Human Reproduction vol.13 no.6 pp.1620–1624, 1998

Is hydrosalpinx fluid cytotoxic?

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Accumulation of oviductal fluid in the ampullar lumen as a result of occlusion of the infundibulum is referred to as hydrosalpinx. A low pregnancy rate (10%) after in-vitro fertilization (IVF) in hydrosalpinx patients and a relatively high incidence (50%) of abortions during the first trimester suggested that leakage of this fluid into the uterine cavity may exert a cytotoxic effect on the developing embryo. To examine this possibility, we analysed the composition of the hydrosalpinx fluid and tested its effect on human granulosa cells and embryos. Hydrosalpinx fluids and granulosa cells were collected from IVF patients at ovum pick-up. IVF eggs containing three pronuclei (3PN) were employed for this study. Analysis of hydrosalpinx fluids revealed electrolyte concentrations similar to those in serum with lower amounts of total protein and albumin. No blood cells were detected and bacterial cultures were negative. Granulosa cells incubated in hydrosalpinx fluid-containing medium (diluted 1:1) were not morphologically different and showed a steroidogenic capacity that was higher than that of cells incubated in its absence. Fertilized 3PN eggs incubated in IVF culture medium successfully developed into 6- to 8- and 8- to 16-cell embryos within 48 and 72 h, respectively. This rate of embryonal development was not impaired by hydrosalpinx fluid (at either 50 or 100% concentration). In the absence of a demonstrable detrimental effect we suggest that the low implantation rate in hydrosalpinx IVF patients may not be due to an embryotoxic effect. We further suggest that constant passage of fluid into the uterine cavity in these patients could possibly introduce some mechanical interference that may result in implantation failure.

Key words: cytotoxicity/embryos/granulosa/human/hydrosalpinx

Introduction

In-vitro fertilization (IVF) was originally designed to by-pass the impediment created by oviductal obstructions that prevent in-vivo sperm–egg interaction. Such oviductal pathology that involves occlusion of the infundibulum, leading to accumulation of the oviductal fluid in the ampullar lumen, is referred to as hydrosalpinx (Harper, 1994). Previous studies have demonstrated that the rate of pregnancies in IVF patients with hydrosalpinx is very low (up to 10%) as compared with that in other IVF patients (30%). Half of the pregnancies in hydrosalpinx patients end in abortion during the first trimester. The poor success rate of pregnancy in these patients is apparently due to a very low implantation rate: 3–4% in hydrosalpinx as compared with 10–12% in non-hydrosalpinx patients (Andersen *et al.*, 1994; Strandell *et al.*, 1994; Vandromme *et al.*, 1995; Fleming and Hull, 1996; Katz *et al.*, 1996a). It has been suggested that leakage of fluid from the oviduct into the uterine cavity in these patients, exerts a cytotoxic effect on the developing embryo, interfering with normal implantation (Strandell *et al.*, 1994; Vandromme *et al.*, 1995; Fleming and Hull, 1996; Katz *et al.*, 1996a). This idea gained support from a recent study showing that hydrosalpinx fluid has a deleterious effect on murine embryos (Mukherjee *et al.*, 1996). Assuming that the influences of human hydrosalpinx fluid on mice and human are not necessarily identical, we further undertook the task of examining its effect on the steroidogenic capacity of human granulosa cells and on the developmental rate of human embryos.

Materials and methods

Hormonal treatment protocol of IVF patients

Patients were treated with 900 μg/day of the gonadotrophin releasing hormone (GnRH) analogue buserelin acetate (Suprefact, Hoechst AG, Frankfurt, Germany) from the 2nd to the 17th day of the menstrual cycle to achieve pituitary down-regulation and endogenous gonadotrophin depletion. When serum 17β oestradiol concentrations decreased to <200 pmol/l, daily doses of 150–225 IU of human menopausal gonadotrophin (HMG; Pergonal, Teva, Petah-Tikva, Israel) were administered for stimulation of follicular growth. Upon sonographic detection of at least two 18 mm lead follicles, 10 000 IU of human chorionic gonadotrophin (HCG; Teva, Petah-Tikva, Israel) was administered for induction of oocyte maturation.

