Inhibitory effect of human hydrosalpingeal fluid on mouse preimplantation embryonic development is significantly reduced by the addition of lactate

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Implantation and pregnancy rates following in-vitro fertilization-embryo transfer are reduced in the presence of hydrosalpinges, but the basis of the inhibition is unknown. We examined the effect of hydrosalpingeal fluid on preimplantation development of mouse embryos. Embryos cultured in 100% hydrosalpingeal fluid were significantly inhibited developmentally as compared to embryos cultured in 100% defined medium, which served as controls. In contrast, embryos cultured in 50% hydrosalpingeal fluid/50% defined medium reached the blastocyst stage at the same frequency as the controls. When lactate (final concentration 10 mM) was added to 100% hydrosalpingeal fluid, the percentage of cultured embryos that reached the blastocyst stage was significantly increased as compared to 100% hydrosalpingeal fluid, although the percentage was slightly lower than that observed for embryos cultured in the 100% defined medium. A similar but less pronounced effect occurred when pyruvate was added to hydrosalpingeal fluid. These results do not support the concept that a potent embryotoxic agent is commonly present in hydrosalpingeal fluid. Rather, they are consistent with the notion that the inhibitory effect of hydrosalpingeal fluid on embryonic development is due to the absence of essential factors, and that this deficiency can be largely corrected by the addition of energy sources.

Key words: embryo/hydrosalpinges/infertility/in-vitro fertilization/mouse

Introduction

In-vitro fertilization (IVF)-embryo transfer was initially developed as a technique for the treatment of infertility caused by tubal damage. Several recent studies have suggested that implantation and pregnancy rates following IVF-embryo transfer are reduced in the presence of hydrosalpinges (Andersen et al., 1994; Kassabji et al., 1994; Strandell et al., 1994; Vandromme et al., 1995; Akman et al., 1996; Fleming and Hull, 1996; Katz et al., 1996), although others have not found such an effect (Sharara et al., 1996).

Several factors may be involved in the reduction in IVF-embryo transfer implantation and pregnancy rates in the presence of hydrosalpinges. Mansour et al. (1991) described accumulation of fluid in the uterine cavity of three patients with hydrosalpinges undergoing IVF treatment. Attempts to aspirate the fluid in two patients were initially successful but the fluid re-accumulated. Thus, hydrosalpingeal fluid may flow into the uterine cavity and result in a mechanical barrier to successful implantation of the embryo.

The initial inflammatory insult leading to the formation of hydrosalpinges may result in damage to other portions of the reproductive tract which might reduce implantation and pregnancy rates. Strandell et al. (1994), however, reported that women with unilateral or bilateral hydrosalpinges still had significantly lower pregnancy rates following IVF-embryo transfer as compared to women who had inflammatory tubal damage but who did not have hydrosalpinges. Fleming and Hull (1996) made a similar observation in a study of patients with inflammatory tubal damage with or without hydrosalpinges. Additionally, hydrosalpinges may increase in size during ovarian stimulation (Hill et al., 1986). This could theoretically impede ovarian growth or reduce its blood supply. Akman et al. (1996) observed the effects of hydrosalpinges on natural cycle IVF with cryopreserved/thawed embryos. Patients with hydrosalpinges had lower implantation and pregnancy rates. In all studies in which significant reductions in implantation and pregnancy rates were associated with hydrosalpinges, the number of oocytes retrieved and transferred was unaffected.

Finally, several authors have suggested that an embryotoxin may be present in the hydrosalpingeal fluid, which gains entrance to the uterine cavity and has deleterious effects on embryo development and implantation. In support of this view, Mukherjee et al. (1996) reported a significant embryotoxic effect of human hydrosalpingeal fluid on mouse embryo development in vitro. These authors and others (Andersen et al., 1994; Strandell et al., 1994; Vandromme et al., 1995; Fleming and Hull, 1996; Katz et al., 1996) have recommended correction of the hydrosalpinges either by salpingectomy or by tubal occlusion prior to IVF-embryo transfer treatment. Shelton et al. (1996) observed the effects of salpingectomy in patients with hydrosalpinges who had undergone failed IVF-embryo transfer, and demonstrated a significant improvement in pregnancy and implantation rates after surgery.

