The effects of follicular fluid from patients with different indications for IVF treatment on the binding of human spermatozoa to the zona pellucida

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This study compared the effects of human follicular fluid (hFF) from women with endometriosis, tubal factor and male factor on the zona binding capacity of human spermatozoa. Samples of hFF were collected from 30 patients, 10 patients for each of the indications of infertility, at the time of oocyte retrieval in an in-vitro fertilization/embryo transfer programme. The hemizona binding assay (HZA) was used to assess the effect of these hFF on the zona binding potential of human spermatozoa. The mean numbers of spermatozoa bound to the zona pellucida after treating the spermatozoa with hFF from endometriosis, tubal factor and male factor were 90.5 ± 20.9, 108.9 ± 22.3 and 101.2 ± 13.4 respectively. These were significantly lower than their corresponding controls, the spermatozoa of which were incubated with Earle’s balanced salt solution (endometriosis 238.7 ± 34.7; tubal factor 210.8 ± 41.6; male factor 205.4 ± 26.3; P <0.002). The hemizona binding index (HZI) was similar between male factor samples (52.0 ± 6.7) and tubal factor samples (53.8 ± 4.2). Spermatozoa incubated with hFF from endometriosis patients (36.0 ± 4.1) had an HZI that was significantly lower than those treated with hFF from tubal factor patients (P <0.01). Probably due to small sample size, the differences in HZI between endometriosis samples and male factor samples did not reach statistical significance (P = 0.076). These data suggest that there was a stronger sperm–zona binding inhibitory effect of hFF from patients with endometriosis than from those without the disease. Key words: endometriosis/follicular fluid/hemizona binding assay/male factor/tubal factor

Materials and methods

Collection of hFF
Human follicular fluid was obtained during oocyte retrieval from 30 women attending our assisted reproduction programme; they were aged 25–35 years. Ten of them were regular cycling women with endometriosis. These women had superficial endometriosis on either the peritoneum or the ovaries, with or without filmy adhesion of the ovaries or the Fallopian tubes. All the cases of endometriosis were stage I according to the American Fertility Society criteria. The remaining patients were 10 with tubal factor and 10 with male factor as the only indication for infertility. Eight patients with tubal factor had blocked tube, while the other two had dense tubal adhesion. All the wives of the patients with male factor infertility were found to be normal after diagnostic laparoscopy.

Gonadotrophin-releasing hormone (GnRH) analogues, human menopausal gonadotrophin (HMG) and human chorionic gonadotrophin (HCG) were used for ovarian stimulation in these patients. The details of the protocol have been reported elsewhere (Yeung et al., 1996). Only hFF from follicles with diameter of 18–29 mm, without blood contamination and which contained an oocyte was collected. hFF (1 ml) from each sample was centrifuged at 600 g for 10 min to remove cellular debris. The hFF was sterilized by filtration and stored at –20°C. Before experimentation, hFF was thawed and diluted with Earle’s balanced salt solution/bovine serum albumin (EBSS/BSA) to a concentration of 25%, for easy comparison with our previous report (Yao et al., 1996a) and other studies on follicular fluid (De Jonge et al., 1993). The inhibitory effect of hFF was dose-dependent, and an effect was detected when the concentration of hFF was >10% (Y.Q.Yao, W.S.B.Yeung and P.C.Ho).

Semencollectionandsperm preparation
Semen samples were collected from men referred to our clinics for routine semen analysis by masturbation after 3–7 days of abstinence.

Introduction
Follicular fluid is found in the Fallopian tube (Edwards, 1974) and it is known that human follicular fluid (hFF) can induce the acrosome reaction (Tesarik, 1985; Suarez et al., 1986; Mortimer and Camenzind, 1989; Siegel et al., 1990; Tarlatzis et al., 1993; Zhu et al., 1994) and enhance motility of human spermatozoa (Mbizvo et al., 1990; Mendoza and Tesarik, 1990; Falcone et al., 1991; Kulin et al., 1994; Zhu et al., 1994).
Only semen samples with normal parameters according to World Health Organization (WHO, 1992) criteria were recruited. After complete liquefaction, spermatozoa were separated by two-step Percoll density centrifugation as previously described (Yeung et al., 1994). Briefly, spermatozoa were prepared by centrifugation through a Percoll gradient (45 and 90% Percoll) at 300 g for 20 min. The resulting sperm pellet was washed and resuspended with EBSS.

