Early history of *in vitro* fertilization

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Although *in vitro* fertilization (IVF) is used widely for a variety of purposes, it is often not appreciated how this technology was developed. A large number of experiments beginning in 1878 contributed to the first successful reports of IVF over 75 years later. The discovery of sperm capacitation in 1951 was central to the development of IVF technology, and it was rapidly followed by the first convincing reports of IVF in several species. The ability to fertilize oocytes *in vitro* has allowed major advances to be made into understanding the mechanisms involved in fertilization and early development, and IVF now supports reproductive biotechnology in animals and in humans. This article is a historical review of key experiments that helped to provide the basis for present day IVF procedures, placed into context with current practice.

In vitro fertilization (IVF) is a well-established technology with a variety of applications in basic and applied sciences. The technology supports the production of embryos used for research investigations, for treating human infertility, for enhancing the productivity of food animals, and for conservation of endangered mammals. For most species, IVF protocols are available that routinely provide high or at least acceptable proportions of fertilized ova, leading to the production of viable embryos that can develop into normal offspring after transfer to recipient females. In view of the extraordinary advances in IVF technology during the past few decades, and its current role in many areas of basic and applied science, it is easy to ignore the origins of IVF in basic research studies conducted as long ago as 1878, and to overlook the enormous significance of the discovery of sperm capacitation. For example, it is not well recognized that the basis for the first documented human IVF success in 1969 can be traced back to two groundbreaking articles with hamster gametes (Yanagimachi and Chang, 1963, 1964); these studies were the first to report mammalian IVF using spermatozoa capacitated *in vitro*. An earlier study by Chang (1959) showing that rabbit eggs fertilized *in vitro* could develop into normal young was also crucial to the acceptance starting 20 years later of human IVF as a clinical infertility treatment. Moreover, many of the key studies underlying the development of IVF were published before electronic databases such as Medline began cataloguing articles, and some reports appeared in sources not represented in these databases.

The present review attempts to describe the contributions to IVF made by numerous pioneering scientists up to 1971 and to illustrate some of the difficulties encountered in this

research. This cutoff date is used because most of the review was written as the Introduction of the author's unpublished PhD dissertation (Bavister, 1971). That Introduction has now been expanded and updated to recognize the current status and practice of IVF. The 1950s and 1960s were the 'golden age' of IVF, when, after the discovery of sperm capacitation, the first convincing successes were reported for several important species including the rabbit, mouse, hamster, rat and human. It seems timely to present the earliest work to remind present-day scientists how much is owed to the IVF pioneers, and how much the routine, protocol-driven IVF technology relies heavily on the information they provided.

The focus of this review is on spermatozoa and the changes they need to undergo before becoming able to fertilize an oocyte. It does not address the complexity of changes undergone by oocytes as a consequence of sperm penetration, in part because the author's early research focus was on sperm capacitation, and in part because so little was known before 1971 about parallel events occurring in mammalian oocytes during fertilization. Since the advent of IVF technology, many detailed reviews and articles have been published on the responses of oocytes to activation by spermatozoa (for example, Yanagimachi, 1994; Fissore *et al.*, 1998; Hewitson *et al.*, 2000). The present review does not address embryonic development subsequent to IVF, about which a very large number of articles have also been published (see Bavister, 1995; Niemann and Wrenzycki, 2000; Khosla *et al.*, 2001). The cited publication list is not exhaustive but includes those articles that were considered by the author in 1971 to be most relevant to sperm capacitation and IVF up to that time, as well as some representative contemporary references to illustrate how the field has progressed in theory and in practice since then.

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Early *in vitro* fertilization studies

In spite of intensive research efforts conducted since the latter half of the 19th Century, by 1971, knowledge of the physiology, biochemistry and morphology of mammalian fertilization and embryonic development was still sparse and inadequate, compared with the wealth of knowledge about that of amphibians and sea-urchins. The progress of research was hindered by the internal site of mammalian fertilization, which means that events involved in fertilization of mammalian eggs and early embryonic development cannot be investigated readily in their natural environment. Therefore, information concerning the relationship between the egg or the developing embryo and the immediate maternal environment is difficult to obtain. A potentially useful technique is to recover fertilized eggs or early embryos from the female reproductive tract, and to study their subsequent development *in vitro*. This approach has been used widely, but provides little information about the preceding events *in vivo*. Moreover, the timing of ovulation and of fertilization *in vivo* cannot be predicted accurately, which particularly restricts the investigation of rapidly occurring events, such as the penetration of spermatozoa through the egg investments and the changes undergone by the gametes during the early stages of fertilization.

Information can be derived much more readily from the study of eggs that are fertilized and then develop *in vitro*. Not only can the process of fertilization be closely observed, but also factors contributing to normal and abnormal fertilization and development can be examined. The progress of fertilization or embryogenesis can be frequently, if not continuously, observed and the conditions of culture can be varied to examine their effects on development. Thus, a wealth of information is available from studies *in vitro*, given the technical ability to accomplish them.

Early attempts to fertilize mammalian eggs *in vitro*

Between 1878 and 1953, numerous attempts were made to fertilize mammalian eggs *in vitro*. Although many reports were published claiming successful results, in the light of modern knowledge, most of these claims seem unjustified. Fertilization is a progressive, continuous process, beginning with the penetration of an egg by a spermatozoon, which results in the extrusion of the second polar body from the egg. This extrusion is followed by the formation, growth and juxtaposition of the pronuclei, and the process of fertilization culminates in the union of maternal and paternal chromosomes (syngamy), restoring the diploid state. Some authors still refer to the timing of specific events as occurring 'post-fertilization' when they actually mean 'post-insemination' of the oocytes. For example, erroneous and contradictory statements such as 'pronuclear formation begins [several] hours post-fertilisation' have been published.

Several early investigators attempted IVF using ovarian oocytes that were probably not mature enough to undergo

fertilization (Schenk, 1878; Pincus and Enzmann, 1935; Pincus, 1939). Other workers were aware of this problem. Rock and Menkin (1944) and Menkin and Rock (1948) cultured human ovarian oocytes for a time before insemination but, in retrospect, the time allowed for maturation of oocytes *in vitro*, which was based on the data of Pincus and Saunders (1939), was probably inadequate, judging from the work of Edwards (1965). Although it is possible that maturation of some oocytes could have been completed within the period during which the spermatozoa and eggs were incubated together, this still does not provide evidence that fertilization was accomplished *in vitro*.

Most of the criteria used to judge the results of IVF experiments can be misleading: for example, extrusion of the second polar body and development of eggs through the early cleavage stages may also be evoked by parthenogenetic activation, or mimicked by fragmentation. Eggs of the rabbit, the most commonly used species for the earliest IVF experiments, can be activated readily in culture by a variety of stimuli other than sperm penetration (Pincus, 1936, 1939; Thibault, 1949; Chang, 1954). The results of a study by Dauter and Thibault (1956) indicated that the incidence of parthenogenetic activation of rabbit eggs in culture was increased by the presence of spermatozoa. However, cleavage of unfertilized rabbit eggs in culture was reported to be much less common if the eggs were incubated with scrapings of Fallopian tube mucosa (Smith, 1951). Before the study of Smith (1951), only Schenk (1878) had used reproductive tract mucosa in the culture system. Parthenogenetic activation may be induced by cooling the eggs of some species; for example, the rat eggs used by Long (1912) are particularly susceptible in this respect (Austin, 1956a,b). With the exception of Smith (1951), none of the investigators in this field from Schenk (1878) to Shettles (1953) maintained the eggs at 37°C to preclude the possibility of cooling-induced activation. It is also possible that incubating eggs for prolonged periods at less than normal body temperature limited or even destroyed their ability to undergo normal fertilization and development.

