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Follicle–oocyte–sperm interactions *in vivo* and *in vitro* in pigs

M. A. Sirard, A. Dubuc, D. Bolamba, Y. Zheng and K. Coenen

Department of Animal Science and Ontogeny–Reproduction Unit, CHUL Research Center, Université Laval, Québec, G1K 7P4 Canada

In vitro culture has provided new information on the mechanisms involved in fertilization where two completely different cells fuse together. At the same time, results obtained *in vitro* have led to new questions. Does the follicle influence the final maturation process of the oocyte and does the oviduct regulate the normal behaviour of spermatozoa? Recent studies indicate a critical influence of both the follicular compartment and the oviduct on the normal fertilization process. Oocytes matured *in vivo* are more competent to induce pronuclear formation, but are still susceptible to polyspermy. Oocytes matured *in vitro* can develop fully after fertilization, but require the presence of follicular factors during culture to enhance their developmental competence. Fresh or frozen spermatozoa can penetrate oocytes *in vitro*, but their conditioning by differential centrifugation or oviductal cells influences the rate of polyspermy. The understanding of these influences is a prerequisite to enhancing *in vitro* production of pig embryos.

Introduction

Mammalian embryos result from a complex series of events taking place in a rapidly changing environment and with the participation of numerous tissues. At least three types of maturation process are involved in fertilization: follicular maturation, oocyte maturation and sperm maturation. In the female, follicular growth is central to the timing process leading to oestrous behaviour and ovulation. At the follicular level, two types of influence are required: the influence of hormones, mainly steroids, on receptive tissues such as the brain, the oviduct or the uterus, and the influence of intrafollicular factors acting on the oocyte. The importance of hormones at the ovarian or systemic level is well known, but the influence of the follicle on the oocyte is poorly understood. The importance of follicular maturation on the capacity of oocytes to achieve fertilization or early embryonic development can now be studied by attempting to reproduce some crucial events *in vitro*. In many mammals, *in vitro* fertilization is now possible and allows the evaluation of oocyte competence following different maturation procedures. In pigs, the evaluation of the effects of *in vitro* maturation protocols on the viability of oocytes has been limited by the difficulties in inducing normal fertilization *in vitro*. Several factors are involved: first, the high levels of variation between boars or ejaculates (Wang *et al.*, 1991), second, the block to polyspermy in pig oocytes seems to be working on a different scale compared with other mammals (Hunter, 1991) and third, the specific conditions prevailing in the pig oviduct at the time of fertilization are not well known (Nichol *et al.*, 1992). It is quite obvious that the conditions prevailing *in vivo* must be better understood to achieve a high number of viable embryos with the objective to introduce new techniques in reproduction management in pigs. This review will address the relation between what is known of events *in vivo* and the resulting effect observed *in vitro* when spermatozoa and oocytes are put together.

Table 1. The effect of the maturation procedure on pronuclear formation following *in vitro* or *in vivo* fertilization in pigs

Culture time (h)	Culture system	Maturation rate	Penetration rate (spermatozoa)	Polyspermy rate	Male pronucleus rate	Reference
22–27	Simple medium	? Mostly matured	63% (*in vivo*)	32%	32%	Motlik and Fulka, 1974
24	Simple medium	60%	0–26% (fresh)	14%	28%	Iritani *et al.*, 1978
29	Simple medium	88%	75% (fresh)	81%	58%	Nagai *et al.*, 1984
—	—	(*in vivo*)	88%	Present	Normal	Toyoda *et al.*, 1984
48	Simple serum + PMSG	80% (*in vitro*)	79%		Abnormal	
32	TCM-199 serum + LH	70%	42% (frozen) (epididymal)	39%	54%	Nagai *et al.*, 1988
46	Everted follicle serum + LH	—	80% (fresh)	78%	43%	Mattioli *et al.*, 1988
48	Porcine follicular fluid	88%	50% (fresh)	—	81%	Naito *et al.*, 1988

PMSG: pregnant mares' serum gonadotrophin.

