In-vitro Maturation of Human Oocytes

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In-vitro Maturation of Human Oocytes
Basic science to clinical application

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Foreword

In-vitro maturation comes of age

Since its clinical outset 30 years ago, human in-vitro fertilization (IVF) has depended on expensive gonadotrophins to induce ovarian stimulation and oocyte maturation, at prices virtually one-half of total costs for this infertility treatment. The in-vitro maturation of human oocytes (IVM) now offers a much cheaper alternative. Invented more than 60 years ago, it has recently been developed as a far simpler approach to IVF, preimplantation diagnosis (PGD) and the preparation of embryo stem cells (ES cells). Indeed, it is already used in some IVF clinics and will doubtless spread to others. Clinical and scientific aspects of this advance, presented in this book, are published at a most opportune moment. It will be welcomed by investigators worldwide, as successive chapters describe background endocrinology, developmental biology of the ovarian follicles, the formation, growth and maturation of oocytes and modifications of well-tested IVF techniques to suit the needs of IVM. The First World Congress on In Vitro Maturation, held in Montreal in 2004, and a new Society will doubtless give a further stimulus to clinics assessing the role of IVM.

Writing a Foreword for this book is not easy in view of its size and contents. Successive well-written and informative chapters are so detailed that each of them can be mentioned only briefly, so I will divide chapters into successive sections for discussion. An excellent opening chapter by Thomas and Vanderhyden covers oocyte growth and developmental competence, which are essential to understand IVM. They discuss follicle formation during human gestation, fundamental aspects of oocyte growth, and the metabolism of oocytes during growth and maturation, how primordial germ cells enter the fetal ovary, enter meiosis I then arrest in diplotene as their germinal vesicle is formed. The follicular pool thus consists of oocytes arrested in diplotene, which is basically a meiotic I arrest that persists throughout succeeding growth stages until just before ovulation. The authors offer a welter of knowledge on later follicular stages including the biochemistry of oocytes and follicles and their interactions with various hormones and cytokines as they enter their growth stages. They exit the follicular pool, apparently re-awoken under the control of regulatory proteins such as p34cdc2 and cyclin B, to enter their growth stages, which vary metabolically. For example, growth recommences as levels of the oocyte proteins Gpr3 and its receptor decline. Several weeks of growth under the partial control of granulosa cells maintains diplotene oocytes via cAMP and PKA pathways as they synthesise mRNAs and zona proteins. Fully-grown oocytes

enter their maturation phase driven by the LH surge, and meiosis resumes as chromosomes enter diakinesis and complete metaphase I and extrude the first polar body. Active agents during these stages include kit ligand, its receptor, GDF (growth differentiation factor) and BMP-15 (bone morphogenic protein-15). These authors stress the importance of FSH and LH, gap junctions and transzonal factors during these stages of development.

Genetics and biochemistry of oocyte growth and maturation are covered in Chapters 2 and 3. Harris and Picton initially assess the metabolic activities in oocytes and follicles which is essential when devising specific culture media. Little is known about primordial stages, although glycolysis is essential for later stages. Glucose breakdown forms ATP via the reduction of NAD, itself formed via the conversion of pyruvate and an active Krebs cycle. Oocytes of most mammalian species require pyruvate and a little glucose, and their oxidative energy depends on mitochondria inherited from the primodial germ cell. These organelles initially aggregate to form the Balbiani body before dispersing in ooplasm. The authors describe the increasing sensitivity of oocytes to FSH and LH, the former determining varying levels of kit ligand. Preovulatory follicles consume glucose and produce lactate, and accumulate stable RNA species and proteins. These topics are also discussed by Picton et al. in Chapter 3. This team assesses oocyte/follicle interactions and how various nutrients including pyruvate especially and a little glucose supply the great majority of the oocyte's needs. Mitochondria are hence essential for their energy source. Attention also concentrates on the role of gap junctions in coordinating follicle growth and differentiation, and their connexins which are seemingly phosphorylated by LH. Differing connexion subgroups characterise thecal and granulosa compartments, and their knockout arrests follicles in antral stages. Increasing numbers of granulosa cells cross-talk with oocytes via paracrines maintaining extracellular matrices, together with GDF-9, anti-Mullerian hormone, basic FGF, retinoblastoma protein, myc oncogene and c-kit and KL/CSF. Picton et al. stress how oocytes become meiotically competent, gap junctions lose their properties, and cAMP levels decline, perhaps regulated by agents such as 3-isobutyl-1-methylxanthine. All this information is essential to understand how granulosa cell properties are modified, luteinisation commences, hyaluronidase is secreted, prostaglandins are released and intracellular calcium is mobilised.

