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Effect of Human Seminal Plasma and Mouse Accessory Gland Extracts on Mouse Fertilization *in vitro*

P. Quinn[A] *and A. J. Begley*[B]

[A] Department of Obstetrics and Gynaecology, Queen Elizabeth Hospital, Woodville, S.A. 5011. (Address for reprints.)
[B] Department of Obstetrics and Gynaecology, Flinders University of South Australia, Bedford Park, S.A. 5042.

Abstract

The mouse *in vitro* fertilization system was used to investigate the effect of human seminal plasma (HSP) on the fertilizing ability of mouse spermatozoa. The addition of HSP to freshly collected mouse epididymal spermatozoa decreased their fertilizing ability to 30%, compared with 85–90% for control spermatozoa not exposed to HSP or for spermatozoa that had been exposed to the capacitating system for 55–145 min (which allowed capacitation to occur before the addition of HSP). Human seminal plasma from a vasectomized donor was more effective in retarding the acquisition of fertilizing ability in mouse spermatozoa than was seminal plasma from non-vasectomized donors. Fluid from the prostate glands and seminal vesicles of mice also reduced the fertilizing ability of freshly collected mouse spermatozoa to 30%.

Introduction

Successful completion of two sequential pre-fertilization events by mammalian sperm is essential for productive sperm–ova interactions. Capacitation, the first pre-fertilization event, was initially reported independently by Chang (1951) and Austin (1951), but is even now poorly understood. Capacitation, unlike the second pre-fertilization event, the acrosome reaction, is not characterized by drastic structural changes (Friend *et al.* 1977). Techniques involving sperm motility patterns (Yanagimachi 1970; Phillips 1972; Yanagimachi and Usui 1974; Fraser 1977), tetracycline binding capacity of sperm (Ericsson 1967; Dukelow and Chernoff 1969; Saling and Storey 1979), and sperm-binding characteristics with zona-intact and zona-free ova (Inoue and Wolf 1975; Wolf *et al.* 1976) and delayed insemination techniques (Austin 1951; Chang 1951) have been employed to ascertain the temporal requirements of capacitation in various mammalian species. By the use of these techniques, it has been estimated that capacitation of mouse spermatozoa occurs in *c.* 60 min (Braden and Austin 1954; Toyoda *et al.* 1971; Inoue and Wolf 1975; Wolf *et al.* 1976; Fraser 1977).

A distinguishing characteristic of capacitation, albeit putative, is its reversible inhibition by factors of seminal plasma origin (Chang 1957). It has been generally accepted, but not clearly stated, that decapacitation factors are entities of seminal plasma origin which reversibly return capacitated sperm to an uncapacitated state, as well as delaying the capacitation of uncapacitated sperm.

Decapacitation factors have been investigated with either *in vivo* or *in vitro* fertilization assays (Chang 1957; Dukelow *et al.* 1967; Pinsker and Williams 1968; Kanwar *et al.* 1979); but only two studies to date, Reddy *et al.* (1979) and Quinn *et al.* (1982*b*), have used the mouse *in vitro* fertilization assay to determine the presence of the decapacitation factors in human seminal plasma (HSP). However, the effect of HSP on mouse fertilization *in vitro* is still unresolved. Reddy *et al.* (1979) presented data indicating that HSP contains

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factors which reduce the fertilizing ability of capacitated mouse sperm, as would be expected if decapacitation factors were present in HSP. On the other hand, the work of Quinn *et al.* (1982*b*) suggests that HSP inhibits the fertilizing ability of uncapacitated, but not of capacitated, mouse sperm.

There were two aims of this study. First, to investigate the effects that HSP may have on the fertilizing ability of mouse sperm *in vitro* over time, and second, to determine if similar effects could be observed with extracts of male mouse accessory glands.