To investigate whether embryotoxicity may be a factor in the reportedly poor implantation rate in women with hydrosalpinges, we undertook this study to evaluate the effects of human hydrosalpingeal fluid on mouse embryonic development.
Table 1. Effect of hydrosalpingeal fluid (HSF) on preimplantation development of mouse embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of experiments</th>
<th>No. of embryos</th>
<th>% 4-cell (day 2)</th>
<th>% morula (day 3)</th>
<th>% blastocyst (day 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOM</td>
<td>5</td>
<td>98</td>
<td>88.8</td>
<td>80.6</td>
<td>79.6</td>
</tr>
<tr>
<td>50% HSF/50% KSOM</td>
<td>5</td>
<td>126</td>
<td>89.9</td>
<td>87.3</td>
<td>76.2</td>
</tr>
<tr>
<td>100% HSF</td>
<td>5</td>
<td>112</td>
<td>66.1*</td>
<td>48.2*</td>
<td>35.7*</td>
</tr>
</tbody>
</table>

*Significantly less than corresponding value for KSOM ($\chi^2$, P < 0.001).

Materials and methods

Six women who had infertility due to tubal damage and hydrosalpinges and who were undergoing a laparoscopic examination were recruited for the study. Of the four who had known tubal disease before surgery, three displayed a unilateral left hydrosalpinx and one displayed bilateral hydrosalpinges. The fifth patient had a prior ectopic pregnancy and a left hydrosalpinx. The sixth, who had no prior diagnosis of tubal damage, had a right hydrosalpinx. All of the hydrosalpinges measured between 1.5 and 2.5 cm in diameter and all patients underwent surgical correction. At the time of laparoscopy, between 1.5 and 3 ml of hydrosalpingeal fluid was collected from each patient by needle aspiration. Each of the specimens was cultured; both general and chlamydial cultures were negative in all six cases. The specimens were then centrifuged and frozen at -70°C until use.

To obtain embryos, outbred CD-1 female mice were superovulated by an i.p. injection 5 IU of pregnant mares’ serum gonadotrophin (Sigma, Mississauga, Ontario, Canada) followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG; Sigma), and placed with CD-1 males after the HCG injection. The next morning, the females were checked for the presence of a vaginal plug to confirm that mating had occurred. Two-cell embryos were obtained by flushing the oviducts of females killed on day 1.5 (day 0 = day of plugging).

Healthy-appearing 2-cell embryos were washed through three drops of potassium simplex optimization medium (KSOM) culture medium (Lawitts and Biggers, 1993) containing 0.1% (w/v) bovine serum albumin (Boehringer, cat. no. 735078) and then transferred to a 5 µl drop of the appropriate culture medium overlaid with washed and filtered light paraffin oil (Fisher, Montreal, Canada) and cultured at 37°C in an atmosphere of 5% CO2 in air. The culture media were pre-equilibrated for at least 60 min prior to embryo transfer and between 10 and 20 embryos were cultured in each drop of medium.

Each day, the embryos were examined using a dissecting microscope and the cleavage stage of each was recorded.

When lactate- or pyruvate-supplemented hydrosalpingeal fluid was required, this was prepared by adding 1 µl of a 20X solution of lactate (final concentration, 10 mM) or pyruvate (final concentration, 0.2 mM) to 19 µl of hydrosalpingeal fluid and then mixing the solution gently. As a control, 1 µl of water was added to 19 µl of hydrosalpingeal fluid.

Results

To examine the effect of hydrosalpingeal fluid on murine preimplantation development, 2-cell embryos were flushed from the oviducts of pregnant mice and transferred to a 5 µl drop of 100% KSOM culture medium, or a 50% hydrosalpingeal fluid/50% KSOM mixture, or 100% hydrosalpingeal fluid. Between 10 and 20 embryos were cultured in each drop. Embryos were examined daily and the number that reached successive cleavage stages was recorded.

As shown in Table 1, embryos cultured in 100% hydrosalpingeal fluid showed impaired development that was manifested as early as the 4-cell stage. Those embryos that failed to reach the 4-cell stage remained as 2-cell embryos and did not show overt signs of degeneration. The percentage of embryos that reached the 8-cell and blastocyst stages was further reduced, and this impaired development was observed in each of the five experimental trials. In contrast to the arrested development of embryos cultured in 100% hydrosalpingeal fluid, those cultured in 50% hydrosalpingeal fluid progressed through preimplantation development at the same frequency as control embryos cultured in 100% KSOM. These results indicate that 100% hydrosalpingeal fluid inhibits preimplantation development of mouse embryos, beginning as early as the 4-cell stage, and that this effect is not manifested when the hydrosalpingeal fluid is combined in equal proportion with embryo culture medium.

