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Fertilization of pig eggs *in vivo* and *in vitro*

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Introduction

The objective of this review is to set some of the problems associated with attempted in-vitro fertilization of pig eggs in a perspective drawn from the results of studies in live animals. Accordingly, the first part of the paper summarizes a variety of classical and more recent experiments performed *in vivo* whilst the second part examines in-vitro observations and the nature of the experimental conditions imposed. A more general discussion follows in which suggestions are made for future work.

It should be emphasized that this review does not attempt an exhaustive citation of relevant publications. None the less, it is hoped that sufficient references are given to provide access to the main body of literature available up to December 1988. Taken in conjunction with previous reviews having a different orientation (Hancock, 1962; Alanko, 1973; Baker & Polge, 1976; Polge, 1978; Einarsson, 1980; Hunter, 1982), a majority of studies bearing on the process of fertilization in pigs should become accessible.

In-vivo observations

Ovulation and egg transport

The fimbriated extremities of the oviducts completely surround the ovaries at ovulation. Eggs are released from the collapsing follicles in a substantial investment of granulosa cells, together with viscous follicular fluid measuring as much as 0·35–0·4 ml per follicle in gilts. Although this fluid enters the infundibulum, the direction of oviduct fluid flow (bulk flow) at this time is ad-ovarian and the follicular fluid is thought to be displaced into the peritoneal cavity. In gonadotrophin-primed prepubertal gilts, less than 1% by volume of follicular fluid passed down the oviduct towards the isthmus (Hansen *et al.*, 1988).

Individual eggs within their follicular cell investment are propelled across the densely ciliated surface of the infundibulum and aggregate in the ampulla to form a cumulus plug (Hancock, 1961). In this condition, they are transported to the site of fertilization at the ampullary-isthmic junction in 30–45 min or less (Hunter, 1974). Although the cumulus plug persists for some hours in unmated animals, it is rapidly dispersed in those that have mated before ovulation and lingering follicular cells are seldom attached to the zona pellucida later than 6 h after ovulation (Szollosi & Hunter, 1973).

In the normal situation of spontaneous cyclic ovulation, eggs are released from the ovaries as secondary oocytes (Spalding *et al.*, 1955), with a prominent perivitelline space and conspicuous first polar body. Shedding of primary oocytes is an extremely rare event (Hancock, 1961).

Although oviduct fluid is the physiological medium for fertilization and early embryonic development, there have been few studies of such fluids in pigs. Iritani *et al.* (1974) recorded accumulation rates of 6·3 ml/24 h during oestrus falling to 2·1 ml/24 h in the luteal phase. The volume of oviduct fluid was highest on the second day of oestrus around the time of ovulation.

Biochemical studies of the fluid are extremely limited. Engle *et al.* (1968) examined the amino acid composition, but it was Iritani *et al.* (1974) who first emphasized that glycine is the predominant amino acid.

Fertilization

Studies on the process of fertilization in domestic pigs were undertaken 30 years ago, amongst which should be mentioned the works of Pitkjanen (1955), Thibault (1959) and Hancock (1959, 1961). These were closely followed by the experiments of C. Polge & M. C. Chang (see Polge, 1978) which have not been published in full. Using phase-contrast microscopy of whole-mount preparations, Polge & Chang gained a clear insight into the stages of sperm penetration, pronuclear formation, and the first two cleavage divisions. A timetable of these developmental stages, in eggs recovered from a second group of animals with known mating schedules, led to retrospective estimates of the time of ovulation: this was found to be 39–40 h after the onset of spontaneous oestrus. Other studies of pig eggs in Polge's laboratory at this time included those on primary oocytes released in response to gonadotrophin injection after a treatment for synchronization of oestrus. Such eggs frequently showed extensive polyspermy and failure of the sperm heads to evolve into male pronuclei (Polge & Dziuk, 1965). Subsequent reports on the failure of sperm head evolution after in-vitro maturation and attempted fertilization often overlook this key observation, and the related suggestion by Dziuk & Dickmann (1965) that the block to polyspermy develops concomitantly with meiotic maturation of the oocyte.

Experimental situations that might influence the efficiency of fertilization were also studied during the 1960s. They included examination of eggs released after superovulation (Hunter, 1964, 1966; Longenecker *et al.*, 1965), eggs undergoing a phase of post-ovulatory ageing (Hunter, 1967a), and eggs from ovulations induced during the luteal phase of the oestrous cycle (Hunter, 1967b) or in post-partum sows (Hancock & Buttle, 1968). Polyspermic fertilization was noted in many of these situations, suggesting that this abnormality was caused by changes in oviduct fluid composition under the influence of increasing ovarian progesterone secretion (Hunter, 1967b). However, there is still no specific evidence that components of oviduct fluid act to regulate the incidence of polyspermy.

Later in this phase of experimental work, the thesis of Alanko (1973) summarized fertilization and embryonic development in pigs under conditions of artificial insemination. The bearing of insemination techniques themselves on fertilization had been comprehensively studied by Baker *et al.* (1968), especially with reference to the influence of numbers and concentration of spermatozoa on the normality of fertilization. As proposed earlier by Hancock & Hovell (1961), Baker *et al.* (1968) concluded that the concentration of spermatozoa in the ejaculate or inseminate had an important effect on the incidence of fertilization. Transport of live *versus* dead spermatozoa was examined by First *et al.* (1968a, b) and Baker & Degen (1972), whilst the role of seminal plasma in the female tract was pursued by Einarsson & Viring (1973) and Einarsson *et al.* (1980).

Timetables of the different stages of fertilization (Hunter, 1972a) and early embryonic development (Hunter, 1974) provide reference points for other experimental studies.

