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The embryo toxicity of hydrosalpinx fluid is only apparent at high concentrations: an in vitro model that simulates in vivo events

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Objective: To simulate the in vivo model in studying the effect of hydrosalpinx fluid on embryonic development.

Design: Controlled prospective study.

Setting: Academic research center.

Patient(s): Five hundred eighty-seven two-cell murine embryos.

Intervention(s): Embryos were grown under two sets of conditions. Half were cultured using 10% fetal calf serum in RPMI medium in varying concentrations of hydrosalpinx fluid (0, 1%, 10%, 50%, 75%, and 100%). To more closely mimic the in vivo environment, the other half were grown in an endometrial coculture system with the same media and hydrosalpinx fluid concentrations.

Main Outcome Measure(s): Embryonic development.

Result(s): For each stage of embryogenesis, diminished development was noted with increasing concentrations of hydrosalpinx fluid. In the group of embryos grown without endometrial coculture, only at a minimum concentration of 50% hydrosalpinx fluid was diminished development noted for the blastocyst, hatching, and outgrowth stages. When an endometrial coculture system was used, development was not inhibited until exposure to a minimum of 75% hydrosalpinx fluid. Embryogenesis was enhanced when an endometrial coculture system was used for each concentration of hydrosalpinx fluid.

Conclusion(s): When a model is used that more accurately mimics the in vivo conditions of IVF-ET in a patient with hydrosalpinges, it appears that high concentrations of hydrosalpinx fluid are required to significantly impede embryogenesis. The endometrium appears to help detoxify hydrosalpinx fluid. (Fertil Steril® 1999;71:619–26. ©1999 by American Society for Reproductive Medicine.)

Key Words: Embryo, hydrosalpinx, coculture, IVF

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Tubal factor infertility was the original indication for IVF-ET. Recent reports have suggested that patients with tubal factor infertility who have hydrosalpinges have diminished implantation and pregnancy rates (1–6). However, not all studies have concurred with these findings (7).

Hydrosalpinx fluid is known to reflux into the endometrial cavity, and this observation has led investigators to use mouse embryos in experiments that attempt to document the toxicity of hydrosalpinges (8–10). These studies have grown mouse two-cell embryos in culture media and varying concentrations of hydrosalpinx fluid in an attempt to reproduce the in vivo model. Unfortunately, these experiments have been somewhat deficient in their experimental design. In IVF-ET, human embryos transferred to a patient with hydrosalpinges are placed within the endometrial canal. The endometrium is known to actively secrete a variety of cytokines and growth factors that play a role in early embryonic development (11). Prior experiments have lacked an important component of the in vivo system—the endometrium.

In this study, we used an in vitro model that more closely mimics the in vivo environment, allowing us to study the possible toxicity of hydrosalpinx fluid using an endometrial coculture system. In this model, we combined all three components of the in vivo system (the embryo, hydrosalpinx fluid, and endometrium) to construct an in vitro model that more accurately depicts the events that occur after ET in patients with hydrosalpinges.

MATERIALS AND METHODS

Hydrosalpinx Fluid

Hydrosalpinx fluid was collected in an aseptic manner from four patients who were undergoing laparoscopy for infertility and were known to have hydrosalpinges. Each of the patients selected had at least 20 mL of hydrosalpinx fluid aspirated. After collection, the fluid was spun at $400 \times g$ for 20 minutes. The cell-free supernatant was collected and immediately frozen at −20°C until used.

Collection of Mouse Embryos

Mouse embryos were isolated as previously described (12). Briefly, 6–8-week-old female mice (CB6F1/Cr1BR) received intraperitoneal injections of pregnant mare serum gonadotropin (Sigma, St. Louis, MO) and an additional injection of hCG (Sigma) 48 hours later. The morning after hCG administration, the mice were examined for copulation plugs. The females that exhibited copulation plugs were killed 42 hours after hCG administration, and two-cell embryos were flushed from the oviducts into phosphate-buffered saline (Sigma) with 0.35% bovine serum albumin (Sigma). The embryos were washed four times with the phosphate-buffered saline plus 0.35% bovine serum albumin before culture or coculture.

