CHAPTER 16

The first steps in the era of human in-vitro oocyte maturation

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…ova maturation and degeneration then proceed pari passu [at an equal pace], and the extent of the former is limited by the degree of advancement of the latter.

Gregory Pincus and Barbara Saunders, 1939

The purpose of this review is to point to the salient contributions to human immature oocyte collection (IOC) and in-vitro oocyte maturation (IVM) by the pioneers and investigators of our field as they are used in those procedures today. We are grateful to all investigators who have added to this body of knowledge. Any lack of reference to them here does not minimize their contribution.

Between 1935 and 1939, Gregory Pincus and coauthors EV Enzman and B Saunders conducted their studies on the maturation of mammalian oocytes[1,2]. In the rabbit pituitary hormones lead to ovulation of the ovarian follicle and the initiation of oocyte maturation in vivo. This was demonstrated by injecting bovine pituitary extracts into the doe. In vivo, mature ova are shed into the oviduct following copulation. When ova were removed from the follicle experimentally and cultured in vitro in the absence of hormones, they spontaneously resumed meiosis and progressed to metaphase II at a rate similar to that observed in vivo. When in-vitro fertilization was induced, the resulting zygotes underwent regular cleavage when allowed to develop in the living oviduct. Care was taken to maintain a proper temperature. High concentrations of sperm were required to bring about fertilization in vitro that in many instances led to polyspermy, which was visibly manifested by aborted early cleavage. That ova spontaneously resumed meiosis when removed from the follicle led to the hypothesis that the follicle may produce substances that inhibit maturation[1]. These findings were quickly extrapolated to the woman, studied, and described. Long and Mark (1911) and Long and Evans (1922) were credited by Pincus and Saunders as having observed that the first maturation division is achieved independent of copulation in spontaneously ovulating mammals[2]. Other investigators had isolated human ova previously; however, it was Pincus and Saunders who observed maturation of the human oocyte. Their studies confirmed that removal of human ova from the follicle initiated the resumption of meiosis and that external stimulation of explanted ova was not required. They concluded that the ability to resume

meiosis is not necessarily linked to the ability to complete meiosis[2]. Resumption of meiosis occurs spontaneously upon removal from the follicle, and a 'positive [meiosis] inhibiting factor prevails in vivo.' Finally, oocyte removal from the follicle delays oocyte atresia[1,2]. They believed that the ability of ova to be rescued from advancing follicular atresia might be measured by their ability to mature and fertilize in vitro[2]. This misconception would be held by most investigators for the next 60 years as each would set new developmental thresholds as a measure of complete developmental potential short of live birth. Subsequent studies in years to come would show that the competence to succeed in development is acquired sequentially and, to a limited extent, is temporally associated with egg diameter and follicle size. Embryos derived from IVM ova that fertilize and undergo early cleavage are not all developmentally competent.

In 1965 Bob Edwards, predicted, 'with this technique [IVM] it should eventually be possible to study pre-ovulatory development, and perhaps fertilization and preimplantation development in various species including man'[3]. In his two publications on the topic that year he discussed the importance of selecting appropriate media and introduced TCM-199 to the IVM protocol. TCM-199 is still used widely for the IVM process today and arguably remains the medium of choice. The kinetics of IVM were described in detail for human primary oocytes as well as other species[3,4]. Resumption of meiosis with a synchronous progression, independent of both menstrual cycle day of recovery and gonadotropin support in the IVM medium, was found to occur in over 80% of oocytes. The progression of meiosis as reported is: germinal vesicle breakdown (GVBD) at approximately 25 hours postexplantation (hpe), diakinesis between 25 and 28 hpe, metaphase I between 28 and 35 hpe, and metaphase II between 36 and 43 hpe. Edwards reported that some ova were recovered from the follicle without cumulus and had progressed to metaphase II[4]. This observation suggests that oocytes are triggered to resume, and in some cases complete, meiosis in the atretic follicle. This is in agreement with studies in cattle where it was subsequently found that oocytes within cohort follicles undergoing atresia spontaneously resume and complete meiosis within the follicle, a process termed pseudo-maturation[5]. Possibly the most important observation made was in his last sentence, 'human oocytes can be fertilized after maturation in vitro.'

Lucinda Veeck and coauthors, from the renowned Jones Clinic, reported an ongoing pregnancy and birth from in-vitro matured oocytes in 1983[6]. The resemblance of the stimulation protocol used in that investigation to FSH and hCG priming protocols used today is remarkable[7–9]. It would next be described in the literature in 1994 when used in cattle and again in 2001 in human IVM[10–13]. Starting on day 3, daily injections of human menopausal gonadotropin (HMG) were given to patients until follicle size reached 12–15 mm as determined by ultrasound. Ten thousand mIU of hCG were administered 50 h after the last hMG injection and followed by egg recovery via laparoscopy 36–38 h later. Immature oocytes were cultured for 22 to 35 hours and inseminated. Frequencies of IVM, fertilization, and the number of cleaving zygotes were at least 80%[6]. Their report suggests but does not specifically state, that many immature oocytes identified at the time of recovery matured within 24 h of in-vitro culture in gonadotropin free medium. This may be the first recorded observation indicating that maturation may progress from the germinal vesicle stage to metaphase II within 24 h of in-vitro culture if hCG is administered to the patient 36 h prior to IOC. The rate of maturation observed would agree with that reported by Chian et al. 16 years later using hCG priming[8]. This observation indicates that hCG administration does not lead to overt IVM in small to medium sized follicles. However, over the course of the ensuing 36 h before recovery it prepares the oocyte for what may appear to be accelerated maturation in-vitro. This suggests that gonadotropin

