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Polyethylene glycol-induced Attachment of Human Spermatozoa to Zona-free Rat Ova *in vitro*

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Abstract

When human spermatozoa are incubated with zona-free ova in the presence of polyethylene glycol (PEG), the spermatozoa attach to the ova. Although the binding occurs when bovine serum albumin (BSA) is omitted from the incubation medium, the spermatozoa are better maintained and bind in higher numbers in the presence of both PEG and BSA. No spermatozoa bind to zona-intact ova or to zona-free ova in the absence of PEG. The number of spermatozoa bound to ova is dependent on the concentration of PEG (optimum between 10 and 15% w/v), time of incubation (increases linearly) and concentration of spermatozoa (increases linearly).

Introduction

The paucity of living human ova preclude their use in extensive studies of human fertilization, although non-living ova collected from cadavers have been used to assess whether human spermatozoa are capable of binding to and penetrating the zona pellucida of the human ovum (Overstreet and Hembree 1976). It would be useful, however, to have a means of assessing the fertilizing capacity of human spermatozoa. Zona-free hamster ova have recently been used to test the fertilizing capacity of human spermatozoa (Yanagimachi *et al.* 1976; Barros *et al.* 1978), but this test cannot be widely applied because the hamster is not yet available in some countries or laboratories. The ability of human spermatozoa to fuse with zona-free rat and mouse ova has been evaluated but no evidence of fusion and penetration of human spermatozoa into the rodent ova was found (Quinn 1979). In view of the reports of virus-assisted fusion of mammalian gametes (Ericsson *et al.* 1971; Lin and Glass 1975), the present study was undertaken to assess the ability of polyethylene glycol (PEG), which has now largely replaced the use of inactivated Sendai virus in cell fusion (reviewed by Lucy 1977), to induce the attachment of human spermatozoa to rat ova.

Materials and Methods

Spermatozoa

A highly motile sample of human spermatozoa was prepared by overlying neat, liquefied semen with Whitten's medium (WM_1) containing purified bovine serum albumin (BSA), as previously described (Quinn 1979; Quinn and Stanger 1980). The numbers of spermatozoa were counted using a haemocytometer and the motility of each sample was assessed in WM_1 medium by using a phase-contrast microscope. More than 98% of the spermatozoa collected by the overlay method showed vigorous forward motility. The concentration of motile spermatozoa was adjusted to 5×10^6/ml by diluting with fresh WM_1 medium.

Ova

Prepubertal Wistar albino rats (3–6 weeks old) were maintained and exposed to light from 0730–2130 h daily and induced to superovulate by intraperitoneal injections of 10 i.u. pregnant mare serum gonadotrophin (Folligon, Intervet, Artarmon, N.S.W.), and human chorionic gonadotrophin (hCG; Chorulon, Intervet) given 46–50 h apart. The oviducts were removed from the females 14–19½ h after the hCG injection and the swollen ampullae punctured to release the cumulus masses into WM_1 medium containing 300 units/ml of hyaluronoglucosaminidase (hyaluronidase, EC 3.2.1.35, bovine testes, Type 1, Sigma Chemical Co., St Louis, Mo.). After 10–20 min, ova had lost most of their cumulus cells and were washed twice (1 ml per wash) in fresh WM_1 medium. For removal of the zona pellucida, ova were placed in WM_1 medium containing 0·1–0·2% (w/v) pronase (B grade, Calbiochem Pty Ltd, Carlingford, N.S.W.) and 3 mg/ml BSA for 2–5 min. At the end of this time most of the ova had a considerably thinned zona which could be easily removed by rapid pipetting in a narrow-bore pipette. The zona-free ova were then washed twice (1 ml per wash) in fresh medium.

Media

All glassware, instruments and media were prepared as described previously (Quinn and Harlow 1978). The BSA in the WM_1 medium was purified by the procedure of Lui *et al.* (1977) and has been found to maintain the motility and integrity of human spermatozoa for prolonged periods, even in the presence of seminal plasma (Quinn and Stanger 1980). Medium containing PEG was prepared by making a 1 : 1 (v/v) dilution of double-strength WM_1 medium with a double-strength solution of the appropriate concentration of PEG (Sigma or Koch-Light 6000 mol. wt fraction) which had been sterilized by autoclaving.