Endometrial Coculture

Endometrium was obtained from one patient with proven fertility and a known history of tubal disease (secondary to a history of pelvic inflammatory disease). This endometrium was used for all the experiments described herein. The endometrium was obtained by a luteal phase endometrial biopsy using a Pipelle Endometrial Suction Curette (Unimar, Wilton, CT). The biopsy was performed 5 days after the LH surge was detected by a urinary ovulation predictor kit. The digestion and separation of the endometrium has been described previously (13).

In brief, the tissue then was minced into small pieces (1–2 mm^3) and washed with Hanks' Balanced Salt Solution (GIBCO BRL, Grand Island, NY) supplemented with 5,000 μg/100 mL of penicillin-streptomycin (GIBCO BRL) to remove excess red blood cells and mucus.

Incubation of the tissue pieces for 5 minutes at 37°C in a shaking water bath in 10 mL of Hanks' balanced salt solution containing 0.2% collagenase type 2 (Sigma) then was performed. Cell clumps were dispersed by brisk aspiration through a sterile transfer pipette. The digested tissue pieces then were allowed to settle by differential sedimentation at unit gravity for 5 minutes. After sedimentation, the supernatant, containing a mixture of single stromal cells and small intact glands, was transferred into a separate 15-mL polyethylene test tube and centrifuged at $400 \times g$ for 5 minutes. The pellet was resuspended in RPMI medium 1640 (GIBCO BRL) supplemented with 10% patient's serum (RPMI/10% serum) and 5,000 μg/100 mL of penicillin-streptomycin.

These steps were repeated four times, resulting in a combined 4 mL of single stromal cells mixed with small glands. This sample of stroma and small glands underwent another differential sedimentation at unit gravity for 45 minutes to separate most of the small glands from the single stromal cells remaining in solution. The supernatant, containing the stroma-enriched fraction, was centrifuged at $400 \times g$ for 5 minutes and the cell pellet was resuspended in RPMI/10% patient serum. A small aliquot of the sample was diluted 1:1 with trypan blue stain 0.4% (GIBCO BRL) and cell yield and viability were determined quantitatively on a hemocytometer. Tissue culture flasks (25 cm^2) were seeded with approximately 5×10^5 cells.

The tissue pieces, which remained after the four digestions, contained predominantly intact glands mixed with undigested connective tissue and stromal clumps. Concurrently, the glands were purified further by resuspension in 10 mL of Hanks' Balanced Salt Solution. After approximately 30 seconds, the largest fragments (stromal clumps and undigested tissue) settled to the bottom of the 15-mL test tube, and the top 8 mL, which had a "snowflake" appearance (glands and single stromal cells), was transferred to another 15-mL test tube and allowed to settle for 30 minutes at unit gravity. This sedimentation allowed most of the glands to form a pellet at the bottom of the test tube while leaving the remaining single stromal cells in the supernatant that was removed and discarded. This glandular-enriched pellet then was resuspended in RPMI medium with 10% fetal calf serum and plated onto one 25-cm^2 tissue culture flask.

The seeded tissue flasks were maintained at 37°C in a 5% CO_2 air atmosphere and the culture medium was changed every 2–3 days. After approximately 1 week, the cells reached confluence and were released with trypsin-ethylenediaminetetraacetic acid (GIBCO BRL). The cells were cryopreserved in a 15% glycerol solution, frozen at −70°C overnight, and transferred to liquid nitrogen storage.