Follicular Maturation

The female pig has a favourable genetic characteristic compared with other domestic species: large numbers of ovulations or constant superovulation conditions. In ruminants, considerable efforts are invested in stimulating the ovary to produce large numbers of oocytes per ovulation and to counteract the dominance phenomenon leading to atresia. In pigs, several growing follicles will survive up to ovulation and even the atretic ones are now considered to play a role in that process (Westhof *et al.*, 1991). Does this mean that all growing follicles contain healthy oocytes that can become a viable embryo and fetus? There is certainly a size limitation in terms of competent follicles. Motlik *et al.* (1984a) measured small antral follicles of cyclic gilts and showed that follicles smaller than 0.7 mm contained oocytes incapable of completing meiotic resumption. This capacity was acquired in a heterogeneous way since some oocytes from the follicles between 0.8 mm and 1.6 mm were capable of maturing *in vitro* (17%), but not all from follicles larger than 1.7 mm were at the metaphase II stage after 48 h of culture. Even the larger follicles (> 3 mm) contain some oocytes that cannot reach the normal configuration in culture (Motlik *et al.*, 1984a). This last observation has also been shown by Iritani *et al.* (1978), Nagai *et al.* (1984, 1988) and Naito *et al.* (1988, 1989). The capacity to achieve meiotic maturation does not ensure the capacity of an embryo to develop, as those competences may be acquired in a stepwise process as shown in other species (First *et al.*, 1988). The size of the oocyte also varies with follicular size and growth is almost complete in 1.8 mm follicles (oocytes of 115 μm) even if the maximum mean diameter (120 μm) is reached in follicles above 5 mm (Motlik and Fulka, 1986). RNA synthesis is another indication of growth in pig oocytes; incorporation studies revealed a good inverse correlation to growth as shown above (Motlik *et al.*, 1984b). The ability to complete the metaphase I to metaphase II transition following incubation in culture coincides with the cessation of nucleolar transcriptional activity during growth (McGaughey *et al.*, 1979). If only fully grown oocytes are competent to become embryos following fertilization, this could indicate that a limiting factor is accumulated to a critical concentration or at the end of the growth phase. Since oocytectomy and transfer to surrogate preovulatory follicles has never been achieved with oocytes of different follicular size, the answer is not known but *in vitro* culture may provide some clues.

Gamete Maturation and Interaction

Oocyte maturation, early culture experiments

The maturation of the oocyte can be divided into two different aspects: nuclear maturation and cytoplasmic maturation. The spontaneous nature of nuclear maturation was observed in mammals as early as 1935 by Pincus and Enzmann. The nuclear maturation in pigs was described by Edwards in 1965 and proceeded to the metaphase II stage in 43–46 h *in vitro*. Similar results were obtained by Foote and Thibault (1969), but many oocytes remained at metaphase I even after 48 h of culture. The pig oocytes require almost twice the time as do other large animals to transform the prophase nucleus, the germinal vesicle, into condensing chromatin. This delay observed *in vitro* reflects a comparable situation *in vivo* where the time between the LH surge and germinal vesicle breakdown is 20–24 h (Edwards, 1965). Foote and Thibault (1969) hypothesized that the time required for the nuclear events involved in the maturation process could be influenced by the conditions in which the oocytes are cultured. Different media were tested for their capacity to promote nuclear maturation and fertilization (Table 1). If nuclear maturation appears to proceed normally in most oocytes in medium containing BSA or serum, the formation of a normal male pronucleus following fertilization is not completed normally in most oocytes. It is considered that the capacity to decondense the spermatozoa is one of the different components of cytoplasmic maturation as observed in pigs by Motlik and Fulka (1974) and as described by Thibault *et al.* in the cow (1975). It was reported that for normal fertilization to occur, the oocyte must experience germinal vesicle breakdown *in vivo* (Motlik and Fulka, 1974). A similar study in which oocytes were transferred to the oviduct of inseminated recipients at a precise time following hCG injection indicated increasing (58, 61 and 83%, respectively) levels of fertilization at 0, 28 or 38 h and a decreasing rate of polyspermy (57, 26 and 12%, respectively) (Baker and Polge, 1976). This study indicates that if oocytes are immature at the

Table 2. The effect of the maturation procedure on developmental capacity following *in vitro* fertilization in pigs

Culture time	Culture system	Penetration rate (spermatozoa)	Polyspermy rate	Male pronucleus	Development rate	Reference
In vivo	—	89% (fresh)	48–79%	Normal	86% of monospermic cleaved, 19 piglets born out of 206 embryos transferred	Cheng *et al.*, 1986
In vivo	—	79% (frozen) (epididymal)	39%	83%	54% cleaved, one piglet born	Nagai *et al.*, 1988
48 h	Tyrode	— (fresh)	—	—	13% cleaved	Naito *et al.*, 1989
	pff	— (fresh)	—	—	36% cleaved	
44 h	Everted follicle serum + LH	78% (fresh)	53%	—	39% cleaved 66% mor-blast* after 4 days *in vivo*, live birth	Mattioli *et al.*, 1989
36 h	TCM-199 pff + oestradiol gonadotrophins	85% (fresh)	62%	16%	49% cleaved 14% mor-blast* after 4 days *in vivo*	Yoshida *et al.*, 1990
In vivo		9.5% (fresh)	6% of one-cell left	—	32% (509/1586) 26% of the cleaved to mor-blast*, 2 piglets	Rath, 1992