Fluids, cells and embryos: collection and cultures

Hydrosalpinx fluid collection

Oviductal fluids were collected from four hydrosalpinx patients undergoing IVF, who were diagnosed by hysterosalpingogram, laparoscopy and transvaginal sonography, and had provided written informed consent. The fluids were aspirated transvaginally at the time of ovum pick-up (OPU) using an additional 17-G needle that was introduced into the Fallopian tube. Sonographic analysis in all cases demonstrated collapsed hydrosalpinges after fluid aspiration with no further refill two days later, at embryo transfer. The aspirated fluids were analysed for their steroid hormones contents and electrolyte composition and examined for their effect on human granulosa cells and embryos.

Table I. Concentrations of total protein, progesterone and oestradiol in human hydrosalpinx and follicular fluids

Patient no.	Fluids	Total protein (g/dl)	Progesterone (nmol/l)	Oestradiol (pmol/l)
1	Hydrosalpinx	0.12	0.7	143
	Follicle	5.7	58.0×10^3	176×10^4
2	Hydrosalpinx	0.45	2.0	73
	Follicle	6.2	84.0×10^3	194×10^4
3	Hydrosalpinx	0.6	10.0	430
	Follicle	6.2	13.0×10^3	360×10^4
4	Hydrosalpinx	0.4	46.0	177
	Follicle	5.9	73.2×10^3	351×10^4

Cell cultures

Granulosa cells were recovered from aspirated follicular fluids obtained at ovum retrieval from another group of four individual patients. Following centrifugation (300 *g* for 5 min) the cells were resuspended in 1031-IVF culture medium (Medicult, Copenhagen, Denmark), plated on 35 mm Falcon Petri dishes (~0.3 mg total protein/dish) and incubated for four days with IVF culture medium at 37°C in an atmosphere of 5% CO_2. Red blood cells were removed by daily medium replacements. On the fifth day the medium was supplemented with hydrosalpinx fluid (50%) and the cells were incubated for an additional period of 24 h. The morphology of the cells was examined and their steroidogenic capacity evaluated.

Embryos

Fertilized eggs that exhibited 3PN, from women participating in our IVF programme, were employed in this study. These 3PN were incubated in the presence or absence of hydrosalpinx fluids in three groups as follows. Group 1 (control group): eggs (n = 11) were incubated in culture medium in two subgroups: group 1a, eggs (n = 5) were incubated in IVF culture medium for the five days of the experiment; group 1b, eggs (n = 6) were incubated initially in IVF culture medium and transferred, 48 h later, into M3 medium (Medicult, Copenhagen, Denmark). This last medium is known to support the development of 4- to 8-cell embryos to blastocysts. Group 2: eggs (n = 11) incubated in a 1:1 dilution of hydrosalpinx fluid in two subgroups: group 2a, eggs (n = 7) incubated for the five days of the experiment in hydrosalpinx fluid diluted (1:1) with IVF culture medium; group 2b, eggs (n = 4) incubated for 48 h in hydrosalpinx fluid diluted (1:1) with IVF culture medium and then transferred into the same dilution of hydrosalpinx fluid with M3 medium. Group 3: eggs (n = 12) incubated in non-diluted hydrosalpinx fluids. Groups of one to three embryos each were incubated in a total volume of 0.5 ml and placed in one-well culture dishes (Falcon).

The embryos were examined microscopically for their development and the rate of blastomere amplification was monitored. The use of these human embryos was approved by the Helsinki committee of the Kaplan Medical Centre.

Steroid hormone measurements

The steroidogenic capacity of the granulosa cells was evaluated by progesterone determination in medium samples collected at the end of incubation. Progesterone concentrations were determined by radioimmunoassay using the Coat-A-Count Progesterone kit (DPC, Los Angeles, CA), according to the manufacturer's protocol and normalized according to the individual protein content of each sample. Protein contents were measured in cells washed twice in cold PBS and collected in 0.1 ml PBS by scraping of the culture dish (Bradford, 1976).

Progesterone and 17β oestradiol were also determined in samples of hydrosalpinx and follicular fluids by radioimmunoassay using the Coat-A-Count Progesterone kit, according to the manufacturer's protocol.

Electrolyte and osmolarity determinations

Electrolyte levels in hydrosalpinx fluids were determined using a Hitachi 747 system analyser, using standard reagents (Boehringer Mannheim, Germany), according to the manufacturer's protocol.

