in A) and B) GV and, C), D), E) and F) MII stages of maturation.*** In GV-stage oocytes, clusters of mitochondria (M), also containing a clear vacuole (white asterisk), Golgi complexes (G) and cortical granules (CG), are shown in the inner ooplasm, and in the outer ooplasm, the presence of microvilli (m) projecting to the zona pellucida (ZP), vacuoles (V) and elements of smooth endoplasmic reticulum (SER) are evident. The inset in A represents a mitochondria-vesicle complex (MV). In MII-stage oocytes a first polar body (1st PB) in the perivitelline space (PVS), CGs in the subcortex just beneath the surface, m projecting to the PVS with some debris (*), a voluminous aggregate between M and SER, a multivesicular body (MVB) and vacuoles (V) are present. The inset in F represents a cortical region of MII-stage oocytes with the presence of secondary lysosomes (double arrows). O: oocyte, mt: microtubules, ch: chromatin. (Modified by Nottola *et al.*, 2008, Nottola *et al.*, 2009 and Palmerini *et al.*, 2014a).

1.2 The origin of GV-stage oocytes

In 1825, the Bohemian physiologist Jan Evangelista Purkinje, at that time at the University of Breslaw, published a treaty entitled ‘De evolutione vesiculae germinativae’ (on the development of the germinal vesicle). The Czech scientist, five years later published a short account of his findings in Latin (Purkinje, 1830). Using only a hand-held lens, he described the presence and consistency of a vesicular structure in the hen's egg. Within each oocyte or “germ”, Purkinje saw a transparent, liquid-filled sphere, which he named the vesicula germinativa (germinal vesicle) (Farley, 1982). This name emerged because he initially considered this ‘vesicle’ as an entire cell from which an embryo would subsequently arise and not as the cell nucleus. As Vladislav Kruta pointed out, J.E. Purkinje discovered the germinal vesicle in the hen’s egg or that which a decade later would be designated a nucleus (Gilbert, 1991). Indeed, Purkinje considered the GV as the whole egg cell; after that, in 1826, the German Ernst Karl von Baer identified the egg cells in mammals and in 1830, Theodor Schwann argued that the ovum described by von Bear was probably a cell and the GV reported by Purkinje was a nucleus. After a while, in 1833, Coste showed that the egg of the rabbit contains a vesicle corresponding to that discovered in the hen's egg by Purkinje (Baker, 1949). In 1834, a doctorate student of J.E. Purkinje, Adolph Bernhardt, who later become one of the founding fathers of modern histology observed in the mammalian ovum a structure analogous to the germinal vesicle. The notion that it might be a cell nucleus could then hardly be avoided (Harris, 1999; Alexandre, 2001).

In 1835, Rudolph Wagner while studying the Graafian follicle of the sheep, discovered the presence of a ‘spot’ within the germinal vesicle, which he called macula germinativa (germinal spot). Wagner assumed that this 'spot' was the origin or first stage in the development of the germinal vesicle. In 1839, a Purkinje's close collaborator, Gabriel Gustav Valentin was the one who confirmed and introduced for the first time the nucleolus or macula germinativa, according to Purkinje. Later, Valentin, based on Purkinje's and Wagner's descriptions, made his observations and referred to the nucleolus as a ‘rounded, transparent secondary nucleus’ (Harris, 1999).

1.3 An *in vitro* approach of human immature oocytes retrieved after controlled ovarian hyperstimulation (COH) in Assisted Reproductive Technologies (ARTs): utility of GVs, ART techniques and well-preserved GVs, COH: COH protocols; patients-responders; cancer risks, importance of TEM

1.3.1 The issue of GV-stage oocytes utility in ARTs

In ARTs, it is of major importance if an oocyte obtained after controlled ovarian hyperstimulation (COH) can produce a high-quality embryo for transfer and afterwards a term gestation following *in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI). After COH, it is possible to retrieve both mature (MII-stage) and immature (GV-stage, MI-stage) oocytes and it is well known that only mature oocytes can undergo fertilization, while the immature ones are known as "leftover" oocytes. Suspicions have been raised repeatedly as to the impact of COH on the overall yield of high-quality oocytes, focusing on those that would be essential for generating high-quality embryos—these that are capable of leading to pregnancy and/or a baby born. However, are such oocytes simply leftover oocytes? And what effect, if any, could have the external gonadotropin administration on infertile women, thus on these oocytes?

This population of immature oocytes, focusing on GV-stage oocytes, are not simply leftover oocytes, and as reported before, they have assumed special importance for patients unfortunate enough to yield either incompetent for IVF/ICSI mature oocytes or not at all after COH. Regarding these so-called poor responders patients, a second opportunity to achieve oocyte maturity would increase the chances of having a qualified embryo transfer and a possible successful outcome. The usage of not simply leftover GV-stage oocytes as an alternative solution for these patients and for optimizing ARTs techniques such as *in vitro* maturation (IVM) and oocyte cryopreservation is now feasible; even if it is not so advanced and its impact on successful IVF or ICSI is not yet fully determined.

In scientific society, there are still major concerns about the 'dark side' of ovarian hyperstimulation. Specified focus is given to the risks of COH and/or COH different protocols on female patients' health as the risk of developing ovarian hyperstimulation syndrome (OHSS) or cancer etc., as well as on the good quality or on the production of healthy oocytes.

1.3.2 Survival and quality concerns of GVs connected to IVM and cryopreservation

1.3.2.1 IVM process, IVM rates and oocyte quality

In clinical IVM, oocytes retrieval occurs after the aspiration of mid-sized immature follicles along with the accompanied cumulus cells followed by 24–48 h of culture to achieve maturation *in vitro* (Fadini *et al.*, 2013; Coticchio *et al.*, 2016). Ovulatory follicles can be obtained by following the natural ovulatory cycle or after COH. IVM process was first introduced into clinical practice as an alternative treatment option in patients with polycystic ovary syndrome (PCOS) (Cha *et al.*, 1991), but nowadays is expanded and adapted as an option in a width area of infertility. However, the application and the success of IVM are still facing difficulties through the failure of artificial techniques to reproduce the *in vivo* regulated conditions of oocyte maturation, thus the embryos derived from *in vitro*-matured oocytes to show limited developmental potential (Gilchrist *et al.*, 2011; Coticchio *et al.*, 2015). Among different studies, the usual IVM rate of GV-stage oocytes collected from unstimulated and stimulated cycles differs and seems to be strictly associated with the age, the factor of infertility and the presence of cumulus cells (Khalili *et al.*, 2013). In the IVM procedure of human GV-stage oocytes, the oocytes matured on Day 1 were found to be of a significantly higher quality than those matured later on (Son *et al.*, 2005). When epidermal growth factor (EGF) was added to human GV-stage oocytes showed either to induce GVBD and nuclear maturation or to improve along with other growth factors (Areg, Ereg) the maturation rate of human GV-stage oocytes *in vitro* (Ben-Ami *et al.*, 2011). A higher significant percentage of GV oocytes that will complete maturation and extrude PBI has been noticed when GVBD occurs in central nucleolus-GV human oocytes rather than in peripheral nucleolus- GV oocytes, leading to improved IVM success rates (Levi *et al.*, 2013).

1.3.2.2 The debate on the sequencing of oocyte cryopreservation and IVM processes

A major debate has been challenged for years in the ART community, concerning the preservation of the quality of oocytes derived from infertile couples after COH, whether it is better to freeze oocytes at GV-stage before or after IVM at MII-stage. Some studies support the aspect of maturing *in vitro* immature oocytes before cryopreservation due to a negative impact of freezing to the success rates of IVM (Cao *et al.*, 2009; Lee *et al.*,

2013). Indeed, another study demonstrates a 46% of IVM success before vitrification, while after freezing the rate of successful IVM decreased up to 23%, however, fertilization, cleavage, and survival rates did not show any changes depending on vitrification before or after IVM (Fasano *et al.*, 2012). Cryopreservation at GV-stage oocytes produced maturation rates lower (51.3 %) than those after IVM (75.7 %), thus suggesting that the cryopreservation of MII oocytes is recommendable, also because of the high spontaneous activation occurring at the GV-stage (Wang *et al.*, 2012). Regarding the type of GVs cryopreservation, slow freezing *vs* vitrification, there was no difference in survival and maturation rates of the oocytes, but a higher number of mature oocytes showing normal bipolar spindle was detected after vitrification in respect to slow freezing, thus confirming the high efficacy of vitrification in oocyte quality (Combelles *et al.*, 2011).

In 1998, the first birth from *in vitro* matured GV-stage oocytes after cryopreservation from a stimulated cycle had been published. Only two GVs succeed to mature successfully *in vitro*, followed by fertilization, embryo development, and transfer, pregnancy as well as a baby born (Tucker *et al.*, 1998).

1.3.2.3 The importance of presence or absence of cumulus cells around GVs during cryopreservation

The role of cumulus cells during oocyte maturation is to maintain the oocytes under meiotic arrest, to induce meiotic resumption and to take part in ooplasmic maturation. In fact, cumulus cells play an essential role in the *in vivo* process of maturation for providing to the oocyte nutrients and regulatory molecules through intercellular connections as reported here before (Van Soom *et al.*, 2002). During *in vitro* processes, GVs with removed cumulus cells were showed a reduced developmental capacity in comparison with cumulus-enclosed GVs (Mahmodi *et al.*, 2009; Zhang *et al.*, 2012). Nevertheless, the effect of the presence or absence of cumulus cells in GVs prior to oocytes' exposure to cryoprotectants and freezing is still a matter of debate. The reduction in subsequent developmental competence caused by exposure to anisotonic conditions and is more severe in GV-stage oocytes matured with cumulus-oocyte complex (COCs) than those of MII-stage oocytes (Agca *et al.*, 2000; Abe *et al.*, 2005). No difference in survival rates of oocytes following freezing has been noticed under the presence or absence of cumulus cells (Mandelbaum *et al.*, 1988; Chian *et al.*, 2004). However, the presence of cumulus

cells after freezing had a positive impact on oocyte survival (Imoedemhe and Sigue, 1992; Im *et al.*, 1997). The high survival rates of cryopreserved oocytes along with cumulus cells were found to be due to changes in the oocyte membrane's permeability in water and cryoprotectant oocyte membrane (Gook *et al.*, 1995). Based on this the presence of the cumulus cells may offer some protection against sudden osmotic changes and stress that could be induced by rapid influx or efflux of cryoprotectant, during the procedures of equilibration and removal of cryoprotectant in the pre-freeze and post-thaw periods, respectively (Imoedemhe and Sigue, 1992; Fabbri *et al.*, 2001). However, a study on cryopreservation of human cumulus-free GVs before or after IVM showed that when oocytes are frozen as cumulus-free GVs exhibited decreased maturation and increased spontaneous activation, proposing that it is best to freeze cumulus-free mature oocytes (Wang *et al.*, 2012).

1.3.3 Controlled ovarian hyperstimulation

COH has been an integral and fundamental part of the success of ART procedures for the last three decades. COH facilitates the recruitment of multiple oocytes in each ovarian cycle and, therefore, can allow more than one embryo to transfer or the possibility to cryopreserve oocyte or supernumerary embryos for future usage, i.e. in a subsequent cycle, thus avoiding the repetition of ovarian stimulation (Hamdine *et al.*, 2014). However, recently it has become evident that COH can negatively affect oogenesis, embryo quality, endometrial receptivity and probably even in perinatal outcomes (Santos *et al.*, 2010).

The hyperstimulation of the ovary occurs with exogenous gonadotropins that promote the synchronized growth of multiple follicles in the preovulatory phase by interfering with the physiological mechanisms, able to ensure the selection of a single dominant follicle (Santos *et al.*, 2010). In natural cycles, the stimulation of ovary initiates from GnRH that stimulates the secretion of FSH and LH from the anterior pituitary gland, which in turn regulates ovarian follicular development and the selection of a single dominant follicle. The LH surge in the middle of the menstrual cycle then determines ovulation (Fig. 2) (Alper and Fauser, 2017). Nowadays two different classes of COH protocols are available, depending on the usage of GnRH analogue, the more classic one that uses GnRH agonists and the more recent one that uses GnRH antagonists instead (Itskovitz-Eldor *et al.*, 2000; Zhang *et al.*, 2010). GnRH is a small decapeptide made and secreted

by the hypothalamus in the portal circulation intermittently, stimulating the pituitary to synthesize and secrete FSH and LH. Clinically safe GnRH agonists have been obtained by replacing one or two amino acids in the original decapeptide structure, making these compounds more hydrophobic and more resistant to enzymatic degradation (Macklon *et al.*, 2006). The classical treatment regimens are based on the administration of these agonists and high doses of FSH of urinary or recombinant derivation (rFSH). The standard doses of gonadotropins used vary between 150 and 450 IU / day, but, recent work shows that several randomized trials have not shown that an increase in the dose of gonadotropins necessarily increases the chances of pregnancy (Verberg *et al.*, 2009). In fact, these treatment regimens present several weak points, due to daily injections for a long period of time and continuous echographic monitoring to evaluate the response to treatment, which can lead to high rates of therapy abandonment and pelvic pain (Aboulghar and Mansour, 2003). One of the major risks is the onset of OHSS, characterized by an increase in capillary permeability with consequent phenomena of generalized transudation, haemoconcentration, and hypovolemia (McClure *et al.*, 1994). In addition, several studies showed that excessively high levels of estrogen linked to stimulation could cause negative effects on endometrial receptivity and consequently reduce the probability of embryonic implantation (Devroey *et al.*, 2004; Haouzi *et al.*, 2010). This decade, a study of 19,146 women undergoing IVF has shown an effective increase in the risk of developing ovarian tumors, compared to the general population (van Leeuwen *et al.*, 2011). In this regard, years before, in 1996, Edward and the co-workers were the first that advocated a less aggressive approach, called mild-stimulation, with the aim of developing COH protocols more tolerated by patients and safer without compromising the rate of pregnancy.

1.3.3.1 Conventional or full ovarian hyperstimulation (fCOH) and mild-hyperstimulation (mCOH)

Clinical high-dose fCOH protocols intend to abolish the physiological decline of FSH and simultaneously to increase the serum FSH levels beyond the threshold required for follicle development over a prolonged period, thus allowing growth and maturation not only of one but of the entire cohort of follicles that reached the stage of FSH-dependent development (Fauser *et al.*, 2005). Using fCOH, daily administration at low doses of GnRH agonists initiated in the mid-luteal phase of the previous cycle, until the end of the

stimulation, aiming to determine a pituitary blockade. About 15 days later and after the menstruation, gonadotropins (150-450 IU / day) are administered to obtain the simultaneous growth of various follicles (Fig. 5). However, the stimulation of the growth of multiple follicles leads to the production of serum levels of supra-physiological estradiol inside follicles, which, through positive feedback to the pituitary gland, can cause a premature LH surge and therefore lead to early luteinization and ovulation. Due to this, the exogenous administration of FSH is accompanied by the administration of GnRH analogues that leads to hypersecretion of gonadotropin, followed by desensitization of the pituitary gland, with consequent to gonadotropin suppression and prevention of a premature LH surge (Santos *et al.*, 2010). Extensive evidence for the supremacy of the GnRH agonist protocol has led to its widespread use in IVF. Advantages of GnRH agonist treatment include the ease of programming the IVF cycles, since the beginning of gonadotropin treatment can be established prior; a low cycle cancellation rate due to poor ovarian response; a high number of recovered oocytes as well of embryos to transfer among with a higher possibility of selecting good quality embryos (Table 2).
In contrast, mCOH is based on the "FSH window" concept, according to which the follicular growth of the dominant follicle is time-dependent and not dose-dependent FSH. Therefore, this protocol is characterized by a continuous but moderate increase in FSH levels during the mid-late phase of folliculogenesis, so as to obtain an extension of the "window" period and the selection of more follicles. This scheme is generally associated with the use of GnRH antagonists in order to inhibit spontaneous ovulation in these patients, without previously desensitizing the pituitary gland. GnRH antagonists prevent the premature LH peak by blocking the GnRH receptor competitively, and they do not induce the initial flare-up effect like the GnRH agonists, but result in rapid suppression of endogenous gonadotropin secretion. Due to this, the administration of low-dose gonadotropins can be postponed to the medium-late follicular phase, a couple of days prior to the antagonist administration, following the physiological follicular recruitment and, at the same time, avoid the reduction of concentrations of endogenous FSH and resulting in a monofollicular selection (Fig. 5) (Verberg *et al.*, 2009).

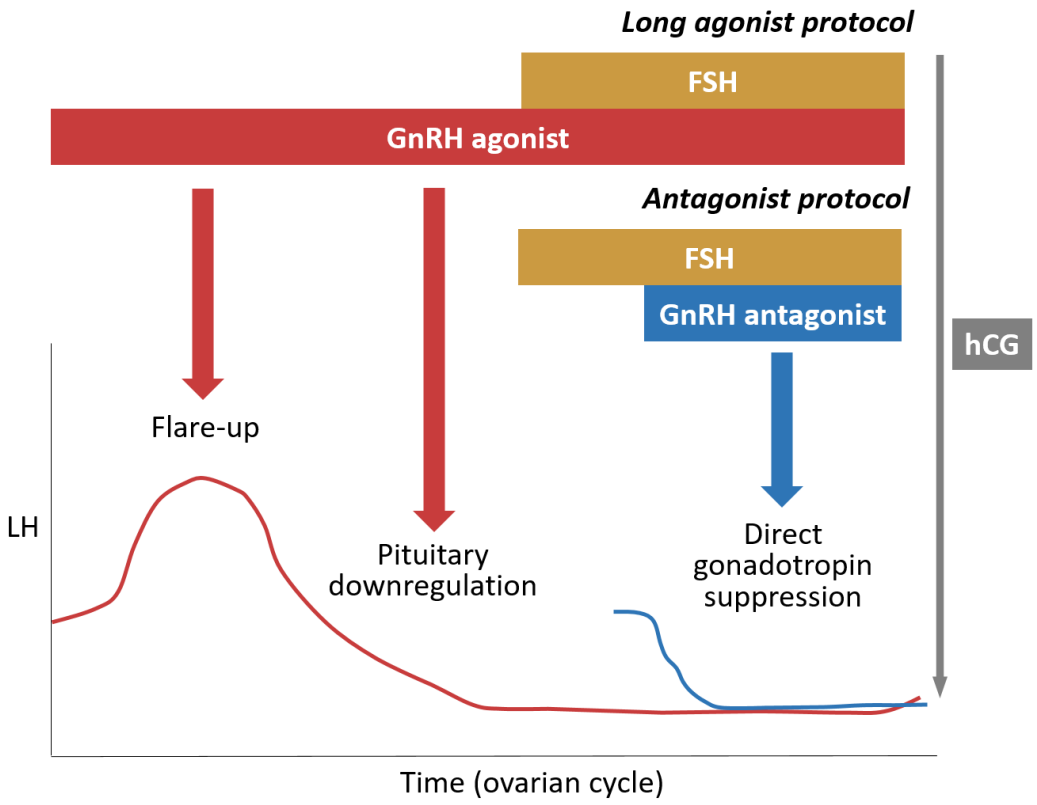


Figure 5. ***Differences in ovarian response to COH treatment between GnRH agonist (full) and GnRH antagonist (mild) stimulation protocols used in ARTs.*** (Modified by Vloeberghs *et al.*, 2011)**.**

In fact, the antagonists do not totally suppress the physiological release of endogenous FSH, as the pituitary function is recovered about 24 hours after their administration and, consequently, a lighter administration and/or for a shorter duration of exogenous FSH may be required in association with these drugs (Santos *et al.*, 2010; Mahajan, 2013). Several studies tried to establish the most appropriate day to initiate ovarian stimulation to obtain the most optimized response. It is showed that starting the stimulation on the 7th day of the cycle a monofollicular selection was obtained in 30% of the cases and a multi-follicular in 70%, on the other hand, a higher percentage of multifollicular selection has been found when the administration started on the 5th day of the cycle (Hohmann *et al.*, 2001). Concerning the dose of gonadotropin, the use of 150 IU / day of rFSH was the most effective dose for follicular selection (de Jong *et al.*, 2000). In recent years, several GnRH antagonists have been developed, later applied in clinical practice. The replacing of histidine in position 2 and tryptophan in position 3 were the model of which first-generation antagonists were obtained, but these compounds had low power. In second-generation compounds, the activity was increased due to the incorporation of a D-amino acid in position 6 of the primary structure; however, the use of these GnRH antagonists

caused frequent anaphylactic responses due to histamine release. To overcome this, introducing further substitutions in position 10, third-generation compounds have been developed, such as ganirelix (developed by Syntex Research, Palo Alto, CA) and Cetrotide (developed by Asta Medica, Frankfurt, Germany), both safe and effective for use in IVF treatments (Macklon *et al.*, 2006). Regarding the clinical outcome, expressed in percentage of pregnancies, a prospective study showed that the effectiveness of the stimulation protocol at 150 IU / day, started on the 5th day of the cycle, can be compared to that of the full conventional protocol (Hohmann *et al.*, 2003). COH treatment with GnRH antagonists is advantageous and was associated with a lower risk of complications such as OHSS and a lower burden of treatment, as reduced dose and adaptable/patient-dependent days of stimulation (Al-Inany and Aboulghar, 2002). Indeed, the introduction of the GnRH antagonist has allowed for the development of more patient-friendly protocols. The general advantages and disadvantages of both fCOH and mCOH used in IVF treatments are shown in Table 2.