*Morula or blastocyst.
pff: pig follicular fluid.

time of transfer to the oviduct, their fertilization is abnormal, but this does not show that oocytes removed from their follicle before 38 h after hCG are incompetent if matured outside in normal fertilization and development. Asynchronous development of the male and female pronuclei, and delay or abnormal transformation of the sperm head into a pronucleus were reported by Iritani (1978) and resulted in the general assumption that pig oocytes matured *in vitro* were deficient in some cytoplasmic factors. Toyoda *et al.* (1984) were among the first to compare the oocytes matured *in vivo* with oocytes matured in culture, and even if penetration rates were similar after *in vitro* fertilization, the oocytes matured *in vitro* resulted in abnormal pronuclei formation (Table 1). More recently, the culture conditions were modified by the addition of follicular fluid or portions of the follicle and pronuclear formation improved, supporting the active role of the follicle in the cytoplasmic maturation of the oocyte. Cytoplasmic maturation remains a concept of numerous changes occurring in the RNA content before the resumption of nuclear maturation leading in post-transcriptional activation later in development (Moor *et al.*, 1990). Normal development is therefore not limited to the formation of the male pronucleus, even if this event has been used as an indication of a potential for development, since it is the first visible event that varies according to the source of oocyte (Table 1).

The effect of the maturation procedure on developmental capacity

Is the attainment of full size sufficient for an oocyte to be fertilized and to proceed to development or is the final influence of the follicle on a fully grown oocyte a prerequisite for normal development beyond the pronuclear stage? It is important to use similar fertilization and developmental conditions to investigate the competence of oocytes. The method of *in vitro* fertilization can be used to evaluate this aspect. One of the largest studies in which *in vivo* matured oocytes recovered from superovulated gilts where used for *in vitro* fertilization was by Cheng *et al.* (1986) (Table 2). At the ultrastructural level they observed a decrease in cortical granule reaction in oocytes fertilized *in vitro* rather than *in vivo*. A fertilization period greater than 8 h increased polyspermy, even with *in vivo* matured oocytes. Eighty-six per cent of the embryos reached the two- to four-cell stage by 40 h after insemination. The transfer of 206 cleaved eggs to 15 recipients following maturation *in vivo* and fertilization *in vitro* resulted in six pregnancies and 19 piglets, but since no control (*in vivo* matured and fertilized) embryos were transferred using the same conditions, it is difficult to conclude that there was a decreased survival capacity (Cheng *et al.*, 1986). Yoshida *et al.* (1990) reported that about half of the *in vitro* fertilized embryos reached the two-cell stage 28 h after insemination and four piglets were born from one sow. The oocytes matured *in vivo* are therefore competent to proceed throughout development following fertilization, but their cortical granule release and sensitivity to polyspermy are affected by *in vitro* conditions. A possible compromise between *in vivo* maturation and *in vitro* maturation can be achieved by dissecting the follicular wall around the oocyte and culturing them together. High embryonic development and some piglets were obtained after 44 h of culture using oocyte–follicle co-culture (Mattioli *et al.*, 1989) (Table 2). Pig follicular fluid, exogenous gonadotrophins and oestradiol in combination can result in early embryonic development, but at a lower rate than with the follicular section (Yoshida *et al.*, 1990) (Table 2).

The effects of the maturation procedure on penetration, male pronucleus formation and polyspermy

There are a number of recent studies in which different procedures were applied during *in vitro* maturation of pig oocytes to study the development of the ability to form normal pronucleus but also to prevent excessive polyspermy during *in vitro* fertilization. In general, high penetration rates result in high polyspermy rates and even if male pronucleus formation is present, more than one male pronucleus can result in polyploiidy. It was mentioned that follicular wall co-culture can improve cytoplasmic maturation compared with similar culture of cumulus-enclosed oocytes, but at the same time, almost all fertilized eggs are polyspermic (Mattioli *et al.*, 1988) (Table 3). The ability of the co-culture system to induce pronuclei formation is improved by LH but not by FSH, indicating a specific role of the follicular wall (Mattioli *et al.*, 1991) (Table 3). Recently Ding and Foxcroft (1992) used follicular shells from follicles isolated 36 or 72 h after equine chorionic gonadotrophin (eCG) injection. They observed a significant effect of follicular size

Table 3. The effect of the maturation procedure on penetration, male pronucleus formation and polyspermy in pigs