Osmolarity of these fluids was measured by the freezing point depression method using a digital osmometer (3D3 Advanced Osmometer; Advanced Instruments Inc., Norwood, MA, USA).

Results

Composition analysis of hydrosalpinx fluid

Concentrations of total protein, progesterone and oestradiol were analysed in the hydrosalpinx fluids and compared with those of fluids aspirated from large antral follicles of the same patient. Comparison of these values revealed that for each individual, concentrations of the two steroid hormones, progesterone and oestradiol, were higher in the follicular fluid by three and four orders of magnitude than in the hydrosalpinx fluid. Total protein contents were at least 10-fold higher in follicular fluids than in the hydrosalpinx (Table I). These results confirmed that the fluids which were subsequently used for egg incubations indeed originated from the hydrosalpinges and were not the result of aspiration from the large antral follicles.

Concentrations of electrolytes determined in different samples of hydrosalpinx fluid are presented in Table II and compared with the range of concentrations of these same electrolytes normally present in human serum. These results revealed that the concentrations of urea and potassium in the hydrosalpinx were within the normal range of serum concentrations of these electrolytes. Levels of other electrolytes analysed were somewhat lower than their normal serum concentrations. Neither red nor inflammatory white blood cells were detected in the hydrosalpinx fluid and bacterial cultures were negative. The pH value of non-diluted hydrosalpinx fluids was 8.6–8.7, and this was reduced to 7.30 after dilution of fluids to 50% with culture medium. Osmolarity values of the hydrosalpinx fluids (268–280 mOsm) were within the physiological range and similar to that of the IVF culture medium (282 mOsm).

Effect of hydrosalpinx fluid on granulosa cells

The effect of hydrosalpinx fluid on the viability of cultured granulosa cells was evaluated from both morphology and

Table II. Electrolyte concentrations in human hydrosalpinx fluids compared with reference concentrations of human serum

Electrolyte	Patient #1	Patient #2	Patient #3	Patient #4	Mean ± SD	Normal serum
Urea (mg/dl)	18	15	24	21	20 ± 3	12–43
Sodium (mEq/l)	142	139	144	125	138 ± 7	153–145
Potassium (mEq/l)	4.0	4.8	4.9	3.2	4.2 ± 0.7	3.4–5.2
Calcium (mg/dl)	5.8	9.1	4.5	5.0	6.1 ± 1.8	8.5–10.5
Magnesium (mg/dl)	0.66	0.51	0.74	1.02	0.73 ± 0.19	1.7–2.8
Inorganic phosphates (mg/dl)	0.7	0.1	0.1	0.3	0.3 ± 0.2	2.7–4.5

Table III. Effect of hydrosalpinx fluids on progesterone production by human granulosa cells

	Progesterone (pmol/mg protein/24 h)			
Hydrosalpinx fluid	Patient #1	Patient #2	Patient #3	Patient #4
–	12	11	22	4.3
+	18	13	30	5.6
Increase in progesterone production (%)	50	18	36	30

Table IV. Effect of hydrosalpinx fluid on early embryonic development of human 3PN eggs

	Concentration of hydrosalpinx fluid in the incubation medium		
Embryonic developmental stage	None (Control, $n = 11$)	50% ($n = 11$)	100% ($n = 12$)
8–16 cells	11	10	11
Blastocyst	–	1	1

steroidogenic capacity. Granulosa cells incubated in hydrosalpinx fluid-containing medium were not morphologically different from control cells incubated in its absence. Surprisingly, cells incubated in the presence of hydrosalpinx fluid produced a higher concentration of progesterone (mean ± SD increase 33.5 ± 11.5%) than was generated by control cells incubated in its absence (Table III).

Effect of hydrosalpinx fluid on human embryos

The possibility that hydrosalpinx fluid exerts a cytotoxic effect on the developing embryo was examined by analysing the rate of development of human embryos incubated in hydrosalpinx fluid-containing and control media.