Capacitation

The involvement of capacitation in the preliminaries to fertilization in pigs first received comment from Thibault (1959) and Hancock (1961), with the observation that the 5·5–6-h interval between mating and fertilization represented a necessary delay, perhaps including a period of capacitation. More specific experiments were performed by C. Polge & M. C. Chang (see Polge, 1978), involving surgical insemination of small aliquants of ejaculated semen directly into the oviduct ampulla shortly after ovulation. Once again, the conclusion was reached that capacitation of boar

spermatozoa required a period of 5–6 h (Polge, 1978). In some conflict with these views, however, were the results of Hunter & Dziuk (1968) in which animals were inseminated intra-cervically so that the eggs would be approximately 0, 4, 8 or 12 h old at the time of sperm penetration. In this experimental situation, penetration of the zona pellucida by ejaculated spermatozoa was found within 2 h of insemination, and penetration of the vitellus with resumption of the second meiotic division within 3 h of insemination. Hence, capacitation after a peri-ovulatory insemination could occur twice as fast as previously thought. Satisfactory explanations for this apparent divergence of results followed in the early 1970s, when it was discovered that the uterus and oviducts acted synergistically to accelerate the process of capacitation (Hunter & Hall, 1974a), primarily by the removal of seminal plasma from spermatozoa swimming through the complex utero-tubal junction. Cell-free seminal plasma, derived from the same boar as was used for mating, deposited in 0·02–0·1 ml volumes directly into the oviduct isthmus delayed fertilization by 2 h or more (Hunter & Hall, 1974a).

The observation that pig eggs transplanted to the uterus show a very low incidence of fertilization, or none at all if oviduct fluids are prevented from reaching the uterus (Baker & Polge, 1973), may be related to the requirement for boar spermatozoa to be exposed sequentially to the uterus and then the oviduct for completion of capacitation.

Focussing on the origin of seminal plasma constituents and their regulatory role in the process of fertilization, later studies showed that suspensions of epididymal spermatozoa deposited directly into the oviducts varied in the time required for capacitation according to their region of origin along the epididymal duct (Hunter *et al.*, 1976). In particular, sperm suspensions prepared from the upper corpus region could fertilize eggs 2 h sooner than those prepared from the cauda epididymidis of the same boar (Hunter *et al.*, 1978). Moreover, cell-free seminal plasma prepared from the cauda epididymidis added to a suspension of spermatozoa from the upper corpus epididymidis of the same animal acted to delay fertilization by 1–2 h (Hunter *et al.*, 1978). These observations therefore correspond with the traditional idea of stabilizing factors in seminal plasma being acquired in the epididymal duct which must then be neutralized or removed in the female tract before spermatozoa can become fully capacitated.

Functional sperm reservoirs

A related line of study has involved the rate of transport and site of storage of the fertilizing population of spermatozoa in the female tract. Sufficient spermatozoa to fertilize, in due course, a proportion of the eggs have already entered the oviducts within 15–30 min of completion of mating (Hunter & Hall, 1974b; Hunter, 1981), a figure that contrasts strikingly with the rate of sperm transport in ruminants. It should be recalled, however, that ejaculation is almost directly into the uterus in pigs. A sperm suspension usually containing $1–2 \times 10^8$ cells/ml can therefore bathe the utero-tubal junction by the completion of mating (Mann *et al.*, 1956). The fact that a vanguard of spermatozoa can negotiate this junction to enter the oviducts within 15 min should not therefore cause surprise. However, the subsequent distribution of viable spermatozoa in relation to the time of ovulation is remarkable, not least in view of the 26–28 cm length of oviduct in these animals.

In an extensive study involving placement of ligatures at various intervals after mating at the onset of oestrus, and therefore progressively closer to the time of ovulation, coupled with a subsequent examination of the eggs for fertilization, it became apparent that the fertilizing population of spermatozoa was arrested in the caudal 1–2 cm of the oviduct isthmus until ovulation was imminent (Table 1). This region was therefore interpreted as the functional sperm reservoir (Hunter, 1984), rather than the utero-tubal junction as highlighted in the studies of du Mesnil du Buisson & Dauzier (1955) and Rigby (1966). In fact, spermatozoa on the uterine side of the utero-tubal junction would remain vulnerable to the extensive post-coital invasion of polymorphonuclear leucocytes (Lovell & Getty, 1968), suggesting that this would not be an appropriate location for prolonged sperm storage.

Table 1. The influence of transecting the oviduct isthmus of pigs 1·5–2·0 cm proximal to the utero-tubal junction at increasing intervals after mating at the onset of oestrus on the proportion of eggs subsequently fertilized (6 animals/group). (Modified from Hunter, 1984)

Interval from mating to transection (h)	Condition of ovaries at transection	Transected isthmus			Control isthmus*		
		Eggs recovered	No. of eggs fertilized	(%)	Eggs recovered	No. of eggs fertilized	(%)
3	Preovulatory	34	0	(0)	32	32	(100)
6	Preovulatory	40	0	(0)	35	33	(94)
12	Preovulatory	42	0	(0)	41	41	(100)
24	Preovulatory	50	0	(0)	41	39	(95)
30	Preovulatory	53	0	(0)	33	32	(97)
36	Preovulatory	51	1	(2)	41	41	(100)
38	Preovulatory	39	2	(5·1)	49	49	(100)
40	Preovulatory	48	19	(39·6)	35	35	(100)
42–44	Postovulatory	46	46	(100)	34	34	(100)
Total		403	68	(16·9)	341	336	(98·5)

*Double ligatures were placed around the control oviduct and then removed.

The caudal region of the oviduct isthmus acts to influence sperm cells in a conspicuous manner. Not only are boar spermatozoa largely trapped in this region for 36 h or more after mating at the onset of oestrus, but the motility of such spermatozoa is suppressed and the sperm surface remains intact as monitored by scanning electron microscopy (Fig. 1a); there is no indication of an acrosome reaction (Fléchon & Hunter, 1981; Hunter *et al.*, 1987). As the time of ovulation approaches, spermatozoa are released and become hyperactive with a whiplash pattern of flagellar beat. They proceed in finely-regulated numbers to the site of fertilization at the ampullary–isthmic junction, although this physical control of numbers is relaxed within 1–2 h of fertilization. Many spermatozoa are then able to confront the eggs, but such accessory spermatozoa arrive too late to cause abnormal forms of fertilization.