As the eggs of some mammalian species may undergo parthenogenetic development in culture, the occurrence of apparently normal activation and cleavage of eggs *in vitro* is insufficient evidence for fertilization. The demonstration of sperm penetration into eggs *in vitro*, together with the formation of pronuclei and the second polar body, provides more convincing evidence for fertilization. No studies conducted before 1954 seem to have achieved this.

In some of the early experiments in rabbits, sperm heads that were apparently within the egg cytoplasm may have been superimposed during histological preparation (Moricard and Bossu, 1949; Moricard, 1950). Shettles (1953) reported that perivitelline spermatozoa were found in five human eggs that were inseminated *in vitro*, but the photographic evidence supporting this claim is by no means convincing and, in some cases, there appear to be breaks in the continuity of the zona pellucida through which spermatozoa might have gained entry. Smith (1951) claimed that 10 of 35

tubal rabbit eggs were penetrated by spermatozoa after finding sperm nuclei or developing male pronuclei in the egg cytoplasm; however, photographic evidence was not submitted to support this claim. One additional egg cleaved, but no sperm penetration was observed, and so parthenogenetic activation cannot be excluded.

The evidence for achievement of fertilization *in vitro* presented by Pincus and Enzmann (1934) appears more convincing. These workers obtained offspring after incubating rabbit eggs and spermatozoa *in vitro* and transferring the eggs, after washing them, to a recipient doe. However, these eggs may have been fertilized after transfer to the oviduct of the recipient animal. The removal of all adherent spermatozoa from freshly ovulated, cumulus-enclosed eggs is difficult (Chang, 1968), so it is likely that some spermatozoa escape the washing procedure and can be transferred into the oviducts along with the eggs. In the study by Pincus and Enzmann (1934), the interval between the start of gamete co-incubation *in vitro* and transfer of the eggs was only 20 min, so spermatozoa attached to oocytes were probably still capable of effecting fertilization *in vivo*. This criticism also applies to the experiments of Venge (1953), in which two litters of rabbits were obtained. In these experiments, the time interval between insemination of eggs *in vitro* and their transfer to recipients (3.5 h) was still insufficient to exclude the possibility of *in vivo* fertilization. This inherent problem is highlighted by the later adoption of this procedure as a treatment for infertile women, in which spermatozoa and eggs are mixed together *in vitro* and then transferred rapidly into the oviduct (Asch *et al.*, 1986). The high pregnancy success rates achieved with this 'gamete intra-Fallopian transfer' procedure indicate that the early claims for IVF success using this approach in animals were unwarranted.

In retrospect, it seems that most of the claims to have fertilized mammalian eggs *in vitro* made before 1954 were inadequately substantiated or were probably based on misinterpreted results. This conclusion is not surprising in view of the many experimental pitfalls discussed above; however, the possibility that IVF was obtained in some cases cannot be ruled out.

Discovery of capacitation

The probable cause of the lack of success of most of the early IVF experiments emerged with a major discovery by Austin (1951) and Chang (1951). These workers found that if spermatozoa were introduced into the oviducts of rabbits shortly after the predicted time of ovulation, very few eggs were fertilized, despite rabbit eggs remaining fertilizable for up to 8 h after ovulation (Chang, 1952; Adams and Chang, 1962a). In contrast, a high proportion of eggs was fertilized if oviductal insemination was performed several hours before ovulation. Observations carried out on rats showed that when spermatozoa were deposited directly into the ovarian capsule after ovulation, no penetrated eggs were recovered until about 4 h later, after which the number of

eggs undergoing fertilization increased rapidly (Austin, 1951). This significant delay before egg penetration was not an artifact of experimental interference, as in oestrous rats permitted to mate naturally but only after ovulation had occurred, penetration of eggs still did not start for about 2 h, even though spermatozoa rapidly reached the oviducts (Austin, 1952).

Both Austin and Chang postulated that the spermatozoa of some mammalian species need to reside for some time within the female reproductive tract before acquiring the capacity to penetrate eggs, and Austin (1951) coined the term 'capacitation' to refer to the change that spermatozoa undergo during this time. The need for rabbit spermatozoa to undergo capacitation almost certainly contributed to the failure of most attempts made before 1954 to achieve IVF in this species. In these experiments, spermatozoa were invariably recovered directly from the male reproductive tract. Today, it is known that spermatozoa from many species can be capacitated *in vitro*, but in several key animals, including rabbits, the spermatozoa must be treated in special ways before they can take part in fertilization (see section on 'Mechanisms of capacitation and the acrosome reaction').

Fertilization of eggs *in vitro* after the discovery of capacitation

Renewed interest in the fertilization of mammalian eggs *in vitro* followed the discovery of capacitation. Within a few years, rabbit eggs were fertilized *in vitro* for the first time, using spermatozoa recovered from the uterus (Dauzier *et al.*, 1954; Thibault *et al.*, 1954; Dauzier and Thibault, 1956). Many other workers confirmed these results, at first using spermatozoa capacitated *in vivo* (Brackett and Williams, 1965, 1968; Brackett, 1968). However, almost 20 years elapsed after the first rabbit IVF before this was repeated using spermatozoa capacitated *in vitro* (Ogawa *et al.*, 1972; Brackett and Oliphant, 1975; Brackett *et al.*, 1978; Hosoi *et al.*, 1981).

In a landmark study, Chang (1959) showed that *in vitro*-fertilized eggs could develop normally. After transfer of IVF rabbit eggs to foster mothers, living young were born that resembled their parents in coat colour. The observation that eggs showed morphological signs of fertilization (including cleavage) before transfer (Chang, 1959) precludes the possibility that eggs were simply fertilized *in vivo* by attached spermatozoa, which was not excluded in earlier studies. Numerous other studies duplicated these results (for example, Thibault and Dauzier, 1961; Brackett, 1969; Brackett and Oliphant, 1975).

During the 'golden age' of IVF, good morphological evidence was provided for success in several other important mammals. Technical aspects of IVF in a wide variety of species are thoroughly described and evaluated by Rogers (1978). Sperm penetration, emission of the second polar body and formation of pronuclei were all seen in golden (Syrian) and Chinese hamster eggs inseminated *in*

vitro (Yanagimachi and Chang, 1963, 1964; Barros and Austin, 1967; Pickworth and Chang, 1969). In one study, 45% of golden hamster eggs inseminated *in vitro* were fertilized and cleaved to the two-cell stage in culture (Yanagimachi and Chang, 1964). However, in spite of intensive efforts, none of these eggs developed further *in vitro* as a result of the 'two-cell block', which held up progress with cultured hamster embryos for 25 years after the landmark articles of Yanagimachi and Chang (1963, 1964) (Schini and Bavister, 1988), and it was not until 1992 that the first IVF hamster pups were born (Barnett and Bavister, 1992).