Culture time (h)	Culture system	Penetration rate (spermatozoa) (%)	Polyspermy rate (% penetrated)	Male + female pronucleus (%)	Reference
46	TCM-199 Serum + LH	47 (fresh)	45	2	Mattioli *et al.*, 1988
	Everted follicle Serum + LH	80 (fresh)	78	43	
44	Everted follicle	77 (fresh)	78	48	Mattioli *et al.*, 1991
	+LH	85		73	
	+FSH	71		44	
	+FSH + LH	77		74	
46–48	10 oocytes (large follicle)	80	—	67	Ding and Foxcroft, 1992
	With 2 shells (small follicle) FCS + Gn + PRL[a]	86	—	77	
36	FCS + H[b]	90	84	51	Yoshida *et al.*, 1992a
	pff total	82	73	59	
	pff frac G1	64	49	67	
36	TCM-199 + 10% pff	87	—	63	Yoshida *et al.*, 1992b
	Waymouth + 10% pff	94	—	91	
	mTLP + 10% pff	94	—	47	

[a]Gn + PRL: prolactin plus FSH and LH.
[b]H: gonadotrophins and oestradiol combined.
pff: pig follicular fluid.

on male pronucleus formation but no specific effect of the time following eCG injection. The effect of the follicle on the capacity to form a pronucleus does not seem to be associated with an effect in reducing polyspermy, as polyspermy is normally high when oocytes are preincubated with follicle cells (Tables 2 and 3). However, the removal of cumulus cells during maturation induces a premature migration of cortical granules leading to an increase in exocytotic events and a decrease in penetrability compared with cumulus-enclosed oocytes (Galeati *et al.*, 1991). It has also been shown that absence of cumulus cells results in a lower membrane potential than in cumulus-enclosed oocytes and the same decrease can be induced in cumulus-enclosed oocytes by LH, but not by FSH or the combination of both (Mattioli *et al.*, 1990). It is suggested that when maturation is triggered by the appropriate gonadotrophin stimulation, namely LH or hCG, the membrane potential undergoes a progressive depolarization either *in vivo* or *in vitro*. Artificial activation of oocytes matured *in vitro* with electrical current before fertilization did not prevent multiple penetration as expected, but resulted in a decrease or an important delay in male pronucleus formation (Funahashi *et al.*, 1993). These results could imply that a single calcium release may induce the cell cycle precociously, but could not induce sufficient exocytosis of cortical granules to prevent penetration. The action of the follicular fluid on nuclear maturation depends on one or more heat labile fractions and the effect on pronuclei formation could be attributed to a molecule between 10 and 200 kDa (Yoshida *et al.*, 1992a). When diluted to 10%, pig follicular fluid can promote pronuclei formation at a similar rate to that reported above if the medium used for culture is the Waymouth medium (Yoshida *et al.*, 1992b). It is not clear yet whether there is an effect of the time oocytes spent in culture before fertilization, since 36 to 44 h were used successfully by different investigators (Tables 2 and 3). In our group, the effects of different sources of serum, follicular fluid or portion of the follicular wall were compared to evaluate their effects on penetration, polyspermy and pronuclear formation (Zheng and

Table 4. The effect of BSA, oestrous serum and follicular wall explants on penetration, male pronucleus formation and polyspermy in pigs

Culture time (h)	Culture system	Second 24 h culture	Penetration rate (spermatozoa) (%)	Polyspermy rate (% penetrated)	Male + female pronucleus (%)
46	FCS + H[a]	FCS + H[a]	81	71	10 mono[b]
		(cumulus free)	63	50	4 mono[b]
	FCS + H[a]	BSA + H[a]	85	52	18 mono[b]
	BSA + H[a]	BSA + H[a]	22	80	0 mono[b]
	FCS + H[a]	BSA + H[a]	80	60	12 mono[b]
		(cumulus free)	60	45	4 mono[b]
	EGS + H[a]	EGS + H[a]	80	60	12 mono[b]
	Follicle + H[a]	follicle + H[a]	56	54	25 mono[b]

H[a]: gonadotrophins and oestradiol combined.
[b]mono: only one male pronucleus present without additional sperm heads.
Data from Zheng and Sirard (1992).