Incubation Procedures and Assessment of Results

Washed ova were placed on watch-glasses in 0·5 ml of medium overlayed with 2 ml of equilibrated paraffin oil (Fisher Lightweight, Saybolt viscosity 125/135, New Jersey) and an aliquot of sperm preparation (10–100 μl), depending on the concentration of spermatozoa required, was then added. The watch-glasses containing the gametes were shaken (60 rpm) on a gyrorotator shaker (New Brunswick Scientific Co., New Jersey) at 37°C in an anaerobic jar gassed with 5% O_2 : 5% CO_2 : 90% N_2 (Hoppe and Pitts 1973).

At the completion of incubation, which continued for up to 3 h in some experiments, the motility of the spermatozoa was assessed on a scale from 0 (immotile) to +++ (highly motile). The ova were removed in a minimal volume of medium and washed thoroughly in two changes of fresh WM_1 medium (1 ml per wash), devoid of PEG, to remove accompanying and weakly adherent spermatozoa (Inoue and Wolfe 1975). The number of spermatozoa firmly bound to the ova was then counted by mounting the ova on a slide, covering with a square coverslip supported by a thin smear of Vaseline along two parallel sides, and observing under a phase-contrast microscope. Further confirmation of the numbers of spermatozoa bound was obtained in some instances by observing the ova under a Normarski interference-contrast microscope and also by staining the ova with lacto-aceto-orcein (Toyoda and Chang 1974). The stained ova were also examined for the presence of pronuclei but these were never observed.

The results were analysed by analysis of variance of the unweighted means (Keppel 1973).

Results

Throughout this study, human spermatozoa were never observed to bind to zona-free rat ova in the absence of PEG (see appropriate controls, Tables 1, 2 and 3), or to zona-intact ova, whether or not these ova were incubated in medium containing PEG.

The time course of binding of human spermatozoa to zona-free rat ova in different concentrations of PEG is given in Table 1, The highest concentration of PEG tested (90% w/v) caused precipitation of the albumin from the medium and the gametes were immediately lost from view in the very viscous and opaque medium. Within

Table 1. Attachment of human spermatozoa to zona-free rat ova *in vitro* in the presence of polyethylene glycol (PEG) at various times from the commencement of incubation

Combined data from two to three experiments presented. Medium contained bovine serum albumin at a concentration of 3 mg/ml and 100 000 spermatozoa in all cases

Time (h)	PEG concn (% w/v)	Spermatozoal motility	n/N [A]	Spermatozoa per ovum[B]
0·5	0	+++	13/13(100)	0(0)
	10	++ to +++	16/17(94)	0(0)
	30	+ to ++	12/14(86)	1·3(0–4)
	90	—[C]	—[C]	—[C]
1	0	+++	13/13(100)	0(0)
	10	++ to +++	17/18(94)	0·1(0–1)
	30	+ to ++	16/16(100)	2·1(0–7)
	90	—[C]	—[C]	—[C]
3	0	+++	11/11(100)	0(0)
	10	+++	16/18(89)	0(0)
	30	+	11/15(73)	10·6(1–20)
	90	—[C]	—[C]	—[C]

[A] n, number of ova recovered at end of incubation; N, number of ova incubated. Ratio expressed as a percentage given in parentheses.
[B] Range of values given in parentheses.
[C] Precipitation of medium; no observations possible.

