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Pioneering contributions by Robert Edwards to oocyte in vitro maturation (IVM)

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12 ***Abstract***

The history of IVM of mammalian oocytes, especially of human oocytes, holds a special place for Robert Edwards. He was the first to comprehensively examine and demonstrate maturation of human oocytes in vitro and in so doing he changed the course of medicine by fertilising them in vitro. In reviewing his contribution, we have examined the state of the field at the time and discuss his pioneering insights into mammalian oocyte biology. We will also discuss how some of the major concepts and challenges identified by Edwards 50 years ago remain amongst the major challenges facing IVM today.

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Introduction

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In vitro maturation (IVM) of oocytes is a technique that has always offered great promise for maximising the potential uses of the female gamete. It offers opportunities to preserve fertility and treat infertility in women, especially those that are unable to access gonadotrophin treatment for ovarian hyperstimulation, due to ovarian hypersensitivity, cost or other social reasons. Oocyte IVM is widely employed in animal breeding, primarily to increase genetic selection or introduce diversity in farmed and companion animal species, and to a lesser extent, IVM is used in the conservation of rare breeds and species. All of these potential future applications of IVM were recognized by Edwards in his landmark 1965 *Nature* publication (Edwards, 1965b). IVM is simple in concept and is elegantly described

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by Edwards in 1962; here in the context of discussing the practical benefit of IVM compared with in vivo endocrinological manipulation of oocyte maturation:

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“Fortunately, there is an alternative technique which promises to supersede the endocrinological control of ovulation in most species. This is to stimulate the oocytes directly in vitro after their liberation from the follicle. In addition to being a most attractive technique in that it avoids the use of hormone injections in vivo and permits the use of small pieces of ovary, it has also proved to be an unusually simple technique when used in several species. All that is necessary is to liberate the oocytes into a simple culture medium by puncturing the follicles; many oocytes then immediately resume their maturation” (Edwards, 1962).

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This definition of IVM remains relevant even today, just as it was when first described by Pincus and Enzmann (Pincus and Enzmann, 1935). Notably, the procedure described above, is still the practice used universally in domestic animal and veterinary IVM. However, in the human reproductive medicine sphere, IVM terminology has become decidedly opaque over the past decade or more; the term IVM has been used variably to describe the culture of completely immature (germinal vesicle, GV-stage) oocytes from unstimulated ovaries (as defined above), or oocytes of mixed meiotic status following a range of ovarian hyperstimulation regimes, or, even more concerning, used to describe the culture of arrested immature GV oocytes recovered from hyperstimulated cycles (“Rescue IVM”). It is well documented that embryos generated from Rescue IVM oocytes are genetically abnormal (Nogueira *et al.*, 2000; Jones *et al.*, 2008). This confusion in IVM terminologies requires resolution by the discipline. For the purposes of this review, we will use the understanding of IVM as founded by both Pincus and Edwards (Pincus and Enzmann, 1935; Edwards, 1962), which is the in vitro maturation of immature, GV-stage, cumulus-oocyte complexes (COCs) collected from antral follicles of unstimulated ovaries.

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Oocytes readily reach metaphase II (MII) of meiosis spontaneously in vitro (i.e. without hormonal stimulation), and MII oocytes can be fertilized for immediate embryo production or alternatively, mature oocytes can be stored by vitrification in liquid nitrogen for later use. IVM is practiced widely for commercial breeding opportunities in beef and dairy cattle, where global production of IVM-derived embryos is approaching half a million each year (Stroud, 2012). It is also sufficiently successful for practical purposes in other species, such

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68 as sheep, deer, goats, pigs, cats and mice. In contrast, IVM is difficult in primates and its
application in human reproductive medicine has been limited. This is largely due to the
poorer outcomes relative to gonadotrophin treated, traditional IVF cycles. This includes
poorer oocyte maturation rates, implantation rates, increased miscarriage rates and therefore
72 poorer pregnancy outcomes per cycle initiated (Gilchrist *et al.*, 2011). Today, human clinical
IVM occupies a niche role in providing a treatment option for women with polycystic ovaries,
fertility preservation for cancer patients and also as a cheaper and simpler alternative to IVF
which has proved attractive in developing countries particularly in East Asia.