Sirard, 1992) (Table 4). Our results are supportive of a regulating role of follicular explants on penetration and polyspermy (Table 4) but, for this experiment, no selection was used for the follicles indicating a less specific requirement for a normal 'maturation factor'. We also showed that polyspermy could be significantly reduced by preincubation of the cumulus-enclosed oocytes in medium containing BSA instead of fetal calf serum (FCS) and that when BSA was the only protein source maturation rates were unaffected, if used only in the second 24 h of maturation (Table 4). The removal of the cumulus at that time (24 h of maturation) did not affect the maturation rate, but did reduce penetration compared with FCS for the complete (46 h) maturation period. The effect of BSA in the prevention of polyspermy led us to speculate about its possible role on the hardening of the zona pellucida as shown by De Felici *et al.* (1985) in mice. In rats (Vanderhyden and Armstrong, 1989), hamsters and cows (Leibfried-Rutledge *et al.*, 1986), the penetration rates are reduced in BSA-supplemented medium. The mechanism of zona pellucida hardening appears to be the conversion of the zona pellucida glycoprotein ZP2 to $ZP2_f$ by a protease from precociously released oocyte cortical granules (Ducibella *et al.*, 1990). Fetuin, a major glycoprotein in FCS, inhibits zona pellucida hardening during mouse oocyte maturation by preventing the proteolytic conversion of ZP2 to $ZP2_f$ (Schroeder *et al.*, 1991). The pig zona pellucida undergoes an increase in protease resistance during fertilization and this transformation is believed to result from the decrease in a 90 kDa protein of the ZP2 family following the cortical granule release (Hatanaka *et al.*, 1992). A similar sensitivity to hardening could explain the results of Galeati *et al.* (1991) with denuded oocytes during maturation, as mentioned above. The importance of the blocking procedure is known to be essential in mammals, but in birds, for example, the female pronucleus associates with only one of the many male pronuclei formed by polyspermic fertilization (for a review, see Cran and Esper, 1990).

From the above studies, it is clear that the oocyte plays a role in ensuring a normal fertilization, but the capacity to respond to the first sperm cell may be different from the capacity not to respond to a second sperm cell encountered a few minutes later. As reported earlier, the capacity to release cortical granule content may be impaired in culture conditions, but the number of spermatozoa and the physiological status of spermatozoa are factors that are potentially different during *in vitro* culture. Very few *in vivo* studies have addressed the effect of the oocyte on polyspermy. Hancock and Buttle (1968) used hCG to induce premature ovulation and observed a decrease in the number of blastocysts and an increase in the number of unfertilized oocytes. In this study, it was suggested that the problem was the incapacity of the oviduct to provide adequate handling of the ovulated ova. If spermatozoa and oocytes were mixed *in vitro* for 4 h before transfer to oviducts, only 33% of the recovered oocytes were penetrated (Baker and Polge, 1976). *In vitro* fertilization or fertilization in oviducts maintained in organ culture of these mature

Table 5. Variations observed among different boars using fresh spermatozoa

Male	Date	Oocytes (*n*)	Penetration rate (spermatozoa) (%)	Polyspermy rate (% penetrated)	2 Pronuclei only of penetrated (%)
Boar 1	6 Jan 1993	33	9	50	0
Boar 2		24	43	0	31
Boar 3	13 Jan 1993	33	6	0	50
Boar 4		37	62	78	9
3 + 4	Washed only	37	78	76	17
3 + 4	Percoll[a]	35	86	93	3

[a]The spermatozoa of both boars were combined following a Percoll (65–70%) separation.

follicular or tubal oocytes did not lead to normal fertilization or successful development (Baker and Polge, 1976; Hunter, 1990), even if a large number of sperm cells did attach to the zona pellucida. These results, taken together, indicate a possible role of the oviduct in promoting normal fertilization or, alternatively, an inadequate medium for interaction of pig gametes *in vitro*.

The effect of sperm treatment on in vitro *fertilization and polyspermy*

In addition to the polyspermy problem encountered with *in vitro* fertilization, it is common to observe large variations from individual to individual in terms of penetration and rates of polyspermy. For example, two experiments from our laboratory are described (Table 5). The results obtained illustrate the large variations among boars, but we have also observed variations between ejaculates from the same boar (results not shown). A simple solution to remove some of the observed variations is to combine the spermatozoa of two individuals, as shown in Table 5, but this type of approach normally results in nearly 100% polyspermy even at reduced sperm concentration.

Different approaches have been reported for manipulating the male gamete variables: sperm source, sperm number, separation procedure, capacitation status and interaction with oviduct cells *in vitro* (Table 6). Both ejaculated and epididymal spermatozoa have been used *in vitro* and resulted in piglets (Cheng *et al.*, 1986; Nagai *et al.*, 1988; Table 2). Percoll gradient centrifugation had been successfully used to separate highly motile spermatozoa from fresh boar semen with some reduction of the polyspermy, and piglets have been produced following embryo transfer (Mattioli *et al.*, 1989). However, polyspermy is again high compared with *in vivo* fertilization or *in vitro* fertilization in other species (Parrish *et al.*, 1986). Initially Nagai *et al.* (1988) compared ejaculated with epididymal spermatozoa following freezing and thawing and only epididymal spermatozoa resulted in significant penetration rates (Table 6). More recently, Wang *et al.* (1991) successfully obtained *in vitro* penetration with frozen–thawed ejaculated boar spermatozoa, but the concentration of spermatozoa needed to achieve a high penetration rate was 25×10^6 cells ml^{-1} and no preincubation was used, in contrast to the experiment of Nagai and co-workers mentioned above. The principal explanation for such a large number of cells is related to the low motility (5–20%) of cells at thawing. Large variations from individual males were noted in both survival and penetration rates in these experiments as mentioned earlier for fresh spermatozoa. The quality of frozen semen is influenced not only by the donor but also, to a greater degree, by the freezing procedure used. Variations in cooling velocity, thawing velocity and cryoprotectant concentration may greatly influence the survival of spermatozoa after thawing. When optimal cooling and warming rates are used, increasing the glycerol concentration improved motility, but, at the same time, the percentage of spermatozoa with a normal apical ridge gradually decreases (Fiser and Fairfull, 1990). Different glycerol concentrations, separation procedures and sperm concentrations were compared in our laboratory (Fig. 1). The concentration of glycerol did not significantly influence the normal apical ridge rate after thawing, but the