Ovarian regulation of oviduct physiology

The regulation of sperm storage, capacitation, hyperactivation and progression in the oviduct has been discussed extensively elsewhere (Hunter, 1987, 1988, 1989). In particular, a model has been proposed whereby the tubal epithelium transduces endocrine information from the preovulatory Graafian follicles (Fig. 2). In part, at least, this proposed means of hormonal regulation has a local countercurrent transfer basis from the ovarian vein to the oviduct branch of the ovarian artery (Hunter *et al.*, 1983). Such a route would permit incisive programming of the oviduct epithelium, and indirectly thereby of sperm physiology (Fig. 2), by supplying higher concentrations of follicular hormones than would arrive by the systemic circulation. Temperature gradients between the two ends of the oviduct could also play an important role in regulating storage of spermatozoa and their subsequent activation (Hunter & Nichol, 1986), and the temperature differentials also seem to be subject to ovarian endocrine regulation. An influence of viscous secretions has been considered essential to this phase of sperm storage, and substances such as the epididymal protein immobilin have been discussed (Hunter & Nichol, 1986; Hunter *et al.*, 1987). Underlying such interpretations is the observation that the pattern of follicular hormone secretion changes critically as ovulation approaches, enabling co-ordination of oocyte release with hyperactivation and redistribution of the male gametes.

Ultrastructural observations

Apart from the scanning electron microscopy studies referred to above (Fléchon & Hunter, 1981; Hunter *et al.*, 1987) and those of Stalheim *et al.* (1975) and Wu *et al.* (1976), there have

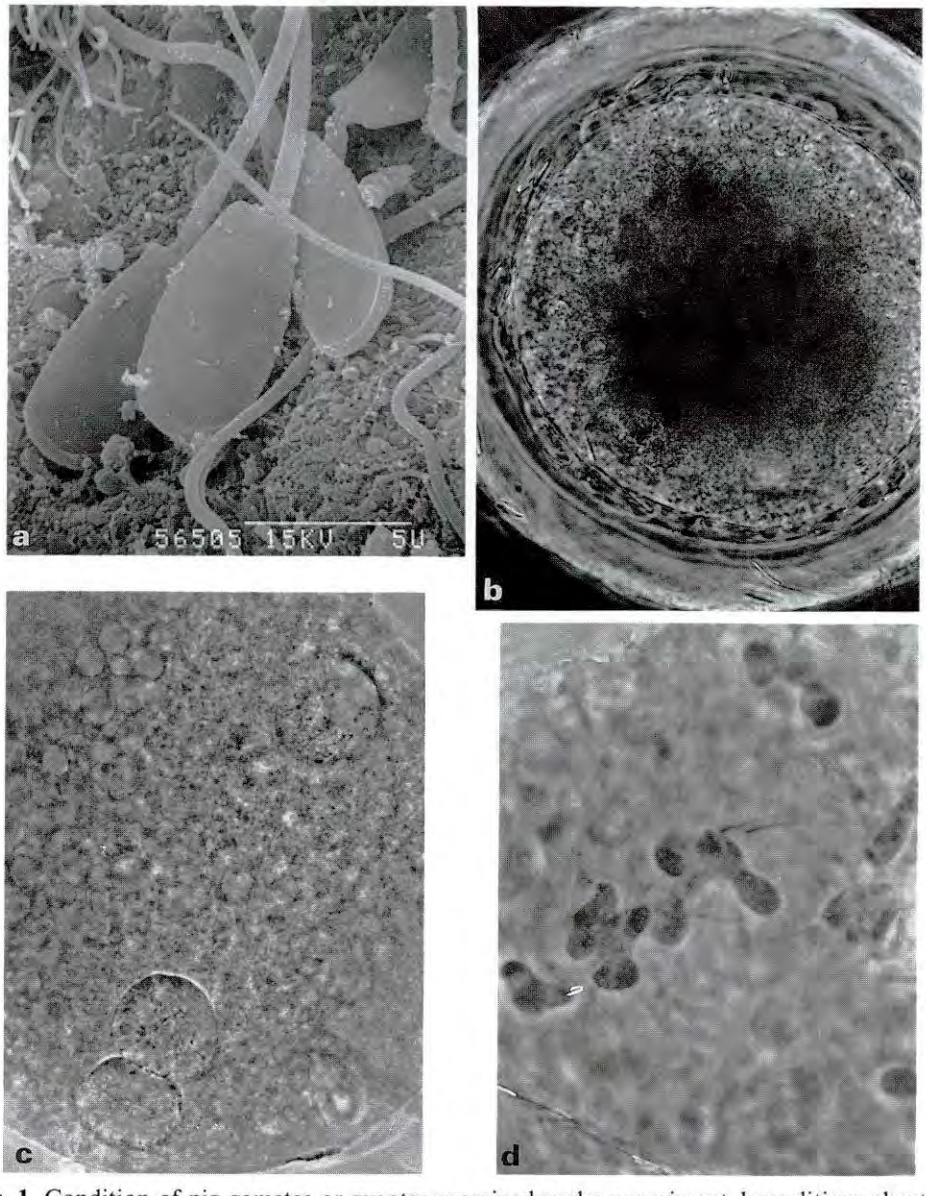

Fig. 1. Condition of pig gametes or zygotes examined under experimental conditions shortly before or after the time of ovulation. **(a)** Ejaculated boar spermatozoa viewed in the caudal portion of the oviduct isthmus. In this preovulatory storage site for fertilizing spermatozoa, the cells are still intact with no suggestion of an acrosome reaction or loss of sperm head membranes. Adapted from Hunter *et al.* (1987). **(b)** Whole-mount preparation of recently-ovulated pig egg showing large numbers of spermatozoa in the perivitelline space, together with spermatozoa in the substance of the zona pellucida. The morphology of the cytoplasm suggests the occurrence of a cortical reaction, yet the disposition of the perivitelline spermatozoa, with no obvious attachment to the plasmalemma, indicates that pig eggs can establish a vitelline block to polyspermy as well as a zona reaction. See Hunter & Nichol (1988). **(c)** Phase-contrast view of a fixed and stained whole mount. The egg is dispermic, showing the female and a single male pronucleus uniting in one hemisphere of the egg, and an accessory male pronucleus in the opposite hemisphere. The location of the pronuclei is characteristic of this condition in pig eggs. **(d)** Phase-contrast view of a highly polyspermic pig egg in which coalescing sperm heads have formed chromatin aggregates. Although the mid-pieces have separated, there is little evidence of progressive nuclear decondensation and pronuclear formation in a majority of instances. (a) $\times 4500$; (b) $\times 500$; (c) $\times 650$; (d) $\times 550$.

