The Presence of Cytokines and Growth Factors in Hydrosalpingeal Fluid¹

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Purpose: To examine the presence of cytokines and growth factors in hydrosalpingeal fluid.

Methods: Eighteen hydrosalpingeal fluids were compared with 15 follicular fluids and serum samples regarding the presence of interleukin-8 (IL-8), IL-12, IL-1 α , epidermal growth factor (EGF), granulocyte macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), tumor necrosis factor- α (TNF α), interferon- γ (IFN γ), and transforming growth factor- β_2 (TGF β_2).

Results: IL-8 and EGF were detected in all the hydrosalpinx samples. IL-8, IL-12, IL-1 α , TNF α , TGF β_2 , GM-CSF, and LIF were detected to a significantly larger extent in hydrosalpingeal than follicular fluids ($p < 0.01$). The same cytokines, with the exception of IL-8, TGF β_2 , and LIF, were also more frequently present in comparison with serum.

Conclusion: The abundant presence of cytokines in hydrosalpingeal fluid suggests an increased expression from the tubal epithelium. Whether high concentrations have a negative influence on embryo development and implantation needs further investigation.

KEY WORDS: Cytokine; hydrosalpinx; implantation; in vitro fertilization.

INTRODUCTION

The presence of hydrosalpinx is nowadays an established factor with negative impact on in vitro fertilization (IVF) outcome. Several clinical studies of retrospective character have demonstrated a significant reduction in pregnancy and birth rates, and meta-analyses have further strengthened the association between the presence of hydrosalpinx and poor IVF outcome (1,2).

Although the results of the meta-analyses are unambiguous, there is still a lack of knowledge as to how the hydrosalpinx exerts its negative effects. The main theories have focused on the hydrosalpingeal fluid and its action through possible embryotoxic proper-

ties, on mechanical leakage into the uterine cavity causing endometrial alterations hostile to embryo implantation and development, or on a simply mechanical washout of embryos (3–6). Other theories have included simultaneous damage to the endometrium at the time of the original tubal infection, influence of inflammatory substances transported through the blood or lymphatic system, and impairment of ovarian function and oocyte quality by the original infection or by the influence of toxic substances. Several studies have shown negative bacterial cultures from hydrosalpinx fluid (7–9), but other agents than microorganisms, such as lymphocytes, cytokines, prostaglandins, leukotrienes, catecholamines, and peptidergic transmitters, have been suggested to be responsible for the negative action (10,11).

The important role of the hydrosalpingeal fluid itself has been further stressed in clinical studies examining the effect of surgical intervention prior to IVF. A randomized clinical trial of salpingectomy has demonstrated that birth rates improved significantly if salpingectomy was performed, particularly evident

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in patients with large hydrosalpinges that were visible on ultrasound (12).

The underlying mechanisms including the theories focusing on the fluid and its content are sparsely investigated. We wanted to focus on cytokine content in the hydrosalpingeal fluid, to examine levels of inflammatory cytokines based on the hypothesis that high concentrations as an effect of previous infection would be associated with poor prognosis for fertilization, embryo development, and implantation. We also wanted to explore the presence of cytokines that are normally expressed in a cycle-dependant manner in the ovary, fallopian tube, and endometrium. The specific aim of this study was to compare hydrosalpingeal fluid with controls from follicular fluid and serum concerning the presence of inflammatory cytokines and growth factors known to be expressed in the human female reproductive tract.

MATERIALS AND METHODS

Patients

Hydrosalpinx fluid was collected from 18 tubes in 17 patients (mean age 35.5 years, range 27.4–45.8) undergoing laparoscopic salpingectomy for presence of hydrosalpinx. Aspiration of hydrosalpinx fluid was performed as the first intra-abdominal procedure during surgery. Great care was taken to avoid blood contamination in the aspirates. The hydrosalpinx fluids were immediately aliquotted into tubes and frozen and stored at -70°C .

Controls from serum and follicular fluid were taken at the time of oocyte retrieval from 15 patients (mean age 33.1 years, range 21.9–38.7) without hydrosalpinx undergoing IVF. The indications for IVF were male ($n = 5$), tubal without hydrosalpinx ($n = 4$), endometriosis ($n = 3$), unexplained infertility ($n = 4$), and polycystic ovary syndrome ($n = 1$). These patients were downregulated by intranasal administration of a gonadotrophin releasing hormone-(GnRH)-agonist, 1.2-mg buserelin acetate per day (SuprecurTM, Aventis Pharma, Strasbourg, France) and were subsequently stimulated with 150–225 IU per day of recombinant follicle stimulating hormone (rec-FSH) (Gonal-F, Serono, Rome, Italy, or Puregon, Organon, Holland). Oocyte maturation was induced by 10,000 IU hCG (Profasi; Serono). Follicular fluid was obtained from three to six follicles from each patient. Both blood samples and follicular fluids were centrifuged and supernatants were frozen and stored until analysis.

