

Relationship between cytokines and the embryotoxicity of hydrosalpingeal fluid*

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Objective: The exact chemical composition of hydrosalpingeal fluid is unknown. The objective of this study was to characterize cytokines in hydrosalpingeal fluid (HSF) and examine their possible role in the embryo development.

Study Design: HSF was aspirated at laparoscopic salpingectomy in eight infertile women. Levels of IL-1 β , IL-13, IL-8, IL-6 and TNF- α in the HSF were determined by quantitative immunoassay kits. Two-cell mouse embryos were incubated with 0, 25, 50 and 75% concentrations of HSF. The blastocyst development rate (BDR) of mouse embryos was measured at each HSF concentration.

Result(s): The embryotoxicity of HSF was concentration dependent. An increase in the HSF concentration resulted in significant decrease in % BDR ($p < 0.01$). IL-1 β was present in six of the eight HSF samples with a mean (\pm SD) concentration of 0.9 ± 0.8 pg/mL. IL-13 was not detected in any of the HSF samples. IL-8, IL-6 and TNF- α were detected in all samples with a mean (\pm SD) concentration of 4741.2 ± 6554.4 pg/mL, 204.8 ± 132.8 pg/ml and 12 ± 12.8 pg/mL respectively. IL-6 was positively correlated with BDR ($r = 0.53$; $p < 0.04$).

Conclusion(s): We demonstrated for the first time the presence of IL-1 β , IL-8, IL-6 and TNF- α and the absence of IL-13 in human hydrosalpingeal fluid. IL-6 was positively related to the BDR.

KEY WORDS: Cytokines; embryotoxicity; Hydrosalpinx.

INTRODUCTION

Lower pregnancy and implantation rates have been reported by many investigators in patients with

hydrosalpinges undergoing IVF-ET. The adverse effect of hydrosalpinges was reversible by salpingectomy prior to IVF (1–5). The flow of hydrosalpingeal fluid (HSF) into the endometrial cavity may lead to mechanical flushing of the embryos from the uterus (6). Meyer *et al.* (7) have shown that HSF reduced endometrial integrins which may facilitate implantation. These integrins were restored to normal following the excision of the hydrosalpinges. The tubal epithelium may secrete cytokines, leukotrienes or prostaglandins into the sequestered fluid that could directly alter endometrial function (8). It has been also speculated that the negative influence of HSF

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may be due to toxic substances in the fluid (9–14). Others have reported no adverse effect of hydrosalpinges on IVF-ET (15).

The study of cytokines in reproductive diseases is a growing science. Recently, the presence of various cytokines in a number of reproductive tissues and body fluids has sparked an interest in studying the relation of cytokines with different female and male aspects of infertility (16). The role of cytokines in reproductive tissues, peritoneal fluid in endometriosis patients and follicular fluid of patients undergoing IVF, has been documented (16,17). Because the exact mechanism by which HSF induces its embryotoxic effect is unknown, we hypothesized that a cytokine mediated mechanism may be involved in this phenomenon. This study was designed to examine the presence of IL-1 β , IL-13, IL-8, IL-6 and TNF- α in HSF and to correlate these parameters to HSF induced embryotoxicity using a mouse embryo model.

MATERIALS AND METHODS

Following the approval of the Institutional Review Board, eight infertile women with known hydrosalpinges undergoing laparoscopic salpingectomy before IVF were enrolled. At the time of laparoscopy, hydrosalpingeal fluid was obtained from each patient by needle aspiration before the tube was excised. In patients with bilateral hydrosalpinges, the fluid was pooled into one sample. The remaining fluid was centrifuged at $300 \times g$ for 7 min to remove the cellular debris and stored at -70°C pending the analysis for IL-1 β , IL-13, IL-8, IL-6 and TNF- α levels and mouse embryotoxicity testing.

Detection of Cytokines in HSF

IL-1 β , IL-6, IL-8, IL-13 and TNF- α were determined in HSF using commercially available cytokine-specific enzyme-linked immunosorbent assays (ELISA) (R & D systems Inc., Minneapolis, MN, USA). Frozen HSF samples were thawed and analyzed. Samples from each patient were always measured in parallel and in duplicate to avoid interassay variance. The sensitivities of the IL-1 β , IL-6, IL-8, IL-13 and TNF- α ELISA kits were 1, 0.7, 10, 32 and 4.4 pg/mL respectively with standard curve ranges of (3.9–250), (3.12–300), (31.2–2000), (62.5–4000) and (15.6–1000) pg/mL respectively.