Materials and Methods

Female F_1 hybrid mice (C57BL × CBA), 4–6 weeks of age, were injected intraperitoneally with 7·5 i.u. of pregnant mare serum gonadotrophin (Folligon, Intervet, Sydney), and 48 h later they were injected intraperitoneally with 7·5 i.u. of human chorionic gonadotrophin (hCG, Chorulon, Intervet). Ova were obtained from mice 12–15 h after hCG injection. The oviducts were freed of connective and fatty tissue, excised, and then blotted to remove any blood. With the aid of a dissecting microscope, the wall of the ampullae was pierced with a 26-gauge needle to release the cumulus clot into high bicarbonate Tyrodes medium containing hyaluronoglucuronidase (E.C. 3.2.1.35, 300 i.u./ml., Sigma Chemical Co., St Louis, U.S.A., Quinn *et al.* 1982*a*), which was then incubated for 10 min at 37°C. Cumulus-free ova were collected and washed twice in high bicarbonate (52 mM) Tyrodes medium containing 15 mg/ml fraction V bovine serum albumin (V-BSA; Sigma, Prod. No. A 9647) to remove any remaining cumulus cells and the hyaluronoglucuronidase. The Tyrode's medium used was similar to the one described by Quinn *et al.* (1982*a*), except that the concentration of $NaHCO_3$ was increased to 52 mM and the concentration of NaCl reduced to 74·0 mM. The medium which had an osmolarity of 280–285 mOsmol/kg and a pH of about 7·5 when 15 mg/ml V-BSA was added, was incubated under an atmosphere of 5% CO_2 : 5% O_2 : 90% N_2. The ova were then placed in 0·5 ml of high bicarbonate Tyrodes medium in insemination dishes containing diluted samples of spermatozoa (see below). Only unfertilized ova of normal morphological appearance, as judged by the following criteria, were used. Firstly, the perivitelline space was uniform. Secondly, the cytoplasm was translucent and non-vacuolized. Thirdly, only a few, if any, cumulus cells remained attached to the ova after treatment with hyaluronoglucuronidase.

Spermatozoa were obtained from the cauda epididymides of two sexually mature F_1 fertile males. The left cauda epididymis from one male and the right epididymis from the other male were excised, blotted and immersed in 0·5 ml of the high bicaronate Tyrodes medium containing 15 mg V-BSA/ml, 10^{-3} M isoniazid (Sigma) (Quinn *et al.* 1982*b*) and pierced with a 26-gauge needle. Isoniazid was included in the medium to overcome any adverse effects of the interaction of spermine present in seminal plasma with diamine oxidase (E.C. 1.4.3.6) present in some preparations of BSA (Quinn *et al.* 1982*b*). The epididymides were gently squeezed with a pair of fine forceps to release the spermatozoa and then discarded. The spermatozoal suspension was incubated at 37°C for 10 min under a humidified atmosphere of 5% CO_2 : 5% O_2 : 90% N_2 to allow the spermatozoa to disperse. Sperm numbers were estimated using an haemocytometer and an aliquot was added to the insemination dishes so that the sperm concentration was 2×10^6/ml. The time required for the collection of a neat preparation of epididymal spermatozoa and the subsequent dilution of an aliquot into the insemination dishes was 20–25 min. As we consider that the spermatozoa begin capacitation immediately after epididymal release, the 20–25 min time lag was taken into account when estimating capacitation time.

Semen was collected from normal donors by masturbation into sterile containers and allowed to liquify at 37°C. After liquification was complete the semen was centrifuged at 1700 *g* for 20 min. The supernatant, free of spermatozoa, was aspirated and stored in a sterile container at −20°C until required. HSP from separate donors was used for different replicates to assay the inhibition of mouse fertilization *in vitro*. Seminal vesicles and prostate glands were removed from F_1 male mice and added to high bicarbonate Tyrodes medium containing isoniazid (1 mM). The volume of medium was then adjusted to give 5% (w/v) suspension of the tissues. The tissue was then hand-homogenized, centrifuged at 1000 *g* for 10 min to remove particulate matter, and the supernatant was filtered, sterilized and stored at −20°C in *c.* 1-ml aliquots. Extracts of mouse uterus and kidney were prepared and stored in a similar fashion.

In all experiments the fertilization medium was high bicarbonate Tyrodes medium containing 15 mg V-BSA/ml and 10^{-3} M isoniazid, overlaid with 3 ml of equilibrated paraffin oil (BDH, Poole). Either 5% (v/v) HSP from a vasectomized donor (vHSP), 5% HSP from a non-vasectomized donor (nvHSP), 5% murine prostate homogenate or 5% murine seminal vesicle homogenate was added to

epididymal sperm (i) 20–25 min after epididymal release or (ii) at 15, 30, 60 and 120 min later than this time. As a control, epididymal sperm which had been released from the epididymis 20–25 min earlier, were incubated a further 120 min. At this time 20–30 cumulus-free ova were added to all treatments and controls. The gametes were then incubated for 4–5 h, and the ova subsequently assessed for fertilization. Ova were considered to be fertilized when they contained a decondensing sperm head or a male pronucleus with accompanying sperm tail and the female chromosomes had recommenced meiosis or had progressed to the pronuclear stage. None of the substances added to the system adversely affected motility.

