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A second look at the embryotoxicity of hydrosalpingeal fluid: an in-vitro assessment in a murine model*

M.K.Koong[1,3], J.H.Jun[2], S.J.Song[2], H.J.Lee[2], I.O.Song[1] and I.S.Kang[1]

[1]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology and [2]Infertility Research Laboratory, Samsung Cheil Hospital and Women's Healthcare Center, College of Medicine, Sungkyunkwan University, 1–19 Mookjung-dong, Chung-ku, Seoul, 100–380, Korea

[3]To whom correspondence should be addressed

The purpose of this study was to evaluate whether hydrosalpingeal fluid (HSF) is toxic to the mouse embryo as assessed by the blastocyst development rate (BDR) and by cell counting *in vitro*. HSF was collected from nine patients undergoing salpingoneostomy to correct hydrosalpinx. Two-cell embryos were obtained from superovulated ICR mice. T6 medium and T6 + 0.4% bovine serum albumin (BSA) were used as control media. T6 medium containing 10% or 50% HSF and 100% HSF from each patient were used as test media. Nine to 15 embryos were cultured in microdrops prepared from each of these media. The BDR was examined after 72 h of culture in these media. To assess the total cell number within each blastocyst, the blastocysts were fixed and stained with Hoechst 33342 to facilitate cell counting. The BDR was affected adversely only by 100% HSF and not in media containing 10% or 50% HSF. The mean BDR using T6 medium and T6 + BSA were 88.7% and 85.3%, respectively. The mean BDR using media containing 10% HSF or 50% HSF were 90.0% and 89.4%, respectively. Mean BDR using 100% HSF was 75.2% ($P < 0.05$). The overall mean cell counts (± SEM) using T6 medium and T6 + BSA were 86.9 ± 3.2 and 91.0 ± 3.8 respectively. Mean cells counts were decreased significantly only in blastocysts cultured in 100% HSF (63.3 ± 4.6; $P < 0.01$) but not in blastocysts cultured in 10% or 50% HSF (90.8 ± 4.2 and 81.9 ± 6.1 respectively). Thus, it is concluded that HSF has no embryotoxic effect but has a mildly negative effect on embryonic growth and development.

Key words: cell count/development/embryotoxicity/hydrosalpinx/mouse

Introduction

Tubal infertility is one of the classic indications for in-vitro fertilization (IVF)–embryo transfer. Recently, several studies have reported that patients with hydrosalpinx also demonstrate reduced pregnancy rates following IVF–embryo transfer (Anderson *et al*., 1994; Kassabji *et al*., 1994; Strandell *et al*., 1994; Vandromme *et al*., 1995; Fleming and Hull, 1996). Several groups of investigators have shown that surgical correction of hydrosalpinx can significantly improve pregnancy rates following IVF–embryo transfer (Poe-Zeigler *et al*., 1995; Shelton *et al*., 1996; Koong *et al*., 1997). The mechanism by which hydrosalpinx reduces pregnancy rate is presently not understood. Based on their results using a murine experimental model, Mukherjee *et al.* (1997) have suggested that hydrosalpingeal fluid (HSF) is embryotoxic. They reported that the apparent embryotoxic effect of HSF could be blocked by surgical interventions designed to attenuate embryo contact with HSF, e.g. salpingectomy or proximal tubal occlusion. However, other researchers using similar study designs have reported contrasting results (Katz *et al*., 1996; Koong *et al*., 1996; Murray *et al*., 1996). Furthermore, the increased rates of ectopic pregnancy in patients with hydrosalpinx would also argue against an embryotoxic effect of HSF (Dubuisson *et al*., 1991; Vasquez *et al*., 1991; Zouves *et al*., 1991). We have suggested previously (Koong *et al*., 1996) that HSF exerts only mildly adverse effects on mouse embryo growth and development. As an extension of our previous study, we have now made a second-look evaluation as to whether or not HSF has embryotoxic effects on mouse embryo development *in vitro* by assessing the blastocyst development rate and cell number in the blastocyst.