Whittingham (1968), Iwamatsu and Chang (1969, 1970) and Toyoda *et al.* (1971a,b) reported IVF of mouse eggs using spermatozoa capacitated *in vitro*. Whittingham (1968) transferred *in vitro*-fertilized mouse eggs at the two-cell stage to recipient mice, and subsequently recovered normal 17-day-old fetuses that genetically resembled their parents. Cross and Brinster (1970) and Mukherjee and Cohen (1970) confirmed this result with mouse eggs that were both matured and fertilized *in vitro*.

Toyoda and Chang (1968) were able to fertilize rat eggs *in vitro*, but only after removal of the zona pellucida. Miyamoto and Chang (1973) reported IVF of intact eggs with rat spermatozoa recovered from the uterus of mated females. Subsequently, *in vitro* capacitation and fertilization in the rat were accomplished by Toyoda and Chang (1974a,b).

Thibault and Dauzier (1961) reported the fertilization of a few sheep eggs and one pig egg *in vitro*, but many years elapsed before protocols were devised that supported consistent IVF results in these species and offspring were produced (Bondioli and Wright, 1980; Crozet *et al.*, 1987; Slavik and Fulka, 1991; Abeydeera, 2002).

Cat eggs that were inseminated *in vitro* with spermatozoa incubated *in utero* cleaved as far as the 16-cell stage (Hamner *et al.*, 1970). However, the first IVF using spermatozoa capacitated *in vitro* was not reported until 1977 (Bowen, 1977). IVF using spermatozoa capacitated *in vitro* has been used more recently by several laboratories to produce embryos for conservation of endangered species (Roth *et al.*, 1994a,b; Pope *et al.*, 2000, 2001).

Guinea-pig eggs were fertilized *in vitro* using epididymal spermatozoa (Yanagimachi, 1972), although it took up to 18 h before penetration began. It was later shown that guinea-pig sperm capacitation is retarded by glucose and by magnesium ions; in their absence, the time required for sperm capacitation was shortened to only 2–3 h (Rogers and Yanagimachi, 1975, 1976; Rogers *et al.*, 1979).

Fertilization of dog eggs *in vitro* was reported by Mahi and Yanagimachi (1976) and the time needed for capacitation of canine spermatozoa *in vitro* was estimated at 7 h. It was reported by Rogers (1978) that dog spermatozoa could become capacitated and undergo acrosome reactions in the absence of any egg or cumulus factors.

Fertilization of human eggs *in vitro* was first convincingly

achieved in 1969, as evidenced by sperm penetration, polar body emission and formation of pronuclei (Bavister *et al.*, 1969; Edwards *et al.*, 1969). This advance was based on modifications of the culture medium protocol used by Yanagimachi and Chang (1963, 1964), so it may be claimed that hamster IVF paved the way for human IVF (Bavister, 2002). In a classic understatement, Edwards commented that 'Human oocytes have been matured and fertilized by spermatozoa *in vitro*. There may be certain clinical and scientific uses for human eggs fertilized by this procedure.' (Edwards *et al.*, 1969). Some IVF human eggs were capable of undergoing apparently normal cleavage and differentiation in culture (Edwards *et al.*, 1970; Steptoe *et al.*, 1971). However, the first human birth from an IVF embryo was not reported until 1978 (Steptoe and Edwards, 1978), apparently because the ovarian stimulation regimens may have interfered with establishment of pregnancy or produced incompetent eggs. Since 1978, the clinical applications of human IVF have become widespread, so that probably more than a million IVF babies have been born worldwide. Originally proposed by Edwards and Steptoe as a treatment for infertility in women, human IVF and its derivative intracytoplasmic sperm injection (ICSI) are now used to treat male infertility, and even azoospermia.

Progress with IVF in non-human primates was slower than it was in humans. Successful IVF in the squirrel monkey was first reported by Gould *et al.* (1973) and by Kuehl and Dukelow (1975). However, in these studies, the presence of fertilizing sperm tails in the eggs was not shown, and the developmental ability of the IVF eggs was very low. Unequivocal evidence for IVF in rhesus macaques was reported by Bavister *et al.* (1983), who showed the presence of sperm tails in eggs, and considerable post-fertilization development. Stimulation of spermatozoa with caffeine and cyclic AMP was essential for supporting sperm fertilizing ability (Boatman and Bavister, 1984). Non-human primate IVF, mostly using macaque species, is now used by several laboratories world-wide to investigate primate fertilization and early embryonic development. In all cases, spermatozoa capacitated *in vitro* have been used. There appears to be only one report of IVF in chimpanzees (Gould, 1983). Oocytes were inseminated *in vitro* using Ham's F10 medium supplemented with serum and caffeine–cyclic AMP but it is not known whether these chemical stimulators are essential for sperm capacitation in chimpanzees. Of 18 oocytes that were mature at the time of collection, 11 were fertilized *in vitro* and five cleaved to about the three-cell stage.

Relationship between capacitation and the acrosome reaction

In most of the IVF studies carried out between 1954 and 1971, spermatozoa were either capacitated *in vivo* before insemination of the eggs, or it could be demonstrated or inferred from the timing of egg penetration that capacitation was required and had occurred *in vitro*. The spermatozoa of

mammals must undergo an acrosomal change called the acrosome reaction before penetration (Yanagimachi, 1994). The acrosome reaction was first demonstrated by Austin and Bishop (1958a,b), who used the novel technology of phase-contrast microscopy to discover that the spermatozoa of guinea-pigs, hamsters and Libyan jirds lost their acrosomes as they penetrated the zona pellucida. Later studies using the electron microscope, first with hamster spermatozoa, showed that this loss is restricted to the outer acrosomal membrane and acrosomal contents (Barros *et al.*, 1967; Franklin *et al.*, 1970). The acrosome reaction was found also to be prerequisite for fertilization in rabbits (Bedford, 1967, 1968), rats (Pikó and Tyler, 1964) and pigs (Dickmann and Dziuk, 1964), and bull spermatozoa were also found to undergo an acrosome reaction (Blom, 1963; Saacke and Almquist, 1964; see also Colwin and Colwin, 1967). In light of all this evidence, it was assumed that the acrosome reaction is a common characteristic of mammalian spermatozoa (Yanagimachi, 1994).