Data for percentage fertilization for each treatment were transformed to angles and analysed by analysis of variance. Difference between treatments were tested by the Student–Newman–Keuls multiple-range test (Zar 1974).

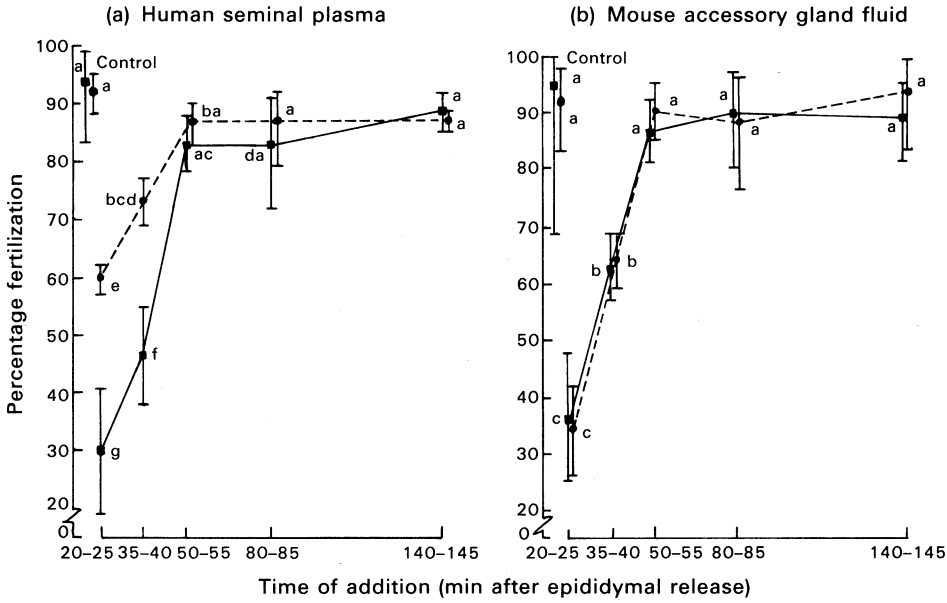

Fig. 1. Percentage fertilization of cumulus-free mouse ova by mouse spermatozoa (concentration, $2 \cdot 0 \times 10^6$/ml) treated at varying intervals with 5% (v/v) of either (*a*) vHSP (■——■) or nvHSP (●— —●), or (*b*) mouse seminal vesicle extract (■——■) or mouse prostrate extract (●— —●). Each point represents the mean of four (HSP) or three (mouse accessory gland fluid) replicates; the bars indicate ±s.e. In each replicate, HSP from a different donor was used. HSP and the extracts were added at the times indicated after sperm had been released from the cauda epididymis. Ova were added to all treatments and controls at 140–145 min p.e.r. All points showing the same letter are not significantly different ($P > 0 \cdot 05$), based on the Student–Newmans–Keuls multiple range test.

Results

The maximal anti-fertility effect of HSP occurred when 5% (v/v) HSP was added to a sperm suspension containing $2 \cdot 0 \times 10^6$ sperm/ml. The fertilizing ability of mouse spermatozoa was significantly ($P < 0 \cdot 05$) altered by both the type of HSP used and the time at which the spermatozoa were exposed to the HSP (Fig. 1). The effect of nvHSP on the fertilizing ability of the spermatozoa followed the same trends obtained with vHSP. Sperm that had been released from the epididymis for 50–55 min or longer before the addition of either vHSP or nvHSP had a similar ability to fertilize ova to that of the control spermatozoa not incubated with HSP. However, compared with the controls, epididymal spermatozoa which had been exposed to either vHSP or nvHSP at 20–25 and 30–40 min post epididymal release (p.e.r.) gave significantly reduced fertilization rates.

The vHSP added at 20–25 min and 35–40 min p.e.r. had significantly greater anti-fertility effects (30 and 47% fertilization respectively) than nvHSP added at these times (60 and 73% fertilization). The addition of vHSP and nvHSP to spermatozoa at 35–40 min p.e.r. significantly increased fertilization rates compared with addition at 20–25 min p.e.r. However, these increased fertilization rates were significantly lower than those of the controls (47 and 73% *v.* 94 and 92%).