Although the inhibitory effect of 100% hydrosalpingeal fluid on embryonic development could be due to the presence of an embryotoxin, the fact that 50% hydrosalpingeal fluid had no detectable inhibitory effect on embryonic development, and the fact that developmentally arrested embryos in 100% hydrosalpingeal fluid showed no overt degenerative changes, are strong arguments against the presence of such a potent embryotoxin in the hydrosalpingeal fluid.

An alternative explanation for the inhibitory effect of 100% hydrosalpingeal fluid could be that it lacked factor(s) necessary for embryonic development in vitro. According to this explanation, embryos cultured in 50% hydrosalpingeal fluid/50% KSOM were able to develop normally because these factors were supplied by the KSOM. Most mouse embryo culture media, including KSOM, are relatively simple salt solutions that also contain energy sources, typically pyruvate and lactate. Pyruvate is the main energy source utilized during the early cleavage divisions. Near the morula stage, embryos switch to glucose, which is not required for the early cleavages (Leese et al., 1993; Leese, 1995; Houghton et al., 1996). Lactate is taken up by embryos and may be utilized for amino acid biosynthesis (Kaye, 1986). In the second part of this study, we therefore tested whether the addition of pyruvate and lactate to 100% hydrosalpingeal fluid would enhance embryonic development.

Two-cell embryos, obtained as in the previous experiment, were cultured in 100% hydrosalpingeal fluid supplemented with either pyruvate (final concentration, 0.20 mM), lactate (final concentration, 10 mM), or water. The pyruvate and lactate concentrations that were chosen correspond to those that are present in KSOM medium (Lawitts and Biggers,
Two explanations may be proposed to account for these percentages of embryos which develop to the blastocyst stage. The hydrosalpingeal fluid is mixed 1:1 with embryo culture by the addition of lactate.

It has long been known that preimplantation development of mouse embryos in vitro requires the presence of energy sources such as pyruvate or lactate (Brinster, 1967; Biggers et al., 1967; Wales and Whittingham, 1974). In humans, biochemical analysis of reproductive tract fluids has revealed that lactate is present at a concentration of 6.1 mM in follicular fluid (Leese and Lenton, 1990) and at 8.6 mM in tubal fluid (Dickens et al., 1995), but at a concentration of only 2.0 mM in hydrosalpingeal fluid (Gott et al., 1990). The concentration of pyruvate is similar in the three types of fluid. We were unable to obtain a sufficient quantity of hydrosalpingeal fluid to permit a biochemical analysis of the specimens analysed in the present study. If it is assumed that their biochemical composition is similar to the published values, then it is reasonable to postulate that the inability of hydrosalpingeal fluid to support complete preimplantation development of mouse embryos is in large part due to the relatively low lactate concentrations. Thus, addition to hydrosalpingeal fluid of either KSOM medium, which contains lactate, or lactate alone is sufficient to rescue embryo development. The reason why pyruvate is less effective than lactate at promoting blastocyst development is unknown. However, the ion pump associated with blastocoel formation is likely to be a major consumer of energy (Leese et al., 1993), and pyruvate uptake declines in older rodent embryos (Leese and Barton, 1984; Brison and Leese, 1992).

Even in the presence of added lactate, however, embryos cultured in 100% hydrosalpingeal fluid were developmentally inhibited compared with those cultured in KSOM. This indicates that lactate was unable to completely overcome the inhibitory effect manifested by hydrosalpingeal fluid. This may indicate that other factors essential for development are also missing from hydrosalpingeal fluid. Alternatively, a mildly embryotoxic activity may have been present in the fluid. Finally, independent of a specific toxic activity, the composition of human hydrosalpingeal or tubal fluid may not be ideal for mouse embryo development. As we were unable to obtain tubal fluid from normal patients, these possibilities could not be evaluated.

Our results may be compared with those of Mukherjee et al. (1996), who suggested that hydrosalpingeal fluid contained a potent embryotoxin that was effective even at a dilution of 1 part in 100. The biochemical composition of the hydrosalpingeal fluid in their study was not reported. At least two explanations may be proposed to explain the difference between our results and those of Mukherjee et al. (1996). First, embryos

1993). Control groups of embryos were cultured in 100% KSOM or 100% hydrosalpingeal fluid supplemented with water. As shown in Table II, preimplantation development was impaired in the water-supplemented hydrosalpingeal fluid as compared with 100% KSOM.