At first, there was considerable confusion over nomenclature concerning the changes that spermatozoa undergo before penetrating eggs. Austin and Bishop (1958b) suggested that the acrosome reaction constituted a part of the process of capacitation, whereas Yanagimachi (1969a) considered that the acrosome reaction '... must represent the final phase of sperm capacitation'. However, an acrosome reaction that is basically similar to that undergone by mammalian spermatozoa is exhibited by the spermatozoa of many marine invertebrates, including annelids, molluscs and echinoderms, and by spermatozoa of two other vertebrate groups, lampreys and sturgeons (Dan, 1956, 1967; Colwin and Colwin, 1965, 1967). However, capacitation seems peculiar to mammals. In agreement with the views of Bedford (1968) and Pikó (1969), it seems most appropriate to consider that capacitation is a discrete physiological change that enables mature spermatozoa to respond to appropriate stimuli, and thereby to undergo the acrosome reaction. This is the most logical way to view the changes undergone by spermatozoa within the female reproductive tract. Although the acrosome reaction is a fundamental change in structure undergone by the spermatozoa of animals from widely diverse groups, capacitation may be regarded as a process that has evolved to suit some peculiar feature of mammalian reproduction (for a review, see Pikó, 1969). Most likely, capacitation represents a conditioning process that prepares spermatozoa to undergo acrosome reactions very rapidly once they are exposed to mature oocytes within the oviduct. However, in the absence of appropriate stimuli (that is, zona pellucida or cumulus oophorus components), most capacitated spermatozoa would not automatically undergo acrosome reactions *in vivo*, thus maintaining them in a stable configuration for several hours, even days, before the arrival of ovulated oocytes. Thus, capacitation may be viewed as a mechanism for coordinating acrosome reactions with the availability of unfertilized oocytes, which has evolved together with internal fertilization. This view is

consistent with the apparent lack of capacitation in animals exhibiting external fertilization, in which spermatozoa are deposited in direct proximity to the oocytes, undergo acrosome reactions almost immediately, and have very short functional lifespans, usually encompassing only a few minutes. In contrast, it appears that mammalian spermatozoa may be sustained within the female reproductive tract without losing their fertility for many hours or even days.

Capacitation in different species

The evidence derived from early studies of a need for capacitation in mammals was necessarily circumstantial, being largely derived from the timing of penetration of eggs *in vivo* (Austin, 1951; Chang, 1951, 1969; Bedford, 1970a) or *in vitro* (Yanagimachi and Chang, 1963, 1964). Nevertheless, the sheer volume of this evidence in a wide variety of mammals (Austin, 1969; Yanagimachi, 1994) provided strong support for the concept of capacitation, even though very little was known about its mechanism.

In rabbits, a large mass of information was gathered concerning the need for spermatozoa to undergo capacitation before penetrating eggs (for a review, see Chang, 1969); however, much of these data were conflicting and confusing with regard to the conditions needed for capacitation. Most of the information related to capacitation in the rabbit uterus, but the oviduct was shown to be the major site of capacitation (Bedford, 1970a,b), although capacitation was accomplished in the shortest time (5–6 h) when spermatozoa passed from the uterus to the oviduct (Chang, 1955; Adams and Chang, 1962b). Bedford (1970a) provides the best discussion on the state of knowledge regarding capacitation in the rabbit and in other animals during the 'golden age' of IVF.

Further studies on the time relationships of fertilization *in vivo* in rats (Austin, 1952; Noyes, 1953; Austin and Braden, 1954) confirmed the original observation (Austin, 1951) that capacitation was required in this species. In golden hamsters, the need for capacitation was firmly established from the timing of fertilization *in vivo* (Strauss, 1956; Chang and Sheaffer, 1957; Yanagimachi, 1966; Hunter, 1969), together with IVF data (Yanagimachi and Chang, 1963, 1964; Yanagimachi, 1966, 1969a; Barros and Austin, 1967; Barros, 1968). In early IVF studies, high percentages of golden hamster eggs were fertilized *in vitro* with epididymal spermatozoa that had not been exposed to the female reproductive tract (Yanagimachi and Chang, 1963, 1964; Barros and Austin, 1967; Barros, 1968). Barros (1968) reported a delay of about 3 h before egg penetration took place *in vitro*. This delay was reduced to less than 30 min by preincubating spermatozoa with oviductal fluid containing ova in cumulus. These results were interpreted as indicating that golden hamster spermatozoa can be capacitated *in vitro* by incubation with cumulus oophorus fluids. Barros and Austin (1967) showed that hamster follicular eggs could be fertilized *in vitro*, and that follicular contents could induce the acrosome reaction in hamster spermatozoa.

These workers concluded that hamster epididymal spermatozoa could be capacitated *in vitro*, in the absence of any contribution from the female reproductive tract, as reported by Yanagimachi and Chang (1963, 1964). Later work by Yanagimachi (1969a) amply supported this conclusion. It became accepted that capacitation of hamster spermatozoa is a change undergone normally within the female reproductive tract, but which may occur *in vitro* under special conditions. Follicular fluid is able to provide these conditions (Yanagimachi, 1969a,b), and the presence of this fluid was essential in these early studies to allow hamster spermatozoa to undergo the acrosome reaction and penetrate eggs *in vitro*. Alternatively, follicular fluid can be replaced by serum albumin (Bavister, 1969) or by the fluid component of the cumulus oophorus (Bavister, 1982). Although the need for capacitation was firmly established by 1971 in rabbits, rats and golden hamsters, the demonstration of capacitation in other mammalian species was less well documented. After their introduction into the tubal ampulla via the ostium, ferret spermatozoa did not penetrate eggs within the oviduct until 3.5 h had elapsed (Chang and Yanagimachi, 1963). This delay presumably represents the time needed for capacitation of ferret spermatozoa in the oviduct. The time relationships of hybrid fertilization of rabbit eggs by snowshoe hare spermatozoa indicated that capacitation of these spermatozoa was necessary and took 6–10 h in the rabbit oviduct; this timing is very similar to that for oviductal capacitation of rabbit spermatozoa (Chang *et al.*, 1971). In a study of the time relationships of fertilization in the Mongolian gerbil and in the deer mouse, animals were artificially inseminated after the time of induced ovulation (Marston and Chang, 1966). The time interval between insemination and the earliest recovery of penetrated eggs was about 4 h for the gerbil and 3 h for the deer mouse. These data were consistent with the idea that capacitation occurs in both of these species. The time needed for IVF of a few Chinese hamster eggs by epididymal spermatozoa was approximately the same as that required for golden hamster eggs (Pickworth and Chang, 1969).

In mice, the time relationships for fertilization *in vivo* indicated that either capacitation occurred within a very short time (about 1 h) or else capacitation was not required in this species (Braden and Austin, 1954). However, Iwamatsu and Chang (1970) subsequently reported that although 1 h or more was needed for untreated epididymal spermatozoa to penetrate eggs *in vitro*, spermatozoa that had been preincubated in heterologous follicular fluid were able to effect penetration in only 20 min. This result was interpreted as indicating that capacitation of mouse spermatozoa was required, and occurred within about 1 h. This timing is consistent with later estimates made using a quantitative fluorescence assay of between 30 and 90 min for mouse sperm capacitation (Ward and Storey, 1984).

The IVF experiments of Hamner *et al.* (1970) indicated that cat spermatozoa require about 0.5–2.0 h of incubation *in utero* for capacitation to occur. This supposition has been

amply confirmed using IVF with spermatozoa capacitated *in vitro* in domestic and endangered felids (Andrews *et al.*, 1992; Roth *et al.*, 1994a,b; Pope *et al.*, 2000, 2001). The results of several early studies indicated a need for capacitation of ram spermatozoa. When ejaculated spermatozoa were introduced into the oviducts of ewes shortly after ovulation, no penetrated eggs were recovered until 3–5 h later. After incubation for several hours in the ligated uterus, spermatozoa were able to penetrate eggs 1.5–2.0 h after deposition into the oviduct (R. Moore, C. Polge and L. Rowson, unpublished). However, Mattner (1963) found that tubally inseminated spermatozoa needed only 1.5 h to penetrate eggs even without prior incubation *in utero*. Dautz and Thibault (1959) were able to fertilize sheep eggs *in vitro* with spermatozoa recovered from the uterus of a mated ewe, but not with ejaculated spermatozoa. In retrospect, this failure was probably due to unsuitable culture conditions for supporting capacitation *in vitro*, because several authors have since reported successful IVF in sheep using spermatozoa taken directly from the male reproductive tract (Bondioli and Wright, 1980; Crozet *et al.*, 1987; Slavik and Fulka, 1991) and the need for ovine sperm capacitation is now well established.