Materials and methods

Preparation of HSF and embryos

Hydrosalpingeal fluid was collected from nine infertile patients undergoing salpingoneostomy to correct hydrosalpinx. Each HSF sample was centrifuged at 1160 *g* for 10 min to remove cell debris. The supernatant from each sample was collected and stored at –20° C. Adult outbred female Institute of Cancer Research (ICR) mice were injected with 7 IU of pregnant mare serum gonadotrophin (PMSG; Sigma Chemical Co., St Louis, MO) and, 47 h later, with 7 IU of human chorionic gonadotrophin (HCG; Sigma Chemical Co., St Louis, MO, USA). The following morning the mice were checked for the presence of copulation plugs to verify successful mating. Successfully mated mice were killed 48 h after HCG injection and their oviducts were removed. Two-cell-stage embryos were obtained by flushing the oviducts with phosphate buffered saline.

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Table I. Comparison of blastocyst development rates (%) using media with and without hydrosalpingeal fluid (HSF) and HSF alone by patient from which HSF was obtained

Patient no.	T6	T6 + 0.4% BSA	T6 + 10% HSF	T6 + 50% HSF	100% HSF
1	100 (10)	82 (11)	91 (11)	100 (11)	90 (10)
2	100 (10)	90 (10)	100 (10)	91 (11)	73 (11)
3	67 (12)	92 (12)	92 (13)	92 (13)	77 (13)
4	83 (12)	83 (12)	91 (11)	82 (11)	73 (11)
5	100 (14)	80 (15)	93 (14)	87 (15)	73 (15)
6	100 (9)	91 (11)	80 (10)	100 (10)	80 (10)
7	80 (10)	90 (10)	100 (10)	64 (11)	73 (11)
8	70 (10)	70 (10)	80 (10)	91 (11)	67 (12)
9	100 (10)	91 (11)	82 (11)	100 (11)	75 (12)

Data in parentheses indicate the number of mouse embryos used.
T6 medium = control culture medium; BSA = bovine serum albumin; T6 + BSA = control culture medium.

Preparation of culture media

HSF was sterilized by filtration (0.22-μm filter, Millipore, Molsheim, France) before use. For each patient, T6 medium and T6 medium containing 0.4% bovine serum albumin (BSA; Gibco Life Technologies, Gaithersburg, MD, USA) were prepared as control culture media, and T6 medium containing 10% or 50% HSF or 100% HSF were prepared as test media. Each of these five solutions was prepared as microdrops which were covered by a layer of mineral oil (Sigma Chemical Co.) in culture dishes (Falcon, Becton Dickinson Inc., Franklin Lakes, NJ, USA). Prior to addition of the collected mouse embryos, the microdrops were equilibrated for 6 h in an atmosphere of air/5% CO_2 at 37°C. Both pH and osmolarity (5500 Vapor Pressure Osmometer; Wescor Inc, Logan, UT, USA) were monitored before and after equilibration of HSF.

Blastocyst development rate (BDR)

The embryos obtained from each animal were randomly distributed among microdrops containing each of the five different media. For BDR assessment, nine to 15 embryos were cultured in each microdrop (see Table I). The development stage of each embryo was determined after 72 h in culture. We defined 'embryotoxic' to mean that most of the embryos did not develop to a more advanced stage in certain media (Mukherjee *et al*., 1996; Sachdev *et al*., 1997). If the percentage of embryos which developed into blastocysts was significantly lower than that of the control, then this was defined as an inhibitory or adverse effect on embryo development (Beyler *et al*., 1997; Murray *et al*., 1997; Rawe *et al*., 1997).