**Hemizona binding assay (HZA)**

Zonae pellucidae from unfertilized oocytes after intracytoplasmic sperm injection (ICSI) were used in this study. The vast majority of the oocytes were at metaphase II; they were stored in salt solution before use. The procedure for HZA was that by Burkman et al. (1988) with modifications by Yao et al. (1996b). Oocyte bisection was performed by micromanipulator (Nikon, Tokyo, Japan) assembled with Beaver Micro-Sharp blade (Becton Dickinson Acutecare, Franklin Lakes, NJ, USA), resulting in two identical hemizona. Spermatozoa were incubated with 25% hFF in EBSS at 37°C under 5% CO2 in air. The incubation lasted for 3 h, during which 25% hFF was known not to affect sperm motility (Yao et al., 1996a). The treated spermatozoa were washed twice with EBSS/BSA after treatment. The zona binding capacities of the washed spermatozoa were compared with those incubated with EBSS under the same conditions. In the assay, each hemizona was incubated with $2.5 \times 10^5$ motile spermatozoa in 100 µl EBSS with oil-overlay for 4 h at 37°C in an atmosphere of 5% CO2 in air. The loosely bound spermatozoa were then removed by pipetting with a 200 µm diameter glass micropipette. The hemizonae with the bound spermatozoa were observed under an inverted microscope with the image projected onto a monitor. The tightly bound spermatozoa were marked on a transparency attached to the monitor and counted. The coefficient of variation of counting was ~3.5% even when the number of tightly bound spermatozoa was over 100. The hemizona binding index (HZI) was calculated as:

$$\text{HZI} = \frac{\text{no. spermatozoa bound in test droplet}}{\text{no. spermatozoa bound in control droplet}} \times 100$$

The intra-assay variation of the assay was determined by incubating matching hemizonae with the same sperm sample in EBSS. The mean HZI after 8 determinations was 101.0, and the standard deviation was 10.3.

**Statistical analysis**

Results were expressed as mean ± SEM. In order to eliminate the variation in sperm binding due to difference in the degree of maturity of the unfertilized oocytes, the zona binding potential of hFF treated spermatozoa was compared with the untreated control using matching hemizona, and only HZI was compared among different infertility causes. Probably due to the small sample size, some of the results did not follow a normal distribution. Therefore, Wilcoxon Signed Rank test (Sigmastat, Jandel Scientific, USA) was used to compare the effects of hFF on the zona binding capacity of spermatozoa with or without hFF treatment. Comparison of HZI among samples with different indications of subfertility was done by Mann–Whitney Rank Sum Test.

**Results**

The effect of hFF from endometriosis on the zona binding capacity of spermatozoa was shown in Table I. The number of spermatozoa bound to the zona was significantly lower than that of the control spermatozoa (90.5 ± 20.9 versus 238.7 ± 34.7; $P < 0.002$). hFF from tubal factor also decreased the number of tightly bound spermatozoa on the zona pellucida (108.9 ± 22.3 versus 210.8 ± 41.6; $P < 0.002$). The number of tightly bound spermatozoa after treating with hFF from male factor patients was 101.2 ± 13.4, which was significantly less than that of the control (205.4 ± 26.3; $P < 0.002$). There was no difference in the HZI between the male factor samples (52.0 ± 6.7) and the tubal factor samples (53.8 ± 4.2). The HZI for endometriosis samples (36.0 ± 4.1) was significantly lower than that for tubal factor samples ($P < 0.01$). Probably due to the small sample size, the difference in HZI between the male factor and endometriosis did not reach statistical significance ($P = 0.076$).

There was no difference in the IVF rate of the three groups of subjects from whom hFF was collected for this study. The fertilization rates for the male factor, tubal factor and endometriosis group were 76.3 ± 5.7, 75 ± 6.5 and 82.8 ± 8.5 respectively.

**Discussion**

This study shows that hFF from patients with all the three indications of infertility contains sperm–zona binding inhibitory activity. This confirms our previous results (Yao et al., 1996a). The present report further demonstrates that hFF from patients with endometriosis contains stronger sperm–zona binding inhibitory activity than those from patients with tubal factors, and probably those with male factors. The latter did not reach statistical significance, presumably due to small sample size.