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IVM prior to the 1960's

IVM as we recognize it today was first reported by Pincus and Enzmann (Pincus and
80 Enzmann, 1935). They made a number of observations about the spontaneous nature of
meiosis when rabbit ova were removed from antral follicles. They carefully described events
such as germinal vesicle breakdown (GVBD) and MII formation during in vivo oocyte
maturation and noted that these events were mirrored in vitro, without the need for
84 supplemental hormones. Pincus and Saunders (Pincus and Saunders, 1939) made similar
observations with human oocytes. In both publications, oocytes were inseminated in vitro in
an attempt to fertilize them. This was pronounced as successfully achieved, but later
questioned by others as dubious, as there was no supporting evidence that sperm capacitation
88 had occurred (as discussed in Biggers; (Biggers, 2012)). As experienced by Edwards in later
years, Gregory Pincus was increasingly criticised in the media of the time for his work on
maturing human oocytes in vitro and attempting to fertilize them. By 1939, in the briefest of
letters to the journal *Science*, he announced he was withdrawing his activity from the area:

92 *“In certain press reports of a recent paper on meiosis in explanted human
ovarian ova, the statement has appeared that I plan to carry on this work to the
extent of attempting to discover if human offspring can be produced by the
methods that we employ in ovum culture. This statement is incorrect. My work
96 with human ova ended with these studies of maturation and I have no intention
whatsoever to continue them”* (Pincus, 1939).

In the intervening years prior to the 1960's, attention shifted away from IVM itself to the
challenges of fertilizing mature oocytes and the discoveries made surrounding sperm
100 capacitation and subsequent embryo development (see companion reviews in this series).

Nevertheless, Chang (Chang, 1955) provided confirmatory evidence to support Pincus and Enzmann's original observations of the timing of meiosis in rabbit oocytes during IVM (9-11 hours to reach MII), and that this occurred independent of any hormonal treatment. He also
104 found that if neat follicular fluid was used to mature oocytes, instead of diluted serum, progression of MII was delayed "*probably due to the presence of an inhibiting factor in the follicular fluid of the unmated rabbit*" (Chang, 1955), a concept that is uncannily relevant today with the current significant interest in delaying oocyte meiosis during IVM.

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Robert Edwards' contribution to IVM

In the late 1950's, Edwards' interest and considerable expertise were in the areas of the
112 genetics of aneuploidy in oocytes, oocyte meiosis and superovulation of mice (Johnson, 2011). Although he had been at the forefront of developing gonadotrophin treatments to induce multiple ovulation and pregnancies and he recognized the capacity of exogenous gonadotrophin treatments to deliver oocytes at all stages of meiosis, nonetheless he became
116 interested in determining if he could establish an in vitro system to examine meiotic progression in human oocytes, as he noted that to do so using exogenous hormonal regulation would be "*...difficult to apply*" (Edwards, 1962).

120 Edwards' contribution to IVM principally lies within three reports in the journal *Nature* (Edwards, 1962; Edwards, 1965b; Edwards *et al.*, 1969) and associated *Lancet* paper (Edwards, 1965a). The 1965 *Nature* publication remains one of the seminal contributions to the field of IVM and is his 2nd most cited publication behind the 1978 *Lancet* publication
124 announcing the birth of Louise Brown (Steptoe and Edwards, 1978). Edwards' work on IVM was largely restricted to the decade of the 1960's, and can be roughly divided into two halves; the first half of the 1960's where he focused on achieving meiotic maturation of animal and human oocytes using IVM, and in the late 1960's, where he focused intensely on achieving
128 fertilisation in vitro of the IVM oocytes. It is noteworthy that, unlike much of his subsequent scientific career, during this early period of the 1960's, he largely worked alone and was responsible for driving the direction of his research, overcoming substantial barriers to access research tissues (especially human), and performing the experiments himself with support
132 from his staff. Perhaps as a consequence, Edwards was particularly passionate about oocyte maturation and he remained an advocate of IVM for the rest of his life.

136 The first report, published on November 3rd 1962 (Edwards, 1962), demonstrated that
spontaneous oocyte maturation was at least initiated (GVBD) in vitro in several species
(mouse, rat, hamster, monkey), although it seems he only cultured the rodent oocytes in vitro
for 6-8 hours, most likely taking the timing of rabbit IVM described earlier by others (Pincus
and Enzmann, 1935; Chang, 1955). Other species attempted in this study were dog, baboon
140 and human, of which there was little evidence of GVBD in vitro, probably as he was too
hasty to examine evidence of maturation (for example, he only allowed 20 hours of IVM for
human oocytes). Nevertheless, he correctly asserted that oocytes, if already denuded of
somatic cells when liberated from follicles and/or already displayed condensed chromatin or
144 a polar body, were likely derived from atretic follicles (Edwards, 1962).