Blastocyst cell counting

To determine total cell numbers in each blastocyst, cell counts were conducted using a modification of the method of Ebert *et al.* (1985). The blastocysts were fixed in a solution of 1% glutaraldehyde in phosphate buffered saline. Next, the embryos were stained with bisbenzimide solution (10 μg/ml; Hoechst 33342, Sigma Chemical Co.). The stained blastocysts were transferred to a clean slide and a cover glass was applied. The slides were examined under a fluorescent microscope (Optiphot-2, Nikon, Tokyo, Japan) with a 330–380 nm band pass excitation filter and a 420-nm long pass barrier filter (UV-2A). A single photograph was taken of each blastocyst. Using this photograph, the number of intact nuclei as well as the number of metaphase figures were counted as the number of cells in the blastocyst (Papaioannou and Ebert, 1988; Summers *et al*., 1995; Sherbahn *et al*., 1996). Among embryos progressing to the blastocyst stage, total cell counts were highly variable. For example, the total cell count in one blastocyst was 38 whereas, in another, the total cell count was 110 (Figure 1). Accordingly, we assessed the effects of HSF on blastocyst cleavage rates using mean cell counts.

Statistical analysis

The percentages of embryos developing into blastocysts in each of the five media were compared by the χ^2 test (Leppens *et al*., 1996). In order to compare the mean cell counts of blastocysts cultured in each of the media, analysis of variance followed by Duncan's multiple range test was performed using SPSS for Windows software (version 6).

Results

The osmolarity of media with HSF ranged from 277 to 297 mOsm with a mean (± SD) of 283.8 ± 8.3 mOsm. These values were thus within a physiological range and had not changed significantly by the end of the incubation period. The mean pH of media with HSF was 8.20 (range 7.9–8.4) and 7.47 (range 7.37–7.51) before and after equilibration, respectively. The post-equilibration values were considered to be physiological. BDR using each of the five media are shown in Table I. Which of the nine patients the HSF was obtained from had no significant effect on BDR ($P = 0.34$). The mean BDR in media containing T6 medium ($n = 97$) or T6 + BSA ($n = 102$) were not significantly different (88.7% and 85.3%, respectively) and nor were mean BDR in media containing 10% HSF ($n = 100$) and 50% HSF ($n = 104$) significantly different (90.0% and 89.4%, respectively). The mean BDR using 100% HSF alone ($n = 105$) was 75.2% ($P < 0.05$, Figure 2). Thus, the mean BDR was not significantly different among controls, 10% HSF- and 50% HSF-containing media. However, culture in 100% HSF produced a statistically significant decrease in BDR when compared with the other media groups. These data thus suggest that HSF is not toxic to mouse embryos *in vitro* unless used in an undiluted (100%) state. Culture in 100% HSF produced a mildly adverse effect on BDR.

Mean cell counts in blastocysts cultured in each of the five different media are shown in Figure 3. There is a general trend toward decreasing cell counts in inverse proportion to HSF concentration except in media prepared using HSF from patient nos. 4 and 5. Mean cell counts (± SEM) in blastocysts cultured in T6 medium and T6 + BSA were 86.9 ± 3.2 ($n = 81$) and

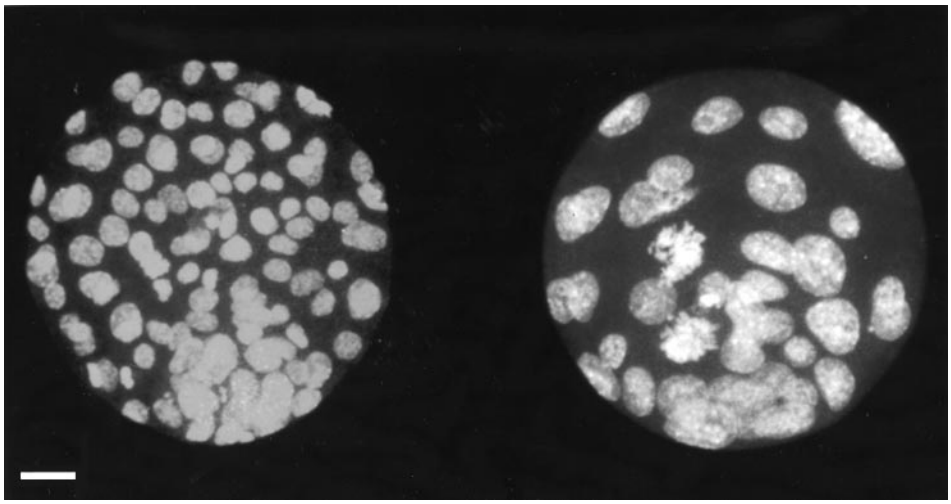

Figure 1. Two blastocysts stained by Hoechst 33342. Even though both represent blastocysts, the number of stained nuclei/metaphase figures for the blastocyst shown in the left panel was 110 and was 38 for the blastocyst shown in the right panel. Scale bar = 25 μm.