The few critical observations available on the timing of fertilization in the pig up until 1971 were consistent with a need for capacitation in this species. Boar spermatozoa introduced into the oviducts soon after ovulation did not penetrate eggs until 4 or 5 h later (Polge, 1969). Hunter and Dziuk (1968) found that only 2 h elapsed between the time of artificial insemination and the beginning of egg penetration. This delay presumably represents the time needed for capacitation and the acrosome reaction, as the time needed for sperm transport to the oviducts is only 15 min in this species (First *et al.*, 1968). In view of the observation made by Polge (1969), capacitation does not appear to proceed optimally in the oviduct alone. Although production of viable pig embryos by IVF is now quite routine using *in vitro*-matured (IVM) oocytes, the problem of polyspermic fertilization remains and the aetiology of this defect is not understood (Abeydeera, 2002). Moreover, embryos produced by IVM and IVF have low average developmental competence *in vitro*, which may also be the result of inadequate maturation of the egg cytoplasm (Niemann, 2001).

The early observations of Ericsson (1967a) and Lauderdale and Ericsson (1970) indicated that capacitation was necessary for bull spermatozoa, and an unpublished result of Edwards (quoted by Austin, 1969), concerning the time needed for the penetration of cow eggs *in vitro*, supported this suggestion. However, Mahajan and Menge (1966) were unable to demonstrate any advantage of uterine-incubated bull spermatozoa over control spermatozoa in terms of ability to fertilize eggs *in vivo*. Thus, the need for capacitation of bull spermatozoa was not convincingly demonstrated by these early studies. However, it has since been shown unequivocally that bull spermatozoa, like those of all other mammals studied, do require capacitation,

which can be achieved *in vitro* under special conditions (see 'Mechanisms of capacitation and acrosome reaction'). The technique of IVF in cattle (*Bos taurus*) is among the most successful of all species; 40% or more of inseminated IVM oocytes can develop into blastocysts, and many calves have been born that were derived from these processes (Brackett *et al.*, 1978; Hasler, 1998).

It was uncertain from the early studies whether primate spermatozoa require capacitation. In humans, the IVF experiments of Edwards *et al.* (1969) showed a delay of about 7 h between insemination of the eggs and the beginning of penetration; it seemed likely that this delay reflected a need for sperm capacitation. Unfortunately, insufficient material was available at that time to investigate this possibility more thoroughly. The observations of Marston and Kelly (1968) on the time relationships of fertilization in rhesus monkeys did not allow any conclusions to be drawn regarding the need for capacitation in this species. Now, it is clear that primate spermatozoa do need a period of capacitation. Macaque monkey spermatozoa require treatment with chemical mediators to acquire the capacity to undergo acrosome reactions and to bind and then penetrate the zona pellucida (Boatman and Bavister, 1984). This finding is curious because human spermatozoa require only washing and incubation for a few hours in a culture medium, without any specific chemical treatments, to become capable of fertilizing oocytes *in vitro* (Mortimer, 1994, 2000).

It has also been claimed that some amphibian spermatozoa undergo capacitation. Shivers and James (1970) showed that untreated frog spermatozoa penetrated very few eggs from which the jelly-coats had been removed; however, spermatozoa that had been preincubated with uterine eggs (that is, with intact jelly-coats) were subsequently able to penetrate a high proportion of de-jellied eggs. These workers inferred a need for capacitation of frog spermatozoa. In addition, Wolf and Hedrick (1971) found that *Xenopus laevis* spermatozoa needed to react with jelly-coat materials before penetrating eggs, and concluded that capacitation was required. However, these studies do not indicate whether the short time-interval between insemination and penetration of eggs (5 min in frogs; 20–30 min in *Xenopus*) includes a period of capacitation, similar to that in mammals, or whether it represents only the time needed for an acrosome reaction to take place. If capacitation does not occur, the prolonged time needed for acrosome reactions in amphibian spermatozoa may represent an interesting evolutionary intermediate between the spermatozoa of some invertebrates in which the acrosome reaction occurs immediately on contact with the egg capsule (Dan, 1956; Colwin and Colwin, 1967), and the spermatozoa of mammals, which need a period of capacitation usually requiring several hours.

It can be concluded that capacitation of spermatozoa is peculiar to mammalian reproduction and is a prerequisite for fertilization. Capacitation has not been found to be unnecessary in any mammal so far investigated, although it

may occur very rapidly in some species (for example, mice), so it may be assumed that capacitation is a universal phenomenon in mammals.

Hyperactivated motility of spermatozoa

Concomitant with the completion of capacitation *in vitro*, golden hamster spermatozoa exhibit 'whiplash' flagellar activity that can easily be observed under a phase-contrast microscope (Yanagimachi, 1970). After 3–4 h incubation under capacitating culture conditions, most motile spermatozoa show this vigorous 'hyperactivated' pattern of activity characterized by high amplitude flagellar bending, whereas in the early IVF studies, spermatozoa incubated under non-capacitating conditions never showed activation. Hyperactivated sperm motility was also observed through the wall of the oviductal ampulla in mated hamsters and mice, and hyperactivated spermatozoa have been flushed from ampullae of several other species near the time of fertilization (Katz and Yanagimachi, 1980, 1994; Demott and Suarez, 1992). These observations indicate that this change in flagellar activity is a natural event in capacitation that is involved in sperm penetration through the egg investments (Yanagimachi, 1970; Katz *et al.*, 1989; Suarez *et al.*, 1991) and is perhaps also necessary for spermatozoa to reach the site of fertilization (Shalgi *et al.*, 1992). Hyperactivated motility of spermatozoa incubated under capacitating conditions also occurs in many other species (Yanagimachi, 1994). Although the precise functional significance of hyperactivation is still not clear, this marked change in motility could be a useful indicator of capacitation in individual spermatozoa. However, hyperactivation can also occur under non-capacitating conditions. When golden hamster spermatozoa are incubated in the absence of serum albumin and cumulus oophorus factors, after 3–4 h they begin to exhibit hyperactivated motility that appears identical to that seen under capacitating conditions. However, these spermatozoa incubated in the absence of serum albumin and cumulus oophorus factors are not capacitated, do not undergo spontaneous acrosome reactions and cannot fertilize oocytes (Bavister, 1981, 1982).