supplementation of the IVM medium as used by subsequent investigators may not be required if the trigger to resume meiosis is initiated in vivo. Of note was that the recovery of immature oocytes occurred in the absence of a dominant follicle, a significant point that would recur and become a dominant theme in subsequent successful protocols. Assisted reproductive technology took a distinct turn away from IVM at this point to favor the more common strategy of recovering and fertilizing mature oocytes. In the late 1990s several groups would similarly report pregnancies from stimulated patients with and without administration of hCG[14–16]. Nonetheless, the threshold of producing a child from an IVM oocyte had been crossed.

Cha and coworkers (1991, also as a prize paper reported at American Society for Reproductive Medicine 1989) made the link between IVM protocols used in sheep and cattle to an experimental protocol used for the treatment of human infertility[17]. In their original work, oocytes were recovered from patients undergoing gynecologic surgery and subsequently donated to their IVF program. Performed in situ, aspiration of antral follicles occurred using a 21-gauge needle attached to a syringe containing medium supplemented with 10% fetal cord serum[17]. If oophorectomy was performed, oocytes were recovered by repeatedly slicing and subsequently rinsing the cortical tissue (personal communication). The maturation protocol used Ham's F10 medium supplemented with either 20% fetal cord serum or 50% mature follicular fluid (heat inactivated)[17]. Subsequent protocols from the same laboratory would use TCM-199 supplemented with pregnant mare serum gonadotropin with hCG or rFSH with hCG and estradiol[18]. A triplet pregnancy occurred after transfer of five embryos to a patient with premature ovarian failure. The paper was of particular importance as it renewed interest in using IVM as an infertility treatment modality[17].

Alan Trounson foresaw the significance of the IVM technology as it might apply to the treatment of infertile patients suffering from polycystic ovarian syndrome (PCOS)[19]. His enthusiasm for the technique and its possibilities was the inspiration for many lectures around the world during the 1990s. His historic understanding of IVM as it is applied to livestock gave him immediate insight into procedure development[20]. In cooperation with Cook Australia, Trounson, Carl Wood, and other colleagues at Monash IVF defined a protocol for ultrasound guided follicle aspiration of human immature oocytes. During their initial investigation, they developed a more rigid needle with a thicker wall and shorter bevel. The increased rigidity facilitated penetration of the ovarian cortical stroma of PCO patients; the shortened bevel allowed the entire lumen of the needle to be placed inside the antral cavity of small follicles. The aspiration pressure used at oocyte collection was reduced to half that used in standard IVF (7.5 kPa vs 15 kPa). This was believed to reduce the amount of granulosa cells that would be stripped from the oocyte during aspiration. Aspirates were recovered in Hepes buffered HTF medium supplemented with heparin[19]. Cumulus–oocyte complexes (COCs) were isolated by filtration through an embryo-concentrating filter, methodology originating from the bovine embryo transfer industry. Fresh medium was used to rinse away red blood cells and other debris then the filter retentant was poured into a 100 mm dish for COC isolation using a dissecting microscope. The collection and isolation protocol described by Trounson et al. has been used extensively in subsequent studies in other laboratories. The observation times used and rate of maturation observed were similar to that first reported by Edwards in 1965[4]. GVBD occurred in greater than 80% of oocytes at 21 to 22 h, with 81% mature by 48 to 54 h. Initially, insemination was performed at 44–54 h. It was noted that some oocytes were mature after only 23 to 25 h of culture. Postulating that early maturing oocytes would have the best developmental competence, subsequent insemination times were moved to ~35 h. Maturation and fertilization frequencies

were similar across ovulatory and anovulatory polycystic ovary syndrome (PCO(S)) groups and ovulatory patients. The rate of embryo development in PCO patients was retarded, suggesting suboptimal culture conditions or intrinsic defects in developmental competence[19]. Their publications provided the essential methodology used in immature oocyte collection and IVM around the world today.

In a series of two publications, Barnes et al. from the Monash IVF laboratory would reintroduce the use of TCM 199, as first used by Edwards, to the maturation protocol[4,21,22]. They would also introduce intracytoplasmic sperm injection (ICSI), blastocyst culture, and assisted hatching and characterize embryo development of PCO(S) and ovulatory non-PCO(S) patients. Their publication indicated that IVM oocytes from ovulatory non-PCO(S) patients had equivocal early cleavage development when compared to stimulated patients where maturation occurred in vivo. A single pregnancy and live birth was reported from the study series; the outcome clearly indicated that good embryo morphology is not an adequate measure of oocyte developmental competence[21,22].