Ethical permission for all procedures including aspiration of hydrosalpingeal fluid, follicular fluid, and serum samples had been obtained from the Human Research Ethical Committee at Göteborg University.

Laboratory Procedures

Individual hydrosalpinx fluids were initially thawed and centrifuged at 794 g for 10 min (Biofuge 15R, Heraeus Sepatech). The fluid samples were subjected to assay employing a quantitative sandwich enzyme immunosorbent assay (ELISA) for detection of the following cytokines: interleukin-8 (IL-8), IL-12, IL-1 α , epidermal growth factor (EGF), granulocyte macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), tumor necrosis factor- α (TNF α), interferon- γ (IFN γ), transforming growth factor- β_2 (TGF β_2) (R&D Systems, Inc, Minneapolis, MN).

The sensitivities of these cytokine assays are 10 pg/mL for IL-8, 5.0 pg/mL for IL-12, 1.0 pg/mL for IL-1 α , 0.7 pg/mL for EGF, 3.0 pg/mL for GM-CSF, 8.0 pg/mL for LIF, 4.4 pg/mL for TNF α , 8.0 pg/mL for IFN γ , and 7.0 pg/mL for TGF β_2 . The intraassay variability for all cytokines ranged from 1.5 to 7.1%.

Statistics

The presence of cytokines, rather than the absolute values were considered to be more important and thus were the comparative analyses performed accordingly using dichotomous variables. Comparison was made between hydrosalpinx fluid and controls regarding the presence of cytokines by means of Fisher's exact test. Comparison of concentrations was performed when the cytokine was present in at least half of the control samples, given the concentration was > 0 , by means of Mann-Whitney U test.

RESULTS

EGF was the only cytokine that was detected in all samples of hydrosalpinx and controls. IL-8 was present in all the hydrosalpinx and serum samples, but only in one follicular fluid. IL-12 was present in the 15 hydrosalpinx fluids but only in 2 serum samples and in 1 follicular fluid. IL-1 α was expressed in 14 samples of hydrosalpinx fluid at tenfold higher levels than in the one sample each of serum and follicular fluid. GM-CSF appeared in 12 hydrosalpinx fluids but in none of

Table I. Presence and Concentrations (Given the Concentration > 0) of Cytokines in Hydrosalpinx (hsx) Fluid ($n = 18$) and in Comparison with Presence in Follicular Fluid and Serum (Fisher's Exact Test)

Cytokine	Number detected (%)	Concentration (pg/mL)			Presence in hsx vs. follicular fluid (p)	Presence in hsx fluid vs. serum (p)
		Mean	Median	Range		
IL-8	18 (100)	11400	606	12–74641	0.000	—
IL-12	15 (83)	6.7	3.1	0.77–35.8	0.000	0.000
IL-1 α	14 (78)	4.1	2.0	0.25–18.5	0.000	0.000
IFN γ	5 (28)	8.1	4.2	1.2–25.8	0.049	0.19
TNF α	10 (56)	5.7	7.0	0.23–13.8	0.001	0.001
TGF β 2	14 (78)	7170	3180	311–29849	0.000	0.11
LIF	9 (50)	88	17	0.13–500	0.009	0.09
EGF	18 (100)	37	29	8.1–128	—	—
GM-CSF	12 (67)	18	15	7.5–38.6	0.000	0.000

the controls. TGF β 2 appeared in 14 hydrosalpinx fluids, in all serum samples, but was not detectable in any follicular fluid. TNF α was present in 10 hydrosalpinx fluids and absent in all controls. A detailed description of cytokine levels in hydrosalpinx fluids and controls is given in Tables I–III.

A comparison of the presence of the different cytokines in hydrosalpinx fluids with controls revealed that IL-8, IL-12, IL-1 α , TNF α , TGF β 2, LIF, and GM-CSF were detected to a significantly larger extent in hydrosalpinx than in follicular fluids ($p < 0.01$) (Table I). The same cytokines, with the exception of IL-8, TGF β 2, and LIF, were detected significantly more often also in comparison with that in the serum.

Cytokines frequently present in either of the control fluids are displayed with their concentration distribution in comparison with that in the hydrosalpinx fluids in Fig. 1. Hydrosalpinx levels of IL-8 and TGF β 2 were significantly higher than the serum levels. EGF concentrations expressed significant differences from serum to hydrosalpinx to follicular fluid. LIF concentrations were similar in hydrosalpinx and follicular fluids.