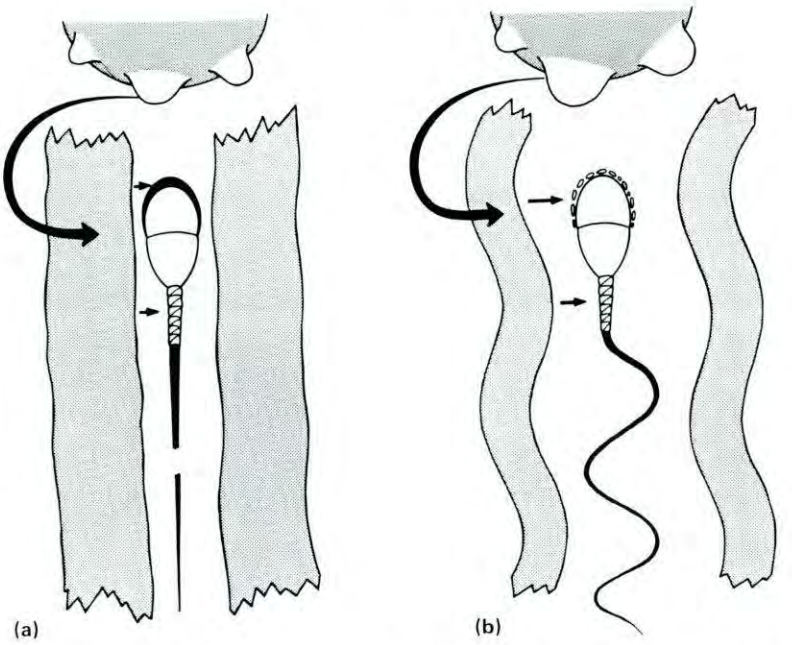

Fig. 2. Model to illustrate the manner whereby the endocrine activity of pre- or peri-ovulatory Graafian follicles acts locally to programme the membrane configuration and motility of spermatozoa in the lumen of the oviduct isthmus. Gonadal hormones (from the follicles) act on the oviduct epithelium whose transudates and secretions in turn influence the nature of the luminal fluids. Expression of capacitation is reasoned to be a peri-ovulatory event, at least in the large farm species with a protracted interval between the gonadotrophin surge and ovulation (Hunter, 1987). **(a)** Intact, relatively quiescent spermatozoon under the overall influence of pre-ovulatory follicles. Membrane vesiculation on the anterior part of the sperm head is suppressed, as is the development of whiplash activity in the flagellum—presumably due to local molecular control mechanisms. The lumen of the oviduct isthmus is extremely narrow and contains viscous secretions, and myosalpingeal contractions are reduced. **(b)** An acrosome-reacted, hyperactive spermatozoon under the influence of Graafian follicles on the point of ovulation. The oviduct isthmus has become more patent, enabling expression of the whiplash pattern of flagellar beat. Progression of such spermatozoa to the site of fertilization is also aided by enhanced contractile activity of the myosalpinx.

also been specific transmission electron microscope studies of fertilization in pig eggs. Notable amongst findings here was the absence of a true acrosome reaction until spermatozoa were actually penetrating between cells of the corona radiata or in contact with the surface of the zona pellucida (Szollosi & Hunter, 1978). Thus arose the suggestion that capacitated boar spermatozoa may be especially sensitive to some factor associated with or emanating from the egg coats, this factor acting to promote the membrane vesiculation characteristic of the acrosome reaction (see Barros *et al.*, 1967). In addition was the observation that the acrosome of boar spermatozoa swells and liberates free acrosomal contents whilst the plasma and outer acrosomal membranes are still apparently intact.

Observations on the functional state of the cortical granules are also relevant to the second part of this paper for, although a classical cortical granule reaction occurs under conditions of fertilization *in vivo* (Szollosi, 1967; Szollosi & Hunter, 1973), this reaction may be incomplete *in vitro*, especially after procedures of culture for inducing oocyte maturation (see Cran & Cheng, 1986). Such evidence for membranous incompetence may be an important factor underlying the elevated concentrations of polyspermy frequently reported *in vitro*.

Polyspermic penetration

In this summary of fertilization *in vivo*, it may be instructive to reconsider observations on polyspermy. Polyspermic penetration of the vitellus is a pathological condition in mammals (Beatty, 1957), and polyploid embryos usually die at a very early stage of development (Bomsel-Helmreich, 1961). Although, as mentioned above, studies involving post-ovulatory insemination or insemination during the luteal phase of the oestrous cycle suggested that the high incidence of polyspermy might be a consequence of altered oviduct biochemistry, this is no longer accepted. On the other hand, diverse experiments have indicated that polyspermy of pig eggs *in vivo* is primarily due to abnormally high numbers of competent spermatozoa reaching the egg surface more or less simultaneously. Multiple sperm penetration through the zona pellucida (Fig. 1b) and into the vitellus (Figs 1c and d) may therefore occur before the block to polyspermy has become fully established.

Experimental models in support of this view of polyspermy include: (i) surgical insemination directly into the oviducts (Polge *et al.*, 1970; Hunter, 1973a, b; Polge, 1978); (ii) resection of the oviduct isthmus to remove the sperm gradient imposed by this portion of the tract (Hunter & Léglise, 1971); (iii) reducing the oedematous condition and muscular contraction of the utero-tubal junction and caudal isthmus, once more removing the sperm gradient, by local microinjection of a solution of progesterone under the serosal layer (Hunter, 1972b) or by large doses of a progesterone solution given systemically (Day & Polge, 1968).