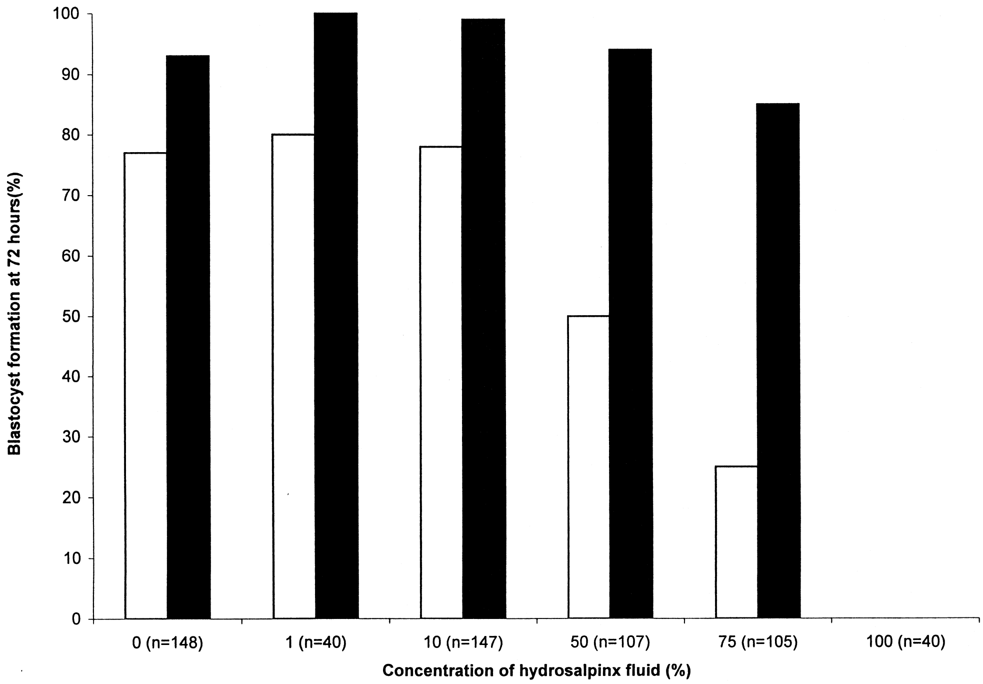
Approximately equal mixtures of the glandular and stromal cells were thawed 3 days before placement of the mouse embryos on the coculture cells. Cell count and viability were determined, and approximately 3×10^5 cells were seeded into a 96-well tissue culture plate containing 100 μL of RPMI medium with 10% fetal calf serum. In general, approximately 95% confluence was achieved when the mouse embryos were placed into the coculture system.

Experimental Design

Each experiment used one patient's hydrosalpinx fluid. For each experiment, 160 two-cell mouse embryos were obtained. The embryos were grown in varying concentrations of hydrosalpinx fluid diluted with RPMI medium with

FIGURE 1

Blastulation rate at 72 hours. The *open bar* represents embryos grown in hydrosalpinx fluid only without endometrial coculture cells. The *solid bar* represents cells grown in hydrosalpinx fluid with endometrial coculture cells. Seventy-seven percent (57/74) of embryos grown without coculture or hydrosalpinx fluid were blastocysts at 72 hours. Not until a 50% hydrosalpinx fluid (50% media) mixture was used was the blastulation rate lower in the embryos grown without coculture (57/74 vs. 27/54; $P = .003$; χ^2 analysis). In the coculture model, 93% (69/74) of the embryos were blastocysts at 72 hours. This was not different from the 75% hydrosalpinx fluid (25% media) group (69/74 vs. 45/53; $P = .21$). In 100% hydrosalpinx fluid, no embryo from either the coculture or the noncoculture group reached the blastocyst stage of development. For each dilution of hydrosalpinx fluid, embryos grown in coculture were more likely to reach the blastocyst stage.



10% fetal calf serum. For the first experiment, the following concentrations were used: 0, 1%, 10%, and 100%. For the subsequent experiments, the following concentrations were used: 0, 10%, 50%, and 75%. Each embryo was grown in an initial volume of 100 μL with an additional 50 μL added at 120 and 168 hours after the beginning of the experiment. Before exposure to the embryos, the fluid was equilibrated for 12 hours in an environment of 37°C and 5% CO_2.