Genetic and developmental factors unique to oocytes must be understood when practising IVM. Ursula Eichenlaub-Ritter provides a stage-by-stage description of biochemical patterns in growing and mature oocytes. She describes in detail the massive increases in mitochondrial numbers, chromatin remodelling, and various forms of RNA and protein synthesis. In oocytes, MPF and cytostatic factor regulate development to metaphase II which is very different to mitotic cells where anaphase is triggered as chromosomes attach to the spindle. Errors in the complex factors controlling the synaptonemal complex, gene recombination and DNA repair must be understood to understand, and perhaps one day control, chromosomal non-disjunction which leads to embryonic aneuploidy and the death of many fetuses. In these stages, ribosomal and other RNAs are synthesised together with transcription factors such as the homeobox gene *Nobox* which sustains downstream developmental genes such as *Oct-4, BMP15*. Post-translational mechanisms are involved as polyadenylated mRNA is translated and numerous mRNAs are recruited. Continuing these themes, Swain and Smith identify successive mechanistic events as oocytes mature such as meiotic I resumption, polar body extrusion, meiotic II re-arrest, and chromatin condensation. They relate to the actions of developmental factors such as laminin proteins, cAMP and protein kinase C, aurora A kinase, protein phosphatase-1, Cdc25 and p34cdc2 kinase and cyclin B with the roles of granulosa cells. Fortier and Trasler are concerned

with a detailed analysis of epigenetic phenomena during oocyte growth. Uniparental mouse embryos, for example, are nonviable because their disordered epigenesis can impair placental and embryonic development. They are formed as methylated sites decline in primordial germ cells migrated to the genital ridge, single-copy genes being rapidly demethylated in both sexes to make them epigenetically equivalent. Reports have appeared of imprinting syndromes in rare IVF children, which may be due to the imbalanced methylation of maternal but not paternal genes. For example, parthenogenetic mice may die in utero through variations in methylation, which can be overcome by isolating the large chromosomal domain between *H19* and *Igf2*. Such defects in the maternal component may also account for human hydatidiform moles and teratomas. Fortier and Trasler are intent on describing how imprints involve DNA methyltransferases (DNMT) which are absent in fertilised eggs and appear in the 8-cell stage. They take us even deeper into the mysteries of methylation by analysing the roles of isoforms, including DNMT1 which is expressed at day 11.5, as imprints are being erased. They also describe two variant DNMT3s; namely, 3a and 3b, with differing activities; e.g., the former possessing methyltransferase activity but not the latter. All this knowledge is essential to understand how the inclusion of various sera in culture media may modify demethylation and remethylation in fertilised eggs and produce children with imprinting syndromes such as Angelman's. Brief mention is finally made of sperm imprinting which may reduce sperm counts, and on imprinting defects in infertile patients with Angelman syndrome.

Three chapters on the physiology of IVM are opened as Ronit Abir et al. concentrate on successive development stages in primordial human follicles which peak at 7×10^6 by mid-pregnancy. These early aspects of follicular development are highly significant for understanding the formation of the ovary when considering oocyte and ovarian donation. Both tissues can be cryopreserved and thawed, although poor results to date indicate the need for greater knowledge on growth factors and endocrinology in fetal stages. Hreinsson et al. stress that live young have nevertheless been born in certain mammalian species, and human births from ovarian grafts have been reported. They consider strategies for preserving female fertility, and again stress the need for improved knowledge on the formation and growth of follicles. Various technical details are also discussed such as using FSH to reduce atresia in vitro, GDF-9 and insulin-like growth factors to promote follicle growth, and designing optimal culture media. New therapies for various afflictions, and the restoration of fertility to cancer patients and post-menopausal women depend on such improvements in this field.

Endocrinology is a basic aspect of ovarian and follicular development. Six chapters are devoted to it, covering ovarian hyper- and hypostimulation and the polycystic ovary. Broekmans and Fauser review ovarian endocrinology in detail, with attention to the roles of gonadotrophins and steroids and the roles of receptors expressed in thecal and granulosa cells as follicles grow and mature. They describe the role of TGFβin sustaining germ cells in the yolk sac and their migration to the genital ridge. They stress how 2 mm follicles can respond to FSH to form Graffian and dominant follicles highly responsive to gonadotrophins. The roles of FSH and LH in follicular recruitment must obviously be understood together with the roles of granulosa cells in steroidogenesis. They also stress how intraovarian modulators of follicle growth; for example, IGF and EGF, regulate the ovarian follicular pool and the selection of a dominant follicle in association with factors such as GDF and BMP-15. Clinical implications such as the polycystic ovary syndrome and malfunctions in these development systems are discussed by Jeffrey Russell. Research in the 19th and 20th centuries produced the Stein-Leventhal operation in 1935, and a better understanding of effects of diminishing levels of ovarian androgens. They