A finding that may prove to be of special significance was that the incidence of polyspermy in most of these studies was between 20 and 35% (Table 2). Because such figures correspond closely to the extent of embryonic mortality recorded in 'Western' breeds of pig, susceptibility to polyspermy under experimental conditions may be a means of revealing incompetent or subnormal oocytes (Hunter, 1979). This remark is restricted to the in-vivo situation. There is no reason to suppose that the repeated observation of polyspermy *in vitro* (see below) necessarily reflects culture of an enhanced population of incompetent oocytes.

Table 2. The incidence of polyspermic fertilization exhibited in mature pigs in various experimental situations after mating or insemination at the time of oestrus (after Hunter, 1979)

Treatment	No. of eggs examined	Polyspermic eggs		Reference
		No.	%	
Delayed mating	53	6	11·0*	Thibault (1959)
Delayed mating	41	12	29·2	Hancock (1959)
Delayed insemination	149	23	15·4	Hunter (1967a)
Tubal surgery	34	11	32·4	Hunter & Léglise (1971)
Progesterone micro-injections	198	64	32·3	Hunter (1972b)
Tubal insemination	77	26	33·8	Hunter (1973a)

*A further 21% of eggs were considered digynic, giving a total of 32%.

The polyspermic condition itself has been used to examine the spatial disposition and fate of accessory male elements in the vitellus (Hunter, 1973a) and the formation of chromatin aggregates between adjoining sperm heads that have failed to decondense (Hunter, 1976; Fig. 1d). The incidence of polyspermy under specific conditions of surgical insemination has also been used to monitor the capacitating potential of individual oviducts in relation to the time of ovulation (Hunter & Nichol, 1988).

Zona composition and sperm penetration

Gametes of the domestic pig have been much used in recent years in two areas of research bearing directly on fertilization, both areas having a strong biochemical orientation. One concerns

the composition of the zona pellucida (Dunbar, 1983), and the nature of its sperm binding sites (Gwatkin *et al.*, 1980). The other has focussed on the spectrum of acrosomal enzymes and their role in penetration of the egg investments (e.g. Brown & Cheng, 1985; Jones *et al.*, 1988).

The zona pellucida is known to be a chemically complex, acellular glycoprotein matrix, the surface of which is highly irregular (Fig. 3). Although some form of sperm-head binding is considered as the initial step in the species-specific penetration of this matrix, the surface of the zona may also act purely physically to arrest a motile spermatozoon. If so, then other more specific forms of contact interaction might seem unnecessary for penetration to proceed. Moreover, the distinction between contact, binding, and penetration by the highly motile fertilizing spermatozoon is not obvious, for these events represent a continuum.

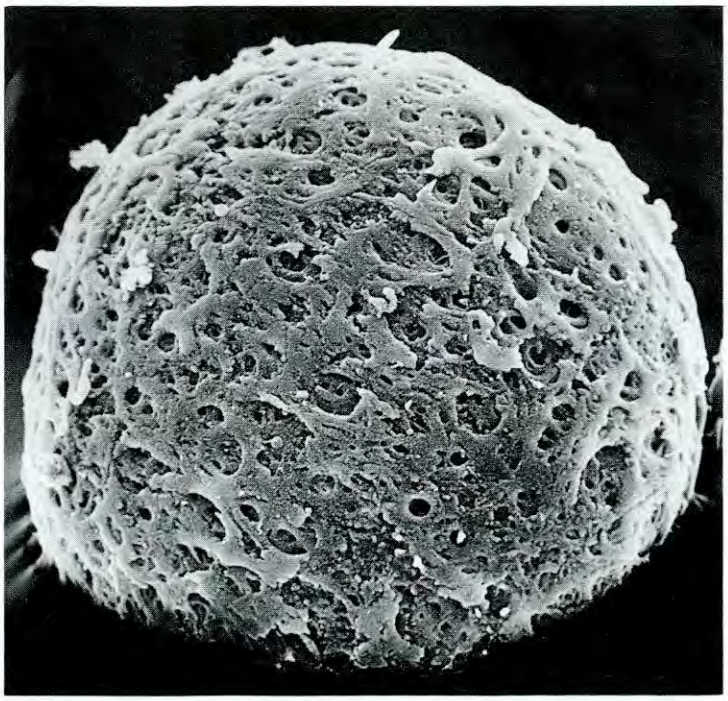

Fig. 3. A scanning electron micrograph of a pig egg denuded of granulosa cells to show the uneven, loose and spongy outer surface of the zona pellucida. Such a surface may facilitate initial contact reactions with the highly motile fertilizing spermatozoon and with subsequent accessory spermatozoa. ×950. (Courtesy of Dr J. E. Fléchon).

Despite characterization of the pig zona pellucida in several laboratories, the zona glycoprotein composition may be altered after ovulation. Brown & Cheng (1986) reported that significant amounts of pig oviducal glycoproteins were able to bind firmly to the zona, even in the presence of the cumulus oophorus. The secretory activity of the cumulus cells themselves should not be overlooked, for there is ultrastructural evidence for a continued deposition of material on the surface of the zona after ovulation.

As to the role of the boar sperm protease, acrosin, in the process of zona penetration, the evidence remains equivocal. Working with freshly-ovulated pig eggs, Brown & Cheng (1985) reported that such acrosin had asserted only a limited and selective proteolysis, a view they maintained in a later publication (Brown & Cheng, 1986). On the other hand, boar sperm pro-acrosin, the zymogen form of the protease, was able to recognize and bind to carbohydrate moieties

of zona pellucida glycoproteins (Jones *et al.*, 1988), suggesting an important function in the determinants of species specificity. Even so, the focus should not remain simply on acrosin, for other lytic enzymes may contribute actively to penetration of the zona pellucida.

In-vitro observations

The purpose of this section is to discuss attempts to obtain not only fertilization *in vitro* but also successful embryonic development following transplantation of penetrated eggs. Previous reviews dealing with the subject in farm animals include those of Baker & Polge (1976), Betteridge (1977), Hunter (1980, 1988), Wright & Bondioli (1981), Brackett & Bousquet (1984) and First & Parrish (1987).

Unpublished work

As an introduction to experiments specifically on pigs, reference will be made to the author's unpublished studies of 1968–1970 whilst working in the laboratory of Professor C. Thibault at Jouy-en-Josas, France. Although these experiments were unsuccessful, some of the observations may none the less be instructive.