Advantages	
fCOH	mCOH
High number of retrieved oocytes	Lower medication consumption
Maximal pregnancy rates	Shorter stimulation timings
Higher number of oocytes/embryos for cryopreservation	Lower cost application «per cycle»
	Lower risk of complications
	Low rate of drop-out from IVF program
	Lower pharmacological impact on oocyte quality
	Lower impact on endometrial receptivity
	Lower incidence of severe OHSS
Disadvantages	
fCOH	mCOH
Longer stimulation timings	Fewer retrieved oocytes
High-cost application «per cycle»	Lower pregnancy rates
High risks and complications for patients	Fewer oocytes/embryos for cryopreservation
Non-physiological estrogen levels	
Higher incidence of severe OHSS	
High rate of drop-out from IVF program	
Uncertain long-term consequences	

Table 2. ***Advantages and disadvantages of «full» and «mild» ovarian hyperstimulation in the clinical application.*** (Modified by Revelli *et al.*, 2011 and Orvieto *et al.*, 2017)**.**

However, since many conflicts have been raised regarding the terminology of mCOH stimulation, in 2007, a current approach is based on the proposal of Rotterdam-based International Society for Mild Approaches in Assisted Reproduction (ISMAAR) Consensus Group on Terminology for Ovarian Stimulation for IVF. ISMAAR association defined as "mild" IVF cycle either (a) a ovarian stimulative scheme including exogenous gonadotropin administration at a lower-than the conventional dose and/or for a shorter duration throughout a cycle (usually as a delayed start) in GnRH antagonist co-treated cycles, or (b) a stimulation including the use of oral compounds (e.g. anti-estrogens or aromatase inhibitors) either alone or combined with gonadotropins and GnRH-antagonists (Nargund *et al.*, 2007). Clomiphene citrate and letrozole are the two most common pharmaceutical compounds administered for mild ovarian stimulation. Nevertheless, the definition of mild stimulation in studies and practice is still variable by usually presenting differences in the response of the chosen stimulation scheme per individual patient (European Society of Human Reproduction and Embryology, 2019a).
Finally, the concomitant administration of a GnRH agonist or antagonist is used to prevent a premature increase in LH, resulting in the development of multiple dominant follicles simultaneously. COH treatments end up with the terminal oocyte maturation and ovulation triggering by a bolus injection of a GnRH agonist, hCG (human Chorionic Gonadotropin, an LH-like hormone, but with a longer half-life) or both (Alper and Fauser, 2017).

1.3.3.2 Patients undergo COH

Female patients undergoing ARTs are driven to the appropriate method in accordance with their cause of infertility and the potential ovarian response to COH treatment. Therefore, the patients are categorized as poor responders, normo-responders, and hyper-responders. Even mCOH uses less aggressive, and more patient-friendly protocols, not all the patients are suitable for this type of stimulation as analyzed before. An individualized approach for COH is currently under discussion in the society of reproductive medicine as a more appropriate and safer treatment for the patients. However, some common suggestions of COH treatments by clinical studies in accordance with the category of the patient are given below.

1.3.3.2.1 Low or poor responder

Bologna criteria are selected to describe poor responders. This may avoid any bias caused by spurious poor ovarian response (POR) definitions connected with the characterization of POR patients. According to the group of Ferraretti, poor responder patients are considered the women who have a poor response to ovarian stimulation-gonadotropin treatment, more specifically they have a reduction in follicular response resulting in a reduced number of retrieved oocytes (Ferraretti *et al.*, 2011). Generally, ≤ 3 follicles on the day of oocyte maturation trigger and/or ≤ 3 oocytes obtained characterize a low response (European Society of Human Reproduction and Embryology, 2019a). At least two of the three following features after max-stimulation are sufficient to classify a patient as poor responder, in the absence of advanced maternal age or abnormal ovarian reserve test (ORT): (i) advanced maternal age (≥40 years) or any other risk factor for POR; (ii) a previous POR (≤3 oocytes with a conventional stimulation protocol); and (iii) an ORT (i.e. AFC <5–7 follicles or AMH <0.5–1.1 ng/ml). In addition, by definition, the term POR refers to the ovarian response. Therefore, one stimulated cycle is considered essential for the diagnosis of POR, as well as the patients with advanced maternal age or abnormal ORT should be defined as expected POR (Ferraretti *et al.*, 2011).
A common treatment trend to be the less effective to poor responders is the scheme that usually includes high doses of exogenous gonadotropins, i.e. GnRH agonist "flare" protocol. However, a recent study suggested that the chosen COH protocol should complement the patient's natural cycles, rather than override them with high doses of gonadotropins, and avoid suppressive hormonal treatments (Gonda *et al.*, 2018).

1.3.3.2.2 Normal responder

As normal ovarian responders, per definition, are consider all women who respond to COH with standard gonadotropin doses and the number of retrieved oocytes is approximately 4 to 15, excluding those who poorly or excessively respond to stimulation. Therefore, these patients could have a good prognostic value for successful IVF. In addition, normal responders' definition could be associated with the chances of achieving a pregnancy with minimal risk of OHSS (Polyzos and Sunkara, 2015). However, the majority of published data failed to demonstrate significant differences in terms of pregnancy rates in their primary analysis, either when comparing GnRH agonist versus

antagonist regimens (Xiao *et al.*, 2014), or different gonadotrophin treatments (Devroey *et al.*, 2009; van Wely *et al.*, 2011; Konig et al., 2013), thus allowing the assumption that less aggressive protocols enable to reduce the adverse effects caused by heavy stimulation, could be preferable (Borges *et al.*, 2017).

1.3.3.2.3 High or hyper responder

Hyper-responders are defined the women who show an exaggerated response to standard doses of gonadotropins. Therefore, they are characterized by the presence of more follicles and/or oocytes than intended (Griesinger *et al.*, 2016). Generally, more than 18 follicles ≥11 mm in size on the day of oocyte maturation trigger and/or 18 oocytes retrieved characterize an excessive ovarian response (Griesinger *et al.*, 2016) defined by a risk increase in OHSS (Griesinger *et al.*, 2016; European Society of Human Reproduction and Embryology, 2019b). Although hyper responders are generally considered a good-prognosis group regarding reproductive success, it is currently debated whether a high ovarian response is related to a decreased chance of successful outcome as compared with a normal response (Arce *et al.*, 2014). Apart from the increased possibility of OHSS in this group of patients, women with PCOS are considered as hyper responders. In fact, PCOS patients characterized by numerous small antral follicles in ovaries capable of responding to gonadotropin triggering (Dumesic *et al.*, 2008). Regarding the COH treatment of hyper responders, a reduced gonadotropin dose is recommended to decrease the risk of OHSS, with GnRH antagonist control to be the most proposed protocol (European Society of Human Reproduction and Embryology, 2019b).

1.3.3.3 COH-related complications

OHSS is rare and the most serious iatrogenic complication of COH applied in IVF techniques, occurring when high doses of gonadotropins used and/or when patients are sensitive to the risk of developing OHSS, as PCOS patients, young women with low body mass index and women who present high levels of estradiol during COH (Rizk and Smitz, 1992; Zivi *et al.*, 2010). The syndrome represents an exaggerated response to the induction of ovulation, involving the secretion of vasoactive-angiogenic ovarian substances that increase capillary permeability and causes accumulation of liquids at the level of the extravascular space. Clinical symptoms of OHSS are usually appeared

following the administration of hCG, during the luteal phase, or after a COH cycle with resulted pregnancy. In most of the cases, the syndrome develops as a self-limiting disorder able to resolve spontaneously (Zivi *et al.*, 2010). In the most severe cases of OHSS may even occur haemoconcentration, hypovolemia, thromboembolism, development of estrogen-dependent tumors and death (Mahajan *et al.*, 2015). Even if the standard protocols of COH still foresee the single-use of gonadotropins, nowadays the increased interest in the use of clomiphene citrate and/or letrozole compounds combined with gonadotropins during COH allow the reduction of OHSS incidence (Haas and Casper, 2017). Indeed, when clomiphene citrate used, the incidence of appearing mild and moderate OHSS were only 13.5% and 8% respectively, while rarely the use of clomiphene citrate is associated with the severe form of the syndrome (Zivi *et al.*, 2010). The use of ovulation-inducing drugs has been hypothesized to increase the risk of several cancers, including ovarian cancer, and there has been concern about the long-term effects of fertility drugs ever since they were first prescribed. A few cases of developing ovarian cancer in women after COH, although, in most of these cases, the tumor was diagnosed soon after IVF treatment and was developed very rapidly, increasing the possibility that COH or other factors, such as oocyte aspiration, accelerate the growth of an existing and/or unexplained tumor (Dor *et al.*, 2002). In mid -60s, the possibility of an association between the gonadotropins used in COH and different types of gynaecological cancer emerged (ovarian, endometrial and cervical), however, the scientific evidence that could confirm this association is still limited. Proofs relating to a direct neoplastic effect of ovarian stimulation drugs are weak and controversial, mainly based on *in vitro* studies (Siristatidis *et al.*, 2012).

In particular, gonadotropins are known to induce a variety of biological effects in the epithelium, including changes in cell proliferation, apoptosis, cell adhesion, and chemosensitivity, together with an increase in estradiol blood concentrations: occurrences that promotes the induction and the development of the tumor. Regarding epithelial ovarian cancer, several theories have been developed on tumorigenesis related to *in vitro* stimulation and fertilization such as incessant ovulation, puncture of trauma during oocyte recovery, depletion of ovarian follicles, inflammation and impaired paracrine activity due to an increase in serum gonadotropins and steroid level. Indeed, a statistically significant association was indicated between COH and increased ovarian cancer risk (Diergaarde and Kurta, 2014). During COH, risk factors for endometrial cancer development (the most common hormone-dependent malignant tumor of the lower female genital

apparatus) are represented by the presence of hyper-estrogenic environment and of the super-physiological levels of gonadotropins (Siristatidis *et al.*, 2013). Cervical cancer is linked not directly to COH, but to several factors that were found to be associated with female infertility, such as pelvic adhesions or tubal stenosis caused by previous pelvic infections and especially to infection by the human papillomavirus. In this regard, in cases where multiple IVF trials have been performed, the infection of virus caused higher rates of infection, associated with a high number of cervical procedures, even if the exact association between cervical cancer and COH is not verified yet (Siristatidis *et al.*, 2012). Lately, concerns have been raised about the potential role of COH drugs in breast tumorigenesis by indicating significant changes in serum estrogen and progesterone levels. However, the relatively short and transient period of increase in circulating estrogens related to an IVF cycle may not be enough to alter the risk of breast cancer substantially. Noteworthy is the fact of the possible protective IVF role and action against breast cancer, probably explained by the so-called "healthy patient effect", so that women seeking infertility treatments are relatively healthier or belonging to a privileged socio-economic class compared to their counterparts in the general population. In fact, protective factors against breast cancer could be more frequent in cases of pregnancies obtained by IVF, such as preeclampsia (a syndrome characterized by clinical signs such as edema, hypertension, and proteinuria) and multiple births (Sergentanis *et al.*, 2014). In addition, it is known that COH treatments may favor the high mitotic activity of granulosa cells in the ovary. So, even if granulosa cell tumors are rare among the gynaecological malignancies of the stromal cells in the ovary, the increased concentration of circulating estrogens, due to the stimulation of ovulation by exogenous gonadotropins, may cause an increase in onset of this type of cancer, indicating a possible relationship between granulosa cell tumor and COH (Yousefi *et al.*, 2018).

1.3.3.4 Optimizing oocyte quality in ARTs: pros and cons of TEM

The positive outcome of ARTs is strictly dependant on the oocyte quality, evaluated in terms of the preservation of full morpho-functional integrity during its complex and long maturative process. In fact, both *in vivo* and *in vitro*, the completion of oocyte maturative changes in the preovulatory period as well as the absence of degenerative alterations in the cytoplasmic microdomains can ensure the competence for fertilization. Electron microscopy (EM) observations are fundamental to assess the structural integrity of single

cells as an oocyte in order to define a proper "oocyte health" status. In fact, EM can highlight not only obvious alterations but also minute subcellular changes in the oocyte microdomains. These minimal changes cannot be defined by other morphological or bio-molecular techniques routinely used in ART laboratories and could be responsible, in some cases, of unexplained failure of ART procedures (Motta *et al.*, 1988). Of all the ultrastructural techniques, transmission electron microscope (TEM) is the gold standard to examine subcellular structures and their preservation. During the past years, the contribution of EM to human ARTs provided useful and objective information about the oocyte sensitivity to cryopreservation (Khalili *et al.*, 2012; 2017; Palmerini *et al.*, 2014a; Nottola *et al.*, 2016; Taghizabet *et al.*, 2018) and IVM (Coticchio *et al.*, 2016).

TEM analysis on immature, leftover oocytes collected from women undergoing ARTs, can be particularly useful for understanding the type and the extension of ooplasmic defects. These can start to appear at the GV-stage, as the formation of vesicles that do not preclude an appropriate nuclear maturation, or can be so deleterious to lead to an *in vivo* maturation failure as the absence of nucleoli, malformations in the ZP (as deep penetration into the ooplasm in different locations) or alterations in the SER network associated to abnormal calcium signalling and subsequent block of the nuclear maturation (Sathananthan *et al.*, 1985).

However, ultrastructural analysis of human oocytes are not extensively applied since TEM is invasive; it requires highly trained technical support and expensive equipment. In fact, TEM can play a crucial role in basic and translational research in reproductive biology and medicine. TEM analysis may be adopted to study morphological changes in human oocytes treated *in vitro* with different substances under different conditions to optimize the outcome of IVF. Therefore, in the present PhD thesis, TEM seemed the most appropriate method for studying the ultrastructural differences in organelles and subcellular compartments from immature, GV-stage oocytes collected by women undergoing different COH treatments.

Part 2: Conception and objective of the study

2.1 Conception and objective of the study

Controlled Ovarian Hyperstimulation (COH) is an integral treatment applied to infertile women undergoing ARTs as IVF or ICSI, aiming to induce ovulation by multiple ovarian follicles for achieving a high number of mature oocytes, possibly competent for the subsequent fertilization. Different COH protocols were developed and evaluated through years and the usage of the 'universal' fCOH one, including high doses of exogenous gonadotropin, started to decrease over time, to be gradually substituted with less aggressive, more individualized protocols to gain more ground in reproductive clinics. Nowadays, both mCOH and fCOH protocols are routinely used, even if recent reports (Almind *et al.*, 2018; Wong *et al.*, 2019) are evidencing a milder impact on women's health and a better effect on the quality of retrieved oocytes after the milder approach, that is also more patient-friendly. The quality of retrieved oocytes was mainly studied in mature oocytes, using various biological techniques under different *in vitro* conditions; however, the leftover immature oocytes, retrieved after COH may represent a unique alternative source of gametes for infertile women, especially for those affected by severe infertility and a history of recurrent failure of ARTs.

Indeed, since COH was associated to aberrant distribution of microfilaments and cortical granules and spindle defects in mature oocytes and embryos (Lee *et al.*, 2006; Van Blerkom and Davis, 2001), **in the present PhD thesis** it was evaluated the effects of two different COH protocols, a GnRH agonist (full, fCOH) and a GnRH antagonist (mild, mCOH) stimulation, on the fine structure of fresh human leftover GV-stage oocytes. The structural features of fCOH and mCOH oocytes, from the outer ZP texture until the inner nucleus morphology and chromatin distribution were analyzed by light microscopy (LM) and transmission electron microscopy (TEM). Moreover, a morphometric analysis of the number and dimension of mitochondria, vacuoles, and cortical granules was performed.

Data here presented from the ultrastructural observations of leftover immature oocytes retrieved after fCOH and mCOH can contribute to a better comprehension of the role exerted by hyperstimulation on the fine structure of human oocytes.

Part 3: Materials & Methods

3.1 Experimental design

The present study evaluated the effects of two different COH protocols on the ultrastructure of fresh GV-stage human oocytes. GV oocytes were obtained from women who underwent IVF for infertility reasons and subjected to ovarian stimulation with either a GnRH agonist, "full stimulation'' (fCOH) or a GnRH antagonist "mild stimulation" (mCOH) protocols. Fourteen and nine GV-stage oocytes were donated from women who followed fCOH and mCOH treatments, respectively. After donation, fresh GVs were washed in phosphate-buffered saline (PBS), fixed in 2.5% glutaraldehyde/PBS and subjected to standard preparative for light (LM) and transmission electron microscopy (TEM). Morphometric analysis was performed on TEM micrographs.