We did not correct for osmolarity or pH changes found in the hydrosalpinx fluid; however, after addition of the media and equilibration in the incubator, these were found to be within the normal range. For each group of embryos grown in a specific concentration of hydrosalpinx fluid, half were grown in an endometrial coculture system and the other half were grown without the presence of endometrial coculture cells. All embryos were grown in 96-well plates in an atmosphere of 37°C and 5% CO_2. The embryos then were analyzed at 72 hours, 120 hours, and 168 hours for development. Embryos were analyzed for the following features: blastocyst formation, hatching, and outgrowth stages as previously described.

Statistical Analysis

Categoric variables were assessed by the χ^2 test, with Yates' correction or Fisher's exact test used in the case of small cell frequencies. $P < .05$ was considered statistically significant.

Institutional Review Board Approval

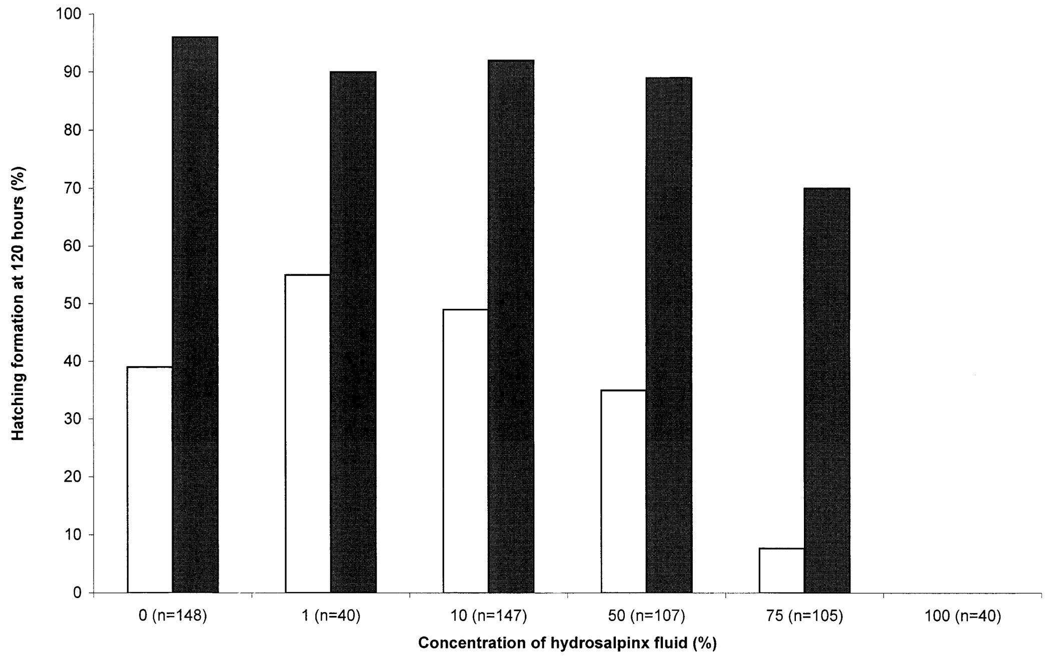
Institutional review board approval was obtained for this study, and the mice were used in accordance with institutional policy.