draw attention to the thin layer of hundreds of small follicles just below the ovarian surface in PCOS patients and their endocrinology. PCOS occurs in 3-7% of women as assessed by diagnoses based on FSH, LH and steroids, and providing that similar symptoms such as premature ovarian failure are excluded. They describe the use of the free testosterone index as a measure of PCOS, stress that obesity is common but not atypical of PCOS cases, that ultrasound helps to assess follicle numbers, and assess the significance of ovarian volume, blood flow and other characteristics. Studies on insulin resistance have led to work on metformin, and letrozole, an inhibitor of aromatase, may also assist with induced ovulation. Despite these findings, OHSS remains today a serious risk for patients and their pregnancies, with serious side-effects leading to hospitalisation.

Adam Balen discusses the diagnostic value of ultrasound in detecting PCOS and hyperstimulation and concludes that it is highly significant. Somewhat in contrast, a recent consensus on PCOS concluded that ultrasound, Doppler and MRI should be restricted to research. Bayrak and Paulson concentrate on methods aimed at predicting and preventing OHSS, and dealing with serious side effects such as ischaemic stroke, myocardial infarction and even death. Pathophysiological studies clarified rises in vascular permeability, capillary leakage and pleural or pericardial effusion, among others. They stress the need for tight controls, since increased permeability may involve oestrogens, histamine, serotonin, prolactin, angiogenic factors and possibly angiotensin. They caution that hCG must be given carefully, whether for ovarian stimulation or to sustain early pregnancy, OHSS may be alleviated by aspirating follicles, administering albumin, maintaining doses of GnRH agonists, and giving methylprednisolone. Unfortunately, many such tests have failed in controlled trials. Kovacs then lists IVF-associated clinical problems other than OHSS, such as errors in FSH dosage, the need for luteal support and continuous ultrasound monitoring of follicles in the follicular phase. He stresses how IVM could help to avoid OHSS. In a final chapter on managing OHSS, Sedler and Balen assess its prevalence and define its mild, moderate and severe forms. For example, VEGF may be a risk factor enhancing capillary permeability, and younger ages involve greater responsiveness to gonadotrophins. Other preventive strategies include aspirating follicles, delaying hCG injections (coasting), abandoning the treatment cycle, and cryopreserving all embryos; and more unusual treatments include antihistamines, inhibitors of prostaglandin synthesis, diuretics and dopamine. Aspirating ascitic fluid may reduce symptoms including pleural effusion in severe cases.

The penultimate section of this book covers details of oocyte maturation. To this end, Ba-Akdah et al. stress the value of various IVM protocols for women with PCOS, and other wishing to avoid treatment with hCG or LH when promoting oocytes maturation. They clarify the benefits of IVM when treating PCOS patients with considerable numbers of small antral follicles. Persisting risks of PCOS may be offset by reducing gonadotrophin dosages or by ultrasound between days 2 and 5 of the menstrual cycle to measure factors such as ovarian volume, the velocity of ovarian stromal blood flow, counts and sizes of follicles, and endometrial thickness. They comment on how a dominant follicle present during IVM does not prevent the aspiration of many oocytes. They also assess the benefits for IVM of small FSH doses in early follicular stages, rapid rates of maturation and its shorter duration, and permitting oocytes to mature in vitro for 24 h or longer. Administering hCG 36 h prior to oocyte collection is also effective, reaching pregnancy rates of >35% per cycle in women <35 years of age when the development of oocytes and endometrium are synchronised. Other technical advances include finer needles for aspiration via the vagina, although they can be blocked by blood, and reducing the need for multiple punctures. Sufficient oocytes can be aspirated,

immature oocytes being matured in maturation medium with FSH and LH, and those with a first polar body being inseminated immediately.