In 1999 Cobo et al.[23] and Mikkelsen et al.[7] simultaneously began focusing on the timing of immature oocyte collection, both having identified the trigger point for retrieval to be before the lead follicle achieves 10 mm. While Mikkelsen et al.[7] principally focused on the effect of FSH priming and would eventually demonstrate the merits of the protocol in subsequent publications, their consideration of follicle dominance in this initial work proved to be insightful. Cobo et al. principally focused on the time of aspiration, correlating it to the emergence of the dominant follicle. They postulated that the visible emergence of the dominant follicle may lead to changes in the follicular endocrine milieu of the cohort that would be detrimental to their subsequent developmental potential. The frequency of oocytes that were atretic, with cumulus cover, matured and fertilized in vitro, did not differ between predominance and postdominance recovery groups. However, significantly more oocytes were recovered and development to blastocyst was improved in the predominance group with cumulus cover[23]. The point was made that early egg and embryo morphology is a poor predictor of developmental potential. The studies of Cobo and colleagues demonstrated that the trigger point for follicle aspiration is a critical component in the IOC/IVM procedure[23]. Mikkelsen and colleagues showed that by using dominant follicle selection to determine the time of IOC, developmentally competent oocytes and live births would result[7]. Both studies were successful for what appears to be the same reason.

Is it possible that oocytes lose developmental competence in the reverse order that they acquire it? Hypothetically, mRNA and proteins that have a specific purpose in early development have temporal limitations while other housekeeping mRNA and proteins are relatively stable. This may lead to the appearance of a reverse loss in the ability to develop. For example: early cleavage, up to the eight-cell stage, is driven by mRNA and proteins derived from the maternal genome. It is likely that embryonic development fails because advanced atresia induces a fundamental decay of these components. Studies using ultrasound (u/s) to evaluate follicle dynamics show that cohort follicles shrink in diameter dramatically within 1 to 2 days of the visible emergence of a dominant follicle[24], suggesting a rapidly advancing atretic process in that population. Therefore embryos derived from postdominance IVM oocytes maintain the ability to resume and complete meiosis, fertilize, and cleave into what we may perceive to be good embryos, but in fact have lost the ability to develop beyond those early cleavage stages. A difference in oocyte recovery of a mere 48 h may be all that separates developmentally competent from incompetent oocytes.

In further exploring the treatment of PCO(S) patients, Chian et al. introduced hCG priming[8]. PCO(S) patients were staged for IOC/IVM with

progesterone induced withdrawal bleeding followed by u/s monitoring to exclude patients with a dominant follicle. Human chorionic gonadotropin was given 36 h prior to egg retrieval on cycle days 10 to 12. The principal finding was that hCG priming increased the number of immature oocytes that would progress from the germinal vesicle stage to the metaphase II stage within the first 24 h of IVM[8]. This suggests again that the signal initiating the resumption of meiosis is initiated in vivo.

There are essentially two methodologies that appear to lead to improved developmental competence of IVM oocytes. The first relies on the ability of an oocyte recovered at the time of dominant follicle selection to spontaneously resume and complete meiosis in vitro within approximately 24–30 h of culture. The second relies on hCG priming, which may recruit oocytes that also resume and complete meiosis in vitro within approximately 24–30 h. The paracrine signal resulting from dominant follicle selection or hCG priming likely prepares for and/or initiates the resumption of meiosis of the cohort in vivo. The resulting in-vitro accelerated maturation rate in both protocols provides a valuable selection method, optimizing oocyte developmental potential.

The first steps in the era of human IVM have, after nearly 70 years, achieved clinical significance. The methodology comes together in a recent work by Soderstrom-Anttiila and colleagues[25], which embraces the ideology of the investigators who preceded them. The ovary contains a large supply of developmentally competent oocytes that, upon explantation from ovarian follicles, can be matured and fertilized in vitro and develop into healthy children. The process of immature oocyte recovery and IVM should be simple and easy to apply and minimally invasive to the patient. Their clinical protocol starts with definitive parameters for when to recover immature oocytes: the day following the observation of a leading follicle (dominant follicle selection) in cycling patients or in PCO(S) patients when endometrial thickness reaches 5 mm following progesterone-induced withdrawal bleeding but before dominant follicle selection. Maturation using TCM-199 supplemented with patient serum and gonadotropins is followed by in-vitro fertilization (by insemination or ICSI) of those oocytes that are mature within 30 h of culture. Finally the transfer of one or two morphologically superior embryos to the prepared endometrium (oral estrogen followed by vaginal progesterone) is performed[25]. As measured by implantation rates of greater than 20%, the success of this protocol would appear to equal that of current IVF protocols.

When selecting human oocytes with the potential for totipotent development, timing appears to be everything. While maturation in vitro can take 36–48 h, it is when oocytes mature within 24 h in vitro that reported pregnancy outcomes are best. The outlined studies suggest that it is when we collect primary oocytes and not how we mature them in vitro that ultimately leads to success. That oocytes acquire developmental competence briefly and lose it in the course of follicle atresia reflects the insight of Pincus and Saunders who stated[2] 'ova maturation and degeneration then proceed pari passu, and the extent of the former is limited by the degree of advancement of the latter.'

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