Approximately 70 separate trials were conducted, each involving a minimum of 8 oocytes, more commonly 12–17. The time of ovulation was controlled in donor Large White gilts by an injection of 500 i.u. hCG given in late pro-oestrus. Ovulation occurred 43–44 h after the injection, significantly later than observed in comparable animals elsewhere (Hunter, 1972c). Animals were killed in the abattoir adjoining the laboratory within 1–2 h of ovulation, and the reproductive tract brought to the culture room (30°C) in warm, sterile, surgical drapes no later than 3–4 min after stunning. In most trials, eggs were recovered in cumulus by flushing the oviducts with 5–10 ml medium at 37°C. Media used for flushing and subsequent culture included TC 199, Tyrode's solution and Brinster's medium, supplemented with bovine serum albumin or fetal calf serum and antibiotics. Osmolality varied from 290 to 310 mosmols. The pH was adjusted to 7·6, and maintained under a gas phase of 5% CO_2 and 95% air.

Ejaculated and epididymal (upper corpus or cauda) sperm suspensions were tested, and also spermatozoa recovered from the oviduct isthmus 6–8 h after mating at the onset of oestrus. Ejaculated and epididymal samples were subjected to mild centrifugation procedures for washing and, on occasions, also to preincubation with or without explants of oviduct ampullary epithelium in an attempt to achieve capacitation. Although progressive motility was usually good in the freshly-prepared and washed samples, hyperactive (whiplash) motility was not observed. Moreover, motility was invariably poor after a 2–3 h preincubation period, although sometimes improved in the presence of preovulatory follicular fluid.

The in-vitro fertilization system used in most experiments consisted of 0·1 ml microdrops under equilibrated oil in Falcon plastic Petri dishes. However, rotating glass tubes containing the culture medium were also tried (Dauzier *et al.*, 1954) and, as a third method, a semi-in-vitro system was prepared. This consisted of the whole oviduct dissected free from its ligaments, and suspended vertically by the fimbria in oxygenated medium in a glass tube. Rhythmic contractile activity continued for several hours. The preparation was made immediately after slaughter within 1 h of ovulation, and the eggs were left undisturbed. Sperm suspensions were introduced into the caudal isthmus.

All eggs in these experiments were subsequently fixed, stained, and sectioned histologically at 8–10 μm. Although one or more nuclei were noted from time to time, there was never evidence of sperm penetration into or through the zona pellucida. However, there was a strong impression from the large numbers of spermatozoa still associated with the surface of the zona, after rinsing and fixing, that some form of attachment had begun. The principal conclusion in these studies was

that inadequate sperm motility underlay the failure to obtain fertilization. A means of maintaining and/or stimulating excellent progressive motility in culture was not discovered. Irreversible damage of the gametes was not evident, for transplantation of the microdrop contents (eggs and spermatozoa) into the oviducts of oestrous recipients after 5–6 h of culture permitted fertilization and cleavage to the 2- or 4-celled stage.

Further lack of success

Baker & Polge (1976) also recorded unsuccessful attempts at in-vitro fertilization of pig eggs. Using 280 in-vivo matured oocytes with expanded cumulus obtained 1–3 h before ovulation, and 267 oocytes obtained from the oviducts 1–4 h after ovulation, they noted large numbers of spermatozoa attached to the zona pellucida but none had penetrated through to the vitellus. Sperm suspensions had been prepared from the sperm-rich fraction of the ejaculate, from surgically-recovered epididymal secretions or from different portions of the reproductive tract of oestrous gilts. Various culture media were examined, with or without the addition of follicular or oviduct fluid. Transplantation of droplets containing the mixture of gametes into the oviducts of oestrous animals after 4 h of incubation *in vitro* yielded only 33% of penetrated eggs. Furthermore, the recovered eggs showed conspicuous degeneration of the cytoplasm (Baker & Polge, 1976), an observation of some relevance to the outcome of subsequent transplant studies (see below).

Sperm penetration of eggs *in vitro*

Apart from isolated instances of in-vitro fertilization of pig eggs (e.g. Harms & Smidt, 1970), significant progress in producing a workable system for generating viable embryos was not reported until the studies of Cheng (1985) and Cheng *et al.* (1986). However, two preceding studies deserve comment. In a quite extensive series, Iritani (1978) recorded low proportions of penetrated oocytes (3–26%) after in-vitro maturation of primary oocytes aspirated from Graafian follicles of 2–3 mm diameter. In an attempt to achieve capacitation, ejaculated or cauda epididymal spermatozoa had been preincubated in a post-mortem preparation of oviduct and uterine tissue. Development of the penetrated oocytes was not tested. Nagai *et al.* (1984) also used cultured follicular oocytes and reacted these with washed, preincubated samples of epididymal or ejaculated spermatozoa. Low concentrations of epididymal spermatozoa during preincubation gave a low incidence of penetration (11%) whereas a high preincubation concentration gave a high incidence of penetration (71–75%) but arrested development of penetrated eggs due to polyspermy. No fertilization was obtained with ejaculated spermatozoa (Table 3).

Zona-free preparations of pig eggs will not be discussed in this review. Although they have proved valuable for studying sperm–egg interactions (Pavlok, 1981), there is no evidence that they could yield viable embryos.

Details of the methodology used in these earlier trials, and in more recent experiments, are summarized in Table 3. As will be noted, most groups did not test the viability of eggs fertilized *in vitro* by subsequent transplantation studies. In fact, by the end of 1988, only 3 groups had reported production of live piglets after procedures of in-vitro fertilization. Litter size was small on each occasion.

The system reported by Cheng (1985) and Cheng *et al.* (1986) contained important new features: (i) freshly-ejaculated spermatozoa were preincubated for 4 h at 37°C at pH 7·8 in medium TC 199 supplemented with fetal calf serum, sodium pyruvate and calcium lactate; (ii) the preincubated spermatozoa were then cultured with ovulated oocytes at 39°C for 6–8 h with 4·7 mM-calcium, leading to 89% of penetrated eggs. Although it is not clear how they were identified (i.e. selected), monospermic eggs cultured in modified BMOC-2 medium (modified Brinster's) for 40 h yielded 86% of 2–4-cell embryos. Transfer of 206 embryos at the 2–4-cell stage to 15 recipient

gilts gave 6 pregnancies and 19 piglets born. Whilst these results have provided encouragement, it remains important to question why the overall yield of viable fetuses (11%) was not higher.