There was no significant difference between the anti-fertility effects exerted by murine prostate and murine seminal vesicle homogenates (Fig. 1*b*). The temporal pattern resulting from these series of experiments was essentially the same as that observed when vHSP was tested. Control experiments using homogenized extracts of mouse uterus and of mouse kidney at 5% (v/v) showed that the fertilizing ability of mouse spermatozoa *in vitro* was not affected if these extracts were added to the spermatozoa at 20–25 min p.e.r. (91±4, 87±6% respectively; mean ± s.e., $n = 4$) or at 140–145 min p.e.r. (95±5, 93±5; $n = 4$).

Discussion

Bedford and Chang (1962) demonstrated that seminal plasma obtained from vasectomized male rabbits exerts an anti-fertility effect on the fertilizing ability of rabbit sperm *in vivo*. However, until now, a direct comparison of the relative potencies of seminal plasma from vasectomized and non-vasectomized donors has not been carried out, either in the rabbit or in the human. Both vHSP and nvHSP lower the fertilizing capacity of uncapacitated but not capacitated mouse sperm. However, nvHSP is not as potent as vHSP in its anti-fertility effect on uncapacitated spermatozoa. The lower potency may be due to reduction of anti-fertility agents in nvHSP by freshly ejaculated human spermatozoa. The ability of the mouse *in vitro* fertilization system to discern a difference in the anti-fertility effect exerted by nvHSP and vHSP indicates that the mouse *in vitro* fertilization system may be applicable to various cases of unexplained male infertility and thus form the basis of an infertility assay. Further work on other types of HSP from various male infertility classes is being carried out in this laboratory to develop such an assay.

This study clearly indicates that factors in HSP and male mouse accessory gland homogenate (AGH) affect the fertilizing ability of mouse sperm in a time-dependent manner. In the control experiments, spermatozoa fertilized 85–90% of the eggs present, demonstrating that these spermatozoa had undergone capacitation successfully. Thus it is assumed that a fertilization rate of 85–90%, in this system, indicates a successfully capacitated sperm suspension. The fertilizing ability of epididymal sperm exposed to a capacitating environment for 50–55 min or longer, was not adversely affected by the presence of either HSP or AGH. Hence it can be assumed that these sperm were successfully capacitated. This estimation of mouse capacitation time is in good agreement with previous estimates which have indicated a time of *c.* 60 min (Braden and Austin 1954; Toyada *et al.* 1971; Inoue and Wolf 1975; Wolf *et al.* 1976; Fraser 1977). Figs 1*a* and 1*b* clearly demonstrate that factors of seminal plasma (human) or accessory gland (mouse) origin do not alter the fertilizing ability of the sperm suspension if the suspension is maximally capacitated. Before this time (i.e. 50–55 min p.e.r.) these factors influence sperm fertilizing ability. This technique, therefore, should provide a useful assay of the capacitation time of mouse epididymal spermatozoa *in vitro* under various experimental conditions. For instance, calcium could be omitted from the pre-incubation medium for 60 min and then the capacitation time of spermatozoa assayed by adding HSP at various times thereafter before the addition of ova. The fact that mouse sperm, once capacitated, are not susceptible to the detrimental effects of HSP or AGH, infers that decapacitation factors, as presently defined, are absent from HSP or AGH.

It is generally accepted that sperm undergo capacitation and the acrosome reaction in an asynchronous manner (Dzuik 1965; Lewis and Ketchel 1972; Rogers and Morton 1973; Fraser 1981). Thus the anti-fertility effect exerted by HSP and AGH added to sperm that

had been released from the epididymis and exposed to a capacitating environment for less than 50–55 min may be a measure of the proportion of sperm in the suspension that are already capacitated.

Previous studies supporting the presence of decapacitation factors in HSP have utilized the rabbit *in vivo* fertilization assay (Chang 1957; Dukelow *et al.* 1967; Pinsker and Williams 1968) and have indicated that the anti-fertility effect of HSP is reversible (Pinsker and Williams 1968). The advantages of using *in vitro* fertilization systems are the reduction of confounding effects and the ability to evaluate many ova at one time. Reddy *et al.* (1979), using the mouse *in vitro* fertilization system, presented evidence of the presence of decapacitation factors in HSP. However, the present study, as well as work by Kanwar *et al.* (1979), Quinn *et al.* (1982*b*), and Hyne and Garbers (1982), which also employed *in vitro* techniques, indicate that there are factors of seminal plasma origin which affect uncapacitated but not capacitated sperm. The presence of such factors may not be discernible in *in vivo* fertilization systems owing to the protective effect of the female reproductive tract on spermatozoa, which allows fertilization to occur at sperm/egg ratios close to unity (Shalgi and Kraicer 1978).

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