RESULTS

The four experiments demonstrated similar trends in toxicity at each level of development, and thus the data were pooled. Figure 1 compares the percentage of embryos that achieved the blastocyst developmental stage after 72 hours. For the embryos grown in media alone without the use of endometrial coculture, 77% (57/74) developed to the blastocyst stage. Using these embryos as a control, embryos grown in a concentration of 50% hydrosalpinx fluid and 50% media demonstrated a decrease in blastulation (50% [27/54],

FIGURE 2

Hatching stage at 120 hours. The *open bar* represents embryos grown in hydrosalpinx fluid only without endometrial coculture cells. The *solid bar* represents cells grown in hydrosalpinx fluid with endometrial coculture cells. Thirty-nine percent (29/74) of embryos grown without coculture or hydrosalpinx fluid were hatching at 120 hours. Not until a 75% hydrosalpinx fluid (25% media) mixture was used was the hatching rate lower in the embryos grown without coculture (57/74 vs. 27/54; $P = .003$; χ^2 analysis). In the coculture model, 96% (71/74) of the embryos were hatching at 120 hours. This was different from the 75% hydrosalpinx fluid (25% media) group (71/74 vs. 37/53; $P = .002$). In 100% hydrosalpinx fluid, no embryo from either the coculture or the noncoculture group reached the hatching stage of development. For each dilution of hydrosalpinx fluid, embryos grown in coculture were more likely to reach the hatching stage.



$P < .05$). For lesser dilutions of hydrosalpinx fluid, no difference in blastulation rates was noted.

For each dilution of hydrosalpinx fluid, the embryos grown in endometrial coculture demonstrated significantly higher rates of blastulation. The blastulation rate for the embryos grown in endometrial coculture without the addition of hydrosalpinx fluid was 93% (69/74). No difference in the blastulation rate was appreciated for the endometrial coculture group until a 100% concentration of hydrosalpinx fluid was used.

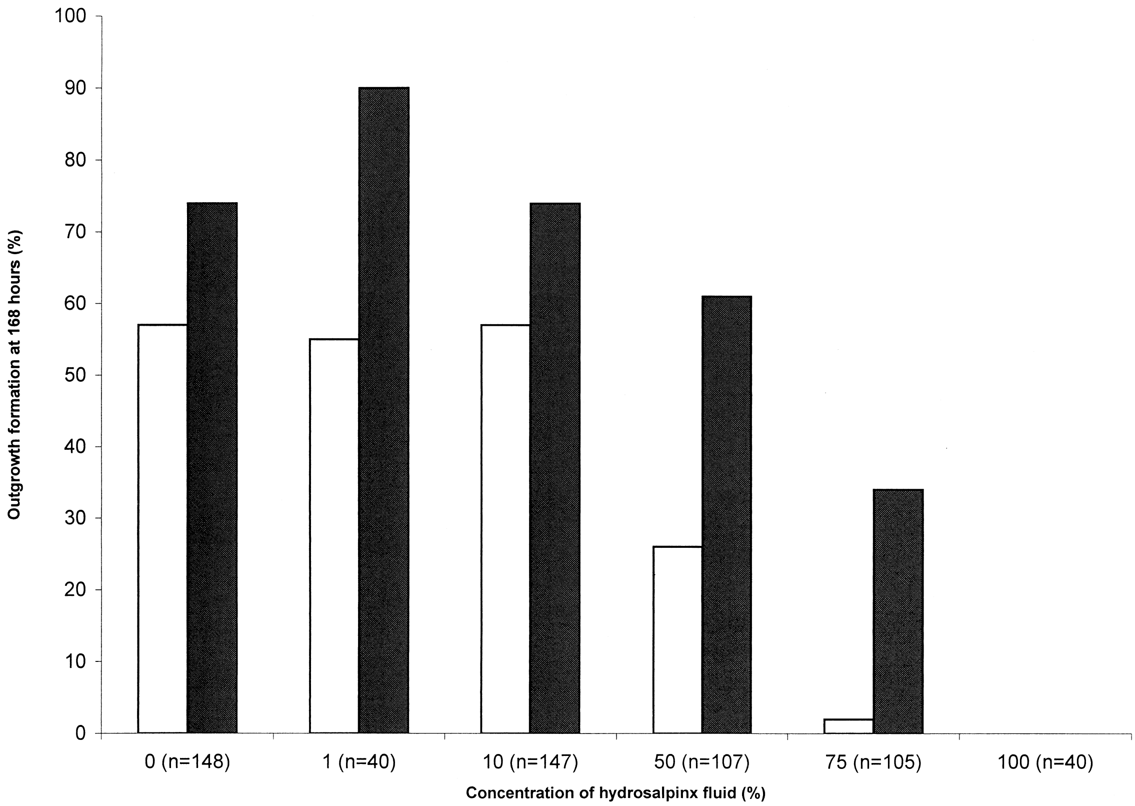
Figures 2 and 3 illustrate the hatching and outgrowth development rates. Similar to what was noted at the blastocyst stage, when the embryos grown in media alone without hydrosalpinx fluid as a control were used, embryos grown in a 50% dilution of hydrosalpinx fluid in media demonstrated a decrease in development. For each concentration of hydrosalpinx fluid, the embryos grown in endometrial coculture demonstrated significantly greater development compared with the embryos grown without the use of endometrial coculture. In the endometrial coculture group, it was not until a 75% concentration of hydrosalpinx fluid was used that a significant decrease in development was noted compared with the control group.