Mechanisms of capacitation and the acrosome reaction

It seems surprising that research into the nature of capacitation proceeded very slowly for 20 years after its discovery (1951–1971). Some of the early developments in this research were reviewed by Austin (1961, 1967) and later developments were reviewed by Yanagimachi (1994). A major obstacle to progress was, and remains, the absence of a suitable means of determining whether individual spermatozoa have been capacitated, other than by observing their ability to penetrate eggs. In a sperm suspension incubated *in vitro*, there will be a heterogeneous population of living and dead, motile and non-motile,

capacitated and non-capacitated spermatozoa, and the relative proportions of all these will change over time. This fluctuating heterogeneity of sperm suspensions *in vitro* makes biochemical studies into the mechanism of capacitation difficult, and it may be unwise to ascribe particular chemical or physical properties found in the population as a whole to those few spermatozoa capable of fertilizing eggs. Bavister (1986) suggested that '...characteristics evaluated in a large sperm population may be more representative of nonfertile than of capacitated spermatozoa'.

In spite of this concern, a large amount of information has been gathered on the changes undergone by spermatozoa during capacitation *in vivo* and *in vitro*. The results of several early studies indicated that, at least in rabbit spermatozoa, no readily detectable morphological change occurs during capacitation (Adams and Chang, 1962b; Austin, 1963; Bedford, 1963, 1964). A report of progressive elevation or loosening of the rabbit sperm plasma membrane during capacitation (Bernstein, 1966) was unsubstantiated by other workers, and the observations probably resulted from fixation artifacts (Bedford, 1969a,b). Nevertheless, some subtle changes do occur in the surface of spermatozoa during capacitation. Spermatozoa within the uterus of the oestrous rabbit were readily engulfed by leucocytes, but in the pseudopregnant uterus, where little or no capacitation occurs, leucocytes failed to ingest intact motile spermatozoa (Bedford, 1965). In addition, a potent substance found in the seminal plasma of a fertile rabbit merely agglutinated ejaculated or epididymal spermatozoa, but immediately immobilized spermatozoa recovered from the uterus (Bedford, 1970c). Furthermore, the net negative charge was reduced on the surface of rabbit spermatozoa after incubation in the oestrous uterus (Vaidya *et al.*, 1971).

In the early studies, a considerable volume of information was accumulated on the metabolic changes that occur in spermatozoa after incubation in the female reproductive tract, or during incubation *in vitro* with secretions from the tract (for a review, see Iritani *et al.*, 1969). It is not known to what extent these metabolic changes are involved with capacitation; they may reflect changes in membrane permeability resulting from capacitation, facilitating the exchange of ions and substrates between the spermatozoon and its environment, or they may be merely contemporaneous with capacitation.

It does seem paradoxical that, whereas capacitation of spermatozoa is accepted as being universally required in mammals, methods for supporting this process *in vitro* differ markedly among species. The mechanism of capacitation, and hence the *in vitro* conditions for capacitating spermatozoa, might be expected to be very similar across species, but this is clearly not so. Mouse spermatozoa can be capacitated simply by diluting epididymal contents into a suitable culture solution and incubating them for as little as 30 min; guinea-pig spermatozoa can be capacitated in the same way by incubation for 2–3 h (Rogers, 1978). This simple approach also works for human and dog

spermatozoa, after removal of seminal plasma by washing or gradient centrifugation (Mahi and Yanagimachi, 1976, 1978; Mortimer, 1994, 2000). Rabbit spermatozoa can also be capacitated *in vitro* by simply washing and incubation, but there must be a second wash step after an initial short period of incubation, probably to eliminate soluble seminal plasma factors from the culture environment (Brackett and Oliphant, 1975; Hosoi *et al.*, 1982). In contrast to human spermatozoa, in another primate genus, the macaque monkeys, spermatozoa require both washing and treatment with caffeine and cyclic AMP to support acquisition of fertilizing ability *in vitro* (Boatman and Bavister, 1984). Golden hamster epididymal spermatozoa under *in vitro* conditions also require treatment with chemical mediators, both to sustain motility (hypotaourine) and to stimulate acrosome reactions (catecholamines such as adrenaline; Leibfried and Bavister, 1982; Bavister, 1989; Boatman *et al.*, 1990). Although hamster, mouse, dog and human spermatozoa thrive on or require glucose in the culture medium (Bavister and Yanagimachi, 1977; Mahi and Yanagimachi, 1978; Fraser and Herod, 1990; Rogers and Perreault, 1990; Travis *et al.*, 2001), glucose inhibits or greatly delays the completion of capacitation in guinea-pig and bull spermatozoa (Rogers and Yanagimachi, 1975; Parrish *et al.*, 1989a; Vredenburg-Wilberg and Parrish, 1995). In bull spermatozoa, this inhibition is the result of glycolysis blocking the normal increase in intracellular pH that accompanies capacitation with heparin (Parrish *et al.*, 1989a). An increase in intracellular pH also appears to accompany the acrosome reaction of mouse spermatozoa (Rockwell and Storey, 2000). Even if they are artifacts of the *in vitro* culture of spermatozoa, these apparently contradictory capacitation requirements need to be resolved if a common mechanism is to be established and the events involved elucidated.

One consistent feature of early culture systems for supporting *in vitro* sperm capacitation and acrosome reactions was the use of biological fluids (usually follicular or cumulus oophorus fluids; Iwamatsu and Chang, 1969; Yanagimachi, 1969a,b; Bavister, 1982). These fluids were later replaced by serum albumin (Bavister, 1969, 1981, 1982, 1989; Miyamoto and Chang, 1973a,b,c; Hoppe and Whitten, 1974; Davis, 1976). In modern IVF protocols, either bovine or human serum albumin is almost always used, although cumulus oophorus fluids may also be present. The way in which serum albumin supports capacitation and acrosome reactions is still not fully understood. At least two mechanisms have been proposed: removal of either lipids or zinc ions from sperm membranes by albumin, leaving them more unstable and thus able to undergo membrane fusion leading to acrosome reactions. Some of the earliest work on this topic was done by Davis *et al.* (1979, 1980) who showed that in a capacitating culture medium, serum albumin depleted cholesterol from rat spermatozoa while transferring phosphatidylcholine to them. Further evidence for involvement of cholesterol depletion in sperm capacitation was provided by Langlais

and Roberts (1985) and Langlais *et al.* (1988). Alternatively or additionally, serum albumin depletes zinc from spermatozoa by chelation (zinc stabilizes membranes and is a counter-ion to calcium, inhibiting many of its functions). Hamster spermatozoa can be capacitated *in vitro* without albumin, using a chemical chelator that depletes zinc concentrations in spermatozoa by 40% or more (Andrews *et al.*, 1994), but these spermatozoa could not undergo acrosome reactions without addition of serum albumin, even in the presence of zona pellucida (Andrews and Bavister, 1989). When albumin was separated from spermatozoa by a semi-permeable membrane, acrosome reactions did not occur (Dow and Bavister, 1989). In spite of much attention, the way in which albumin supports acquisition of sperm fertilizing ability is unknown, in part because of the problem of discriminating which spermatozoa in a suspension are actually capacitated, but also because of fluctuating concentrations of contaminants in commercial serum albumin preparations, such as fatty acids (Chen, 1967) and even a factor that activates the phosphatidylinositol system in a number of cells (Tigyi *et al.*, 1991), which may counteract the biological activities of serum albumin preparations, or which conversely may even be responsible for them. Certainly, the biological activity of these preparations is batch variable, but not particularly species-specific. Alternative methods for capacitating spermatozoa would be particularly useful as there is potential for disease transmission by using bovine or human serum albumin preparations. The induction of acrosome reactions by lysophosphatidylcholine showed that sulphated glycoconjugates, including heparin, could capacitate bovine spermatozoa (Parrish *et al.*, 1989b). Further research into non-serum-derived capacitating compounds is needed in view of the potential for transmitting bovine spongiform encephalopathy or Creutzfeldt-Jakob disease from infected animal or human donors, as well as for elucidating the mechanism of capacitation.