By the early 1960's, superovulation technologies in animals, but not humans, were well
advanced and Edwards had considerable experience with the kinetics of oocyte meiosis in
148 vivo in a variety of animals, based on the timing of human chorionic gonadotrophin (hCG)
administration. With great insight, he reasoned that the differing kinetics of meiosis across
different species in vivo may be paralleled by differing and species-specific meiotic kinetics
in vitro. The time required for human oocyte maturation was unknown. In the more heralded
152 1965 paper (Edwards, 1965b), he extended the culture time for human IVM. With each failed
attempt he further extended the culture duration until eventually he witnessed GVBD of a
human oocyte at 28 hours of IVM. This was a truly triumphant moment for Edwards, as he
recounts in his book:

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*“The next four hours passed slowly, slowly, but when I did examine the final
oocyte I felt as much excitement as I had ever experienced in my life. Excitement
beyond belief. A living, ripening, human egg, unbombarded by any hormones,
160 beginning its programme just as the mouse eggs had done. There, in one egg, in
the last of the group, lay the whole secret of the human program.”* (Edwards and
Stephoe, 1980).

164 Edwards went on to determine that MII is reached at 36 hours in human IVM oocytes. He
also demonstrated that complete spontaneous oocyte maturation could be achieved in various
and disparate species (mice, pigs, cows, sheep, rhesus monkey and human), so long as
sufficient maturation time was allowed, demonstrating the universality of this concept
168 (Edwards, 1965b). His comparative reproductive biology approach in both papers was

important and insightful as he understood that reliance on one model only (i.e. the mouse) may not provide the answers he required to enable reliable IVM for production of embryos in the human. This was his primary goal.

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From this point onwards, Edwards turned his attention to attempting to fertilise the IVM MII oocytes. After a number of years attempting to capacitate human sperm, including in vivo in reproductive tracts of a range of species, he returned to in vitro approaches. At this stage he had recruited Barry Bavister to his lab to work on media for IVF. Bavister's experience with developing hamster IVF with 'Bunny' Austin proved decisive, as adapting those techniques and using their own sperm, in March 1968 they eventually successfully fertilised a human oocyte in vitro (Bavister *et al.*, 1969; Edwards *et al.*, 1969). Notably both these papers used human IVM oocytes collected from unstimulated ovaries and matured in vitro for 36-37 hours in a mixture of three parts follicular fluid and one part "Bavister's medium". At this time Edwards sought out a clinical partner and subsequently teamed up with Patrick Steptoe. From this point onwards, Edwards was immersed in the world-wide race to achieve the first IVF pregnancy and he never returned to IVM research. Notably, despite his expertise and successes with IVM, he incisively turned to in vivo matured oocytes for the IVF program. He justified this decision on the basis that, at the time, embryonic development from IVM animal oocytes was poor. This was an astute observation and this is an ongoing issue for IVM today (see below). While major advances continued in animal IVM which led to the first pregnancies from IVM mouse oocytes (Cross and Brinster, 1970), human IVM took a back seat to hyperstimulated IVF, and it was not until much later in 1991 that the first human IVM child was born from an unstimulated cycle (Cha *et al.*, 1991).

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Insights from the 1960's that remain relevant to the challenges in clinical IVM today

In many respects, clinical practice of human IVM has changed little since the 1960's. Spontaneous maturation of oocytes following recovery from medium sized antral follicles (6-10 mm) continues to this day. Furthermore, the base media formulation used has not altered in four decades. We propose that there are six main challenges for clinical IVM today:

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1. To increase the recovery rate of immature COCs from follicles of unstimulated ovaries,
2. To increase the meiotic maturation rate of IVM oocytes,
3. To increase the developmental competence of IVM oocytes,

- 204 4. To determine whether any in vivo ovarian stimulation is desirable and if so which stimulation regime,
5. To establish a clinical IVM protocol that supports adequate endometrial development and receptivity,
- 208 6. To further verify the safety of IVM by conducting detailed follow up studies on the health of IVM offspring.

In the following sections, we will discuss concepts that emerged from Edwards' work and from that period, that remain relevant to some of these current challenges.

212 Oocyte meiotic competence:

It is highly noteworthy that Edwards reported a MII rate for human IVM of approximately 50% (Edwards, 1965b). This is similar to the earliest descriptions of human IVM by Pincus and 216 Saunders (Pincus and Saunders, 1939). Despairingly to investigators currently working in the field, the efficiency of maturation of human IVM oocytes to MII essentially has not improved. Maturation rates of ~50 – 60% are still the norm today. Edwards reported high rates of oocyte maturation from IVM in mice, sheep and cows, but lower rates (<50%) in pig, rhesus 220 and human oocytes. This is largely consistent with the literature today, where in particular, the low MII rate in human IVM remains a major obstacle to the efficiency of the technology and therefore to the clinical uptake of the approach.