3.2 Patients

Seven patients undergoing ART treatment (IVF/ICSI) program due to different infertility factors (tubal or male factors) donated a total number of twenty-three fresh GV-stage oocytes with their informed consent and according to the current Italian laws. The study was approved by the ethical committee of Province di Chieti and Pescara, convened in "G. d'Annunzio" University of Pescara (protocol approved on 22.03.2018). Fourteen fresh GV-stage oocytes were donated from four women (mean age 32.07 ± 1.54) whose infertility was due to tubal factors, and nine fresh GV-stage oocytes were donated from three women (mean age 35.1 ±2.23) whose infertility was due to male factors, and their ovarian stimulation was carried out with an fCOH and an mCOH protocols, respectively. More than one oocyte from the same patient was included in this study.

3.3 "Full" and "mild" controlled ovarian hyperstimulation protocols

The fCOH of the donors (n = 4) was performed with the widely used daily long GnRH-agonist protocol (Decapeptyl 3.75 mg, Ipsen SpA, Italy) and recombinant rFSH (Gonal F, Merck Serono, Rome, Italy), 150 IU per day. Serum 17β-estradiol measurements and ovarian ultrasonography were applied for monitoring the follicular growth. Final ovulation was triggered with 10,000 IU of hCG (Gonasi HP, AMSA, Italy) when at least 3 follicles with a diameter ≥ of about 17 mm were visible at ultrasound scan monitoring,

and the numbers of the follicles were appropriate according to 17β-estradiol levels. The oocytes were retrieved 34-36 h after hCG administration.

The mCOH of the donors (n = 3) was performed with a GnRH-antagonist protocol. The patients received 150-225 IU/day s.c. rFSH starting on day 2 of the menstrual cycle for the first three days of treatment. This was followed by an individually adjusted daily dose of rFSH from day 5 of the menstrual cycle. Multiple doses (0.25 mg/day) of cetrorelix (Cetrotide; Serono) were administered s.c. from day 6 of stimulation up to the day of hCG. An i.m. a single bolus of 10,000 IU of hCG (Gonasi HP 5000; AMSA, Rome, Italy) was administered when at least 3 follicles reached a mean diameter of about 18 mm. Transvaginal follicular aspiration was carried out 34-36 h after hCG administration (Centurione *et al.*, 2010).

After oocyte pick-up, among the pool of leftover GV-stage oocytes, only those with an evident nucleus and without any sort of dysmorphism, after Phase Contrast Microscopy (PCM) examination, were included in this study. Fresh GV-stage oocytes (n = 14) obtained from patients subjected to fCOH protocol were here indicated as fCOH oocytes; those (n = 9) obtained from patients subjected to mCOH protocol as mCOH oocytes.

3.4 Preparation for light and transmission electron microscopy

The total number of oocytes from both groups, fCOH, and mCOH, included in the current PhD thesis were fixed and processed for LM and TEM analysis as previously described (Nottola *et al.*, 2007; Nottola *et al.*, 2008). According to standard preparation protocol, oocyte fixation was performed in 2.5% glutaraldehyde (SIC, Rome, Italy) in phosphate-buffered saline (PBS) solution. For at least 48h at 4°C after fixation, the samples were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar Scientific, Stansted, UK) in PBS and rinsed in PBS. Oocytes were then embedded in small blocks of 1% agar of about 5 × 5 × 1 mm in size, dehydrated in an ascending series of ethanol (Carlo Erba Reagenti, Milan, Italy), substituted in propylene oxide (BDH Italia, Milan, Italy), before embedding in epoxy resin EMbed-812 (Electron Microscopy Sciences, Hatfield, PA, USA). Semithin sections (1 mm thick) were contrasted with Methylene Blue, examined using a Zeiss Axioskop light microscope and photographed using a digital camera (Leica DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, on a Reichert-Jung Ultracut E ultramicrotome, mounted on copper grids and contrasted with uranyl acetate followed

by lead citrate (SIC, Rome, Italy). They were examined and photographed using Zeiss EM10 and Philips TEM CM100 electron microscopes operating at 80 kV.

The following parameters were evaluated by LM and TEM and taken into consideration for qualitative assessment of the ultrastructural preservation of oocytes, as previously described (Motta *et al.*, 1988; Motta *et al.*, 2003; Coticchio *et al.*, 2010, 2016; Khalili *et al.*, 2012, 2017; Nottola *et al.*, 2016): general features (including oocyte shape and dimensions); morphology of the nucleus, nucleolus, chromatin and nuclear envelope (including nuclear membrane integrity); microtopography, type, and quality of the organelles; mitochondria shape, number, and dimensions; presence and extent of ooplasmic vacuolization; position, number, and dimensions of cortical granules (CGs); integrity of the oolemma and microvillus pattern; appearance of the perivitelline space (PVS) (width, presence of fragments); texture of the zona pellucida (ZP).

3.5 Statistical analysis

Numbers and dimensions of mitochondria, vacuoles, and CGs were evaluated by ImageJ software (http://rsbweb.nih.gov/ij/; version 1.51t), as previously done in other studies (Coticchio *et al.*, 2010, Palmerini *et al.*, 2014b, Leoni *et al.*, 2015, Nottola *et al.*, 2016). For the assessment of the morphometry, low-magnification TEM micrographs taken from at least three equatorial sections per oocyte (distance between the sections: 3-4 μm) were analyzed by Adobe Photoshop and ImageJ software to count and measure mitochondria, vacuoles, and CGs. Images were enlarged on the PC screen for more easily recognizing and evaluating the organelles by Image J software.

Values related to organelle numbers were expressed as the number of mitochondria and vacuoles per 100 μm^2 of the oocyte area, and the number of CGs for 10 μm of the oocyte linear surface profile.

Values related to organelle dimensions were expressed as medium diameter (in μm) of mitochondria, vacuoles, and CGs since all of these structures appeared mostly rounded in shape.

For the selection and classification of vacuoles by TEM, we studied the electron-lucent vacuoles with a diameter of at least 0.2 μm, i.e., corresponding to the size of vacuoles detectable by LM at a magnification of 400X.

The number and dimensions of the organelles were expressed as mean ± standard deviation (SD). Data were compared with the Student's t-test (SIgmaPlot, version 11.0 Build 11.0.075, 2008). Differences in values were considered significant if $P < 0.05$.

Part 4: Results

4.1 General features

LM and TEM allowed analysis and comparison of size, shape and organelle distribution of all GV-stage oocytes included in this study. All fCOH and mCOH oocytes were rounded, approximately 73–86 μm in diameter (ZP excluded), showing a normal ooplasm and a uniform distribution of organelles (Figs. 6A, 7A). In all the observed oocytes, the nucleus and nucleolus were spherical, the ZP was continuous and/or intact and separated by a narrow PVS from the oolemma provided with microvilli (Figs. 6A-B, 7A-B). By PCM, all GV-stage oocytes showed a good morphological appearance.

4.2 Nucleus

All fCOH and mCOH oocytes were provided with a roundish nucleus, centrally located, as evidenced by LM analysis (Figs. 6A, 7A *insets*). Occasionally, some oocytes of the mCOH group, at a late GV-stage, had a nucleus more amoeboid in shape. By TEM, fCOH and mCOH oocytes showed the presence of a prominent, irregularly rounded nucleus, usually containing a single, rounded nucleolus (Figs. 6A-B, 7A-B). In both groups, the nucleolus appeared strongly electron-dense and in close contact with patches of heterochromatin (Figs. 6A-B, 7A-B). The heterochromatin, sometimes, was disposed to form a ring - in section - around the nucleolus (Fig.7A-B). A juxta-nucleolar (or satellite) body occasionally appeared close to the nucleolus, in association with the heterochromatin (Fig. 6A-B).

High magnification micrographs showed differences in the distribution of the heterochromatin around the nucleolus between the two groups of oocytes (Figs. 6B, 7B). A linear and continuous nuclear envelope (nucleolemma) delimited the nucleoplasm of both groups; however, its circumference was regularly circular in the fCOH group and more indented in the mCOH group. The nucleolemma was provided with numerous and regularly distributed nuclear pores (Figs. 6A-B, 7A-B); inside the nucleoplasm, the euchromatin was predominant and finely dispersed (Figs. 6A-B, 7A-B). In fCOH oocytes, the diameter of the nucleolus varied between 2.5 μm and 2.8 μm and in mCOH varied between 1.2 μm and 2.3 μm.

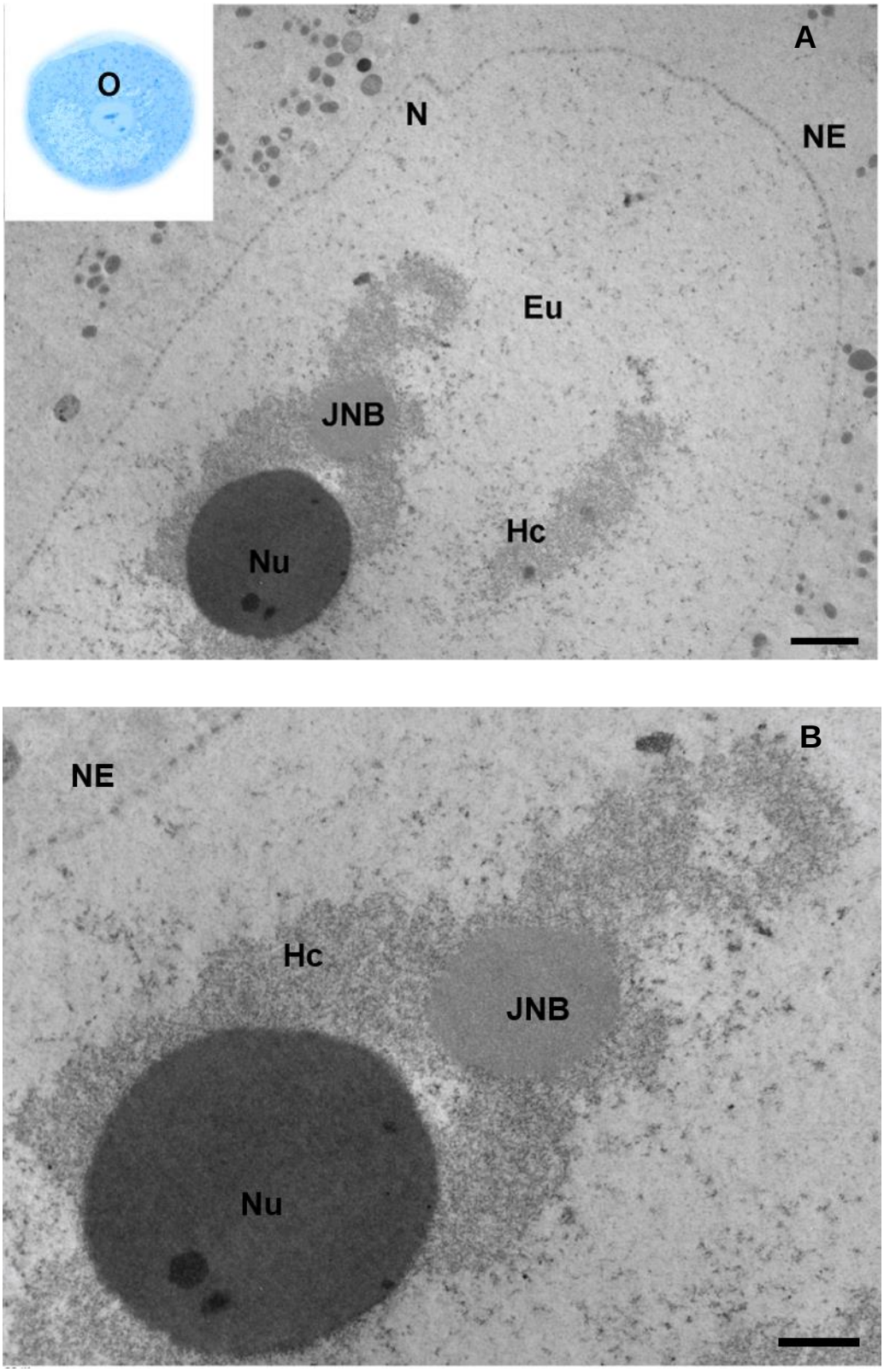


Figure 6. ***Ultrastructure of the nucleus in fCOH GV-stage oocytes.*** A) A nucleus (N) delimited by a continuous and circular nuclear envelope (NE) (bar: 1 µm). Inset in A: a representative image of a semithin section (LM. Mag: 20x). B) High magnification of the nucleus presenting a high electron negative nucleolus (Nu) near to dense heterochromatin (Hc) and a juxta-nucleolar body (JNB) (bar: 1 µm). O: oocyte; Eu: euchromatin.

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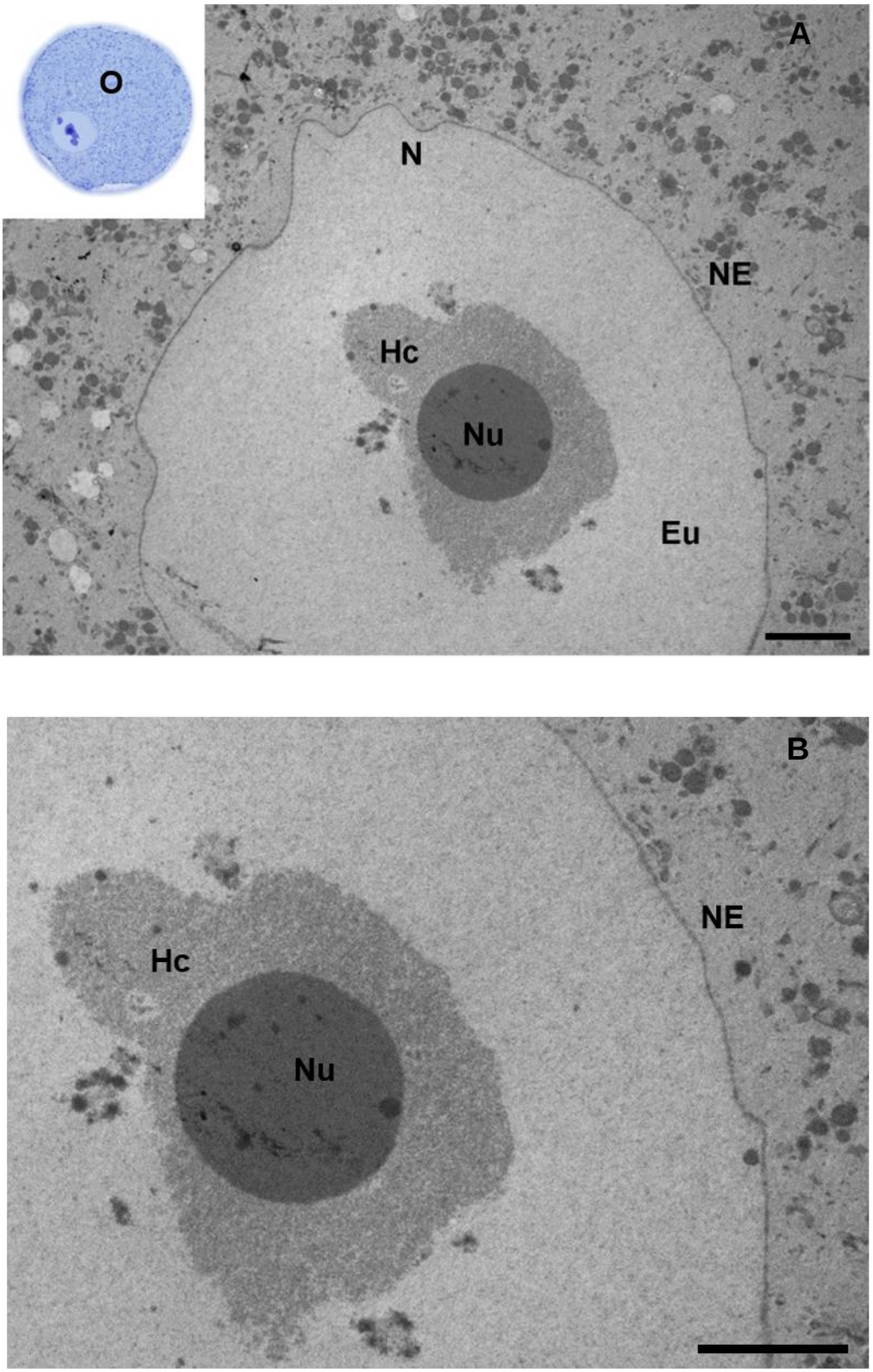

Figure 7. ***Ultrastructure of the nucleus in mCOH GV-stage oocytes.*** A) A nucleus (N) delimited by a continuous circular nuclear envelope (NE) (bar: 1 μm). Inset in A: a representative image of a semithin section (LM. Mag: 20x). B) High magnification micrograph of the nucleus showing a strong electron-dense nucleolus (Nu) located centrally in the nucleus and the distribution of dense heterochromatin (Hc) disposed to form a ring around nucleolus (bar: 1 μm). O: oocyte; Eu: euchromatin.

4.3 Ooplasm

Organelles were homogeneously distributed in the ooplasm of both fCOH and mCOH oocytes, as evidenced by LM. However, TEM evidenced specific differences.

4.3.1 Mitochondria and ooplasmic membranes

TEM showed the presence of spherical-to-ovoid mitochondria, more rarely ellipsoidal, in both groups. Mitochondria were the most numerous organelles mostly located in the inner ooplasm, provided with peripheral or transversal cristae and an electron-dense matrix either in fCOH (Figs. 8A-B, 9A-B, 10A-B) and mCOH oocytes (Figs. 11A-B, 12A-B, 13A-B). Mitochondria of both groups were often organized in clusters surrounding small electron-negative vesicles (and forming the so-called "MV-complexes") (Figs. 9B, 13B *insets*). MV-complexes were frequently found in perinuclear position. Mitochondria vacuolization was occasionally identified, limited to some protrusions of the external mitochondrial membrane. In both groups, isolated smooth endoplasmic reticulum (SER) tubules and/or SER networks were also observed in the cortical ooplasm (Figs. 9B, 13B). Cisternae of the Golgi complexes were also found scattered in the cortex of mCOH oocytes (Fig. 12A). Annulate lamellae (cytomembranes derived from the nuclear envelope shedding that may be transformed in cytoplasmic membranes) were detected in the oooplasm of both fCOH and mCOH oocytes (Figs. 10A, 12B).

4.3.2 Vacuoles, lysosomes, multivesicular bodies

Numerous vacuoles, round, electron-negative and membrane-bounded found in both groups (Figs. 8A-B, 9A, 10A, 11 A-B, 12A-B, 13A), although apparently more abundant in fCOH than in mCOH oocytes (Figs. 8A, 11A). In both oocyte groups, a few vacuoles were observed in the periphery of the ooplasm, but the majority were located in the inner part, in the region surrounding the nucleus (Figs. 8A, 11A). Vacuoles appeared either isolated (Figs. 12A, 13A) or arranged in groups (Figs. 9A, 10A), sometimes fused each other and showing interrupted membranes (Figs. 8B, 9A, 10A, 11B). Secondary lysosomes were occasionally detected in both groups (Figs. 10B, 13A). Multi-vesicular bodies (mvbs), with a vesicular content of heterolysosomal nature, were filled with a

flocculent, slightly electron-dense debris; they were mainly found scattered in the inner ooplasm, near the vacuoles and around the nuclei of fCOH oocytes (Fig. 10A).