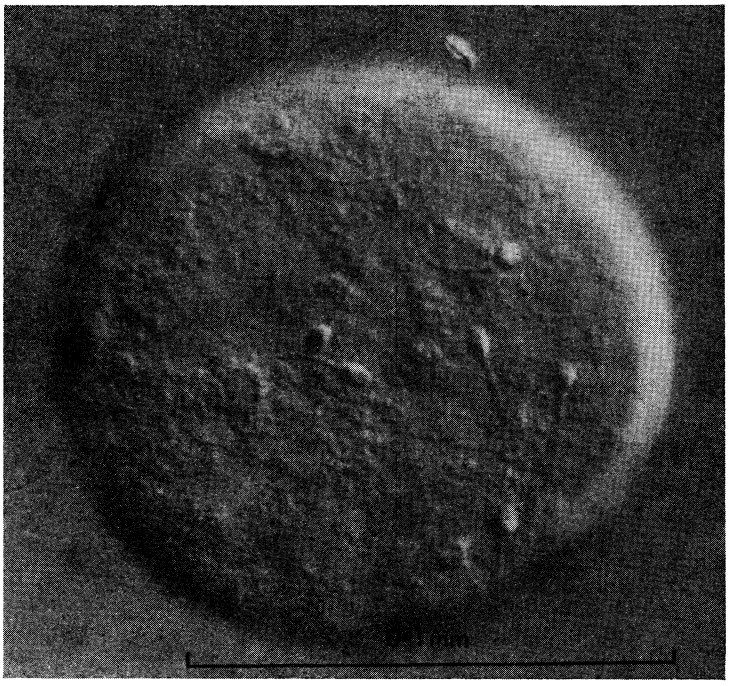

Fig. 1. Human spermatozoa attached to the surface of a zona-free rat ovum after 1 h incubation in medium containing 30% (w/v) polyethylene glycol. Normarski–interference optics.

30 min from the start of incubation, however, some spermatozoa were bound to ova in the presence of 30% (w/v) PEG (Fig. 1). The spermatozoa were mainly bound by their heads, but on occasions, a spermatozoon would become attached by its tail. Some spermatozoa attached to the vitelline surface and became relatively immotile in a short period, as occurs in normal intra-species fertilization of zona-free ova (see Yanagimachi 1977). Other spermatozoa, however, maintained a vigorous thrashing movement whether they had become attached to the ova by their heads or tails. Even though there was a progressive decline in the motility of the spermatozoa with increasing PEG concentration and incubation time, the number of spermatozoa bound to ova increased significantly ($P < 0{\cdot}001$) with both increasing PEG concentration and incubation time.

Table 2. Attachment of human spermatozoa to zona-free rat ova in the presence or absence of PEG (15% w/v) and bovine serum albumin (BSA 3 mg/ml) after 1 or 3 h incubation in the presence of 500 000 spermatozoa

Combined data from two to three experiments

Time after commencement of incubation (h)	Presence of PEG	Presence of BSA	Spermatozoal motility	n/N[A]	Spermatozoa per ovum[B]
1	−	+		Not tested	
	+	−	+	14/23(61)	2·0(0–7)
	+	+	++	22/23(96)	3·3(0–10)
3	−	+	+++	27/31(87)	0(0)
	+	−	+	5/16(31)	0·4(0–1)
	+	+	++	20/23(87)	9·7(1–26)

[A] See Table 1 for explanation of symbols.
[B] Range of values given in parentheses.

From Table 2, it can be seen that human spermatozoa bound to the zona-free rat ova even when the medium did not contain BSA. A significantly ($0{\cdot}05 < P < 0{\cdot}025$) greater number of spermatozoa bound to ova, however, when the medium contained both PEG (15% w/v) and BSA (3 mg/ml) and this medium also maintained spermatozoal motility better than the medium containing PEG but devoid of BSA. As observed in all media containing PEG, many spermatozoa became agglutinated by their heads in a manner indistinguishable from that observed when human spermatozoa are incubated in serum from vasectomized males containing sperm-agglutinating activity (P. Quinn and H. Tinneberg, unpublished observations).