Assessment of sperm fertilizing ability

Egg penetration or fertilization as tests for capacitation and the acrosome reaction

In early studies, in the absence of more suitable methods, most information relating to capacitation was derived from experiments involving penetration or fertilization of eggs. Rabbits were the most widely used animal in such experiments, not only because the need for capacitation had been clearly established in this species, but the relatively short and predictable fertilizable life of rabbit eggs was particularly useful in the capacitation test system (as described by Soupart, 1967). The major disadvantage of this method was that the onset and duration of ovulation in rabbits are highly variable (Walton and Hammond, 1928; Harper, 1961, 1963). Although this objection was largely overcome by the technical refinement of adding recently ovulated eggs to the preincubated spermatozoa instead of

vice versa (Harper, 1970), the method as a whole lacks precision. It is not easy to examine the biochemical conditions prevailing in the female reproductive tract during capacitation of spermatozoa, and it is not possible to control these conditions, except crudely by making drastic hormonal or surgical changes to investigate the effect upon capacitation (for example, see Soupart and Orgebin-Crist, 1966; Bedford, 1970b). In addition, once capacitated spermatozoa are incubated with eggs in the female reproductive tract, little further information can be gained concerning the acrosome reaction and other events associated with egg penetration.

It was realized that more precise and perhaps less conflicting information could be gained by capacitating rabbit spermatozoa *in vitro*, but the validity of early claims to have achieved this (Kirton and Hafs, 1965; Ericsson, 1969; Johnson and Hunter, 1971) is in some doubt because of the lack of confidence in the rabbit capacitation test system (see above). This situation would be improved by fertilizing rabbit eggs consistently *in vitro*. However, the necessary degree of reproducibility was initially difficult to achieve (for example, see Fraser *et al.*, 1971) and reproducible protocols were devised only after methods were developed for capacitating spermatozoa *in vitro* (Brackett and Oliphant, 1975; Hosoi *et al.*, 1982), by which time, IVF in rodents had become the predominant approach for assessment of sperm capacitation and the acrosome reaction.

In vitro fertilization in the hamster

Golden hamsters are highly suitable for the study of sperm capacitation, and a well-defined protocol for achieving IVF in this species has been described by Bavister (1989). There were numerous advantages when hamsters were used for IVF studies: not only were capacitation of spermatozoa and penetration of eggs accomplished easily *in vitro*, but the hamster acrosome reaction (like that in rabbits, guinea-pigs and dogs) is pronounced enough to be discernable under the phase-contrast microscope in living, motile spermatozoa (Yanagimachi and Chang, 1964; Yanagimachi, 1966, 1972, 1994; Mahi and Yanagimachi, 1978), so that the percentage of reacted spermatozoa can be readily assessed at any time.

However, a problem encountered with the early attempts to fertilize hamster eggs *in vitro* was the highly variable outcome of the experiments, ranging from near 0 to 80–90% fertilization of eggs. This variability was the result of differences in the pH of the culture medium used for IVF, which was uncontrolled and altered by the numbers of live spermatozoa present. After pH was controlled using a bicarbonate–CO₂ buffer system, high, reproducible frequencies of IVF were obtained (Bavister, 1969). These early experiments with hamster spermatozoa were the first to show that occurrence of the acrosome reaction, sperm binding to the zona pellucida and fertilization are pH-dependent events. This culture system was then used successfully for the first documented human IVF (Bavister

et al., 1969; Edwards *et al.*, 1969). The sequence of experiments that connected hamster IVF to the first human IVF is described by Bavister (2002). Studies by Miyamoto and Chang (1974) showed that pH was also important for IVF in mice and rats.

In virtually all IVF experiments with hamster gametes, spermatozoa have been obtained from the cauda epididymidis. Not only is it easy to obtain viable spermatozoa in this way, but the time-course of capacitation and penetration of eggs *in vitro* with epididymal spermatozoa (Barros, 1968; Yanagimachi, 1969b) is similar to that observed *in vivo* after normal mating (Strauss, 1956; Chang and Sheaffer, 1957; Yanagimachi, 1966). Moreover, methods for routinely collecting ejaculated hamster spermatozoa have not been developed. Although it is more practical to investigate capacitation using hamster epididymal spermatozoa and the IVF approach, it must be kept in mind that capacitation of epididymal spermatozoa may not be identical to capacitation of ejaculated spermatozoa, that events occurring in culture conditions may not be the same as events after normal mating, and that the process of capacitation in hamster spermatozoa may not be fully representative of capacitation in other mammals. One obvious difference between conditions *in vitro* and *in vivo* is that under most IVF conditions, a very large population of spermatozoa is present but only a small proportion seems capable of penetrating zonae pellucidae in a timely manner, whereas *in vivo*, a small number of spermatozoa is present at the site and time of fertilization but most of the spermatozoa appear to be capable of fertilizing oocytes (Austin, 1951; Zamboni, 1970; Yanagimachi and Mahi, 1976). The total number of spermatozoa in a typical IVF culture drop often exceeds the number of eggs present by three or more orders of magnitude. However, when the culture conditions for hamster sperm survival and capacitation *in vitro* were optimized, very small numbers of spermatozoa (≤ 5) could be used for gamete co-incubation and were capable of fertilizing eggs (Bavister, 1979).

Alternative sperm fertility assays

The ability of spermatozoa to penetrate the zona pellucida and to fertilize eggs is the only unequivocal test for completion of capacitation and the ensuing physiological acrosome reaction. However, IVF is far from satisfactory as a means to evaluate sperm fertilizing ability in a large sperm population, as it introduces large experimental variations, such as differences in the fertilizability of eggs, and yields no information on the frequency of capacitation. One obvious reason for this problem is the rapid operation of the block to polyspermy (Austin and Braden, 1954; Stewart-Savage and Bavister, 1988), such that only one spermatozoon, or at most several, can gain access to the oocyte before the properties of the zona pellucida are altered, preventing further sperm entry. One way to overcome this limitation to testing sperm fertilizing ability is to inhibit the block to polyspermy, for example, by storing oocytes for a time in

concentrated salt solutions (Yanagimachi *et al.*, 1979; Boatman *et al.*, 1988; Yoshimatsu *et al.*, 1988). When the block to polyspermy is destroyed in this way, many, perhaps hundreds of, spermatozoa can penetrate each zona pellucida, thus increasing the efficiency of this 'capacitation detection' system by two or more orders of magnitude (Boatman *et al.*, 1988). This method has also been used to assess the fertility of spermatozoa from endangered species using oocytes from related common animals (Andrews *et al.*, 1992). A different approach is to count the proportion of spermatozoa able to respond to a challenge that induces acrosome loss, such as lysophosphatidylcholine (Parrish *et al.*, 1988, 1989b), after capacitation treatments, although how accurately this procedure mimics the natural acrosome reaction is not clear.