4.3.3 Cortical granules

Round CGs showed a moderate to high electron-density. They were detected frequently aligned as a discontinuous layer just beneath the oolema, either in fCOH (Fig. 14A, C) and mCOH oocytes (Fig. 15A-B); however, in both groups, often some of them appeared scattered throughout the inner ooplasm (Figs. 9B, 13A-B).

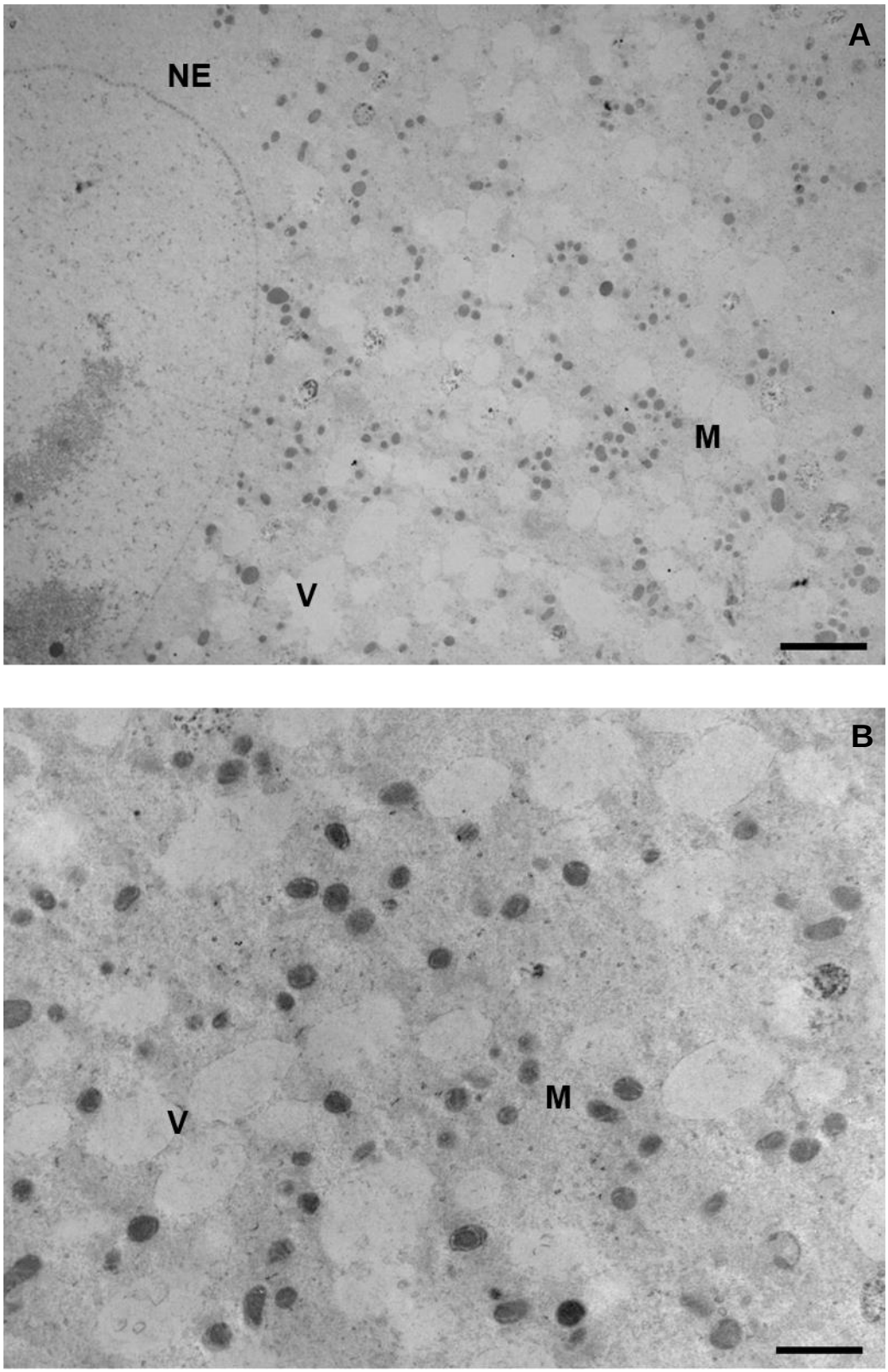

Figure 8. ***Representative ultrastructure of the subcortical region in fCOH oocytes***. A) Abundant mitochondria (M) and vacuoles (V) are distributed around the nucleus (bar: 2 μm). B) High magnification micrograph evidenced numerous electron-dense mitochondria and electron-negative vacuoles (bar: 1 μm). NE: nuclear envelope.

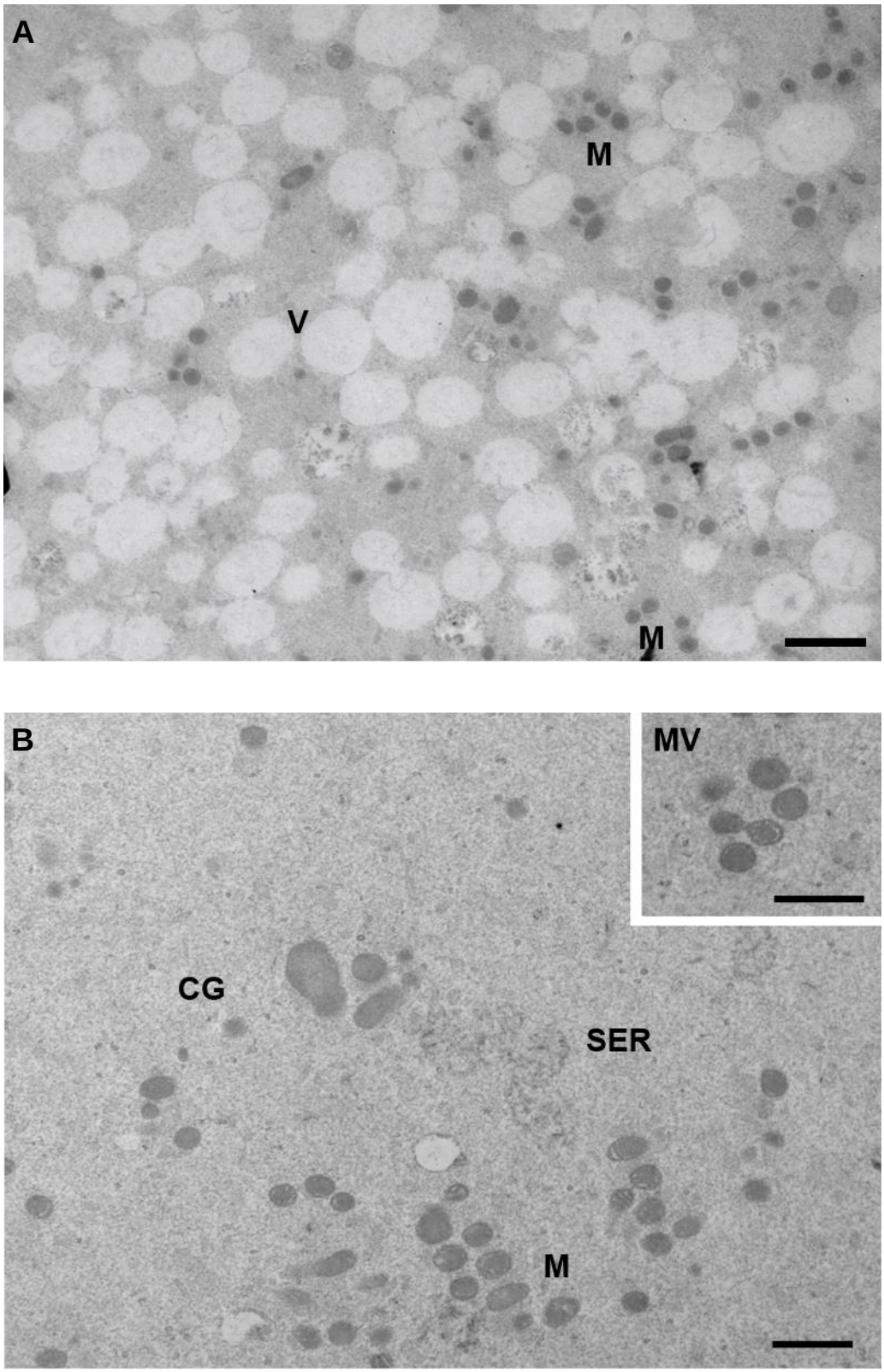

Figure 9. ***Ultrastructure of the subcortical region in fCOH oocytes***. A) An ooplasmic area with numerous isolated or grouped vacuoles (bar: 1 μm). B) High magnification micrograph showing isolated smooth endoplasmic reticulum (SER) tubules and subcortical located cortical granules (CG) (bar: 1 μm). Inset in B shows a mitochondria-vesicle (MV) complex (bar: 1 μm). M: mitochondria.

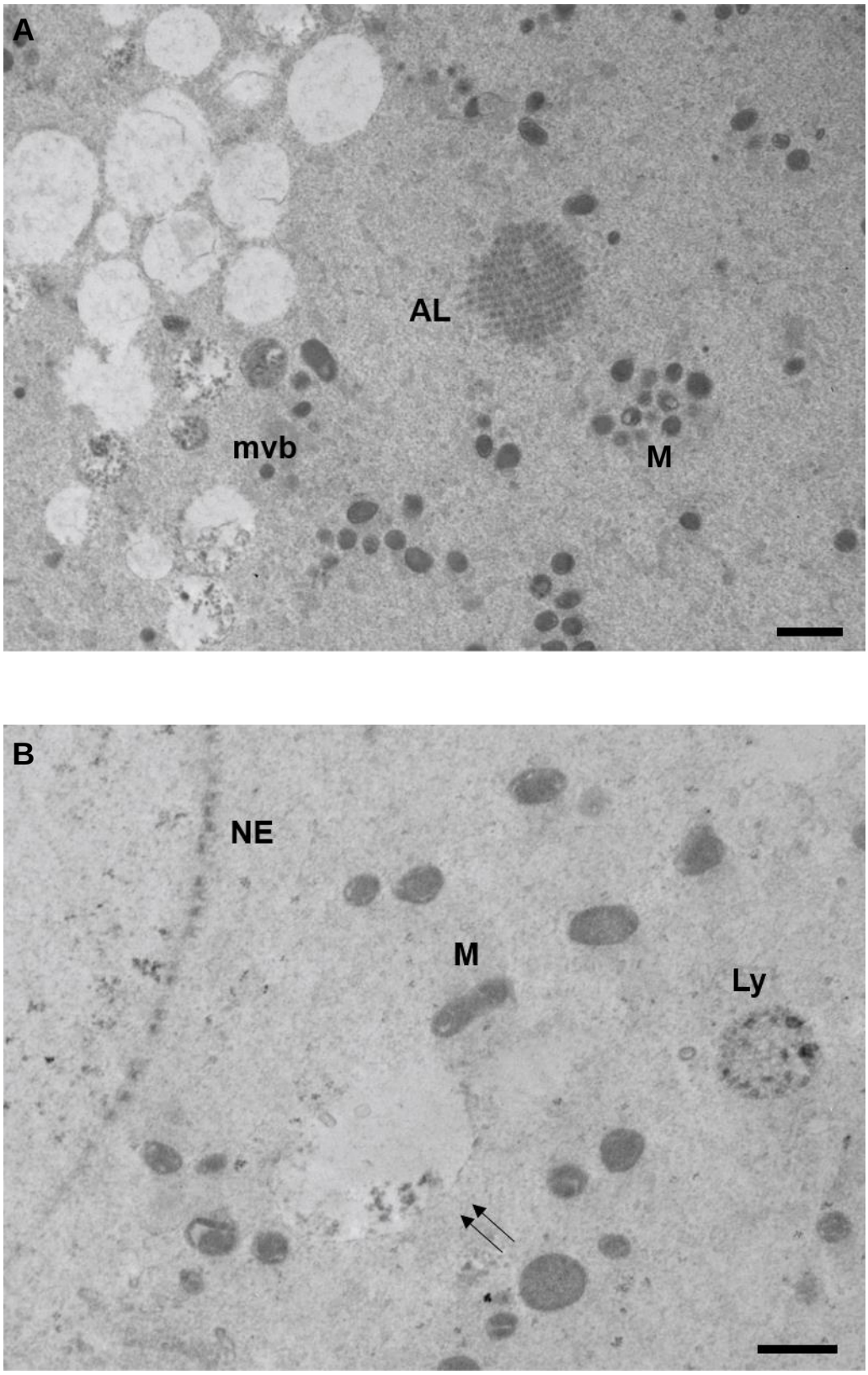

Figure 10. ***Ultrastructure of the subcortical region in fCOH oocytes.*** A) Clusters of mitochondria, annulate lamellae (AL) and a multivesicular-body (mvb), in the inner ooplasm (bar: 1 μm); B) Ooplasmic patch (double arrows) and round/elongated mitochondria near the nucleus (bar: 0,8 μm). Ly: lysosome; NE: nuclear envelope.

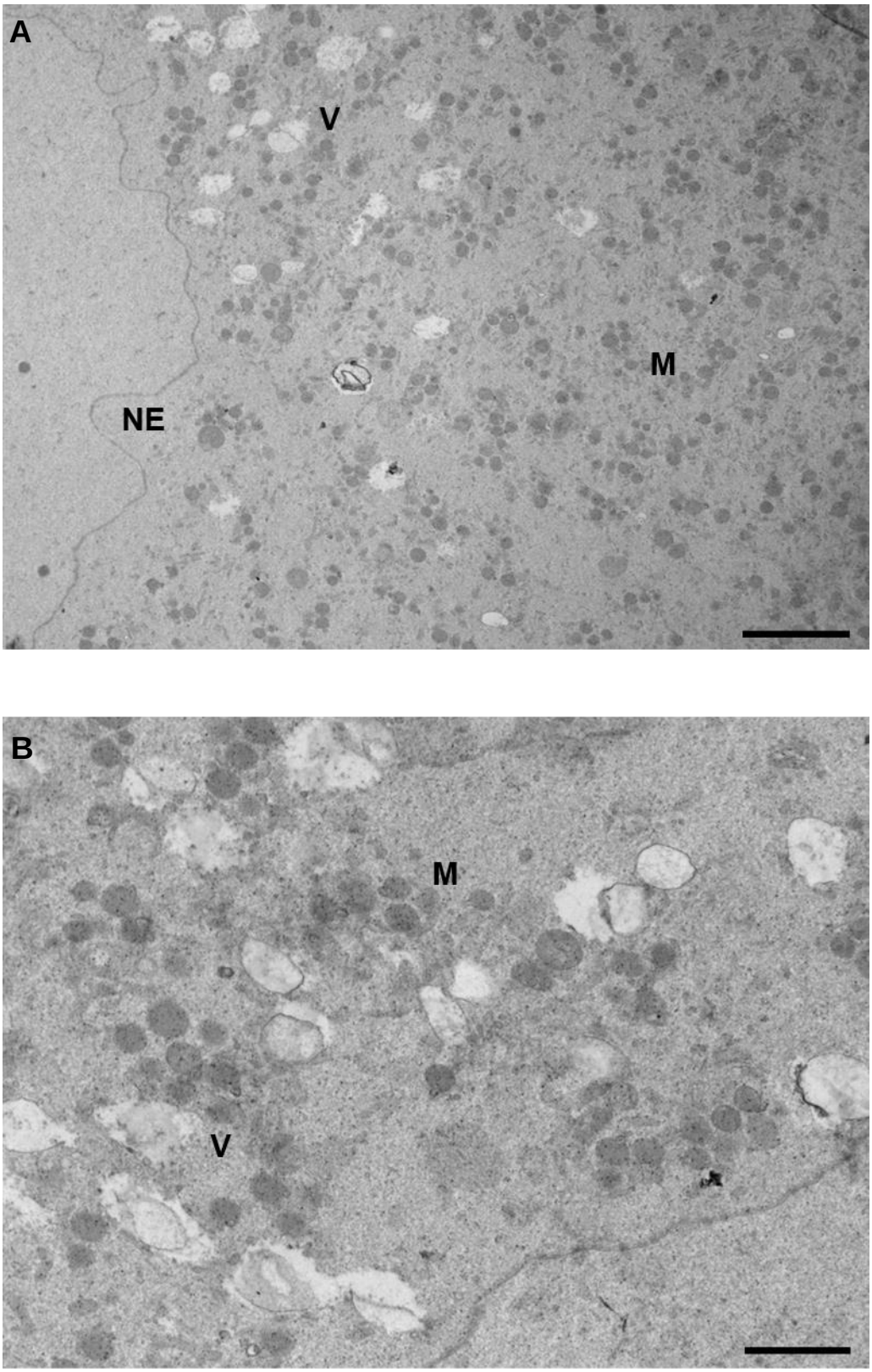

Figure 11. ***Ultrastructure of the subcortical region in mCOH oocytes.*** A) Vacuoles (V) and abundant mitochondria (M) are distributed close to the nucleus (bar: 2 μm). B) High magnification micrograph shows numerous highly electron-dense mitochondria and electron-negative vacuoles (bar: 1 μm). NE: nuclear envelope.

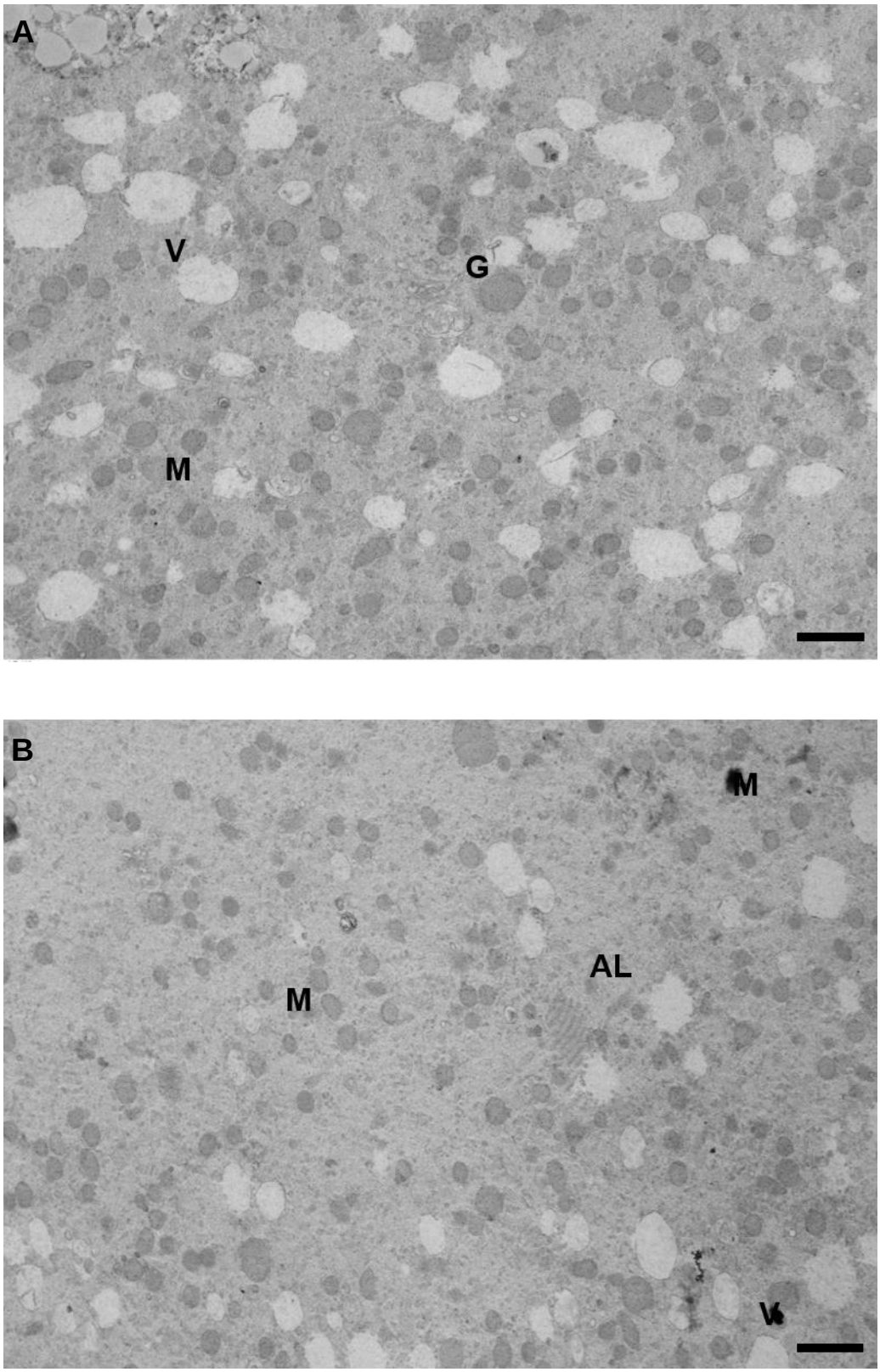

Figure 12. ***Ultrastructure of the subcortical region in mCOH oocytes.*** A) Among mitochondria and vacuoles, a Golgi complex (G) is visible in the inner oocyte ooplasm (bar: 1 μm). B) Annulate lamellae (AL) and numerous mitochondria distributed in the ooplasm (bar: 1 μm). M: mitochondria; V: Vacuoles.