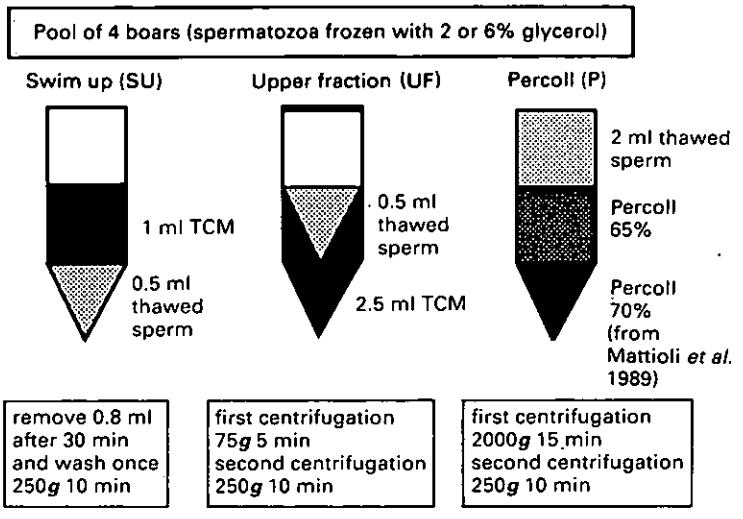

Fig. 1. The different separation procedures used in a first series of experiments with ejaculated frozen–thawed spermatozoa. The cells were thawed and prepared as described before using a swim-up method or using different centrifugation parameters or medium.

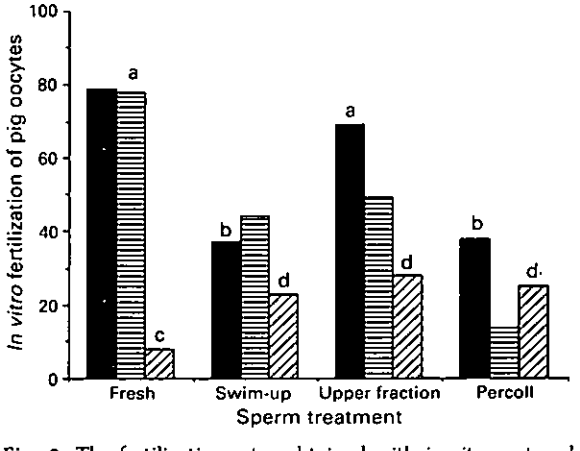

Fig. 2. The fertilization rates obtained with *in vitro* matured pig oocytes using the separation procedures described in Fig. 1 and a minimum of three replicates and 75 oocytes per treatment. Different letters indicate significant differences ($P < 0.05$) among treatments for each variable measured: (■) penetration, (▤) polyspermy and (▨) two pronuclei. Two pronuclei represent a normal fertilization (no additional sperm heads).

separation procedure did (Zheng *et al.*, 1992). Our first attempt to use Percoll to increase the rate of motile spermatozoa following thawing was inspired from the method of Mattioli *et al.* (1989) (Fig. 1). Use of differential centrifugation with or without Percoll resulted in an increase in the acrosome integrity compared with simple washing or swim up technique as used in cattle (Parrish *et al.*, 1986). Penetration rates reflected the normal apical ridge rates (Zheng *et al.*, 1992) and polyspermy was reduced with Percoll and swim up (Fig. 2). In this first series of experiments, the upper fraction method seemed to be the most

Table 6. The effect of sperm treatment on *in vitro* fertilization and polyspermy in pigs