Why such poor viability of in-vitro fertilized embryos?

The answer to this question presumably lies in a divergence from physiological conditions during the procedures of gamete recovery and preparation *in vitro*, and likewise during the steps of actual in-vitro fertilization and culture of the newly-formed zygotes. The paragraphs that follow involve speculation. If the shortcomings in the in-vitro system were clearly understood, then these could be modified and a significant improvement in results might be obtained.

Until recently, actually obtaining fertilization *in vitro* of pig eggs was the major problem, with the inference that, in the absence of polyspermy or digyny, the zygote was potentially viable. This view cannot be justified. There is ample evidence that damage to the gametes may not impede a successful union, yet the zygote may fail to develop normally and show derangements at an early stage. As an example, X-irradiation of ejaculated rabbit spermatozoa (20 000 r) did not prevent them from competing successfully with non-irradiated spermatozoa from the same buck after insemination of the mixed sperm suspension. However, eggs fertilized by the irradiated spermatozoa showed a delayed first cleavage, and further embryonic development failed (Bedford & Hunter, 1968). Whether this kind of failure usually corresponds closely with the onset of expression of the embryonic genome is yet to be resolved. More recent studies in rats also underline the influence of lesions in the gametes on early embryonic failure (Setchell *et al.*, 1988).

For in-vitro fertilization of pig eggs, the question must therefore be asked whether preincubation of spermatozoa at elevated temperatures and/or in a hyperosmotic medium leads to subtle but irreversible damage to the nucleus which may not prevent fertilization but which acts to compromise normal development. The same question must be asked of any pretreatment of oocytes, and likewise of the in-vitro fertilization system itself. For example, exposure of the gametes to incubation temperatures of 39°C may be more traumatic than hitherto realized and, in the case of the oocyte, perhaps damaging not only to the nucleus but also to the cytoplasmic organelles. Cheng *et al.* (1986) emphasized that the relatively high level of calcium (4·7 mM) was essential for inducing capacitation of boar spermatozoa *in vitro* and likewise for successful fertilization. None the less, the delayed cleavage reported for in-vitro generated pig embryos by Yoshida (1987) might be an expression of nuclear and/or cytoplasmic damage. The delay is certainly significant when compared with the in-vivo situation, in which the first cleavage is found 14–16 h after sperm penetration and the second cleavage occurs approximately 6–8 h later (C. Polge & M. C. Chang, unpublished; Hunter, 1974).

Quite apart from the preceding suggestions, other sources of potential damage could reside in laboratory methods used to date (Table 3). After collecting ovaries at the abattoir, subjecting them to temperature variations and anoxia *en route* to the laboratory, in due course extracting oocytes from follicles far smaller than the preovulatory diameter, and culturing them for various times in microdrop systems overburdened with cells, the fact that viable embryos cannot be generated at an incidence comparable with the in-vivo situation is not surprising. And what actually happens in the culture system? The notion that such systems are chemically defined is an illusion. The number of spermatozoa added is quite unphysiological, large numbers of cells will die, degradation products will be released into the medium, and its chemical composition will change significantly. And what is the gas tension at the base of a microdroplet under oil in a plastic Petri dish? *In vivo*, the system within the oviducts is sensitive and dynamic with micro-currents, ciliary and muscular movements, and a constant adjustment of the medium. The epithelium and underlying vascular supply cannot be taken for granted, nor perhaps macromolecular secretions that interact with the surface of gametes and embryos.

So what is the way forward? A logical, if painstaking, means of testing these ideas would be to subject gametes to the in-vitro fertilization procedures favoured in recent studies, and then to

Table 3. Summarized details of the methodology and degree of success after various procedures for attempting in-vitro fertilization of pig eggs

Reference	Source and preparation of oocytes	Source and preparation of spermatozoa	System for in-vitro fertilization	Results
Iritani *et al.* (1978)	Oocytes aspirated from 2–3 mm diam. follicles; cultured for at least 24 h at 37°C in modified KRB; 60% maturation to second metaphase	Ejaculated or cauda epididymal spermatozoa washed in KRB and preincubated for 4–5 h in a post-mortem specimen of pig uterus and oviduct	10 µl sperm suspension (0·5–1 × 10^6 cells/ml) into 0·4 ml medium containing 10–15 oocytes; cultured for 17–20 h at 37°C	Very low proportion of oocytes penetrated; range 3–26% in all experiments
Pavlok (1981)	Zona-free eggs from preovulatory follicles of gonadotrophin-treated animals; 0·2% pronase used to dissolve zona	Washed ejaculate; best conc. for preincubation was 0·2–1·4 × 10^9 cells/ml in protein-supplemented medium at 37·5°C; < 1·0 × 10^6 cells/ml gave very low incidence of penetration	100 µl medium with zona-free eggs + sperm suspension to give final concentration of 0·5–2·0 × 10^6 cells/ml	Zona-free eggs penetrated by preincubated spermatozoa within 4 h; similar eggs penetrated by freshly-washed spermatozoa 8 h after insemination; extensive polyspermy
Nagai *et al.* (1983)	Oocytes aspirated from 2–5 mm diam. follicles; cultured for 28 h at 37°C in modified KRB; 48% maturation to second metaphase	Cauda epididymal spermatozoa, kept at room temperature for 3–26 h; preincubated in isolated uterine horn for 4 h at 37°C; used a 1-ml volume between ligatures containing 4 × 10^8 spermatozoa	5–20 µl sperm suspension (1–200 × 10^4 cells/ml) into 0·4 ml medium containing 10 oocytes; cultured for 20 h at 37°C	Incidence of penetration correlated with sperm concentration; 100–200 × 10^4 spermatozoa/ml → 74–76% penetration; 5–10 × 10^4 ml → 47–48% penetration; 82% polyspermy at the higher concentrations
Nagai *et al.* (1984)	Oocytes aspirated from 2–5 mm diam. follicles; cultured for 28–29 h at 37°C in modified KRB	Ejaculated or cauda epididymal spermatozoa; preincubated in modified KRB for 4 h at 37°C; whiplash motility observed in epididymal but not ejaculated spermatozoa	0·4 ml droplets of modified KRB medium; final sperm conc. of 2 × 10^6 cells/ml; cultured for 20 h at 37°C	Incidence of penetration with epididymal spermatozoa depended on preincubation concentration; 4–16 × 10^8 cells/ml → 71–75% oocytes penetrated; 0·8 × 10^8 cells/ml → 11% oocytes penetrated; 84% oocytes denuded of cumulus penetrated