Effect of Hydrosalpingeal Fluid on Mouse Embryo Development

A 2-cell mouse embryo culture system was used to evaluate the embryotoxicity of each HSF specimen. Cryopreserved embryos (Embryotech, Wilmington, MA) were grown in gradually increasing concentrations of HSF diluted with human tubal fluid (HTF) media (Irvine Scientific, Santa Ana, CA) containing 0.5% bovine serum albumin (BSA) to a final volume of 1 mL. The culture dishes containing 1 mL HTF with 0.5% BSA were incubated for overnight equilibration at 37°C and 5% CO_2 . The HSF was equilibrated for 2 h at 37°C and 5% CO_2 before exposure to the embryos. Forty embryos were used to test the embryotoxicity of HSF of each patient's specimen ($n = 8$). The embryos were cultured in 0, 25, 50 and 75% concentrations of HSF in a final volume of 1 mL in an atmosphere of 5% CO_2 at 37°C . A total of 320 embryos were used. The embryos were examined following 72 h incubation and the number of embryos progressing to the blastocyst stage was recorded. The blastocyst development rate (BDR) was calculated by dividing the number of blastocysts by the total number of embryos incubated.

Statistical Methods

The relationship of each of the measurements with BDR was assessed with logistic regression, and odds ratios (with 95% confidence intervals) were calculated for significant variables. Because the data is "clustered" (i.e., several embryos within each trial), generalized estimating equation (GEE) methodology was utilized, which adjusts for the fact that each embryo is a repeated measure. Each of the cytokine measures was base-10 log-transformed to normalize their distribution. Due to the relatively small sample size, the effect of cytokines on BDR would need to be relatively large to achieve statistical significance. This study has 90% power to detect whether, as a cytokine's levels increased 10-fold, the odds of development increased or decreased by four times.

All cytokines and HSF concentration were included in the multivariate analysis, so that evaluation of significance of each cytokine is adjusted for the effect of HSF concentration and other cytokines. To illustrate the degree of relationships between variables, Spearman rank-correlations and their significance were assessed. Summary statistics are expressed as median with interquartile ranges (25th and 75th percentiles) also provided. Significance was assessed with two-tailed tests at $p < 0.05$,

Table I. Hydrosalpingeal Fluid Cytokines and Multivariate Odds Ratios of their Effect on Blastocyst Development Rate

Cytokines (pg/mL)	Median (25th percentile, 75th percentile)	Odds ratio (95% CI) ^a	p-value ^a
IL-1 β	0.7 (0.3,1.5)		0.14
IL-13	0.0 (0.0, 0.0)		1.00
IL-8	1593.9 (894.1, 8824.4)		0.06
IL-6	300.0 (66.5, 300.0)	4.35 ^a (1.32–14.15)	0.04
TNF- α	6.4 (3.4, 21.0)		0.22

^aEffect of each factor on BDR using logistic regression; odds ratio of blastocyst development after adjusting for HSF concentration based on a 10 \times increase in IL-6 levels.

and computed with SAS version 8.1 (SAS Institute, Inc., Cary, NC) software.

RESULTS

Hydrosalpingeal Fluid Cytokines

The embryotoxicity of HSF was concentration dependent. An increase in the HSF concentration resulted in significant decrease in the percentage of BDR ($p < 0.01$). IL-1 β was present in six of the eight HSF samples with a mean (\pm SD) concentration of 0.9 ± 0.8 pg/mL. IL-13 was not detected in any of the HSF samples. IL-8, IL-6 and TNF- α were de-

tected in all samples with a median concentration of 1593.9 pg/mL, 300.0 pg/mL and 6.4 pg/mL respectively (Table I). IL-6 was positively correlated with BDR ($r = 0.53$; $p = 0.04$)(Table I).

Blastocyst Development Rate of Mouse Embryos

The median BDR of the mouse embryos was 93.8, 60.0% and 45.0% at HSF concentrations of 25, 50 and 75% respectively. The BDR of the control group was 96.7%. As HSF concentration increases, BDR tends to decrease. The samples with IL-6 values greater than 300 pg/mL tended to have higher BDRs than the samples with IL-6 levels less than 300 pg/mL (Fig. 1).