DISCUSSION

All the chosen cytokines were detected in hydrosalpinx fluid, which is not surprising since they are known to be involved in inflammatory reactions or expressed in the female reproductive tract. A consistent problem with the analysis of hydrosalpinx fluid content is to find an appropriate control group. The ideal control fluid is a normal tubal fluid, which is difficult, if not almost impossible, to retrieve in sufficient amount *in vivo*. We choose to use serum and follicular fluid, which would represent a sample of body fluids retrieved during ovarian stimulation without any sign of ongoing infection or inflammation but with the concurrent effects of GnRH-analogues, FSH, and hCG, known to alter the local expression of cytokines in the ovary, fallopian tube, and endometrium. The control fluid would thus represent the standard situation present at the periovulatory period in IVF.

The distribution of cytokines in the control groups typically showed that only LIF and EGF were commonly present in follicular fluid. EGF was also commonly found in serum, as was IL-8 and TGF β 2.

Table II. Presence and Concentrations (Given the Concentration > 0) of Cytokines in Follicular Fluid ($n = 15$)

Cytokine	Number detected (%)	Concentration (pg/mL)		
		Mean	Median	Range
IL-8	1 (7)	137		
IL-12	1 (7)	6.6		
IL-1 α	1 (7)	0.3		
IFN γ	0	—		
TNF α	0	—		
TGF β 2	0	—		
LIF	14 (93)	27	25	2.0–51.7
EGF	15 (100)	9.4	9.2	4.9–13.7
GM-CSF	0	—		

Table III. Presence and Concentrations (Given the Concentration > 0) of Cytokines in Serum ($n = 15$)

Cytokine	Number detected (%)	Concentration (pg/mL)		
		Mean	Median	Range
IL-8	15 (100)	8.4	8.4	6.3–11.5
IL-12	2 (13)	4.3	4.3	2.1–6.6
IL-1 α	1 (7)	0.4	—	
IFN γ	1 (7)	65	—	
TNF α	0	—	—	
TGF β 2	15 (100)	166	152	111–249
LIF	1 (7)	0.7	—	
EGF	15 (100)	153	124	14–461
GM-CSF	0	—	—

Fig. 1. Cytokines frequently present in either of the control fluids and their concentrations in comparison with hydrosalpinx fluid (Mann–Whitney U test). H = hydrosalpinx; F = follicular fluid; S = serum.

In this series of patients, all but one had a unilateral hydrosalpinx. The patient with a bilateral hydrosalpinx contributes with fluid from both her tubes and they were analyzed as two separate fluids. The reason for not pooling the fluids and performing the analysis on a sole individual basis is that there was not an apparent dependence between the two fluids, which expressed a large variation between themselves.

Primary inflammatory cytokines like IL-8, IL-12, IL-1 α , and TNF α were more commonly found in hydrosalpinx fluid suggesting a retained negative effect of a tubal infection. However, since the normal physiological appearance of these cytokines in healthy tubes is unknown, we cannot truly infer that the detected levels represent a consequence of inflammatory response. IL-12, produced by macrophages in response to infectious agents, is a central mediator

of the cell-mediated immune response. It has been claimed that IL-1 α is not produced by the cells of healthy individual, but only in response to stimuli from inflammatory agents, infections or endotoxins (13). The secretion of these factors may effect the endometrial environment through reflux into the uterine cavity, but this theory still needs to be examined. Indeed, these proteins are multifunctional and although associated with inflammatory processes, their role in early embryo development is not sufficiently investigated.

IL-8 has been observed in the human fallopian tube through immunohistochemistry staining, predominantly in epithelial cells which are likely to secrete IL-8 (14). The cytokine is suggested to play an interactive role with gametes or embryos in addition to a local regulatory role in the tubal epithelium. IL-8 is also present in the human endometrium, where it displays a cyclic pattern throughout the menstrual cycle, acts as a potent angiogenic agent, and stimulates cell growth (15).

Also different isoforms of TGF β occur in the endometrium during the course of the cycle, and TGF β polypeptides have been identified as potential modulators of many endometrial functions. Both TGF β_1 and TGF β_2 are found in the developing oocyte and embryo in mouse studies, with TGF β_2 being most abundant prior to fertilization and diminished in 4- and 8-cell embryos (16). The secretion of TGF β_2 in the human fallopian tube has implied the cytokine to play an important role of gamete maturation and early embryo development also in the human (17).