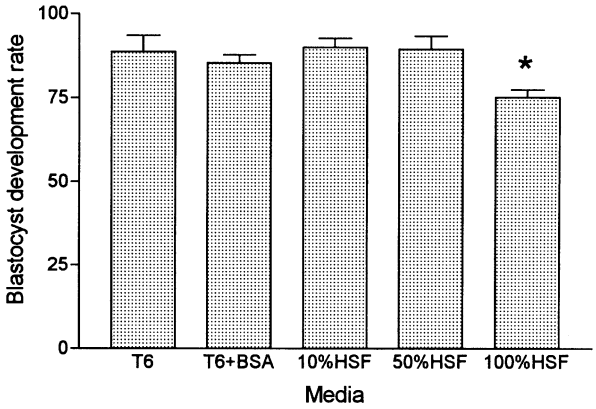

Figure 2. Mean blastocyst development rate for mouse embryos cultured in T6 medium, T6 + 0.4% bovine serum albumin (BSA), T6 + 10% hydrosalpingeal fluid (HSF), T6 + 50% HSF or in 100% HSF alone. Results are expressed as mean ± SEM. *$P < 0.05$ compared with all other groups.

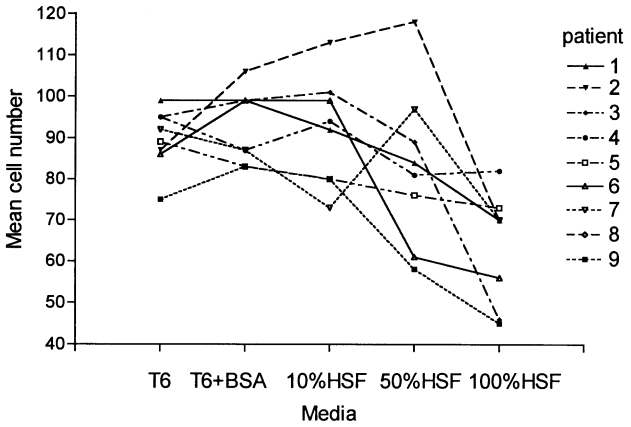

Figure 3. Mean cell counts for blastocysts cultured in T6 medium, T6 + 0.4% bovine serum albumin (BSA), T6 + 10% hydrosalpingeal fluid (HSF), T6 + 50% HSF or 100% HSF alone, relative to each patient. The mean cell counts in each medium were expressed as the average cell counts for all of the blastocysts cultured in a single microdrop.

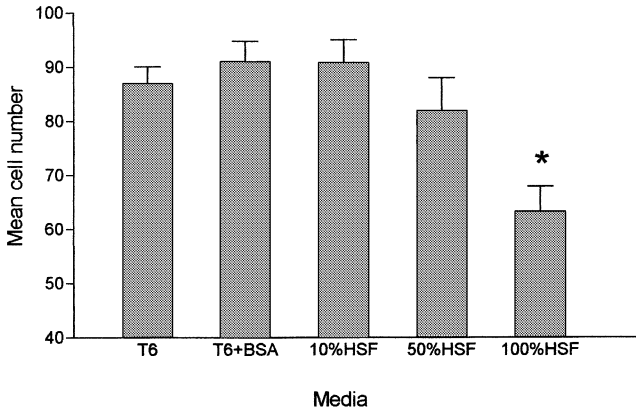

Figure 4. Overall mean cell counts for blastocysts using the nine hydrosalpingeal fluid (HSF) patient samples for each medium preparation. The results are expressed as mean ± SEM. *$P < 0.01$ compared with two control groups, T6 medium, and T6 + bovine serum albumin (BSA).