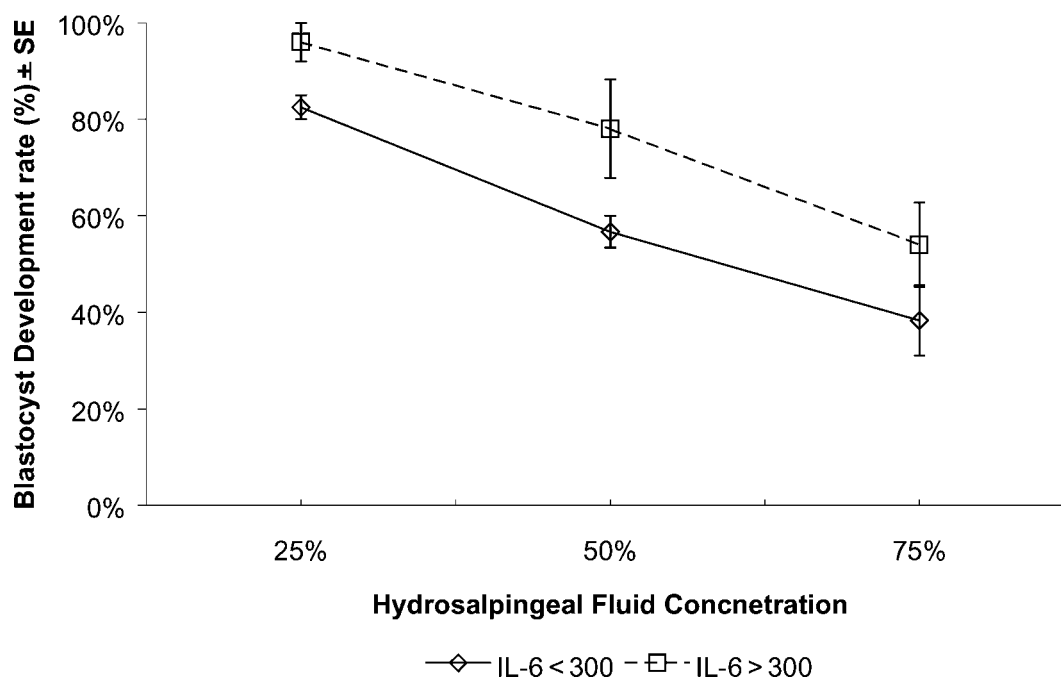


Fig. 1. Relationship of hydrosalpingeal fluid IL-6 levels and blastocyst development rate at different HSF concentration.

DISCUSSION

The exact biochemical nature of HSF is unknown. The dynamic nature and small volumes of fluid within normal fallopian tubes pose obvious difficulties in obtaining fluid to provide standard concentrations of growth factors, cytokines, prostaglandins and reactive oxygen species.

Cytokines may also play a role in the pathophysiology of HSF. Because cytokines have been associated with inflammatory processes and involved in embryotoxic and embryotrophic effects, we hypothesized that their balance in hydrosalpingeal fluid might explain the poor outcome of IVF-ET in women with distally occluded fallopian tubes. Currently, there is no scientific evidence that tubal fluid, comes into contact with the embryos which are placed into the uterus. However, this does not change the low IVF outcome in the presence of hydrosalpinx.

We characterized for the first time the presence or absence of multifunctional proteins (IL-1 β , IL-8, IL-6 and TNF- α and IL-13) in hydrosalpingeal fluid. Although we could not compare these results with a control population of non-obstructed fallopian tubes; our results should provide a better understanding of the biochemical nature of the hydrosalpingeal fluid.

Tumor necrosis factor- α was detected in 100% of the hydrosalpingeal fluid samples. This cytotoxic angiogenic cytokine, originally identified as a product of activated macrophages, is now known to be produced by many types of cells. In humans, the TNF- α transcripts and proteins have been identified in the oviduct and other reproductive tract cells (18). The effects of this cytokine on preimplantation embryo development have not been fully elucidated. Haimovici and Anderson reported that some studies have demonstrated the inhibition of both gamete function and the development of murine embryos, whereas other studies have failed to show detrimental effects (19).

Interleukin-6 is a pleiotropic cytokine produced by a variety of cell types, including monocytes, lymphocytes, fibroblasts, endothelial cells, keratinocytes, and mesangial cells (20). The cytokine, which appears to mediate numerous physiologic and pathogenic processes, acts on a wide variety of cells and regulated immune responses, acute-phase responses of the liver, hematopoiesis, neuronal functions, and osteoclastogenesis (21). The cytokine may also have important functions in reproductive physiology, including the regulation of ovarian steroid production, folliculogenesis, and early events

related to implantation (22,23). The fact that IL-6 is positively correlated with BDR may explain in part, why some hydrosalpingeal fluid samples are not embryotoxic. Also, IL-6 may not directly affect embryogenesis but simply be a marker of tubal secretory function.

IL-8, a cytokine with neutrophil chemotactic activity, is a potent angiogenic factor. It has been observed that increased peritoneal fluid level of IL-8 in patients with endometriosis observed in this study supports the results of previous studies (24).

IL-13 has the capacity to inhibit pro-inflammatory cytokine synthesis by activated macrophages (25) was not detected in any of the HSF samples tested. IL-1 β which is known to inhibit ovarian follicular cell apoptosis (26) was found in all HSF samples tested. Secretory products from healthy tubal mucosa have embryotrophic effects (27–29). Thus, the cytokine levels may be a marker of normal tubal secretory function.

We also noted a concentration dependent embryotoxic effect of HSF but only at high concentrations of 50 and 75%. This may represent a dilutional effect of required media nutrients and not the result of a true embryo toxin. Several investigators did demonstrate HSF induced embryotoxicity even at very low concentrations (i.e. less than 10%) (9–12). Others noted an embryo toxic effect only at high concentrations (i.e. 50–100%), similar to our findings (30–32).

Our study demonstrated the heterogeneous nature of hydrosalpingeal fluid, which may be due to normal inter-patient variations as well as to the cause of the hydrosalpinx. The dynamic nature and microvolumes of fluid within normal fallopian tubes pose obvious difficulties in obtaining fluid to provide data regarding the normal concentrations of cytokines, which would make comparisons to HSF more relevant.

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