As the male factor patients are likely to have normal ovarian function, the presence of the zona-binding inhibitory activity in hFF from these patients suggests that the inhibitory activity may play a role in oocyte development and fertilization. It will be of interest to know if the inhibitory activity is present in hFF from unstimulated cycles. This inhibitory effect is not due to the effect of hFF on sperm motility, as exposure of spermatozoa to hFF for <6 h was shown to affect neither sperm motility nor hyperactivation (Mortimer and Camenzind, 1989; Mendoza and Tesarik, 1990; Zhu et al., 1994). Our previous study also confirmed that sperm motility was not affected after 1 and 3 h of incubation with 25% pooled hFF from patients with different causes of infertility when compared with the untreated control (Yao et al., 1996a). In other studies, progesterone, a component of the follicular fluid, was found to promote sperm–zona binding (Yao et al., 1996a), and to increase polyspermic fertilization in the pig (Day and Polge, 1988).
1968; Hunter, 1972). On the other hand, hFF inhibited the binding of spermatozoa to the zona pellucida despite the presence of progesterone in hFF (Yao et al., 1996a). Addition of porcine follicular fluid to the IVF medium reduced the number of spermatozoa binding to the zona, and decreased the incidence of polyspermy (Funahashi and Day, 1993). Thus the sperm–zona binding inhibitory activity of follicular fluid may modulate the progesterone effect and prevent polyspermic fertilization. The mechanism by which hFF inhibits the zona binding capacity of spermatozoa is unknown.

Endometriosis is one cause of infertility, although its pathophysiology remains unclear. Two possible mechanisms may operate. The first mechanism involves the transport of deleterious substances from the body fluid into the tubal and endometrial environment. Components of the peritoneal fluid may diffuse into the lumen of the reproductc tract, and affect sperm functions and fertilization. Peritoneal fluid from patients with endometriosis affected the motion kinematics (Burke, 1987; Aeby et al., 1996), and decreased the zona-free hamster egg fusion capacity (Aeby et al., 1996) and the HZI (Coddington et al., 1992) of the treated spermatozoa. These effects have been attributed to a 32 kDa protein found in the peritoneal fluid from women with endometriosis (Northick et al., 1994). Recently, serum from endometriosis patients was found to impair the development of mouse embryos (Ito et al., 1996). Oviductal fluid is composed of constituents derived from the plasma together with specific proteins secreted from the oviductal epithelium (Leese 1988). Thus it is possible that serum components may transudate into the oviductal fluid and thus affect embryo development in vivo.

The second mechanism involves a direct effect of endometriosis on ovarian function. Using an oocyte donation model, it has been shown that endometriosis may cause alterations within the oocyte which, in turn, result in embryos of lower quality, and with a lower implantation capacity (Pellicer et al., 1995). Moreover, preovulatory granulosa cells from women with endometriosis were associated with a reduced steroidogenic activity (Harlow et al., 1996). Our data also suggest a direct effect of endometriosis on the ovarian function, and give support to the second hypothesis. The disease increases the sperm–zona binding inhibitory activity of hFF and may contribute to infertility through impairment of gamete inter- action in vivo. But whether this increase in the inhibitory activity is due to excessive production of the inhibitory factor or due to production of other factors detrimental to sperm function remains to be investigated.

Despite the increase in inhibitory activity of hFF from patients with endometriosis, our present data for this group of patients as well as our cumulative data from the assisted reproduction programme suggest that the IVF rate of oocytes from these patients is not affected. Similar observations have been reported by Geber et al. (1995). Two reasons may explain the lack of effect of hFF from these patients upon the conventional IVF rate. Firstly, only a small amount of hFF is present in the insemination medium because the oocyte–cumulus masses are washed with culture medium during oocyte retrieval. Secondly, even if a small amount of hFF is present, the large number of spermatozoa used for insemination will ensure that sufficient number of spermatozoa can bind to the zona pellucida for fertilization. However, Harlow et al. (1996) reported reduced fertilization rate in women with endometriosis. The reason for the observed difference is uncertain.

Recent evidence showed that tubal damage may also impair ovarian function resulting in lower ovarian response, pregnancy rate and take-home baby rate (Csemiczky et al., 1995, 1996). The exact mechanism by which tubal damage affects ovarian response is unknown. The present results show that tubal factor hFF samples have similar sperm–zona binding inhibitory activity when compared with male factor samples. This suggests that tubal factor does not affect the sperm–zona binding inhibitory activity of hFF.

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References


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