91.0 ± 3.8 ($n = 86$), respectively. Mean cell counts (± SEM) in blastocysts cultured in 10% HSF or 50% HSF were 90.8 ± 4.2 ($n = 88$) and 81.9 ± 6.1 ($n = 81$), respectively. Mean cell counts in blastocysts cultured in 100% HSF were 63.3 ± 4.6 ($n = 79$) ($P < 0.01$, Figure 4). Thus, culture of blastocysts in 100% HSF produces a significant decrease in mean cell counts, compared with culture in two control media. These data indicated that undiluted (100%) HSF has a mildly negative effect on cell cleavage in blastocysts. To test for possible type II errors, specific power calculation was performed for comparison between control media groups and the 50% HSF-containing media group. The power to detect significant differences of the magnitude observed in our study, given the sample sizes used in our study, was low (0.10). To increase the power to 0.80 would require a 10-fold increase in the sample size.

Discussion

The adverse effects of hydrosalpinx on successful outcome following IVF–embryo transfer cycles have been reported by

several groups of investigators (Anderson *et al*., 1994; Kassabji *et al*., 1994; Strandell *et al*., 1994; Vandromme *et al*., 1995; Fleming and Hull, 1996). However, the mechanism for this adverse effect remains undefined. Recently, some groups have proposed that HSF might contain a toxic substance which diffuses into the uterine cavity to inhibit embryo development in a murine model (Mukherjee *et al*., 1997; Sachdev *et al*., 1997). However, other research groups using similar design studies have reported different results (Katz *et al*., 1996; Koong *et al*., 1996; Murray *et al*., 1996).

Mukherjee *et al*. (1996) reported that the mouse blastocyst development rate was 5% using 10% HSF and 0% using 100% HSF. Sachdev *et al.* (1997) reported that no embryo survived to the blastocyst stage when maintained in media containing >1% HSF. In our study, 75% of the mouse embryos developed to blastocyst when cultured in 100% HSF; these results are similar to those of Murray *et al*. (1996) who reported that 61% of 2-cell-stage mouse embryos developed into blastocysts when kept in 100% HSF. It is also known that patients with hydrosalpinx tend to have increased tubal pregnancy rates, implying that hydrosalpinx (i.e. 100% HSF) is not toxic to early embryo development (Dubuisson *et al*., 1991; Miyazaki *et al*., 1991; Zouves *et al*., 1991; Vasquez *et al*., 1995). Thus, HSF would appear to have little, if any, embryotoxic activity in a murine experimental model or in humans. Early embryos seem fully capable of developing into blastocysts in the presence of 100% HSF. It is also noteworthy that Schats *et al*. (1997) reported that HSF has no apparent toxic effect on sperm viability and that Sawin *et al*. (1997) reported that HSF actually enhanced human trophoblast viability.

The mechanism for hydrosalpinx-associated decreases in pregnancy rates following IVF–embryo transfer is currently undefined. Several authors have reported the accumulation of intrauterine fluid in patients with hydrosalpinx (Mansour *et al*., 1991; Bloechle *et al*., 1997; Sharara *et al*., 1997). Mere fluid distention of uterine cavity might inhibit contact of the embryo with the endometrial epithelium and thus inhibit successful implantation. On the other hand, Gott *et al*. (1990) has reported that HSF has a low concentration of protein and glucose. Murray *et al*. (1997) observed that the inhibitory effect of HSF on blastocyst survival is attenuated significantly by the addition of lactate. In our data, the only blastocysts cultured in 100% HSF (no media) have decreased BDR and cell numbers. Thus, the effect of hydrosalpinx to reduce pregnancy rates may be related to insufficient substrates required for early embryo growth and development. The effects of HSF on implantation have not been characterized and should be addressed by future studies. Certainly, the results of our study would indicate that HSF does not have a toxic effect on the mouse embryo growth and development, although it may have mildly adverse effects at high concentrations.

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