All of the in-vitro fertilized 3PN eggs, whether incubated in the presence or absence of hydrosalpinx fluid, underwent cleavage and developed into 8- to 16-cell embryos within 48 h. No difference was observed between the two control groups and the results are therefore not broken down (Table IV, Figure 1), showing a similar rate of blastomere amplification. In all but two experiments, development *in vitro* proceeded to the 8- to 16-cell stage with longer incubation. Surprisingly, one of the embryos incubated in 100% hydrosalpinx fluid and one of those incubated in 50% hydrosalpinx fluid:50% IVF culture medium further developed into blastocysts on the fifth day of culture (Table IV). Differentiation into trophectoderm and inner cell mass in these embryos was clearly observed (Figure 1d). The blastocyst in 50% hydrosalpinx fluid continued its developmental process and hatched from its zona pellucida 2 days later (Figure 1e and f).

Discussion

Our experiments suggest that hydrosalpinx fluid may have no cytotoxic effects on early human embryogenesis. The developmental rate of human embryos incubated with this fluid was essentially similar to that of embryos incubated in its absence. In agreement with these findings, hydrosalpinx fluid did not inhibit, but rather promoted, steroidogenic activity of human granulosa cells in culture. Similar to previous reports, composition analysis of this fluid did not reveal the presence of inflammatory elements.

Several laboratories have recently examined the effect of human hydrosalpinx fluid on mouse embryo development. It has been reported that incubation of mouse fertilized eggs in the presence of different concentrations of hydrosalpinx fluid (1–100%) arrested embryonic development and in some cases led to embryo degeneration as early as the 2- to 4-cell stages (Mukherjee *et al.*, 1996). Other work has shown a dose-dependent negative effect of hydrosalpinx fluid on mouse embryo development (Katz *et al.*, 1996b). However, Murray *et al.* (1996) have presented contradictory results demonstrating that mouse embryo growth was not affected by the presence of human hydrosalpinx fluid.