The effect of different concentrations of spermatozoa on the numbers of attached sperm per ovum in different concentrations of PEG at 1 and 3 h after the commencement of incubation is given in Table 3. Sperm attachment (0·9 per ovum) was observed within 1 h of incubation, even at the lowest sperm concentration (50×10^3 per 0·5 ml of incubation medium) at a PEG concentration of 15% (w/v). These conditions also permitted the recovery of 60% or more of ova at the end of the incubation period. With higher concentrations of PEG (20 and 30%, Tables 1 and 3), the ova often became firmly attached to the bottom of the watch glass and were subsequently lysed in attempts to free them. With these higher concentrations of PEG, even freed and washed ova occasionally degenerated on subsequent processing for microscopic

observations. A greater number of ova were usually recovered from the dishes containing 500×10^3 spermatozoa and this was probably due to the greater dilution of the PEG concentration on addition of the 100-μl aliquot of spermatozoa compared with the smaller dilution of PEG when only 10 μl of spermatozoa was added [e.g. 100 μl of 500×10^3 spermatozoa added to 0·5 ml of 15% (w/v) PEG would give a final concentration of 12·5% (w/v) PEG compared with 10 μl of 50×10^3 spermatozoa giving a final concentration of 14·7% (w/v) PEG]. This dilution of the PEG at the higher spermatozoal concentrations is also probably responsible for the improved motility of the spermatozoa in these dishes, especially at the end of 3 h of incubation.

Table 3. Effect of spermatozoal concentration at various times from the commencement of incubation on the attachment of human spermatozoa to zona-free rat ova *in vitro* in the presence of different concentrations of PEG

Medium contained BSA at a concentration of 3 mg/ml on all occasions. Combined data from two to three experiments

Time after commencement of incubation (h)	PEG concn (% w/v)	$10^{-3} \times$ No. of spermatozoa per 0·5 ml	Sperm-atozoal motility	n/N[A]	Spermatozoa per ovum[B]
1	0	500		n.t.[C]	
	10	50		n.t.	
		100		n.t.	
		500		n.t.	
	15	50	+	34/42(81)	0·9(0–3)
		100	++	26/43(60)	2·0(0–7)
		500	++	27/35(77)	4·1(0–16)
	20	50		n.t.	
		100		n.t.	
		500		n.t.	
3	0	500	+++	27/31(87)	0(0)
	10	50	++	24/25(96)	0(0)
		100	++	23/25(92)	0·1(0–1)
		500	+++	47/48(98)	0·04(0–2)
	15	50	+	22/39(56)	3·3(0–9)
		100	+	23/42(55)	6·0(0–17)
		500	++	30/35(86)	9·4(0–29)
	20	50	+	10/25(40)	0·5(0–2)
		100	+	6/25(24)	3·5(0–7)
		500	+	6/25(24)	12·2(7–19)

[A] See Table 1 for explanation of symbols.
[B] Range of values given in parentheses.
[C] n.t., not tested.

The results in Table 3 indicate that with increased spermatozoal concentration, more spermatozoa were bound at all concentrations of PEG tested and also when the time of incubation was increased from 1 to 3 h in 15% (w/v) PEG. In 15% (w/v) PEG, the increase in spermatozoa bound per ovum with time of incubation was almost directly proportional whereas the increase with increasing spermatozoal concentration began to plateau at the level of 500×10^3 spermatozoa per dish.

It would appear from these results that human spermatozoa can be induced to attach to zona-free rat ova in medium containing 10–15% (w/v) PEG. The average

number of sperm bound per ovum can be varied depending on the concentration of spermatozoa incubated and also the length of incubation.