An early attempt to devise a chemical test for capacitation was reported by Ericsson (1967a,b). This test involved the fluorescent labelling of spermatozoa with tetracycline hydrochloride and the subsequent removal of fluorescence during capacitation. Unfortunately, the time courses, sites and conditions of fluorescence removal and of capacitation were not strictly correlated (Vaidya *et al.*, 1969), so the test was of dubious value for the study of capacitation. Nevertheless, this approach was a valuable stimulus to further attempts to develop chemical tests for capacitation. A refinement of this approach uses chlor-tetracycline (CTC) fluorescence to quantitate mouse sperm capacitation (Saling and Storey, 1979); distinct patterns of fluorescence change as capacitation progresses (Ward and Storey, 1984). This assay has also been used with human spermatozoa (Lee *et al.*, 1987).

Johnson and Hunter (1970) used a fluorescent antibody technique to identify rabbit sperm-specific and sperm-coating antigens; the sperm-coating antigens were removed after prolonged incubation in rabbit uteri, re-exposing acrosomal sperm-specific sites. This approach seemed promising, not only as a potential test for capacitation, but also as a method of investigating the nature of this process. Brackett and Oliphant (1975) measured the removal of rabbit sperm surface antigens as a progressive indicator of capacitation taking place *in vitro*.

The occurrence of the sperm acrosome reaction, in the absence of fertilization, has often been used to indicate that capacitation has taken place (Barros and Garavagno, 1970). However, structural changes in the acrosome are not necessarily the result of capacitation of spermatozoa, but can also occur under other circumstances, as degenerative changes (Blom, 1945; Hancock, 1952; Austin and Bishop, 1958c) or 'false' acrosome reactions that are not dependent on previous capacitation (Bedford, 1969b). Thus, the occurrence of an acrosome reaction is an insufficient indication that capacitation has occurred, except in species in which the acrosomes are very large (for example, golden hamsters and guinea-pigs), so that the acrosome reaction (detachment or loss) can be seen clearly in freely motile spermatozoa that do not appear to be moribund (Yanagimachi, 1994).

Two remarkable procedures derived from IVF have been devised for evaluating sperm fertilizing ability: one procedure uses oocytes without zonae pellucidae, and the other uses zonae pellucidae without oocytes. The hamster egg can be fertilized by spermatozoa of other species if the zona pellucida is removed (Yanagimachi, 1994). This 'zona-free penetration assay' is very useful for evaluating human sperm chromosomes (Rudak *et al.*, 1978; Martin, 1985) and has been used as a surrogate test for human sperm fertility (Yanagimachi *et al.*, 1976; Rogers *et al.*, 1979); however, its predictive value is dubious, and most clinical IVF programmes no longer use it. A major problem with this assay is that, in the absence of the zona pellucida, which is a natural stimulator of acrosome reactions, spermatozoa may fail to exhibit acrosome reactions or to fertilize hamster eggs, yet remain capable of effecting fertilization of homologous zona-intact (human) eggs (giving a false negative result). Conversely, spermatozoa from an infertile male may be artificially induced to undergo acrosome reactions by the conditions of the test, such as overnight incubation of the sperm sample at 4°C, and thus become able to fuse with hamster egg plasma membranes and form pronuclei (giving a false positive result). The 'hemi-zona assay' avoids the problems with the hamster egg zona-free penetration assay by using human zonae pellucidae to gauge sperm-binding capability, which should correlate with fertilizing ability. Each zona pellucida is bisected and one half is inseminated with a sperm sample of unknown fertility. The resulting sperm binding frequency is compared directly with sperm binding to the matched half-zona pellucida using a control sample of known fertility (Oehninger *et al.*, 1992; Franken *et al.*, 1993; Coddington *et al.*, 1994). This assay does not measure ability of the spermatozoa to form pronuclei because no egg is present.

A variant of IVF that has gained great popularity in recent years is ICSI, in which a single spermatozoon is selected and injected into the egg cytoplasm with a fine pipette. The first successful human ICSI was reported by Palermo *et al.* (1992) but this approach was first demonstrated with animal gametes many years earlier (Uehara and Yanagimachi, 1977). Uehara and Yanagimachi (1977) showed that hamster spermatozoa from the cauda epididymidis could undergo decondensation and form pronuclei after injection into eggs. This demonstration that testicular spermatozoa could form pronuclei after ICSI pre-dated by many years the use of this technique for azoospermic men (Palermo *et al.*, 1999). ICSI in humans is very effective for addressing 'male factor' infertility (van Steirteghem *et al.*, 1993; Oehninger *et al.*, 1995) but some clinics routinely use ICSI in place of IVF for convenience. Although numerous offspring have been born after ICSI in humans (Palermo *et al.*, 1992, 1999; van Steirteghem *et al.*, 1993; Oehninger *et al.*, 1995; Bonduelle *et al.*, 1999, 2002) and in animals, there are concerns about potential abnormalities during fertilization and even in the offspring themselves as a result of this technique (Kent-First *et al.*, 1996; Wakayama *et al.*, 1998; Hewitson *et al.*, 2000;

Terada *et al.*, 2000) indicating a need for more animal testing with ICSI to provide a more complete understanding of the long-term consequences (Yanagimachi, 1995). Although one major study showed no differences in defects at birth (Bonduelle *et al.*, 2002), it is possible that problems resulting from ICSI will be revealed later in life.

Conclusions (2002)

During the last 30 years, it has become clear that IVF can be achieved in many mammals and has important practical applications in human clinical medicine and in animal breeding. It is apparent from evaluating both the early and the contemporary literature that successful fertilization of mammalian eggs *in vitro* has depended upon the use of spermatozoa that have undergone capacitation, either *in vivo* or *in vitro*. However, precise information concerning the nature of capacitation has been gained by studying the requirements for accomplishing IVF. Therefore, the studies of IVF and of capacitation are complementary, so it is not surprising that knowledge about these processes has advanced in parallel.

The preceding account of early IVF studies illustrates the rather primitive state of knowledge during the first 20 years after the discovery of capacitation about the mechanisms of this process and of the ensuing acrosome reaction, and about the functional significance of these events for fertilization in mammals. In the ensuing 30 years, much has been learned on these topics, and IVF technology is currently being applied routinely in a wide variety of species. However, several key questions remain completely or partially unanswered. For example, what is the stimulus for the initiation of capacitation within the female reproductive tract? What molecular events are involved in capacitation? How exactly does the completion of capacitation lead to the acrosome reaction? How does serum albumin induce capacitation and the acrosome reaction? Considering that it takes only one or a few spermatozoa to fertilize oocytes within the oviduct, why are so many spermatozoa needed to effect fertilization *in vitro*? How is capacitation stimulated within the female reproductive tract in species such as the hamster or rhesus monkey that require chemical stimulators for supporting sperm capacitation *in vitro*? Does the application of ICSI make IVF obsolete or redundant, or does IVF, by preserving the natural mechanisms for selecting against the entry of abnormal spermatozoa into the oocyte, safeguard against defects that may be introduced using ICSI? Full answers to these perplexing questions concerning fertilization should be provided during the next 30 years of research into this fundamental aspect of mammalian reproduction.

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