224 Cytoplasmic maturation in vitro and oocyte competency:

Edwards was working on IVM at a time when the concept of oocyte cytoplasmic maturity was emerging. The concept was first used to describe the capacity for DNA replication and 228 cleavage of amphibian eggs. By the 1970's, it was widely described that oocyte maturation could be divided into nuclear maturation and cytoplasmic maturation, whereby meiotically mature oocytes may not necessarily have undergone complete cytoplasmic maturation. When applied to mammalian oocytes, unfortunately the term cytoplasmic maturation has multiple 232 meanings depending on the context. For example, oocyte cytoplasmic maturation can be used to describe ultrastructural features of the oocyte, including movement of organelles during nuclear maturation. It has been used to describe the capacity of the ooplasm to enable complete meiotic maturation, e.g. as a function of oocyte growth. But most commonly, 236 cytoplasmic maturation was thought to encompass the oocyte's capacity for subsequent

development. More recently, oocyte cytoplasmic maturation is termed “oocyte capacitation” (Hyttel *et al.*, 1997) or “oocyte developmental competence”, describing “*the biochemical and molecular state that allows a mature oocyte to be fertilized normally and develop to an embryo, which on transfer will enable development to term.*” (Gilchrist and Thompson, 2007). This clearly encompasses an enormous field of cell biology and explains why it has proved so elusive, but nevertheless, an appreciation of the hallmarks of oocyte developmental competence is emerging.

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By the mid-1960’s Robert Edwards was acutely aware of the concept of oocyte developmental competence, even if the terminology did not exist. Although the results of his 1965 paper focused entirely on oocyte meiotic competence and meiotic kinetics, his insight extended to the possible role of accumulated mRNA within oocytes having an impact on subsequent embryo development. He noted that synthesis of RNA and “*initiation of heterochromatin*” in the oocyte during the pre-ovulatory period might be important (Edwards, 1965b). This of course has proved entirely accurate and lies not only at the heart of oocyte developmental competence but also in the concept of the developmental origins of adult health having its origins in appropriate epigenetic programming of the developing immature oocyte and early embryo. His prediction is particularly poignant, as it reveals he appreciated the importance of the oocyte’s legacy to embryo development, as he concluded that oocytes maturing in vivo are more competent for further development than IVM matured oocytes (Edwards *et al.*, 1969). This mismatch in competency remains a defining limitation of the IVM-derived oocyte (Gilchrist *et al.*, 2011; Gremeau *et al.*, 2012). An important area of future research is to determine if the lower competence of IVM oocytes affects the health of IVM offspring. This area of research is made more difficult by the relatively few IVM children born so far, but the follow-up studies to date are encouraging in that no major increased health risks have been reported (Shu-Chi *et al.*, 2006; Soderstrom-Anttila *et al.*, 2006; Buckett *et al.*, 2007). Likewise, follow-up studies on mice IVM offspring suggest there is no major cause for concern (Eppig *et al.*, 2009), but nonetheless, more animal and clinical research is needed in this area.

Oocyte developmental competence is largely acquired in vivo during the antral phase of folliculogenesis. However, this competence can be readily “lost” using inappropriate IVM conditions. Edwards appreciated the critical importance of IVM culture conditions to the health of the oocyte. His IVM papers give truly insightful suggestions for improving IVM,

which in subsequent years, were shown by others to significantly impact oocyte
272 developmental competence across several species, including human. For example, in both
papers he experimented with different base media, especially tissue culture medium-199
(TCM199), but also Waymouth's media. Today, TCM199 with Earles' salts remains the gold
standard in IVM across several species, including widespread commercial application in
276 cattle and human IVM. Furthermore, described within the text of the 1962 and 1965 *Nature*
papers is an array of culture condition variables he experimentally tested to determine if they
had an impact on oocyte maturation. These included: incubation with supplemental serum,
follicular fluid, feeder cell monolayers; varying atmospheric gas composition; addition of
280 antioxidants (reduced glutathione and ascorbic acid); addition of vitamins (folinic acid, a
vitamer for folic acid, and ascorbic acid) and addition of ATP directly to cultures, in addition
to other ideas (Edwards, 1965b). Although the details of what was attempted presumably
remains within his laboratory books, the judgement to assess such factors was, in hindsight,
284 years ahead of his time as many of these remain pertinent questions to the improvement of
IVM nuclear maturation and developmental competence nearly half a century later.