Since these cytokines may be normal components of tubal fluid, regulated at specific times during the cycle, they would not have any negative effect during normal conditions on embryo development and on the endometrium, but when concentrated in hydrosalpingeal fluid, a theory is that they may loose their temporal contribution and instead cause adverse effects in the reproductive system. Indeed, the levels of IL-8 and TGF β_2 in individual hydrosalpingeal fluids were detected at levels at which these cytokines are not normally secreted. Of course, this theory is speculative and certainly needs experimental verification. Possibly, the levels in hydrosalpingeal fluid may also serve as markers of reduced implantation ability as a result of the primary infection. Chlamydia trachomatis is considered to be the main cause of tubal infection, but interestingly only five of the hydrosalpinx fluids expressed IFN γ , which has a documented antiprotozoal activity against Chlamydia (18).

Cytokines with a proliferative effect and known to be produced in the human reproductive tract (LIF, EGF, GM-CSF) were also commonly found in hydrosalpinx fluid supporting the theory of growth promoting properties, reported by a few other studies. Indeed, LIF was present in almost all of follicular fluids, suggesting the cytokine to be valuable at least to oocyte maturation. LIF seems to be regulated in the human in the same way as in the mouse, where endometrial LIF secretion is decisive for embryo implantation. In human endometrium, a maximal rise in LIF concentration is observed between cycle days 19 and 25 coinciding with the implantation window (19). Both LIF and GM-CSF have been shown to be beneficial for preimplantation development, by demonstration of an increased proportion of embryos developing to blastocysts in vitro (20,21). In this study, LIF and GM-CSF were present in at least half of hydrosalpinx samples, which raises the question whether the lack of certain growth factors causes the impaired implantation ability in hydrosalpinx patients.

The production of tropho-uteronection (TUN) by human cytotrophoblasts has been studied in order to describe early embryo-integrin interactions. The production of TUN was significantly increased by the presence of hydrosalpinx fluid in an in vitro system (22). The results indicated that hydrosalpinx fluid promoted the production of TUN in a dose-dependent manner. Furthermore, the study by Sawin demonstrated a significant increase in trophoblast cell viability as well as in the production of β -hCG in the presence of hydrosalpinx fluid. Granot *et al.* studied the effect of hydrosalpinx fluid on steroid production and demonstrated that granulosa cells incubated in the presence of hydrosalpinx fluid produced a higher concentration of progesterone than control cells in its absence (8). All these findings indicate that hydrosalpinx fluid may possess growth-promoting rather than embryotoxic properties.

There is one study with similar design as ours, including collection of hydrosalpingeal fluids and examination with respect to cytokines and growth factors (23). Generally, cytokines were detected to a lesser degree as compared to our results and contrary to our findings IFN γ and TGF β_2 were not detected in any of the 10 samples. EGF and TNF α were found with similar concentrations in both studies. The discrepancy between the results supports the concept that hydrosalpinges are individually different, even within an individual with bilateral hydrosalpinges, and might express a variety of properties ranging from

embryotoxicity to growth promotion. The discrepancy might also be an effect of a small sample size ($n = 10$) and a larger sample would certainly give a better estimate of whether hydrosalpingeal fluids are as heterogeneous as suggested. The time lapse since the original infection may also play a role in the composition and properties of the hydrosalpingeal fluid.

In a somewhat larger study, the composition of 39 hydrosalpingeal fluids including the cytokines EGF, LIF, $\text{TNF}\alpha$, and $\text{IFN}\gamma$ was included in a logistic regression analysis for determination of predictors of IVF outcome (24). The cytokine concentration was not predictive of subsequent IVF outcome, but the blastocyst formation rate in a mouse embryo culture model with 50% hydrosalpinx fluid was an independent predictor as was age. Again, this finding supports that individual hydrosalpingeal fluids may be deleterious to embryo growth and/or implantation.

Two other cytokines have been studied as predictors of IVF outcome. The presence of IL-8 and IL-12 in periovulatory follicular fluid in relation to fertilization failure was investigated in 52 women undergoing IVF (25). IL-8 did not affect outcome while failed fertilization was significantly more common among IL-12 positive patients. Although this study did not involve patients with hydrosalpinx, the association between IL-12 and poor IVF outcome can be viewed in relation to the high levels found in hydrosalpingeal fluid in our study as compared with only one case with IL-12 in follicular fluid.

This study is the first to evaluate a large number of cytokines in hydrosalpinx fluid in relation to other body fluids. It can be concluded, that hydrosalpinx fluid displays a variety of factors with possibly both inhibitory and stimulatory properties, suggesting that hydrosalpinges are heterogeneous. The interaction of these factors and their action at different concentrations on embryo growth and implantation needs further evaluation.

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