Discussion

The present observation that polyethylene glycol induces the attachment of human spermatozoa to zona-free rat ova complements the observations of Ericsson *et al.* (1971) and Lin and Glass (1975) of the virus-assisted fusion of mammalian gametes. Lin and Glass (1975) suggested that differences in the relative sizes of spermatozoan heads and the microvilli on the surface of the vitelline membrane may be partly responsible for the differences in the success of cross-species fertilization in mammals. These authors observed that treatment of mouse ova with Newcastle disease virus caused the elongation of the microvilli on the vitelline surface and suggested this as the reason for the improved attachment of hamster spermatozoa to virus-treated, zona-free, mouse ova. Yanagimachi (1977, 1978), however, has proposed that the cortical granule material released upon activation of the ovum may remove the sperm-binding sites from either the vitelline membrane surface or the zona pellucida. Thus, in the hamster, most of these sperm-binding sites are on the surface of the zona and this is probably largely responsible for the ease of cross-species fertilization of zona-free hamster ova (see Hanada and Chang 1978), whereas in the mouse and rat, sperm-binding sites are located on the surface of the vitelline membrane as well as the zona. These differences between species are therefore most likely to be responsible for some of the variations observed in the success of cross-species fertilization, particularly, the inability of human spermatozoa to bind to the vitelline surface of the zona-free mouse and rat ovum (Quinn 1979). Reports of the penetration of human spermatozoa into murine zona-free ova (Aitken 1979) should therefore be viewed with caution, especially if frozen human semen is used. The cryoprotectant agents in the freezing mixtures for spermatozoa, such as glycerol and dimethyl sulfoxide, may be responsible for the attachment of the human spermatozoa to these ova (A. O. Trounson, personal communication). This may also occur if a complex tissue culture medium, such as M199, is used to capacitate human spermatozoa in the presence of mouse zona-free oocytes (Aitken 1979) and in which the fusogenic action of some of the chemical components, such as cholesterol, has not yet been fully evaluated.

Although the penetration of spermatozoa into somatic cells has been observed to occur spontaneously (Johnson *et al.* 1970) and has also been induced using viruses (Bendich *et al.* 1974), reactivation of the spermatozoa has not generally occurred. When spermatozoa have penetrated heterologous zona-free ova, on the other hand, chromosomes of human spermatozoa have been obtained in hamster ova (Rudak *et al.* 1978) and cleavage of the hamster and rat ova to the two-cell stage has been obtained after penetration by human (Yanagimachi 1977) and mouse spermatozoa (Quinn 1979), respectively. The PEG-induced attachment of human spermatozoa to zona-free rat ova observed in the present study might also prove to be useful for this purpose if the sperm head nucleus becomes incorporated into the vitellus and decondenses normally. The rate of sperm head decondensation appears to be dependent on cytoplasmic factors in the egg which vary with different stages of the cell cycle (Yanagimachi 1977, 1978). Similarly, the induction of premature chromosome condensation can be induced in interphase cells fused with mitotic cells using either virus- or PEG-induced fusion (Lau *et al.* 1977). PEG-induced fusion of spermatozoa

with ova and embryos may provide a useful means of studying the phenomenon of sperm head decondensation and its control.

The action of chemical fusogens, such as PEG, in inducing cell membrane fusion is thought to occur by increasing the fluidity of the membranes (see Lucy 1975). The close similarity between the events of normal fertilization and the membrane fusion events which occurred when bull spermatozoa were induced to fuse with hen erythrocytes using glycerol monooleate has been noted (Lucy 1975). Because PEG is a powerful chemical fusogen, however, it could not be used to test the fertilizing capacity of human spermatozoa since it is likely that PEG would induce the attachment of even dead or non-motile spermatozoa to zona-free oocytes. The reason why human spermatozoa were never incorporated into the rat oocytes in the present study is unknown. Perhaps a higher concentration of PEG (e.g. 44% w/w) in the presence of dimethyl sulfoxide (Norwood *et al.* 1976) in medium devoid of calcium (Schneiderman *et al.* 1979) may achieve this end. Differences in the lipid composition of the cell membranes of the gametes would undoubtedly influence their fluidity (Shinitzky and Henkart 1979) and therefore their susceptibility to the action of fusogens. Further studies of the lipid composition of human and rodent gamete cell membranes and attempts to modify their composition may lead to a successful technique for inducing the fusion and incorporation of human spermatozoa into zona-free rodent ova.

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References

Aitken, R. J. (1979). Tubal and uterine secretions; the possibilities for contraceptive attack. *J. Reprod. Fertil.* **55**, 247–54.

Barros, C., Gonzales, J., Herrera, E., and Bustos-Obregon, E. (1978). Fertilizing capacity of human spermatozoa evaluated by actual penetration of foreign eggs. *Contraception* **17**, 87–92.

Bendich, A., Borenfreund, E., and Sternberg, S. S. (1974). Penetration of somatic mammalian cells by sperm. *Science* (Wash., D.C.) **183**, 857–9.