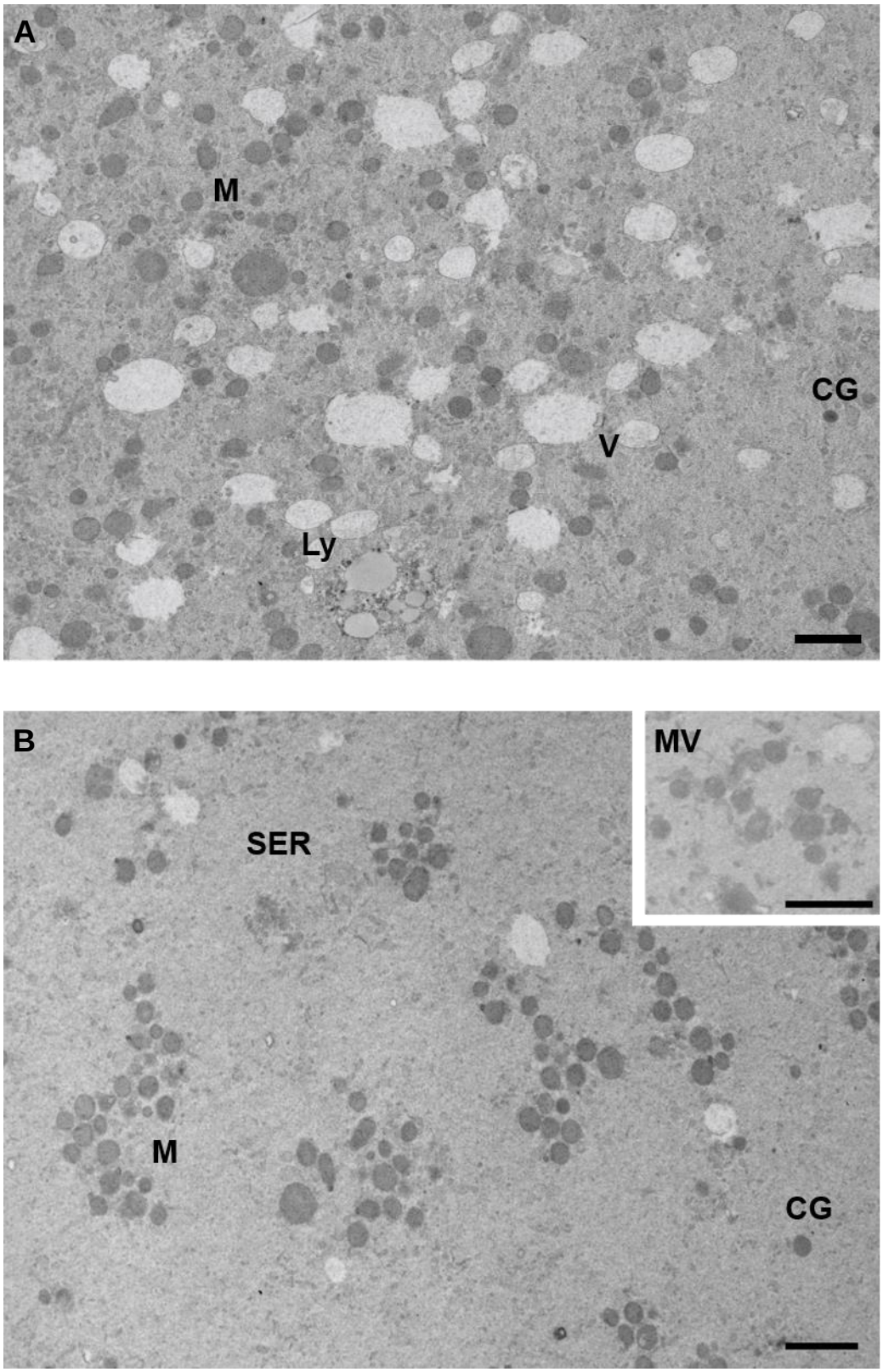

Figure 13. ***Ultrastructure of the subcortical region in mCOH oocytes.*** A) TEM micrograph of the inner ooplasm shows the presence of a secondary lysosome (Ly) and cortical granules (CG) (bar: 1 μm). B) High magnification micrograph shows isolated smooth endoplasmic reticulum (SER) tubules and several mitochondria (M) (bar: 1 μm). Inset in B represents a mitochondria-vesicle (MV) complex (bar: 1 μm). V: vacuoles.

4.4 Oolemma, Microvilli, PVS, ZP texture

The oolemma was continuous and regular oolemma in the fCOH and mCOH oocytes observed. Numerous microvilli of variable length, projecting from the oolemma to the PVS towards the ZP, were found in both oocyte groups (Figs. 14A, 15A). Particularly in fCOH oocytes, some areas of rarefaction of microvilli were occasionally observable (Fig. 14B). In oolemmal and suboolemmal areas, occasionally, was detected the presence of endocytic vesicles (also known as "ooplasmic pouch"), as evidenced by the microvilli still visible inside the vesicle (Figs. 10B, 14A). In the oocytes of the mCOH group, a conspicuous pinocytotic activity was also detected (data not shown). As a narrow space between the oolemma and the ZP appeared the PVS, containing a granular and filamentous matrix with scarce electron density in both groups (Figs. 14B-C, 15B). At higher magnification, electron-negative remnants of transzonal projections extruding from corona radiata cells crossed the ZP were visible in the PVS of both fCOH and mCOH oocytes (Figs. 14A-C, 15B); in some areas of fCOH oocytes, transzonal projections were found still connected to the oolemma through small desmosomes (Fig. 14B). The ZP of both groups appeared intact and continuous with a typical filamentous texture (Figs. 14A, 15A), having a thickness of 7-10 μm in fCOH and 8-11 μm in mCOH oocytes.

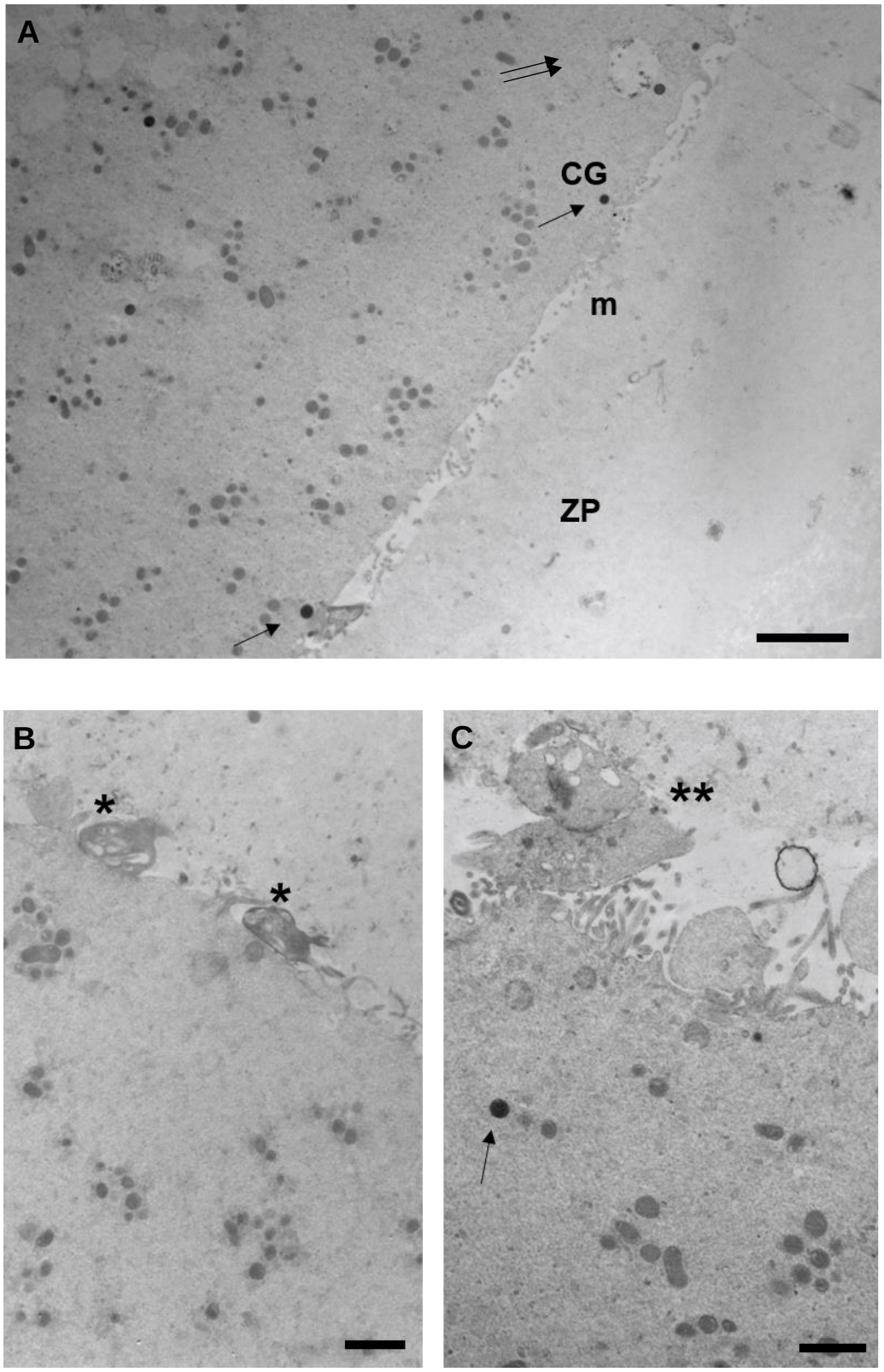

Figure 14. ***Ultrastructure of the cortical region in fCOH oocytes.*** A) Presence of an ooplasmic patch (double arrows) and cortical granules (arrows) aligned in a discontinuous layer just beneath the oolema (bar: 2 μm). Note, at higher magnification: B) the presence of small desmosomes (*) in the perivitelline space (bar: 1 μm) and C) a corona cell ending forming a focal cell (**) (bar: 1 μm). Microvilli: m; CG: cortical granules.

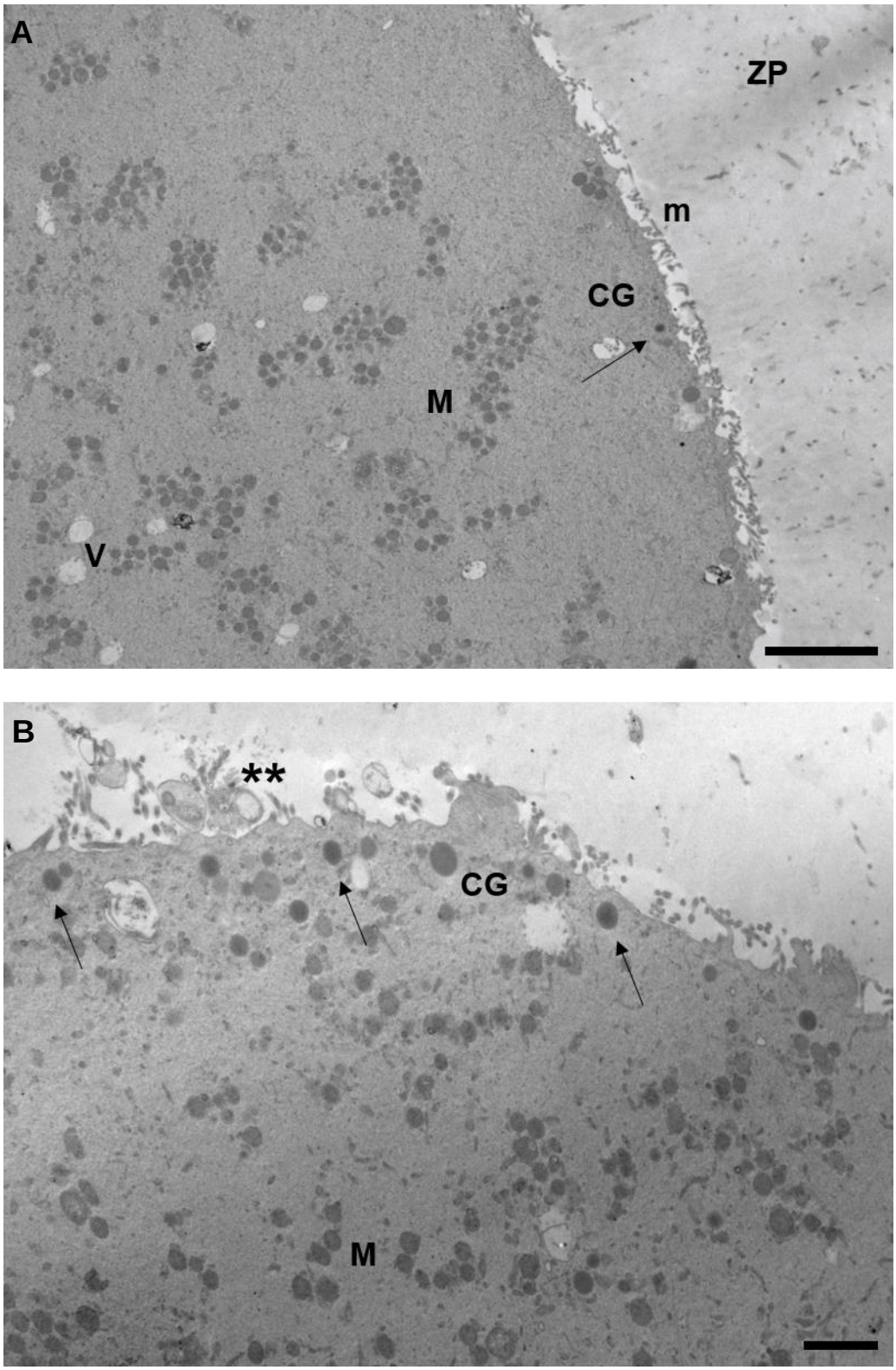

Figure 15. ***Ultrastructure of the cortical region in mCOH oocytes.*** A) TEM micrograph showing the general distribution of microvilli (m) (bar: 2 μm). B) At high magnification, a discontinuous layer of cortical granules (CG) is seen just beneath the oolema (bar: 1 μm). M: mitochondria; V: vacuoles; ZP: zona pellucida; **: corona cell ending.

4.5 Morphometric analysis

Morphometric analysis revealed that the number of mitochondria per 100 μm^2 oocyte area was significantly lower in fCOH than in mCOH oocytes (72.125 ± 8.957 *vs* 94.500 ± 15.067, respectively; P = 0.04333). Conversely, the number of vacuoles did not significantly vary between fCOH and mCOH oocytes (54.750 ± 29.353 *vs* 42.250 ± 19.050, respectively; P = 0.5018). CGs in fCOH oocytes were less numerous than in mCOH. More in detail, the suboolemmal CGs were significantly less in fCOH than in mCOH oocytes (3.000 ± 1.414 *vs* 7.308 ± 2.057, respectively; P < 0.001). Even if the medium diameter of mitochondria did not show significant variations between fCOH and mCOH (0.345 ± 0.096 μm *vs* 0.350 ± 0.077 μm, respectively; P = 0.6301), significantly larger vacuoles were observed in fCOH, respect to mCOH oocytes (1.256 ± 0.313 μm *vs* 0.618 ± 0.198 μm, respectively; P < 0.0001). Irrespective of the CGs number, CG dimensions were similar in both fCOH and mCOH oocyte groups (0.293 ± 0.070 μm *vs* 0.272 ± 0.065 μm, respectively; P = 0.2466). All the data are reported in Table 3.

	Organelles number (N) and diameter (μm)					
GV-stage oocytes	Mitochondria		Small/large Vacuoles		Cortical granules	
	N	μm	N	μm	N	μm
fCOH	72.125 ± 8.957^a	0.345 ± 0.096^a	54.750 ± 29.353^a	1.256 ± 0.313^a	3.000 ± 1.414^a	0.293 ± 0.070^a
mCOH	94.500 ± 15.067^b	0.350 ± 0.077^a	42.250 ± 19.050^a	0.618 ± 0.198^b	7.308 ± 2.057^b	0.272 ± 0.065^a

Table 3. ***Number (N) and dimensions (μm) of mitochondria, vacuoles and cortical granules from fCOH and mCOH oocytes.*** The morphometric analysis of the number of mitochondria and vacuoles was performed on a 100 μm^2 oocyte area and of cortical granules on 10 μm of the oocyte linear surface profile in low-magnification TEM micrographs of at least three equatorial ultrathin sections per oocyte. The values of each group are expressed as mean ± SD. Different superscripts within each column indicate significant differences between the fCOH and mCOH oocyte groups (P < 0.05).