Applied aspects of IVM are now discussed, beginning as Frank Barnes describes the history of IVM from the days of Pincus, Enzmann and Saunders. Early errors delayed progress, although slow but deliberate investigations began as clinical IVF was introduced. Anne Mikkelesen assesses the benefits of FSH priming with 150 IU daily for 3 days before oocyte collection, and confirms high pregnancy rates of 29%, and implantation rates per embryo of 29% (versus 0% in controls). Such advances may have emerged through increased follicular sizes or plateauing FSH levels, and hCG injections (10,000 IU) at 36 h before oocyte retrieval could have raised pregnancy rates by hastening maturation in vitro. She concludes that IVM may replace ovarian stimulation in IVF. Hwang and Lin also discuss the advantages of FSH and hCG stimulation, querying the value of FSH priming but stressing the beneficial effects of hCG despite its misuse in some clinics. FSH coasting seems to be more acceptable. They stress the differences between IVM, routine gonadotrophin stimulation and 'rescue' IVF, especially the timing of hCG injections which are usually given during IVM when follicles reach 12 mm, versus ca. 18 mm for IVF. Despite such differences, IVM has already led to >500 babies free of anomalies. Bulent Gulekli et al. stress the widening use of transvaginal aspirations with spinal or epidural anaesthesia and the continued value of ultrasound for visualisation. They stress again how mature oocytes can be aspirated from small follicles during IVM, using 19-20G needles and strict asepsis. Anne-Maria Suikkari assesses varying methods for endometrial preparation in IVM which differ from those applied during IVF, oocyte donation and transfers of frozen-thawed embryos. Endometrial proliferation must be achieved quickly, oestrogens are delayed and oestrogens plus progesterone are given over weeks 7-12 of gestation for IVM. Laboratory aspects of IVM, natural cycle IVF and IVF with ovarian stimulation are compared by Ri-Cheng Chian who reports that IVM culture media demand attention to serum additives, gonadotrophins, steroids and growth factors. He also stresses that IVM must be timed to particular stages of the menstrual cycle to avoid exposing oocytes to androgens, although, overall, side effects are fewer than with ovarian stimulation.

Embryonic growth, pregnancies and social aspects of IVM are discussed in the final section. Several contributors cover pregnancy rates, opening with David Gardner's review on embryo culture. He describes numerous factors, including media composition, problems with static cultures, the values of various supplements, gas phases, the size of culture droplets, the overlying paraffin oil and quality control. Significant aspects also include embryo grading, sequential scoring, the optimal day for transfer, and attention to embryonic arrest at the 8-cell stage. William Buckett attends to neonatal outcomes after IVM, especially pregnancy complications and neonatal health. He stresses that, despite the risk of imprinting syndromes, multiple pregnancies and low weight for gestational age, overall, current data are reassuring. Antoine Terré and his colleagues from France comment on improved implantation rates when IVM is used for patients with PCOS, IVF failure, oocyte donation, and previous chemotherapy. Their many PCOS patients have poor rates of embryonic growth in vitro, low implantation rates and high rates of loss in early and later pregnancy, some of it ascribed to lifestyle and other matters. Benefits include avoiding OHSS, lower costs, and a simpler treatment. Jiayan Liu and colleagues identify benefits of IVM to patients with diminished ovarian reserve, few small follicles and weak responses to gonadotrophins. Even so, IVM pregnancy rates nevertheless reach 20% and further advantages include the lack of need for high gonadotrophin doses when treating poor responders. Over-responders to hormones who are sensitive to OHSS are discussed by Kyung-Sil Lim and colleagues who conclude that IVM

offers new approaches to the care of patients and that disorders may be preventable. Jin-Ho Lim et al. report on combining natural cycle IVF and IVM. For IVM, they administer 10,000 IU hCG then aspirate follicles of <12 mm diameter to collect mature and immature oocytes simultaneously. Mature oocytes with a first polar body are inseminated immediately, whereas unripe oocytes with germinal vesicles are matured in vitro and inseminated in vitro 3-4 days later to produce implantation rates of 10% per embryo and pregnancy rates of 30%. The final chapter of the book is given to Holzer et al. who discuss IVM and fertility preservation after therapies for malignancies, lupus and premature ovarian failure. They stress again how the reproductive period can be extended through ovarian or oocyte cryopreservation, and how IVM oocytes can also assist this purpose.

It has not been easy to summarise the considerable information in this book. It is spiced with information and provides the personal views of leading investigators. IVM is clearly a new branch of IVF, with special advantages and disadvantages. A book such as this is urgently needed to reveal variations between laboratories, the application of current techniques and the chances of establishing pregnancies. Differences between individual authors are apparent, although there is little doubt that optimal techniques will emerge with further research. In this and other ways, this book is also reminiscent of the early days of IVF when a series of conferences extended the technique worldwide, and we will watch with interest for a similar expansion of IVM.