Culture time (h)	Culture system	Sperm preparation	Penetration rate (spermatozoa) (%)	Polyspermy rate (% penetrated)	Male + female pronucleus (%)	Reference
32	TCM-199	(Epididymal)	42 (frozen)	39	54	Nagai *et al.*, 1988
	Serum + LH	(Ejaculated)	0 (frozen)	0	0	
44	Everted follicle	Percoll 65–70%	78 (fresh)	53	—	Mattioli *et al.*, 1989
	Serum + LH					
48	TCM-199	No pre-incubation	89 (fresh)	81	—	Nagai and Moor, 1990
	Serum + Gn	+oviduct[a] 1 h	94 (fresh)	71	—	
	Follicle cells	+oviduct[a] 2.5 h	84 (fresh)	53	—	
		+oviduct[a] 3.5 h	19 (fresh)	14	—	
36 h	TCM-199	6×10^6	12 (frozen)	40	3	Wang *et al.*, 1991
	Serum + Gn	12×10^6	43 (frozen)	53	16	
		25×10^6	88 (frozen)	62	32	

[a]In presence of oviduct cells in suspension.

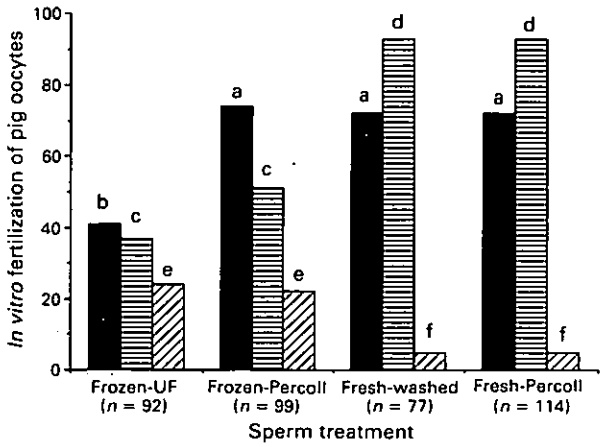

Fig. 3. The fertilization rates obtained with *in vitro* matured pig oocytes using the new separation procedures described in the text and a minimum of three replicates and 77 oocytes per treatment. Different letters indicate significant differences ($P < 0{\cdot}05$) among treatments for each variable measured: (■) penetration, (▤) polyspermy and (▨) two pronuclei. Two pronuclei represent a normal fertilization (no additional sperm heads). UF: upper fraction (see Fig. 1).

effective procedure with respect to oocyte penetration, and although the Percoll gradient appears to be the method of choice because of a low incidence of polyspermy, the total number of oocytes obtained with two pronuclei was highest with the upper fraction method using either 2 or 6% glycerol (Fig. 2). In a more recent experiment, a different concentration of glycerol was used (4% instead of 2 or 6% as in the first experiments) and we investigated the reasons why the motility was not improved as expected with Percoll. With numerous preliminary experiments, we realized that the highest motile fraction using Percoll, 65–70%, was at the bottom, in the pellet. A second series of experiments were therefore performed to evaluate this new separation procedure with fresh or frozen–thawed ejaculated boar spermatozoa. The results (Fig. 3) indicate a penetration rate for frozen–thawed spermatozoa at the same level as fresh spermatozoa using 10^6 motile spermatozoa. Since motility after these new procedures is close to 50%, the normal concentration of spermatozoa is about 2×10^6 (similar to fresh spermatozoa). These results also show that polyspermy is much lower in these conditions with frozen–thawed spermatozoa than with fresh spermatozoa. At this concentration of spermatozoa, the Percoll separation of fresh spermatozoa is not very useful, but it might make a difference at a lower concentration as reported by Mattioli *et al.* (1989). The penetration rate with the upper fraction procedure is lower than in the first series of experiments and there are at least two possible explanations: the different males used or the difference in the glycerol concentration. These results also indicate that boar spermatozoa are very heterogeneous and the separation procedures affect results. Sperm selection also occurs *in vivo* and the oviduct may provide clues about how to separate the male gametes.

Co-culture of oviduct cells can be used to recreate the appropriate conditions for a normal fertilization, since they are easy to prepare and show high survival rates in sheep (Gandolfi and Moor, 1987) and in cattle (Eyestone and First, 1989). Nagai and Moor (1990) used a similar procedure as in sheep to prepare cells to be added to sperm prior (0 to 3·5 h) to fertilization (Table 6). An increase in exposure time reduced polyspermy at a greater rate than penetration indicating a potential regulatory function in these cells. The importance of oviduct secretions and motility on the control of polyspermy has been studied and reviewed in detail by Hunter (1973, 1976, 1990, 1991). An abnormal number of sperm cells at the site of fertilization induces polyspermy, reflecting the sensitivity of this species to critical sperm:oocyte ratios (Hunter, 1973). This cannot be explained by abnormal migration of cortical