These conflicting results leave open the question regarding

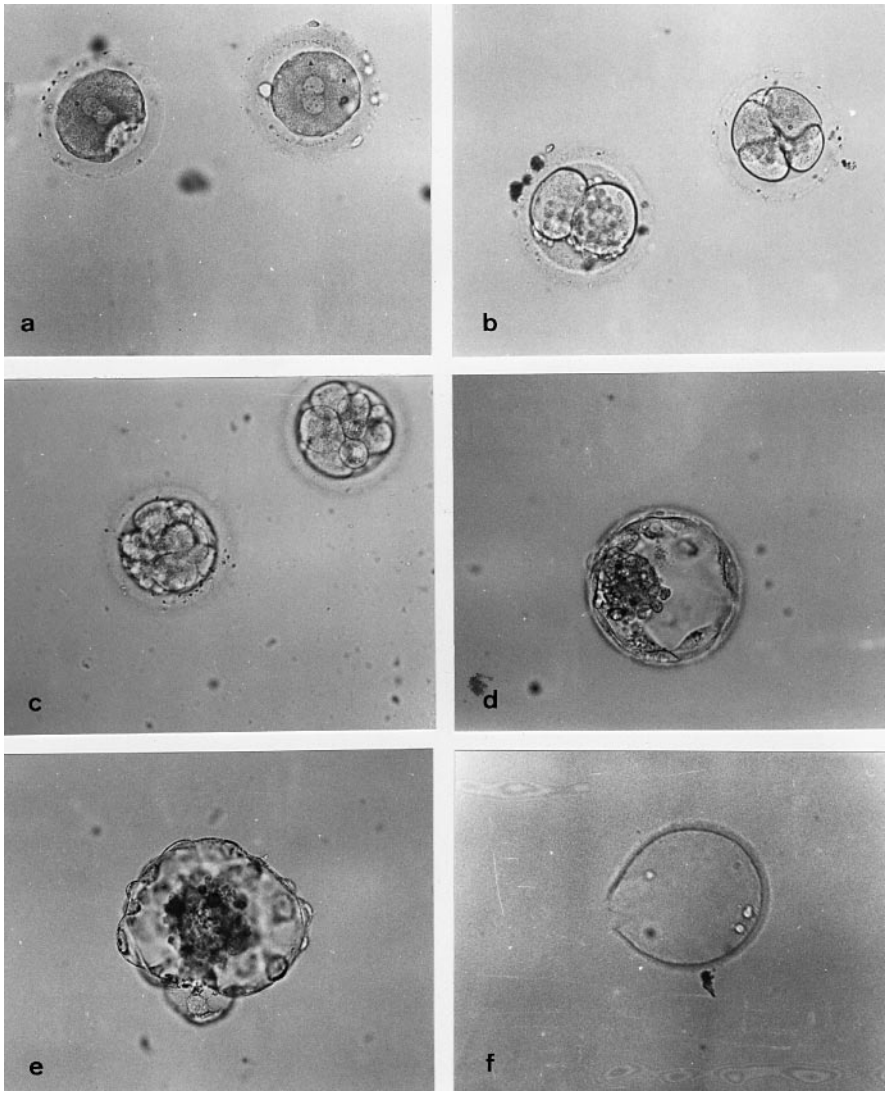

Figure 1. Early human embryogenesis in medium containing hydrosalpinx fluid (50%). (**a**) 3PN fertilized eggs. (**b**) 2- and 4-cell -embryos. (**c**) 6- to 8-cell embryos. (**d**) A blastocyst. (**e**) A hatched blastocyst. (**f**) The empty zona pellucida, after hatching.

the influence of human hydrosalpinx fluid on mouse embryos. However, the definitive solution of this issue may not necessarily be relevant to the possible effect of hydrosalpinx fluid on human embryos. An experimental system that employs normal human embryos is the only appropriate biological model for clarification of this issue. However, legal restrictions in Israel do not allow the use of normal 2PN embryos for experimentation. Under these restrictions, the use of 3PN human embryos represents a reasonable compromise for studying the effect of hydrosalpinx fluid on early human embryonic development. Using this system, we clearly failed to show a detrimental effect of hydrosalpinx fluid on early embryogenesis in humans. Experiments with human ovarian granulosa cells also suggested a lack of cytotoxic effect of hydrosalpinx fluid on human tissues, supporting this conclusion.

Our results, as well as those of previous studies, show that the electrolyte concentrations (David *et al.*, 1969) and the osmolarity (Mukherjee *et al.*, 1996) of hydrosalpinx fluid are similar to those of serum. Chemical analyses of normal oviductal fluids (non-hydrosalpinx), collected from patients undergoing hysterectomy and bilateral salpingo-oophorectomy, also revealed electrolyte concentrations similar to those of serum (Lippes *et al.*, 1972; David *et al.*, 1973; Borland *et al.*, 1980). Taken together, these results suggest a high level of similarity between normal oviductal and hydrosalpinx fluid. Taking into account that the oviductal milieu supports early embryonal development, our results may not be unexpected. It may not be surprising also that embryonic development did not stop at the morula stage, and rather proceeded to the blastocyst stage, but only in the presence of hydrosalpinx fluid (50 and 100%).

The newly formed embryo does not leave the oviduct before its transition from the morula to the blastocyst, that occurs on the fifth day of pregnancy. Thus, the blastocyst, rather than embryos at earlier stages of development, is most appropriate for transfer into the uterus. It is therefore desirable to culture human fertilized eggs to the blastocyst stage under conditions that will support their development *in vitro*. To achieve this purpose, the medium is presently supplemented with pyruvic acid and autologous serum (Hardy *et al.*, 1996). Our findings

appear to imply that hydrosalpinx fluid may be an appropriate supplement in order to support the development of the blastocyst *in vitro*.

In summary, the results of this study are in conflict with the commonly accepted idea that hydrosalpinx fluid is embryotoxic. The low implantation rate in hydrosalpinx IVF patients may result from the constant passage of fluid into the uterine cavity causing mechanical interference with the process of implantation. This possible mechanism of interference has been suggested previously (Andersen *et al.*, 1994, 1996; Akman *et al.*, 1996; Bloechle *et al.*, 1997). Hence, the reported improvement of pregnancy rates in IVF patients with hydrosalpinx after salpingectomy (Vandromme *et al.*, 1995; Shelton *et al.*, 1996) may also result from the elimination of a mechanical interference rather than of an embryotoxic effect of hydrosalpinx fluid.

Acknowledgements

The authors would like to thank to Mrs Malka Chen, Mr Moshe Rosenberg and Mrs Mira Ulman for their assistance in steroid hormone determinations, and Mrs Malka Kopelowitz for her secretarial assistance.

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Received on September 16, 1997; accepted on 4 March, 1998