I have little doubt that IVM will extend to many clinics. The numerous small follicles in most patients can be aspirated for mature oocyte without any need for massive forms of ovarian stimulation as practised with IVF. Enormous advantages could flow; for example working with immature oocytes should avoid the risks of cryopreservation damage which was a serious and long-term impediment to oocyte freezing for IVF. New classes of patients could well be attracted to cryopreserve their immature oocytes for their older ages. It may be much safer to cryopreserve immature oocytes than those which are fully mature. The ability to extract dozens of oocytes, especially in PCOS patients, will also open possibilities of detailed research using enormous banks of oocytes (or follicles). Research such as this should help those patients recovering from cancer therapies or an innate loss of oocytes. The causes of meiotic errors due to anomalies in chromosome pairing in diakinesis, or in the attachment of chromosomes to the meiotic spindle should be assessed. Such knowledge could help to avoid these anomalies, so that embryos are free of aneuploidies or polyploidies without any need for FISH as used today. Lastly, research will be opened into the hidden nature of primordial germ cells and follicles to uncover the fundamentals of early development. In short, scientists and clinicians could be presented with enormous opportunities at present denied to them.

To finish, let me compliment the Editors planning and publishing this book. It is certain to carry IVM into many more clinics.

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Preface

An introduction to in-vitro maturation of human oocytes

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The clinical use of in-vitro matured (IVM) oocytes has come a long way since the initial work of Robert Edwards in the United Kingdom in the 1960s[1–3] and the early clinical successes of Kwang Yul Cha *et al.*[4] in South Korea and Alan Trounson *et al.*[5] in Australia in the early 1990s. There are now successful clinical IVM programs around the world and nearly a thousand children have been born as a result of clinical IVM treatment. In fact the contributing authors to this book are testament to the geographic spread of IVM.

Although the world's first IVF baby, Louise Brown, was conceived within an unstimulated menstrual cycle[6], it soon became obvious that if IVF was to progress from a research tool to a clinical treatment, the use of ovarian stimulation would be necessary. The principle was that ovarian stimulation recruited more of the developing primary follicles to progress to maturation rather than atresia and thus generated more oocytes and ultimately more embryos for transfer. Ovarian stimulation is responsible for IVF reaching the success rate which has enabled it to be readily available throughout the world[7,8].

However, ovarian stimulation also has a cost. Stimulation protocols are associated with side-effects and risks, including ovarian hyperstimulation syndrome (OHSS), as well as with increased direct and indirect costs. The development of IVM allows the benefits of ovarian stimulation – namely more oocytes and more embryos – without these additional risks and costs. IVM appears particularly suited to women with polycystic ovaries (PCO) and polycystic ovary syndrome (PCOS), who seem to do well with IVM treatment and who are at significantly increased risk of OHSS following ovarian stimulation[9,10].

Following the First World Congress on In-Vitro Maturation held in Montreal in 2004 and the establishment of the International Society for the In-Vitro Maturation of Human Oocytes, it became apparent as IVM is increasingly practiced throughout the world that there is a real need for a comprehensive IVM textbook. We hope that this volume will fulfill this need!

We have endeavored to collect contributors with international expertise in all aspects of IVM from the basic scientific teams to the clinical treatment programs from around the world. The book is divided into four parts.

Part I covers the scientific rationale for IVM by outlining the normal oocyte growth, interaction, and maturation in vivo and how these have led to the current understanding and development of protocols for oocyte maturation in vitro. We have also sought to determine possible future scientific developments in IVM and scientific

concerns regarding the effect of IVM on oocyte and later embryo development. Here we have to mention that follicular maturation and oocyte maturation are two totally different concepts. Follicular maturation refers to the process from primordial follicle to preovulatory follicle, in other words it reflects follicular growth. Oocyte maturation refers to the development of the fully grown oocyte from germinal vesicle (GV) to metaphase II (MII) stage, in order to receive sperm at fertilization. Oocyte maturation is triggered by the LH surge in vivo, and occurs spontaneously in vitro with suitable culture conditions. IVM mentioned in this book mainly refers to oocyte maturation in vitro, not follicular development in vitro.

Part II covers the normal ovarian function and the clinical, ultrasound, and biochemical features of PCO and PCOS. It also covers the risks of ovarian stimulation for conventional IVF and the prevention and treatment of OHSS.

Part III covers the clinical application of IVM. It aims to cover all aspects including patient selection, current treatment protocols (including the various priming protocols), immature oocyte retrieval, endometrial preparation, embryo development, all laboratory aspects, and the pregnancy and neonatal outcome following IVM.

Finally, Part IV covers possible new developments – such as how to improve IVM success rates – and also new treatment directions for IVM – such as those with previous poor conventional IVF treatments, the development of natural cycle IVF/IVM, and the use of IVM in fertility preservation.

Although IVM is still relatively new amongst assisted reproductive technologies (ART), we hope that this textbook will contribute towards its increased availability. We all believe that IVM offers many advantages over conventional IVF and that couples who would benefit from this treatment modality should be able to do so.

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