Ericsson, R. J., Buthala, D. A., and Norland, J. F. (1971). Fertilization of rabbit ova in vitro by sperm with adsorbed sendai virus. *Science* (Wash., D.C.) **173**, 54–5.

Hanada, A., and Chang, M. C. (1978). Penetration of the zona-free or intact eggs by foreign spermatozoa and the fertilization of deer mouse eggs in vitro. *J. Exp. Zool.* **203**, 277–86.

Hoppe, P. C., and Pitts, S. (1973). Fertilization in vitro and development of mouse ova. *Biol. Reprod.* **8**, 420–6.

Inoue, M., and Wolf, D. P. (1975). Sperm binding characteristics of the murine zona pellucida. *Biol. Reprod.* **13**, 340–6.

Johnson, R. T., Rao, P. N., and Hughes, H. D. (1970). Mammalian cell fusion. III. A Hela cell inducer of premature chromosome condensation active in cells from a variety of animal species. *J. Cell Physiol.* **76**, 151–8.

Keppel, G. (1973). 'Design and Analysis: A Researcher's Handbook.' Ch. 17. pp. 345–63. (Prentice-Hall: Englewood Cliffs.)

Lau, Y-F., Brown, R. I., and Arrighi, F. E. (1977). Induction of premature chromosome condensation in CHO cells fused with polyethylene glycol. *Exp. Cell Res.* **110**, 57–61.

Lin, T. P., and Glass, R. H. (1975). Interaction of male and female germ cells of two mammalian species in culture. *Som. Cell. Genet.* **1**, 209–14.

Lucy, J. A. (1975). Aspects of the fusion of cells in vitro without viruses. *J. Reprod. Fertil.* **44**, 193–205.

Lucy, J. A. (1977). Cell fusion. *Trends Biochem. Sci.* **2**, 17–20.

Lui, C. W., Cornett, L. E., and Meizel, S. (1977). Identification of the bovine follicular fluid protein involved in the in vitro induction of the hamster sperm acrosome reaction. *Biol Reprod.* **17**, 34–41.

Norwood, T. H., Zeigler, C. J., and Martin, G. M. (1976). Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. *Som. Cell Genet.* **2**, 263–70.

Overstreet, J. W., and Hembree, W. C. (1976). Penetration of the zona pellucida of nonliving human oocytes by human spermatozoa in vitro. *Fertil. Steril.* **27**, 815–31.

Quinn, P. (1979). Failure of human spermatozoa to penetrate zona-free mouse and rat ova in vitro. *J. Exp. Zool.* **210**, 497–506.

Quinn, P., and Harlow, G. M. (1978). The effect of oxygen on the development of preimplantation mouse embryos in vitro. *J. Exp. Zool.* **206**, 73–80.

Quinn, P., and Stanger, J. D. (1980). Effect of purification of bovine serum albumin on the interaction of human semen with mouse ova in vitro. *Biol. Reprod.* **22**, 134–40.

Rudak, E., Jacobs, P. A., and Yanagimachi, R. (1978). Direct analysis of the chromosome constitution of human spermatozoa. *Nature* (*London*) **274**, 911–13.

Schneiderman, S., Farber, J. L., and Baserga, R. (1979). A simple method for decreasing the toxicity of polyethylene glycol in mammalian cell hybridization. *Som. Cell. Genet.* **5**, 263–9.

Shinitzky, M., and Henkart, P. (1979). Fluidity of cell membranes—current concepts and trends. *Int. Rev. Cytol.* **60**, 121–47.

Toyoda, Y., and Chang, M. C. (1974). Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. *J. Reprod. Fertil.* **36**, 9–22.

Yanagimachi, R. (1977). Specificity of sperm–egg interaction. In 'Immunobiology of Gametes'. (Eds M. Edidin and M. H. Johnson.) pp. 255–95. (Cambridge University Press.)

Yanagimachi, R. (1978). Sperm–egg association in mammals. *Curr. Top. Dev. Biol.* **12**, 83–105.

Yanagimachi, R., Yanagimachi, H., and Rogers, B. J. (1976). The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol. Reprod.* **15**, 471–6.

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