When the hydrosalpingeal fluid was supplemented with pyruvate, the percentage of embryos that reached the 4-cell stage was significantly increased compared to the water-supplemented group and was not significantly different from the frequency observed in the KSOM controls. Development to the morula stage was apparently also increased (not significant) compared with the water-supplemented group, although it was lower than in the KSOM controls. The pyruvate-supplemented hydrosalpingeal fluid was unable, however, to support development to the blastocyst stage. These results indicated that addition of pyruvate could partially overcome the inhibitory effects of hydrosalpingeal fluid on embryonic development.

Embryos cultured in hydrosalpingeal fluid supplemented with lactate developed to the 4-cell, morula, and blastocyst stages at significantly higher frequencies than the embryos in the water-supplemented group. These results clearly show that addition of lactate to hydrosalpingeal fluid increased its ability to support all stages of preimplantation development. It may be noted, however, that the percentage of embryos in the lactate-supplemented group that reached the morula and blastocyst was less than in the KSOM controls. This implies that the inhibitory effect of hydrosalpingeal fluid was not completely overcome by the addition of lactate.

### Discussion

We have shown that 100% hydrosalpingeal fluid is unable to support preimplantation development of outbred CD-1 mouse embryos. This inhibitory effect is not observed, however, when the hydrosalpingeal fluid is mixed 1:1 with embryo culture medium. Furthermore, supplementation of the hydrosalpingeal fluid with lactate or, to a lesser extent, pyruvate, increases the percentage of embryos which develop to the blastocyst stage. Two explanations may be proposed to account for these observations. It is possible that hydrosalpingeal fluid contains an embryotoxic agent that is neutralized by 2-fold dilution or by addition of pyruvate or lactate. Alternatively, hydrosalpingeal fluid may lack adequate levels of these energy sources.

It has long been known that preimplantation development

Table II. Effect of supplemented hydrosalpingeal fluid (HSF) on preimplantation development of mouse embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of experiments</th>
<th>No. of embryos</th>
<th>% 4-cell (day 2)</th>
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<th>% blastocyst (day 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOM</td>
<td>5</td>
<td>90</td>
<td>90.0b</td>
<td>86.7b</td>
<td>80.0b</td>
</tr>
<tr>
<td>100% HSF + water</td>
<td>2</td>
<td>35</td>
<td>42.9b</td>
<td>28.6b</td>
<td>0b</td>
</tr>
<tr>
<td>100% HSF + pyruvate</td>
<td>3</td>
<td>63</td>
<td>77.8b</td>
<td>47.6b</td>
<td>1.6b</td>
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<tr>
<td>100% HSF + lactate</td>
<td>3</td>
<td>85</td>
<td>92.9b</td>
<td>70.6b</td>
<td>58.8b</td>
</tr>
</tbody>
</table>

aSignificantly greater than corresponing value for 100% hydrosalpingeal fluid plus water (% 2, P < 0.001).
bSignificantly less than corresponding value for KSOM (χ², P < 0.05).

KSOM = potassium simplex optimization medium.
obtained from different strains of mouse were used, and these may be differentially sensitive to agents commonly present in hydrosalpingeal fluid. Second, the hydrosalpingeal fluid samples were collected from different individuals. As hydrosalpinges are believed to arise following bacterial infection, it is possible that, depending on the nature and severity of the infection, different specimens of fluid may carry different levels of infection-associated toxins. The hydrosalpingeal specimens used in our study tested negative for chlamydial and other cultures, but it is possible that undetected infectious agents could underly the mild embryotoxic effect that remained even in the presence of added lactate. Interestingly, the results of Sharara et al. (1996) indicate that prior treatment with antibiotics may improve IVF outcome in patients with hydrosalpinges. Finally, Mukherjee et al. (1996) did not test tubal fluid specimens obtained from patients without hydrosalpinges to demonstrate that the embryotoxic activity was specifically associated with hydrosalpinges.

In summary, our results do not support the concept that a potent embryotoxin is commonly present in 100% hydrosalpingeal fluid. Rather, they are consistent with the notion that the inhibitory effect of hydrosalpingeal fluid on embryonic development is largely due to the absence of essential factors. Furthermore, this deficiency can be largely corrected by the addition of the energy sources, lactate and pyruvate. As abnormally developing human embryos in vitro display reduced uptake of energy sources (Leese et al., 1993), these data raise the possibility that the deleterious effects of hydrosalpingeal fluid on human fertility may be related to a lack of essential energy sources.

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References

Lactate relieves hydrosalpingeal inhibition of mouse embryo development


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