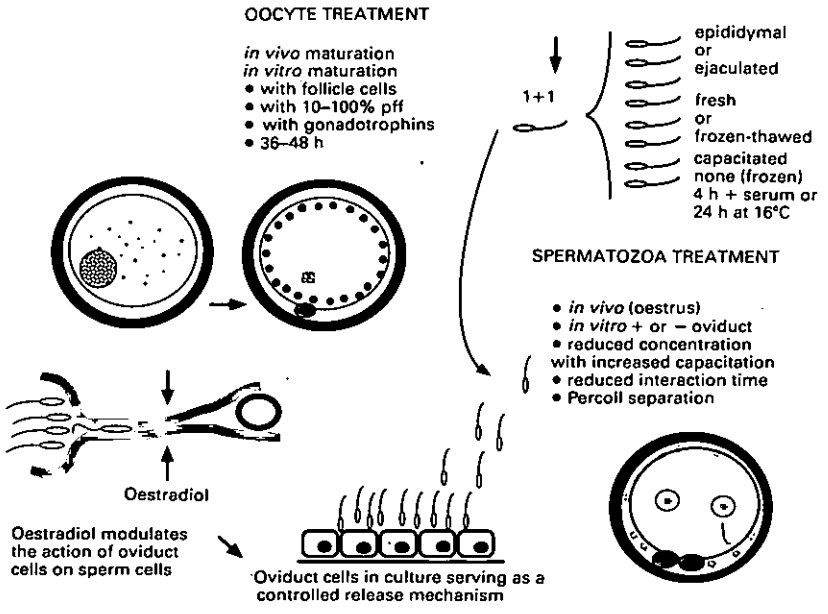

Fig. 4. The conditions for optimal *in vitro* fertilization in pigs. A normal cytoplasmic maturation in the presence of follicular factors ensures a better response to spermatozoa in terms of pronucleus formation. Different types of spermatozoa can be used but repeatability is high with a pool of either fresh or frozen spermatozoa from different boars. The treatment or pretreatment of spermatozoa with oviduct cells or follicular fluid may decrease polyspermy, but investigations to decrease fertilization time with highly capacitated spermatozoa might provide new methods to achieve the same objective.

granules, since contralateral oocytes were normally fertilized. It would seem that time is critical in regulating the number of spermatozoa entering the oocyte and a large number of spermatozoa at the site of fertilization would decrease the time between the first encounter and the second or the third and not leave sufficient time to properly install the blocking procedure. The oviduct could regulate the state of capacitation and the number of spermatozoa coming from the uterus, to ensure that the right conditions are present at the fertilization site. It is known that oviduct cells can bind to spermatozoa under the influence of oestradiol and can release motile spermatozoa about 44 h later. This capacity is observed more readily with tissues from the isthmus than from the ampulla (Raychoudhury and Suarez, 1991). This capacity of oviduct cells is maintained when cells are cultured as vesicles in suspension, as used for co-culture with embryos in cattle (Sirard, unpublished and Gagné *et al.*, 1991) or as monolayers (Pollard *et al.*, 1991). It has been shown that oviductal incubation changes the phospholipids of boar spermatozoa, mainly by increasing phosphatidylinositol content (Snider and Clegg, 1975). It is also known that the oviduct secretes a viscous product at oestrus which would trap sperm cells in the isthmus and indirectly act against polyspermy. This product would be present in smaller quantities during the luteal phase or in progesterone-treated females, resulting in higher polyspermy rates on those occasions (Hunter, 1991). Is the adverse hormonal environment affecting the oocytes or the spermatozoa? There is a possible effect of the oviductal protein on the zona itself as two new radiolabelled glycoproteins can be observed in the zona of ovulated oocytes versus follicular oocytes (Brown and Cheng, 1986). Reciprocal transfer indicated that the observed effect was on the spermatozoa and not on the oocytes, as eggs ovulated during the luteal phase transferred to recently ovulated animals resulted in normal fertilization (Hunter, 1991). The implication of the different factors involved in normal fertilization in pigs are summarized (Fig. 4).

Conclusion

The high polyspermy rates encountered in pigs are probably the result of a precarious equilibrium between the number of spermatozoa released in the ampulla and the speed of reaction of the oocyte to the first cell fusing with the plasma membrane. The vulnerability of individual oocytes to polyspermy is variable *in vivo* as one out of three oocytes will be affected if an excess of spermatozoa is present or if ageing of the oocyte is induced. This variability is amplified during *in vitro* culture and factors present in the follicle are acting during the maturation process both to decrease polyspermy and to enhance normal sperm decondensation. The use of oviductal cells *in vitro* might help to regulate a normal interaction between gametes, by increasing capacitation through specific binding and by the same way limiting the concentration of spermatozoa available per unit of time.

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