Pincus, Chang and Edwards all separately noted that oocytes remain at the GV stage when
288 retained within their follicle, whether in vivo or explanted. They correctly concluded that the
follicle is inhibitory to oocyte maturation and that oocytes will spontaneously resume meiosis
when liberated from the follicle. One theory that emerged to improve cytoplasmic maturation
of fully grown oocytes during IVM was to delay the spontaneous maturation process that
292 Edwards and others had described. In the mouse, several approaches were found to inhibit
GVBD during IVM. Wassarman et al (Wassarman *et al.*, 1976) demonstrated that addition of
dibutryl cAMP was effective at preventing GVBD in mouse oocytes, reflecting the major role
cAMP has in arresting meiosis in vivo. Inhibition of GVBD was found to occur by inhibiting
296 protein synthesis (cycloheximide) or by use of kinase inhibitors (DMAP, roscovitine,
butyrlactone) that target specific cell-cycle regulatory kinases. These treatments can be very
effective at preventing oocyte meiosis in vitro, but collectively all have been found to
negatively affect subsequent development (including of human oocytes; (Anderiesz *et al.*,
300 2000)), demonstrating that inhibiting such proteins directly involved in meiosis is not a sound
strategy. By contrast, follicular somatic cell factors that have been identified as the natural
mediators of oocyte meiotic arrest (i.e. the natriuretic peptides and their downstream effectors;
cGMP and cAMP) have been successfully used in IVM in animal species to control
304 spontaneous meiosis during IVM, as a means to improve oocyte competence (reviewed;

(Gilchrist, 2011; Smitz *et al.*, 2011)). The limited number of studies to date show promising results using human IVM oocytes (Nogueira *et al.*, 2006; Shu *et al.*, 2008).

308 Clinical IVM protocols:

Edwards' pioneering studies on human IVM were all conducted using oocytes collected from unstimulated ovaries or pieces of ovaries collected from gynaecological surgeries (Edwards, 312 1962; Edwards, 1965b; Edwards *et al.*, 1969). As a source of oocytes, this parallels what is currently used with high success in ruminant animal IVM laboratories for producing embryos from abattoir-derived ovaries. However, over the past two decades, attempts to improve human IVM have transgressed into an array of in vivo hormonal interventions. A wide range 316 of clinical protocols exist, including: no stimulation; short duration follicle stimulation hormone (FSH)-priming; hCG-priming; and FSH and hCG stimulation which may more appropriately be called "*short-stim IVF*". Protocols derived by SL Tan and colleagues at McGill University recommended an "hCG-priming" regime of a single large bolus of hCG 320 (10,000 IU), approximately 36 hours prior to oocyte recovery (Chian *et al.*, 1999). The result is mostly a mixture of 1-2 oocytes with expanded cumulus and undergoing meiotic progression, and usually a larger cohort of recovered oocytes with compact cumulus, most of which had not undergone GVBD. A greater proportion of embryos per oocyte retrieved 324 invariably were associated with the activated follicular oocytes. A consequence of this regimen is the need for multiple rounds of intracytoplasmic sperm injection per cycle, as timing of meiotic completion to MII varies markedly. This protocol has been widely used by IVM clinics throughout the 1990s and 2000s, although a number of more recent randomised 328 trials have shown no benefit in overall pregnancy establishment over hormone-free IVM (Fadini *et al.*, 2009; Zheng *et al.*, 2012). A trend is emerging within the human IVM field to move back to the more traditional definition of IVM (i.e. in vitro maturation of immature COCs), either from unstimulated patients or patients mildly stimulated with 1-3 days of FSH. 332 The question of patient preparation remains a vitally important one in IVM and an important area of clinical research.

Concluding remarks

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Robert Edwards conducted groundbreaking IVM research, published in a series of seminal papers that paved the way for the development of a reproductive technology that today

generates millions of embryos and pregnancies annually across a wide array of species. In
340 women, it promises fertility preservation as well as simple, safe and cheaper infertility
treatment, without the need for patient hormonal stimulation. IVM was instrumental in
Edwards' major advances in reproductive medicine. He remained a passionate advocate of
IVM throughout his life and in the twilight of his career he published a review entitled "*Are*
344 *minimal stimulation IVF and IVM set to replace routine IVF?*" (Edwards, 2007). Time will
tell and it remains to be determined if Edwards' enormously successful approach of
translating breakthroughs from animal research to revolutionary clinical medicine, can be
sustained in the modern era, to increase the uptake of clinical IVM.

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Authors' Roles

The authors contributed equally to the writing of this review.

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