Part 5: Discussion & conclusions

5.1 Discussion

Among the numerous protocols of COH used in ARTs, the "milder" one was developed to reduce the detrimental effects connected to the use of exogenous gonadotropins, especially in poor responder patients as PCOS (D'Amato *et al.*, 2018) or those at risk of OHSS induction (Fouda *et al.*, 2012), that are subjected to repeated cycles of hyperstimulation after recurrent failure. Mild stimulation was applied also for ovarian or breast cancer patients (Coyne *et al.*, 2014; Cavagna *et al.*, 2017), to prevent the exposure to excessive estrogens, even if data are still controversial (Taheripanah *et al.*, 2018). In particular, the "mild" approach is usually based on the use of a GnRH antagonist an oral analogue, such as clomiphene citrate, a relatively inexpensive medication with high patient compliance due to oral administration. The mCOH induced often a reduction in the mean number of oocytes retrieved in both the general IVF population and in the poor responders respect to fCOH, less doses of gonadotropins or reduced number of administrations, thus resulting in a 'patient-friendly COH' (Alper and Fauser, 2017; Kamath *et al.*, 2017). However, for many years, the most frequently used COH protocol was the "long" protocol with high daily administration of gonadotropins particularly for the expected poor responders (Shanbhag *et al.*, 2007) and, despite the high number of retrieved oocytes, this protocol significantly failed to improve the final success rate after implantation. Moreover, fCOH determined a relevant increase in economical costs (Lekamge *et al.*, 2008).

Currently, the suggestions for a more individualized, less aggressive ovarian stimulation approach in all responder types of patients are under discussion, focusing on a more efficient and fine-tuned treatment in terms of patients' health preservation.

In addition, a recent attention has been given to the use of "leftover" oocytes, retrieved at GV-stage after COH, and usable as an alternative source of oocytes for ARTs, especially for patients affected by diminished ovarian reserve (DOR), premature ovarian failure (POF) and POR (Barton *et al.*, 2012; Cohen *et al.*, 2015; Mishra and Chirumamilla, 2018). Indeed, the leftover oocytes can increase the yield of total available oocytes and can be used, in association with IVM and cryopreservation, to successfully increase the outcomes after IVF and embryo transfer (IVF-ET) (Gunasheela *et al.*, 2012).

The purpose of the study was to describe the ultrastructure of fresh GV-stage human oocytes retrieved after fCOH (GnRH agonist long stimulation) and mCOH (GnRH

antagonist) protocols, to assess which is less harmful, in terms of alterations on the fine oocyte morphology.

According to data available in the literature, this is the first comprehensive study evaluating, with qualitative and quantitative analysis, the ultrastructural modifications occurring after their retrieval of immature leftover GV-stage oocytes from donor women subjected to fCOH and mCOH protocols. Even if several studies evaluated the ultrastructure of mature oocytes under different *in vitro* conditions (Coticchio *et al.*, 2016; Segovia *et al.*, 2017), up to now the effects of ARTs as COH in human immature oocytes gained restricted attention (Palmerini *et al.*, 2014a). COH protocols caused crucial side effects in oocytes and/or tissues of the female reproductive system from an abnormal expression of genes (Horcajadas *et al.*, 2005; Alvarez Sedó *et al.*, 2012), dysregulations of metabolic pathways in oocytes (Sakiner *et al.*, 2018; Ferrero *et al.*, 2019) up to morphological modifications (Mirkin *et al.*, 2004; Taheri *et al.*, 2018). To this point, it seems essential to define in detail the structural modifications that may occur in immature oocytes, in order to settle a more accurate and patient-friendly COH.

General Features

All fresh GV-stage oocytes did not show evident alterations about shape, size and general structures, respect to qualitative data in literature available on mammals (Hegele-Hartung *et al.*, 1999; Mondadori *et al.*, 2010; Palmerini *et al.*, 2014a) including human (Sathananthan *et al.*, 1993, El Shafie *et al.*, 2000; Makabe and Van Blerkom, 2006; Van Blerkom, 1990) after fCOH or mCOH.

Organelles of both groups were uniformly distributed in the ooplasm, apart from specific subcellular compartments. In fact, organelle-specific differences mainly interested the heterochromatin distribution around the nucleolus, the number of mitochondria and CGs, the abundance of microvilli and the size of vacuoles.

Nucleus

The nucleus, i.e. the most representative organelle to define the meiotic stage, appeared in both groups well preserved, mainly located in the central area of the oocytes and delimited by a slightly undulated and uninterrupted nucleolemma, provided with numerous nuclear pores and well-formed nucleoplasm structures, according to previous

studies (Sathananthan *et al.*, 1993; Makabe and Van Blerkom, 2006; Palmerini *et al.*, 2014b). The exact nuclear position in the cortex is identified as a morphological marker, predictive of oocyte meiotic competence in GV-stage oocytes, since oocytes with a central nucleus have a higher meiotic competence than those with an eccentric or peripheral nucleus (Brunet and Maro, 2007); this criterion of selection is used to improve the success of IVM (Levi *et al.*, 2013). The euchromatin around the nucleoli was abundant and well preserved and the heterochromatin was dispersed close to electron-dense nucleoli in both fCOH and mCOH oocytes, however, the distribution of heterochromatin showed differences between the groups. In contrast, the nucleus of some oocytes of the mCOH group appeared irregular in shape, with more than one nucleolus and a visible reduction in chromatin. This could be a sign of a late response to gonadotropins by immature oocytes with a morphological indication of a resumption of meiotic maturation and consequent progression to the GVBD phase. The increase in the number of nucleoli found in mCOH oocytes at the late GV-stage could be linked to the high ribosomal activity of these growing oocytes necessary to support the increased metabolic demands of the subsequent meiosis phase which is occurred with the GVBD (Shishova *et al.*, 2015). The ring-shaped heterochromatin observed around the nucleolus in some mCOH oocytes, different from the homogenous distribution in the nucleoplasm of the fCOH oocytes, could be linked to the ability to mature and to develop embryos from fresh immature GV-stage oocytes, even if data in the literature are conflicting. Thus, in mice, the presence of a chromatin ring, if associated with a central GV, was for some authors indicative of a good competence in the further development (Bellone *et al.*, 2009; Sánchez *et al.*, 2015; Bui *et al.*, 2017), while for others (Zuccotti *et al.*, 2008; Inoue *et al.*, 2008) or in animals other than the mouse, including the human species (Wang *et al.*, 2009; Levi *et al.*, 2013) was not. The limited number of oocytes used in this study makes necessary to confirm this last consideration with further studies.

Ooplasm

In the ooplasm of immature oocytes, as in the GV-stage oocytes here studied, the organelles were less abundant than in mature oocytes (Nottola *et al.*, 2014); however, some differences were found in the composition of the ooplasm from fCOH and mCOH cycles and above discussed.

Mitochondria and ooplasmic membranes

The numerosity of mitochondria, the richness in lamellar or transversal cristae, the presence of numerous aggregates with small/large vesicles (small MV complexes) and the strong electron-density here observed in both groups, are morphological features indicative of a good mitochondrial preservation, in agreement to previous data (Palmerini *et al.*, 2014b; Sathananthan *et al.*, 2006). However, the significant reduction in the mitochondrial number of fCOH, compared to mCOH oocytes, could be due to a reduced or impaired mitochondrial activity related to ovarian hyperstimulation and fewer copies of mtDNA, lower ATP levels and reduced mitochondrial membrane potential, as described in previous studies (Ge *et al.*, 2012; Cree *et al.*, 2015; Shu *et al.*, 2015).

Vacuoles, lysosomes, multivesicular bodies

Abundant vacuoles were detected around the nuclei of both fCOH and mCOH oocytes, frequently grouped and delimited by an electron-dense membrane, and normally filled by an electronegative content with the exception of the occasional presence of debris, in agreement with the previous data present in the literature (Sathananthan *et al.*, 1993; Makabe and Van Blerkom, 2006). Vacuoles of fresh immature oocytes may derive from the swelling or coalescence of Golgi and/or SER membranes (Sathananthan *et al.*, 1993), probably associated with cytoskeletal defects (Nottola *et al.*, 2008). The morphometric analysis showed that in oocytes retrieved after "full" COH, the vacuoles had a significantly larger size than those of "mild" COH. This result may be related to the increased exposure to gonadotropins in the fCOH protocol. In addition, all the markers of immaturity in fresh GV-stage oocytes, as the presence of a physiological vacuolization around nucleus, small MV-complexes, mvbs, and ooplasmic pouches together with isolated SER tubules or small M-SER aggregates are produced by a system of interconnected and interchanging membranes that change dynamically depending on the age of the oocyte, its stage of maturation, the potential damage derived from cryopreservation and according to the type of applied ART protocol (Nottola *et al.*, 2016).

Cortical granules

In the GV-stage oocytes, CGs originate from the abundant membranes of the Golgi apparatus and during oocyte maturation progressively proliferate to migrate toward the oolemma in the MII-stage oocytes (Sathananthan *et al.*, 1985; Familiari *et al.*, 2006). The results showed that some CGs either in fCOH or mCOH oocytes were typically dispersed within the ooplasm, as already previously verified following an fCOH protocol (Palmerini *et al.*, 2014b). However, occasionally CGs were also observed stratified in the outer ooplasmic cortex, as demonstrated also in other studies of immature human oocytes (Makabe and Van Blerkom, 2006; Segovia *et al.*, 2017), probably in consequence of a premature disorganization of the cortical microfilaments induced by ovarian hyperstimulation (Van Blerkom, 1990). Although CGs were significantly less abundant in fCOH oocytes compared to mCOH, with similar dimensions between the groups, this small difference may be irrelevant from a physiological point of view (Liu, 2011; Palmerini *et al.*, 2014b).

Oolema, Microvilli, PVS, ZP texture

A continuous and intact oolemma was observed in both groups of oocytes, provided with regularly distributed microvilli. The presence of a regular and continuous coverage of microvilli (Sathananthan *et al.*, 1993; Hutanu *et al.*, 2010) is an essential contribution to the success of fertilization, due to its initial role in the interaction and subsequent fusion of the oolemma with the spermatozoon consequent to the presence of adhesion proteins such as CD9 (Nottola *et al.*, 1991; Runge *et al.*, 2007). However, in some fCOH oocytes the microvillar distribution was found to be less abundant, occasionally with some areas of rarefaction of microvilli, sharp and blunt microvilli in comparison to mCOH oocytes, more densely populated by long microvilli, with a curved profile. In fresh GV-stage oocytes, the presence of a continuous layer of numerous long and thin microvilli was often found in mammals including humans (Ebensperger *et al.*, 1984; Wu *et al.*, 2006; Palmerini *et al.*, 2014b). The presence of area with a reduced microvillar coverage in the fCOH oocytes, however, may be case-specific, related to the sectioning or, in the final instance, probably connected to a dynamic adaptation of the oocyte as a response to the exogenous caused stimulation. The presence of numerous transzonal communications in the fCOH oocytes, intact and continuous along the entire area covered by ZP, unlike what

was observed in the mCOH oocytes is an indication of functionality in the connections with the cells of the radiated corona.

5.2 Conclusions

In conclusion, this study on the fine structure of human fresh GV-stage oocytes retrieved after mild and full hyperstimulation showed good overall preservation but evidenced the occurrence of specific ultrastructural damages, probably related to the COH protocols. In particular, GV-stage oocytes retrieved after mCOH, due to the presence of more abundant microvilli, more numerous and larger mitochondria, and smaller vacuoles than those after fCOH seem more idoneous to finalize maturation and to become competent to fertilization, also in association to cryopreservation and IVM.

This study gives an overall point of view on the possible effects connected to the use of hyperstimulation; however, to have a more complete and comparative view of the ultrastructural characteristics of the GV-stage oocytes - so important for providing additional good-quality leftover gametes for ARTs - there is the need to compare the present data with unstimulated GV-stage oocytes derived from natural cycles.

Bibliography

Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppä L, Louhio H, Tuuri T, Sjöberg J, Bützow R, Hovata O, Dale L, Ritvos O. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab. 1999, 84(8):2744-50.

Abe Y, Hara K, Matsumoto H, Kobayashi J, Sasada H, Ekwall H, Rodriguez-Martinez H, Sato E. Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biol Reprod. 2005, 72(6):1416-20.

Aboulghar MA, Mansour RT. Ovarian hyperstimulation syndrome: classifications and critical analysis of preventive measures. Hum Reprod Update. 2003, 9(3):275-89.

Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol. Reprod. Dev. 2000, 55:212-19.

Albertini DF, Carabatsos MJ. Comparative aspects of meiotic cell cycle control in mammals. J Mol Med (Berl). 1998, 76(12):795-9.

Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction. 2001, 121(5):647-53.

Albertini DF. Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. Mutat Res. 1992, 296(1-2):57-68.

Alexandre H. A history of mammalian embryological research. Int J Dev Biol. 2001, 45(3):457-67.

Al-Inany H, Aboulghar M. GnRH antagonist in assisted reproduction: a Cochrane review. Hum Reprod. 2002, 17(4):874-85.

Almind G, Faerch E, Lindenberg F, Lindenberg S. Mild stimulation approach for In Vitro Fertilization treatment: Retrospective data from one Danish Centre. Facts Views Vis Obgyn. 2018, 10(2):81-84.

Alper MM, Fauser BC. Ovarian stimulation protocols for IVF: is more better than less? Reprod Biomed Online. 2017, 34(4):345-353.

Alvarez Sedó C, Baronio M, Lavolpe M, Nodar F, Papier S. Oocyte stress: gene expression study in oocytes and cumulus cells from gonadotropin-stimulated and unstimulated ovaries. Fertil Steril. 2012, 98(3):S154.

Amsterdam A, Rotmensch S. Structure-function relationships during granulosa cell differentiation. Endocr Rev. 1987, 8(3):309-37.

Arce JC, Klein BM, La Marca A. The rate of high ovarian response in women identified at risk by a high serum AMH level is influenced by the type of gonadotropin. Gynecol Endocrinol. 2014, 30(6):444-50.

Ataya K, Rao LV, Lawrence E, Kimmel R. Luteinizing hormone-releasing hormone agonist inhibits cyclophosphamide-induced ovarian follicular depletion in rhesus monkeys. Biol Reprod. 1995, 52(2):365-72.

Bachvarova R. Gene expression during oogenesis and oocyte development in mammals. Dev Biol (N Y 1985). 1985, 1:453-524.

Baker JR. The Cell-Theory: a Restatement, History, and Critique. Q J Microsc Sci. 1949, 90(1):87-108.

Baker TG. A quantitative and cytological study of germ cells in human ovaries. Proc R Soc Lond B Biol Sci. 1963, 158:417-33.

Barton SE, Missmer SA, Berry KF, Ginsburg ES. Female cancer survivors are low responders and have reduced success compared with other patients undergoing assisted reproductive technologies. Fertil Steril. 2012, 97(2):381-6.

Bellone M, Zuccotti M, Redi CA, Garagna S. The position of the germinal vesicle and the chromatin organization together provide a marker of the developmental competence of mouse antral oocytes. Reproduction. 2009, 138(4):639-43.

Ben-Ami I, Komsky A, Bern O, Kasterstein E, Komarovsky D, Ron-El R. In vitro maturation of human germinal vesicle-stage oocytes: role of epidermal growth factor-like growth factors in the culture medium. Hum Reprod. 2011, 26(1):76-81.

Beyer EC, Kistler J, Paul DL, Goodenough DA. Antisera directed against connexin-43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. J Cell Biol. 1989, 108:595.

Beyer EC. Gap junctions. Int Rev Cytol. 1993, 137C:1-37.

Bianchi V, Macchiarelli G, Borini A, Lappi M, Cecconi S, Miglietta S, Familiari G, Nottola SA. Fine morphological assessment of quality of human mature oocytes after slow freezing or vitrification with a closed device: a comparative analysis. Reprod Biol Endocrinol. 2014, 12:110.

Bogolyubova IO, Bogolyubov DS. Oocyte nuclear structure during mammalian oogenesis. In: Perrotte A (ed). Recent Adv Germ Cells Research, pp. 105–133. 2013, New York: Nova Science Publishers, Inc.

Borges E Jr, Zanetti BF, Setti AS, Braga DP, Figueira RCS, Iaconelli A Jr. FSH dose to stimulate different patient' ages: when less is more. JBRA Assist Reprod. 2017, 21(4):336-342.

Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Cook RW, Kipp JL, Shea LD, Mayo KE, Woodruff TK. Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool. Dev Biol. 2006, 298(1):132-48.

Brown JB. Pituitary control of ovarian function--concepts derived from gonadotrophin therapy. Aust N Z J Obstet Gynaecol. 1978, 18(1):46-54.

Brunet S, Maro B. Germinal vesicle position and meiotic maturation in mouse oocyte. Reproduction. 2007, 133(6):1069-72.

Buffet NC, Bouchard P. The neuroendocrine regulation of the human ovarian cycle. Chronobiol Int. 2001, 18(6):893-919.

Bui TTH, Belli M, Fassina L, Vigone G, Merico V, Garagna S, Zuccotti M. Cytoplasmic movement profiles of mouse surrounding nucleolus and not-surrounding nucleolus antral oocytes during meiotic resumption. Mol Reprod Dev. 2017, 84(5):356-362.

Bukovský A, Caudle MR, Keenan JA, Wimalasena J, Foster JS, Van Meter SE. Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein and macrophages during follicular development and atresia, and in human corpora lutea. Biol Reprod. 1995, 52(4):776-92.

Cao Y, Xing Q, Zhang ZG, Wei ZL, Zhou P, Cong L. Cryopreservation of immature and in-vitro matured human oocytes by vitrification. Reprod Biomed Online. 2009, 19:369-73.

Cavagna F, Pontes A, Cavagna M, Dzik A, Donadio NF, Portela R, Nagai MT, Gebrim LH. A specific controlled ovarian stimulation (COS) protocol for fertility preservation in women with breast cancer undergoing neoadjuvant chemotherapy. Contemp Oncol (Pozn). 2017, 21(4):290-294.

Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycle, their culture in vitro and their transfer in a donor oocyte program. Fertil Steril. 1991, 55:109-13.

Channing CP, Kammerman S. Characteristics of gonadotropin receptors of porcine granulosa cells during follicle maturation. Endocrinology. 1973, 92(2):531-40.

Cheon YP. Regulation and 3 dimensional culture of tertiary follicle growth. Clin Exp Reprod Med. 2012, 39(3):95–106.

Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured in vitro following vitrification. J Reprod Dev. 2004, 50(6):685-96.

Cohen J, Chabbert-Buffet N, Darai E. Diminished ovarian reserve, premature ovarian failure, poor ovarian responder--a plea for universal definitions. J Assist Reprod Genet. 2015, 32(12):1709-12.

Combelles CM, Ceyhan ST, Wang H, Racowsky C. Maturation outcomes are improved following Cryoleaf vitrification of immature human oocytes when compared to choline-based slow-freezing. J Assist Reprod Genet. 2011, 28: 1183-92.

Conti M, Chang RJ. Chapter 125 - Folliculogenesis, Ovulation, and Luteogenesis. In: Jameson JL, De Groot LJ, de Kretser DM, Giudice LC, Grossman AB, Melmed S, Potts JT, Weir GC (eds). Endocrinology: Adult and Pediatric (Seventh Edition), pp: 2179-2191. 2016, W.B. Saunders, Elsevier Inc.

Coticchio G, Borini A, Distratis V, Maione M, Scaravelli G, Bianchi V, Macchiarelli G, Nottola SA. Qualitative and morphometric analysis of the ultrastructure of human oocytes cryopreserved by two alternative slow cooling protocols. J Assist Reprod Genet. 2010, 27(4):131-40.