Cheng *et al.* (1986)	Secondary oocytes flushed from oviducts within 1–2 h of ovulation *or* oocytes aspirated from preovulatory follicles 36 h after hCG and cultured for 6 h; > 2000 oocytes tested	Freshly-ejaculated spermatozoa; sperm-rich fraction (a) washed and preincubated for 4–5 h at 37°C in modified TC 199 at 2×10^8 cell/ml or (b) stored at 20°C for 16 h followed by three washes	Oocytes with partly removed cumulus placed in 2 ml diluted sperm suspension; 15–20 oocytes/dish; cultured for 6–8 h in concentrations of 1×10^5 or 1×10^6 spermatozoa/ml; 2 mM-caffeine added	Culture temperature critical; < 1% oocytes fertilized at 37°C; 89% oocytes penetrated at 39°C; high incidence of polyspermy (48–79%); transplanted 206 embryos (2–4-cell) to 15 recipients; 6 became pregnant, giving 19 piglets
Yoshida (1987)	Oocytes aspirated from 7–12 mm diam. follicles at slaughter 39–40 h after hCG in PMSG-primed gilts, or flushed from oviducts; oocytes washed twice and preincubated for 1–3 h	Freshly-ejaculated spermatozoa; sperm-rich fraction diluted in saline + BSA and washed; then further diluted to 2×10^8 cells/ml; preincubated for 4 h at 37°C; 2 mM-caffeine added	10–15 oocytes in 0·2 ml droplets at 38·5°C; sperm conc. in droplet 2×10^6 cells/ml; incubated for 6–7 h, then transferred to 0·1 ml droplets of culture medium for 18–28 h	95% (25/26) oocytes fertilized; variable polyspermy (25–100%); ~ 50% cleaved to 2-cells at 27–28 h; 17 embryos transplanted at 2-cell stage into oviducts of single recipient yielded 4 piglets; 3 other transfers failed
Nagai *et al.* (1988)	Oocytes aspirated from 2–5 mm diam. follicles at slaughter; matured *in vitro* for 32 h in 0·1 ml hormone-supplemented TC 199 at 39°C. Alternatively, oocytes matured *in vivo*, collected 39 h after hCG, and incubated for 3 h	Cauda epididymal sample frozen–thawed or ejaculated sample frozen–thawed; preincubated in modified TC 199 for 4 h at 37°C; suspensions contained 2–4×10^8 cells/ml	In-vitro matured oocytes in 0·4 ml Brackett's solution with preincubated spermatozoa + 2 mM-caffeine; $2{\cdot}4 \times 10^7$ cells/ml. In-vivo matured oocytes for 6 h with spermatozoa at 39°C; then rinsed and cultured for 24 h	0–40% oocytes penetrated with epididymal sample; 0% oocytes penetrated by ejaculated sample; 79% in-vivo matured penetrated by epididymal but 57% were polyspermic; 2-cell eggs transplanted gave 1 litter of 3 piglets
Mattioli *et al.* (1988)	Oocytes aspirated from 3–6 mm diam. follicles that had been cultured for 44 h at 39°C; expanded cumulus mass removed before IVF procedures	Ejaculated spermatozoa, diluted, stored at 16°C for 1–3 days, then washed	Incubated 10–15 oocytes in 2 ml modified TCM 199, containing 10·5 mM-calcium, with final sperm concentration of 10^5 cells/ml; 6–8 h later, oocytes cultured in Brinsters medium	Fertilization of 78% of oocytes, but only 47% were monospermic; 39% of 681 oocytes cultured after IVF were 2–4 cells; 41% of 211 embryos transplanted into oviducts were blastocysts after 4 days

KRB is Krebs–Ringer–bicarbonate solution; BSA is bovine serum albumin.

examine viability by transplanting the mixture of gametes to the oviducts close to the time of ovulation. If normal fertilization and embryonic development were not obtained, then a rigorous and stepwise analysis would have to be made of the in-vitro system. Of course, the ultimate reference point for what is and is not acceptable *in vitro* in terms of temperature, osmolarity, pH and actual composition of a culture medium would be the conditions within an oviduct shortly after ovulation. Unfortunately, not only are most of the relevant measurements not available for pigs, but the techniques for making them might lead to artefacts. None the less, in-vivo measurements of the above values using the best technology would be an advance, and would provide information of great relevance to successful in-vitro fertilization and embryonic development in culture. This Centre (the CRRA) is currently engaged on such a programme of research. The alternative approach of inspired modifications to techniques used in laboratory rodents might yield reasonable levels of success from time to time, but it is unlikely to be widely repeatable and therefore satisfactory in the long term.

Despite this point of view, an interim approach that might yield better results than those reported so far would be to work *in vitro* with oocytes in cumulus together with their bathing fluid aspirated from the site of fertilization shortly after ovulation (see above). The cell suspension would be set up as a microdrop preparation. Vigorous ejaculated or epididymal spermatozoa could be selected by means of washing and subsequent swim-up techniques. To reduce the risk of polyspermy, small numbers of highly motile spermatozoa would be introduced into the microdrops. After 4–5 h of initial interaction, the gamete or zygote preparation would be transplanted to fresh microdrops of post-ovulatory fluid obtained from the oviduct lumen at the ampullary–isthmic junction, together with explants of epithelium from the same region of the oviduct. The emphasis would therefore be on the microenvironment that is thought to play a vital role during fertilization *in vivo*.

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