Coticchio G, Dal Canto M, Fadini R, Mignini Renzini M, Guglielmo MC, Miglietta S, Palmerini MG, Macchiarelli G, Nottola SA. Ultrastructure of human oocytes after in vitro maturation. Mol Hum Reprod. 2016, 22(2):110-8.

Coticchio G, Dal Canto M, Mignini Renzini M, Guglielmo MC, Brambillasca F, Turchi D, Novara PV, Fadini R. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. Hum Reprod Update. 2015, 21(4):427-54.

Coyne K, Purdy M, O'Leary K, Yaklic JL, Lindheim SR, Appiah LA. Challenges and considerations in optimizing ovarian stimulation protocols in oncofertility patients. Front Public Health. 2014, 2:246.

Cree LM, Hammond ER, Shelling AN, Berg MC, Peek JC, Green MP. Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. Hum Reprod. 2015, 30(6):1410-20.

D'Amato G, Caringella AM, Stanziano A, Cantatore C, Palini S, Caroppo E. Mild ovarian stimulation with letrozole plus fixed dose human menopausal gonadotropin prior to IVF/ICSI for infertile non-obese women with polycystic ovarian syndrome being pre-treated with metformin: a pilot study. Reprod Biol Endocrinol. 2018, 16(1):89.

de Jong D, Macklon NS, Fauser BC. A pilot study involving minimal ovarian stimulation for in vitro fertilization: extending the "follicle-stimulating hormone window" combined with the gonadotropin-releasing hormone antagonist cetrorelix. Fertil Steril. 2000, 73(5):1051-4.

de Matos DG, Furnus CC, Moses DF, Martinez AG, Matkovic M. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. Mol Reprod Dev. 1996, 45(4):451-7.

Devroey P, Boostanfar R, Koper NP, Mannaerts BM, Ijzerman-Boon PC, Fauser BC, Investigators E. A double-blind, non-inferiority RCT comparing corifollitropin alfa and recombinant FSH during the first seven days of ovarian stimulation using a GnRH antagonist protocol. Hum Reprod. 2009, 24:3063-72.

Devroey P, Bourgain C, Macklon NS, Fauser BC. Reproductive biology and IVF: ovarian stimulation and endometrial receptivity. Trends Endocrinol Metab. 2004, 15(2):84-90.

Diedrich K, Felberbaum R. New approaches to ovarian stimulation. Hum Reprod. 1998, 3:1–13.

Diergaarde B, Kurta ML. Use of fertility drugs and risk of ovarian cancer. Curr Opin Obstet Gynecol. 2014, 26(3):125-9.

Ding X, Zhang X, Mu Y, Li Y, Hao J. Effects of BMP4/SMAD signaling pathway on mouse primordial follicle growth and survival via up-regulation of Sohlh2 and c-kit. Mol Reprod Dev. 2013, 80(1):70-8.

Dor J, Lerner-Geva L, Rabinovici J, Chetrit A, Levran D, Lunenfeld B, Mashiach S, Modan B. Cancer incidence in a cohort of infertile women who underwent in vitro fertilization. Fertil Steril. 2002, 77(2):324-7.

Dragovic RA, Ritter LJ, Schulz SJ, Amato F, Thompson JG, Armstrong DT, Gilchrist RB. Oocyte-secreted factor activation of SMAD 2/3 signaling enables initiation of mouse cumulus cell expansion. Biol Reprod. 2007, 76(5):848-57.

Dumesic DA, Padmanabhan V, Abbott DH. Polycystic ovary syndrome and oocyte developmental competence. Obstet Gynecol Surv. 2008, 63(1):39-48.

Durinzi KL, Saniga EM, Lanzendorf SE. The relationship between size and maturation in vitro in the unstimulated human oocyte. Fertil Steril. 1995, 63(2):404-6.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology. 2001, 142(11):4891-9.

Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology. 1999, 140(12):5789-96.

Ebensperger C e Barros Dr. C. Changes at the haMSter oocyte surface from the germinal vesicle stage to ovulation. Mol Reprod and Develop 1984, 9(4): 387-397.

Eichenlaub-Ritter U, Peschke M. Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. Hum Reprod Update. 2002, 8(1):21-41.

El Shafie M, Windt M-L, Kitshoff M et al. Ultrastructure of human oocytes: a transmission electron microscopic view. In: El Shafie M, Sousa M, Windt M-L, Kruger TF (eds). An Atlas of the Ultrastructure of Human Oocytes: a Guide for Assisted Reproduction, pp: 119-28. 2000, Parthenon, New York, London.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 2001, 122(6):829-38.

Erickson GF, Magoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cells: a review of structure/function relationships. Endocr Rev. 1985, 6(3):371-99.

European Society of Human Reproduction and Embryology (ESHRE). Guideline on Ovarian Stimulation for IVF/ICSI. 2019a https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Ovarian-Stimulation-in-IVF-ICSI, Last access 27 Nov 2019.

European Society of Human Reproduction and Embryology (ESHRE). Guideline on Ovarian Stimulation for IVF/ICSI: stakeholder review report. 2019b

https://www.eshre.eu/Guidelines-and-Legal/Guidelines/Ovarian-Stimulation-in-IVF-ICSI, Last access 27 Nov 2019.

Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod. 2001, 16(3):411-6.

Fadini R, Mignini Renzini M, Dal Canto M, Epis A, Crippa M, Caliari I, Brigante C, Coticchio G. Oocyte in vitro maturation in normo-ovulatory women. Fertil Steril. 2013, 99(5):1162-9.

Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev. 1995, 42(4):437-42.

Familiari G, Heyn R, Relucenti M, Nottola SA, Sathananthan AH. Ultrastructural dynamics of human reproduction, from ovulation to fertilization and early embryo development. Int Rev Cytol. 2006, 249:53-141.

Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, Richards JS. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science. 2009, 324(5929):938-41.

Farley J. Gametes and spores. Ideas about sexual reproduction, Smith C (ed). 1982, Baltimore, Md., and London, Johns Hopkins University Press.

Fasano G, Demeestere I, Englert Y. In-vitro maturation of human oocytes: before or after vitrification? J Assist Reprod Genet. 2012, 29:507-12.

Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet. 2005, 365(9473):1807-16.

Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L; ESHRE working group on Poor Ovarian Response Definition. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum Reprod. 2011, 26(7):1616-24.

Ferrero H, Corachán A, Aguilar A, Quiñonero A, Carbajo-García MC, Alamá P, Tejera A, Taboas E, Muñoz E, Pellicer A, Domínguez F. Single-cell RNA sequencing of oocytes

from ovarian endometriosis patients reveals a differential transcriptomic profile associated with lower quality. Hum Reprod. 2019, 34(7):1302-1312.

Flaws JA, Abbud R, Mann RJ, Nilson JH, Hirshfield AN. Chronically elevated luteinizing hormone depletes primordial follicles in the mouse ovary. Biol Reprod. 1997, 57(5):1233-7.

Fortune JE, Cushman RA, Wahl CM, Kito S. The primordial to primary follicle transition. Mol Cell Endocrinol. 2000, 163(1-2):53-60.

Fouda UM, Sayed AM, Elshaer HS, Hammad BE, Shaban MM, Elsetohy KA, Youssef MA. GnRH antagonist rescue protocol combined with cabergoline versus cabergoline alone in the prevention of ovarian hyperstimulation syndrome: a randomized controlled trial. J Ovarian Res. 2016, 9(1):29.

Franchi LL, Baker TG. Oogenesis and follicular growth. In: Hafez ESS, Evens TN (eds). Human reproduction, conception and contraception, pp. 53-83. 1973, Harper & Row, Hagerstown, Maryland.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet. 2000, 25(3):279-83.

Ge H, Tollner TL, Hu Z, Dai M, Li X, Guan H, Shan D, Zhang X, Lv J, Huang C, Dong Q. The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. Mol Reprod Dev. 2012, 79(6):392-401.

Gilbert SF (ed). A Conceptual History of Modern Embryology: Volume 7. 1991, Springer Science & Business Media.

Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update. 2008, 14(2):159-77.

Gilchrist RB, Smitz JEJ, Thompson JG. Current status and future trends of the clinical practice of human oocyte in vitro maturation. In: Gardner DK, Rizk B, Falcone T (eds).

Human Assisted Reproductive Technology Future Trends in Laboratory and Clinical Practice, pp: 186-98. 2011, Cambridge: Cambridge University Press.

Gonda KJ, Domar AD, Gleicher N, Marrs RP. Insights from clinical experience in treating IVF poor responders. Reprod Biomed Online. 2018, 36(1):12-19.

Gong X, Dubois DH, Miller DJ, Shur BD. Activation of a G protein complex by aggregation of beta-1,4-galactosyltransferase on the surface of sperm. Science. 1995, 269(5231):1718-21.

Gook DA, Schiewe MC, Osborn SM, Asch RH, Jansen RP, Johnston WI. Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. Hum Reprod. 1995, 10(10):2637-41.

Gougeon A, Chainy GB. Morphometric studies of small follicles in ovaries of women at different ages. J Reprod Fertil. 1987, 81(2):433-42.

Gougeon A, Lefèvre B. Evolution of the diameters of the largest healthy and atretic follicles during the human menstrual cycle. J Reprod Fertil. 1983, 69(2):497-502.

Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev. 1996, 17(2):121-55.

Griesinger G, Boostanfar R, Gordon K, Gates D, McCrary Sisk C, Stegmann BJ. Corifollitropin alfa versus recombinant follicle-stimulating hormone: an individual patient data meta-analysis. Reprod Biomed Online. 2016, 33:56–60.

Gunasheela S, Gunasheela D, Jaykumar A, Hiremath N, Son WY. Live birth after in vitro maturation and vitrification of immature oocytes retrieved from conventional IVF cycle: a case report. J Assist Reprod Genet. 2012, 29(10):1073-6.

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. Biol Reprod. 2000, 62(5):1322-8.

Haas J, Casper RF. In vitro fertilization treatments with the use of clomiphene citrate or letrozole. Fertil Steril. 2017, 108(4):568-571.

Hamdine O, Broekmans FJ, Fauser BC. Ovarian stimulation for IVF: mild approaches. Methods Mol Biol. 2014, 1154:305-28.

Haouzi D, Assou S, Dechanet C, Anahory T, Dechaud H, De Vos J, Hamamah S. Controlled ovarian hyperstimulation for in vitro fertilization alters endometrial receptivity in humans: protocol effects. Biol Reprod. 2010, 82(4):679-86.

Harris H. The discovery of the cell nucleus. In: Harris H (ed), The Birth of the Cell, pp. 76-93. 1999, Yale Univ. Press, New Heaven.

Hegele-Hartung C, Kuhnke J, Lessl M, Grøndahl C, Ottesen J, Beier HM, Eisner S, Eichenlaub-Ritter U. Nuclear and cytoplasmic maturation of mouse oocytes after treatment with synthetic meiosis-activating sterol in vitro. Biol Reprod. 1999, 61(5):1362-72.

Hirshfield AN. Granulosa cell proliferation in very small follicles of cycling rats studied by long-term continuous tritiated-thymidine infusion. Biol Reprod. 1989, 41(2):309-16.

Hodgen GD. Neuroendocrinology of the normal menstrual cycle. J Reprod Med. 1989, 34(1 Suppl):68-75.

Hohmann FP, Laven JS, de Jong FH, Eijkemans MJ, Fauser BC. Low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development. Hum Reprod. 2001, 16(5):846-54.

Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonistcotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. J Clin Endocrinol Metab. 2003, 88(1):166-73.

Homer H. New insights into the genetic regulation of homologue disjunction in mammalian oocytes. Cytogenet Genome Res. 2011, 133(2-4):209-22.

Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S, Simón C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. Mol Hum Reprod. 2005, 11(3):195-205.

http://rsbweb.nih.gov/ij/ Image J software. Last access, 27 November 2019.

Hunzicker-Dunn M, Mayo K. Chapter 20 - Gonadotropin Signaling in the Ovary. In: Plant TM, Zeleznik AJ (eds). Knobil and Neill's Physiology of Reproduction (Fourth Edition), pp: 895-945. 2015, Academic Press.

Hutanu, D., Crăciun, C., Checiu, M., & Checiu, I. Ultrastructural aspects of human immature oocytes. Romanian Biotechnological Letters. 2010, 15(3):14.

Hyttel P, Fair T, Callesen H, Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology. 1997, 47:23–32.

Im KS, Kang JK, Kim HS. Effects of cumulus cells, different cryoprotectants, various maturation stages and preincubation before insemination on developmental capacity of frozen-thawed bovine oocytes. Theriogenology. 1997, 47(4):881-91.

Imoedemhe DG, Sigue AB. Survival of human oocytes cryopreserved with or without the cumulus in 1,2-propanediol. J Assist Reprod Genet. 1992, 9(4):323-7.

Inoue A, Nakajima R, Nagata M, Aoki F. Contribution of the oocyte nucleus and cytoplasm to the determination of meiotic and developmental competence in mice. Hum Reprod. 2008, 23(6):1377-84.

Itskovitz-Eldor J, Kol S, Mannaerts B. Use of a single bolus of GnRH agonist triptorelin to trigger ovulation after GnRH antagonist ganirelix treatment in women undergoing ovarian stimulation for assisted reproduction, with special reference to the prevention of ovarian hyperstimulation syndrome: preliminary report: short communication. Hum Reprod. 2000, 15(9):1965-8.

Jay V. A portrait in history. The legacy of Reinier de Graaf. Arch Pathol Lab Med. 2000, 124(8):1115-6.

Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature. 2004, 428(6979):145-50.

Jones ASK, Shikanov A. Follicle development as an orchestrated signaling network in a 3D organoid. J Biol Eng. 2019; 13:2.

Kamath MS, Maheshwari A, Bhattacharya S, Lor KY, Gibreel A. Oral medications including clomiphene citrate or aromatase inhibitors with gonadotropins for controlled ovarian stimulation in women undergoing in vitro fertilisation. Cochrane Database Syst Rev. 2017, 11:CD008528.

Khalili MA, A Nottola S, Shahedi A, Macchiarelli G. Contribution of human oocyte architecture to success of in vitro maturation technology. Iran J Reprod Med. 2013, 11(1):1-10.

Khalili MA, Maione M, Palmerini MG, Bianchi S, Macchiarelli G, Nottola SA. Ultrastructure of human mature oocytes after vitrification. Eur J Histochem. 2012, 56(3):e38.

Khalili MA, Shahedi A, Ashourzadeh S, Nottola SA, Macchiarelli G, Palmerini MG. Vitrification of human immature oocytes before and after in vitro maturation: a review. J Assist Reprod Genet. 2017, 34(11):1413-26.

Kidder GM, Mhawi AA. Gap junctions and ovarian folliculogenesis. Reproduction. 2002, 123(5):613-20.

Konig TE, van der Houwen LE, Overbeek A, Hendriks ML, Beutler-Beemsterboer SN, Kuchenbecker WK, Renckens CN, Bernardus RE, Schats R, Homburg R et al. Recombinant LH supplementation to a standard GnRH antagonist protocol in women of 35 years or older undergoing IVF/ICSI: a randomized controlled multicentre study. Hum Reprod. 2013, 28:2804-12.

Kumar NM, Gilula NB. The gap junction communication channel. Cell. 1996, 84(3):381-8.

Kumar P, Sait SF. Luteinizing hormone and its dilemma in ovulation induction. J Hum Reprod Sci. 2011, 4(1):2–7.

Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet. 1997, 15(2):201-4.

Latham KE, Wigglesworth K, McMenamin M, Eppig JJ. Stage-dependent effects of oocytes and growth differentiation factor 9 on mouse granulosa cell development:

advance programming and subsequent control of the transition from preantral secondary follicles to early antral tertiary follicles. Biol Reprod. 2004, 70(5):1253-62.

Lee JA, Barritt J, Moschini RM, Slifkin RE, Copperman AB. Optimizing human oocyte cryopreservation for fertility preservation patients: should we mature then freeze or freeze then mature? Fertil Steril. 2013, 99:1356-62.

Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev. 2004, 69(2):159-63.

Lekamge DN, Lane M, Gilchrist RB, Tremellen KP. Increased gonadotrophin stimulation does not improve IVF outcomes in patients with predicted poor ovarian reserve. J Assist Reprod Genet. 2008, 25(11-12):515-21.

Leoni GG, Palmerini MG, Satta V, Succu S, Pasciu V, Zinellu A, Carru C, Macchiarelli G, Nottola SA, Naitana S, Berlinguer F. Differences in the Kinetic of the First Meiotic Division and in Active Mitochondrial Distribution between Prepubertal and Adult Oocytes Mirror Differences in their Developmental Competence in a Sheep Model. PLoS One. 2015, 10(4):e0124911.

Levi M, Ghetler Y, Shulman A, Shalgi R. Morphological and molecular markers are correlated with maturation-competence of human oocytes. Hum Reprod. 2013, 28(9):2482-9.

Li S, Maruo T, Ladines-Llave CA, Kondo H, Mochizuki M. Stage-limited expression of myc oncoprotein in the human ovary during follicular growth, regression and atresia. Endocr J. 1994, 41(1):83-92.

Liu M. The biology and dynamics of mammalian cortical granules. Reprod Biol Endocrinol. 2011, 9:149.

Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr Rev. 2006, 27(2):170-207.

Mahajan N, Gupta S, Sharma S, Rani K, Naidu P, Arora PR. Early onset ovarian hyperstimulation syndrome despite use of segmentation approach and ovarian hyperstimulation syndrome prophylaxis. J Hum Reprod Sci. 2015, 8(4):234-8.

Mahajan N. Should mild stimulation be the order of the day? J Hum Reprod Sci. 2013, 6(4):220-6.

Mahmodi R, Abbasi M, Amiri I, Ragardi Kashani I, Pasbakhsh P, Saadipour Kh, et al. Cumulus cell role on mouse germinal vesicle oocyte maturation, fertilization, and subsequent embryo development to blastocyst stage in vitro. Yakhteh Medical Journal. 2009, 11:299-302.

Makabe S, Van Blerkom J. Atlas of human female reproductive function: Ovarian development to early embryogenesis after in vitro fertilization (First Edition). 2006, Informa Healthcare.

Mandelbaum J, Junca AM, Tibi C, Plachot M, Alnot MO, Rim H, Salat-Baroux J, Cohen J. Cryopreservation of immature and mature hamster and human oocytes. Ann N Y Acad Sci. 1988, 541:550-61.

Marieb EN. Human Anatomy and Physiology, 5th edition. 2001. Benjamin Cummings - An imprint of Addison Wesley Longman, Inc.

McClure N, Healy DL, Rogers PA, Sullivan J, Beaton L, Haning RV Jr, Connolly DT, Robertson DM. Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. Lancet. 1994, 344(8917):235-6.

McNatty K. Ovarian follicular development from the onset of luteal regression in human and sheep. In: Rolland R, van Hall EV, Hillier SG, McNatty KP, Schoemaker J (eds). Follicular Maturation and Ovulation, pp. 1-18. 1982, Amsterdam: Excerpta Medica.

Mirkin S, Nikas G, Hsiu JG, Díaz J, Oehninger S. Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. J Clin Endocrinol Metab. 2004, 89(11):5742-52.

Mishra V, Chirumamilla L. Unexpected repeat immature oocyte response after IVF stimulation: a case report. Gynecol Endocrinol. 2018, 34(2):100-102.

Mondadori RG, Santin TR, Fidelis AA, Name KP, da Silva JS, Rumpf R, Báo SN. Ultrastructure of in vitro oocyte maturation in buffalo (Bubalus bubalis). Zygote. 2010, 18(4):309-14.

Motta PM, Familiari G. Occurrence of a contractile tissue in the theca externa of atretic follicles in the mouse ovary. Acta Anat (Basel). 1981, 109(2):103-14.

Motta PM, Nottola SA, Familiari G, Makabe S, Stallone T, Macchiarelli G. Morphodynamics of the follicular-luteal complex during early ovarian development and reproductive life. Int Rev Cytol. 2003, 223:177-288.

Motta PM, Nottola SA, Makabe S, Heyn R. Mitochondrial morphology in human fetal and adult female germ cells. Hum Reprod. 2000, 15 Suppl 2:129-47.

Motta PM, Nottola SA, Micara G, Familiari G. Ultrastructure of human unfertilized oocytes and polyspermic embryos in an IVF-ET program. Ann N Y Acad Sci. 1988, 541:367-383.

Nakamura M, Minegishi T, Hasegawa Y, Nakamura K, Igarashi S, Ito I, Shinozaki H, Miyamoto K, Eto Y, Ibuki Y. Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. Endocrinology. 1993, 133(2):538-44.

Nargund G, Fauser BC, Macklon NS, Ombelet W, Nygren K, Frydman R; Rotterdam ISMAAR Consensus Group on Terminology for Ovarian Stimulation for IVF. The ISMAAR proposal on terminology for ovarian stimulation for IVF. Hum Reprod. 2007, 22(11):2801-4.

Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, Wang H, Ke H, Nikolaev VO, Jaffe LA. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development. 2009, 136(11):1869-78.

Nottola SA, Albani E, Coticchio G, Palmerini MG, Lorenzo C, Scaravelli G, Borini A, Levi-Setti PE, Macchiarelli G. Freeze/thaw stress induces organelle remodeling and membrane recycling in cryopreserved human mature oocytes. J Assist Reprod Genet. 2016, 33(12):1559-70.

Nottola SA, Coticchio G, De Santis L, Macchiarelli G, Maione M, Bianchi S, Iaccarino M, Flamigni C, Borini A. Ultrastructure of human mature oocytes after slow cooling cryopreservation with ethylene glycol. Reprod Biomed Online. 2008, 17(3):368-77.

Nottola SA, Coticchio G, Sciajno R, Gambardella A, Maione M, Scaravelli G, Bianchi S, Macchiarelli G, Borini A. Ultrastructural markers of quality in human mature oocytes vitrified using cryoleaf and cryoloop. Reprod Biomed Online. 2009, 19(Suppl 3):17-27.

Nottola SA, Familiari G, Micara G, Aragona C, Motta PM. The ultrastructure of human cumulus-corona cells at the time of fertilization and early embryogenesis. A scanning and transmission electron microscopic study in an in vitro fertilization program. Arch Histol Cytol. 1991, 54(2):145-61.

Nottola SA, Macchiarelli G, Coticchio G, Bianchi S, Cecconi S, De Santis L, Scaravelli G, Flamigni C, Borini A. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. Hum Reprod. 2007, 22(4):1123-33.

Nottola SA, Macchiarelli G, Familiari G. Fine Structural Markers of Human Oocyte Quality in Assisted Reproduction. Austin J Reprod Med Infertil. 2014, 1(1): 5.

Oktay K, Briggs D, Gosden RG. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clin Endocrinol Metab. 1997, 82(11):3748-51.

Orvieto R, Vanni VS, Gleicher N. The myths surrounding mild stimulation in vitro fertilization (IVF). Reprod Biol Endocrinol. 2017, 15(1):48.

O'Shaughnessy PJ, McLelland D, McBride MW. Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. Biol Reprod. 1997, 57(3):602-8.

Palma GA, Argañaraz ME, Barrera AD, Rodler D, Mutto AÁ, Sinowatz F. Biology and biotechnology of follicle development. ScientificWorldJournal. 2012, 2012:938138.

Palmerini MG, Antinori M, Maione M, Cerusico F, Versaci C, Nottola SA, Macchiarelli G, Khalili MA, Antinori S. Ultrastructure of immature and mature human oocytes after cryotop vitrification. J Reprod Dev. 2014a, 60(6):411-20.

Palmerini MG, Nottola SA, Leoni GG, Succu S, Borshi X, Berlinguer F, Naitana S, Bekmukhambetov Y, Macchiarelli G. In vitro maturation is slowed in prepubertal lamb oocytes: ultrastructural evidences. Reprod Biol Endocrinol. 2014b, 12:115.

Pan B, Li J. The art of oocyte meiotic arrest regulation. Reprod Biol Endocrinol. 2019, 17(1):8.

Parfenov V, Potchukalina G, Dudina L, Kostyuchek D, Gruzova M. Human antral follicles: oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data). Gamete Res. 1989, 22(2):219-31.

Picton H, Briggs D, Gosden R. The molecular basis of oocyte growth and development. Mol Cell Endocrinol. 1998, 145(1-2):27-37.

Polyzos NP, Sunkara SK. Sub-optimal responders following controlled ovarian stimulation: an overlooked group? Hum Reprod. 2015, 30(9):2005-8.

Purkinje JE. Symbolae ad ovi avium historiam ante incubationem. In: Vossi L (ed). Medical Heritage Library, pp. 1-24. 1830. Leipzig.

Raju GA, Chavan R, Deenadayal M, Gunasheela D, Gutgutia R, Haripriya G, Govindarajan M, Patel NH, Patki AS. Luteinizing hormone and follicle stimulating hormone synergy: A review of role in controlled ovarian hyper-stimulation. J Hum Reprod Sci. 2013, 6(4):227–234.

Revelli A, Casano S, Salvagno F, Delle Piane L. Milder is better? Advantages and disadvantages of "mild" ovarian stimulation for human in vitro fertilization. Reprod Biol Endocrinol. 2011, 9:25.

Reynolds SRM. The vasculature of the ovary and ovarian function. Recent Progr Horm Res. 1950, 5:65.

Rizk B, Smitz J. Ovarian hyperstimulation syndrome after superovulation using GnRH agonists for IVF and related procedures. Hum Reprod. 1992, 7(3):320-7.

Rodgers RJ, Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod. 2010, 82(6):1021-9.

Runge KE, Evans JE, He ZY, Gupta S, McDonald KL, Stahlberg H, Primakoff P, Myles DG. Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. Dev Biol. 2007, 304(1):317-25.

Sadeu JC, Nogueira D. Folliculogenesis and oogenesis in vivo and in vitro, in humans females. In: Physiologie, pathologie et thérapie de la reproduction chez l'humain. 2011. Springer, Paris.

Sakiner L, Bulut ID, Yalcinkaya P, Elmas C. Effects of controlled ovarian hyperstimulation protocols on uterine markers. Ann Med Res. 2018, 25(3): 495-8.

Salmon NA, Handyside AH, Joyce IM. Oocyte regulation of anti-Müllerian hormone expression in granulosa cells during ovarian follicle development in mice. Dev Biol. 2004, 266(1):201-8.

Sánchez F, Romero S, De Vos M, Verheyen G, Smitz J. Human cumulus-enclosed germinal vesicle oocytes from early antral follicles reveal heterogeneous cellular and molecular features associated with in vitro maturation capacity. Hum Reprod. 2015, 30(6):1396-409.

Santos MA, Kuijk EW, Macklon NS. The impact of ovarian stimulation for IVF on the developing embryo. Reproduction. 2010, 139(1):23-34.

Sarma UC, Findlay JK, Hutt KJ. Oocytes from stem cells. Best Pract Res Clin Obstet Gynaecol. 2019, 55:14-22.

Sathananthan AH, Ng S-C, Bongso A, Trounson A, Ratnam S. Visual atlas of early human development for assisted reproductive technology. Singapore: National University of Singapore; 1993. (Chapter 1, Oocyte maturation and the mature oocyte. 1-14)

Sathananthan AH, Ng SC, Chia CM, Law HY, Edirisinghe WR, Ratnam SS. The origin and distribution of cortical granules in human oocytes with reference to Golgi, nucleolar, and microfilament activity. Ann N Y Acad Sci. 1985, 442:251-64.

Sathananthan AH, Selvaraj K, Girijashankar ML, Ganesh V, Selvaraj P, Trounson AO. From oogonia to mature oocytes: inactivation of the maternal centrosome in humans. Microsc Res Tech. 2006, 69(6):396-407.

Schultz GA, Gifford DJ, Mahadevan MM et al. Protein synthetic patterns in immature and mature human oocytes. In: Jones, Jr, HW, Schrader C (eds). In-vitro fertilization and other assisted reproduction, vol 541, pp: 237-47. 1988, Ann NY Acad Sci.

Segovia Y, Victory N, Peinado I, García-Valverde LM, García M, Aizpurua J, Monzó A, Gómez-Torres MJ. Ultrastructural characteristics of human oocytes vitrified before and after in vitro maturation. J Reprod Dev. 2017, 63(4):377-382.

Sergentanis TN, Diamantaras AA, Perlepe C, Kanavidis P, Skalkidou A, Petridou ET. IVF and breast cancer: a systematic review and meta-analysis. Hum Reprod Update. 2014, 20(1):106-23.

Shanbhag S, Aucott L, Bhattacharya S, Hamilton MA, McTavish AR. Interventions for 'poor responders' to controlled ovarian hyperstimulation (COH) in in-vitro fertilisation (IVF). Cochrane Database Syst Rev. 2007, (1):CD004379.

Shishova KV, Lavrentyeva EA, Dobrucki JW, Zatsepina OV. Nucleolus-like bodies of fully-grown mouse oocytes contain key nucleolar proteins but are impoverished for rRNA. Dev Biol. 2015, 397(2):267-81.

Shu J, Xing LL, Ding GL, Liu XM, Yan QF, Huang HF. Effects of ovarian hyperstimulation on mitochondria in oocytes and early embryos. Reprod Fertil Dev. 2015

Simon AM, Goodenough DA, Li E, Paul DL. Female infertility in mice lacking connexin 37. Nature. 1997, 385(6616):525-9.

Siristatidis C, Sergentanis TN, Kanavidis P, Trivella M, Sotiraki M, Mavromatis I, Psaltopoulou T, Skalkidou A, Petridou ET. Controlled ovarian hyperstimulation for IVF: impact on ovarian, endometrial and cervical cancer—a systematic review and meta-analysis. Hum Reprod Update. 2013, 19(2):105-23.

Siristatidis C, Sergentanis TN, Kanavidis P, Trivella M, Sotiraki M, Mavromatis I, Psaltopoulou T, Skalkidou A, Petridou ET. Controlled ovarian hyperstimulation for IVF:

impact on ovarian, endometrial and cervical cancer—a systematic review and meta-analysis. Hum Reprod Update. 2013, 19(2):105-23.

Son WY, Lee SY, Lim JH. Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in in vitro maturation cycles. Hum Reprod 2005, 20:3204-7.

Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. Development. 2008, 135(1):111-21.

Taghizabet N, Khalili MA, Anbari F, Agha-Rahimi A, Nottola SA, Macchiarelli G, Palmerini MG. Human cumulus cell sensitivity to vitrification, an ultrastructural study. Zygote. 2018, 26(3):224-231.

Taheri F, Alemzadeh Mehrizi A, Khalili MA, Halvaei I. The influence of ovarian hyperstimulation drugs on morphometry and morphology of human oocytes in ICSI program. Taiwan J Obstet Gynecol. 2018, 57(2):205-210.

Taheripanah R, Balash F, Anbiaee R, Mahmoodi M, Akbari Sene A. Breast Cancer and Ovulation Induction Treatments. Clin Breast Cancer. 2018, 18(5):395-399.

Tajima K, Orisaka M, Mori T, Kotsuji F. Ovarian theca cells in follicular function. Reprod Biomed Online. 2007, 15(5):591-609.

Tiwari M, Gupta A, Sharma A, Prasad S, Pandey AN, Yadav PK, Pandey AK, Shrivastav TG, Chaube SK. Role of Mitogen Activated Protein Kinase and Maturation Promoting Factor During the Achievement of Meiotic Competency in Mammalian Oocytes. J Cell Biochem. 2018, 119(1):123-129.

Tsafriri A. Follicular development: impact on oocyte quality. In: Fauser BCJM (ed). FSH action and intraovarian regulation, pp 83–105. 1997, Parthenon Press, New York.

Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. Fertil Steril. 1998, 70:578-9.

Van Blerkom J. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. J Electron Microsc Tech. 1990, 16(4):324-46.

van Leeuwen FE, Klip H, Mooij TM, van de Swaluw AM, Lambalk CB, Kortman M, Laven JS, Jansen CA, Helmerhorst FM, Cohlen BJ, WilleMSen WN, Smeenk JM, Simons AH, van der Veen F, Evers JL, van Dop PA, Macklon NS, Burger CW. Risk of borderline and invasive ovarian tumours after ovarian stimulation for in vitro fertilization in a large Dutch cohort. Hum Reprod. 2011, 26(12):3456-65.

Van Soom A, Tanghe S, De Pauw I, Maes D, de Kruif A. Function of the cumulus oophorus before and during mammalian fertilization. Reprod Domest Anim. 2002, 37(3):144-51.

van Wely M, Kwan I, Burt AL, Thomas J, Vail A, Van der Veen F, Al-Inany HG. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. Cochrane Database Syst Rev. 2011, CD005354.

Verberg MF, Macklon NS, Nargund G, Frydman R, Devroey P, Broekmans FJ, Fauser BC. Mild ovarian stimulation for IVF. Hum Reprod Update. 2009, 15(1):13-29.

Vloeberghs V, Blockeel C, Devroey P. Agonists or antagonists for ovarian stimulation? In: Kovacs G (ed). How to Improve your ART Success Rates: An Evidence-Based Review of Adjuncts to IVF, pp. 80-86. 2011, Cambridge: Cambridge University Press.

Wang H, Racowsky C, Combelles CM. Is it best to cryopreserve human cumulus-free immature oocytes before or after in vitro maturation? Cryobiology. 2012, 65:79-87.

Wang HL, Sui HS, Liu Y, Miao DQ, Lu JH, Liang B, Tan JH. Dynamic changes of germinal vesicle chromatin configuration and transcriptional activity during maturation of rabbit follicles. Fertil Steril. 2009, 91(4 Suppl):1589-94.

Wang XN, Greenwald GS. Hypophysectomy of the cyclic mouse. I. Effects on folliculogenesis, oocyte growth, and follicle-stimulating hormone and human chorionic gonadotropin receptors. Biol Reprod. 1993, 48(3):585-94.

Wassarman PM, Florman HM. Cellular Mechanisms During Mammalian Fertilization. In: Terjung R (ed). Comprehensive Physiology. 2011, American Physiological Society.

Wassarman PM, Josefowicz WJ. Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. J Morphol. 1978, 156(2):209-35.

Wassarman PM, Liu C, Litscher ES. Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle. J Cell Sci. 1996, 109(Pt8):2001-4.

Wassarman PM. Fertilization in mammals. Sci Am. 1988, 259(6):78-84.

Westergaard CG, Byskov AG, Andersen CY. Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age. Hum Reprod. 2007, 22(8):2225-31.

Wickramasinghe D, Albertini DF. Cell cycle control during mammalian oogenesis. Curr Top Dev Biol. 1993, 28:125-53.

Williams CJ, Erickson GF. Morphology and Physiology of the Ovary. In: Feingold KR, Anawalt B, Boyce A, et al., (eds.). 2000, Endotext. South Dartmouth (MA): MDText.com, Inc.

Wong KY, Ee TX, Tan HH. Minimal Stimulation Using Letrozole in Poor Responders. J Clin Gynecol Obstet. 2019, 8(3):77-80.

Wu C, Rui R, Dai J, Zhang C, Ju S, Xie B, Lu X, Zheng X. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol Reprod Dev. 2006, 73(11):1454-62.

Xiao JS, Su CM, Zeng XT. Comparisons of GnRH antagonist versus GnRH agonist protocol in supposed normal ovarian responders undergoing IVF: a systematic review and meta-analysis. PLoS One. 2014, 9:e106854.

Yamoto M, Shima K, Nakano R. Gonadotropin receptors in human ovarian follicles and corpora lutea throughout the menstrual cycle. Horm Res. 1992, 37 Suppl 1:5-11.

Yang YJ, Zhang YJ, Li Y. Ultrastructure of human oocytes of different maturity stages and the alteration during in vitro maturation. Fertil Steril. 2009, 92(1):396.e1-6.

Yousefi Z, Khalilifar H, Jafarian AH, Davachi B, Mousavi Seresh L, Babapour N, Shirinzadeh L, Baradaran M. Granulosa-cell tumor after ovarian stimulation: A case report. Int J Reprod Biomed (Yazd). 2018, 16(6):417-420.

Zamboni L, Mishell DR Jr, Bell JH, Baca M. Fine structure of the human ovum in the pronuclear stage. J Cell Biol. 1966, 30(3):579-600.

Zeleznik AJ, Hillier SG. The role of gonadotropins in the selection of the preovulatory follicle. Clin Obstet Gynecol. 1984, 27(4):927-40.

Zeleznik AJ. The physiology of follicle selection. Reprod Biol Endocrinol. 2004, 2:31.

Zelinski-Wooten MB, Hutchison JS, Chandrasekher YA, Wolf DP, Stouffer RL. Administration of human luteinizing hormone (hLH) to macaques after follicular development: further titration of LH surge requirements for ovulatory changes in primate follicles. J Clin Endocrinol Metab. 1992, 75(2):502-7.

Zhang A, Xu B, Sun Y, Lu X, Niu Z, Chen Q, Feng Y, Xu C. The effect of human cumulus cells on the maturation and developmental potential of immature oocytes in ICSI cycles. J Assist Reprod Genet. 2012, 29(4):313-9.

Zhang J, Chang L, Sone Y, Silber S. Minimal ovarian stimulation (mini-IVF) for IVF utilizing vitrification and cryopreserved embryo transfer. Reprod Biomed Online. 2010, 21(4):485-95.

Zivi E, Simon A, Laufer N. Ovarian hyperstimulation syndrome: definition, incidence, and classification. Semin Reprod Med. 2010;28(6):441-7. Fouda UM, Sayed AM, Elshaer HS, Hammad BE, Shaban MM, Elsetohy KA, Youssef MA. GnRH antagonist rescue protocol combined with cabergoline versus cabergoline alone in the prevention of ovarian hyperstimulation syndrome: a randomized controlled trial. J Ovarian Res. 2016, 9(1):29.

Zuccotti M, Merico V, Sacchi L, Bellone M, Brink TC, Bellazzi R, Stefanelli M, Redi CA, Garagna S, Adjaye J. Maternal Oct-4 is a potential key regulator of the developmental competence of mouse oocytes. BMC Dev Biol. 2008, 8:97.