Figure 4A shows a degenerative mouse embryo grown in 75% hydrosalpinx fluid without coculture at 144 hours of development. Conversely, Figure 4B shows an embryo grown in the same concentration of hydrosalpinx fluid, but with the use of endometrial coculture, resulting in a morphologically normal, hatching blastocyst.

To demonstrate that these effects were not due to a dilution effect of the hydrosalpinx fluid on the growth media, we conducted a set of similar experiments in which the control media were diluted with normal saline (0.9%). In these experiments, no differences were found in growth rates to the blastocyst, hatching, or outgrowth stage for dilutions of up to 50% normal saline (50% standard growth media) compared with the control of nondiluted media. Eighty-one percent (13/16), 75% (12/16), and 75% (12/16) of embryos grown in RPMI medium with 10% fetal calf serum without endometrial coculture developed to the blastocyst, hatching, and outgrowth stages, respectively. This was not significantly different from the 75% (12/16), 69% (11/16), and 69% (11/16) of embryos grown in a dilution of 50% normal saline (50% standard growth media) that reached the blastocyst, hatching, and outgrowth stages, respectively.

FIGURE 3

Outgrowth stage at 168 hours. The *open bar* represents embryos grown in hydrosalpinx fluid only without endometrial coculture cells. The *solid bar* represents cells grown in hydrosalpinx fluid with endometrial coculture cells. Fifty-seven percent (42/74) of embryos grown without coculture or hydrosalpinx fluid were outgrown at 168 hours. Not until a 50% hydrosalpinx fluid (50% media) mixture was used was the outgrowth rate lower in the embryos grown without coculture (42/74 vs. 14/54; $P = .002$; χ^2 analysis). In the coculture model, 74% (55/74) of the embryos were outgrown at 168 hours. This was different from the 75% hydrosalpinx fluid (25% media) group (55/74 vs. 18/53; $P = .001$). In 100% hydrosalpinx fluid, no embryo from either the coculture or the noncoculture group reached the outgrowth stage of development. For each dilution of hydrosalpinx fluid, embryos grown in coculture were more likely to reach the outgrowth stage.



DISCUSSION

In this study, we developed an in vitro model that more accurately depicts the in vivo system. This model places the embryo in an environment that allows exposure to hydrosalpinx fluid as well as to endometrial glands and stroma. An important premise in validating this model is that hydrosalpinx fluid can reflux into the endometrial canal, which has been documented in recent reports (14, 15). Although we believe that this model is superior to those previously described, it cannot fully parallel in vivo events. The endometrium contains not only glandular and stromal cells but also immunologic cells not specifically included in our model. Further, endometrial cells grown in vitro do not completely mimic their in vivo function. For example, growth factors or cytokines elaborated by an intact endometrium may not be exhibited in this model.

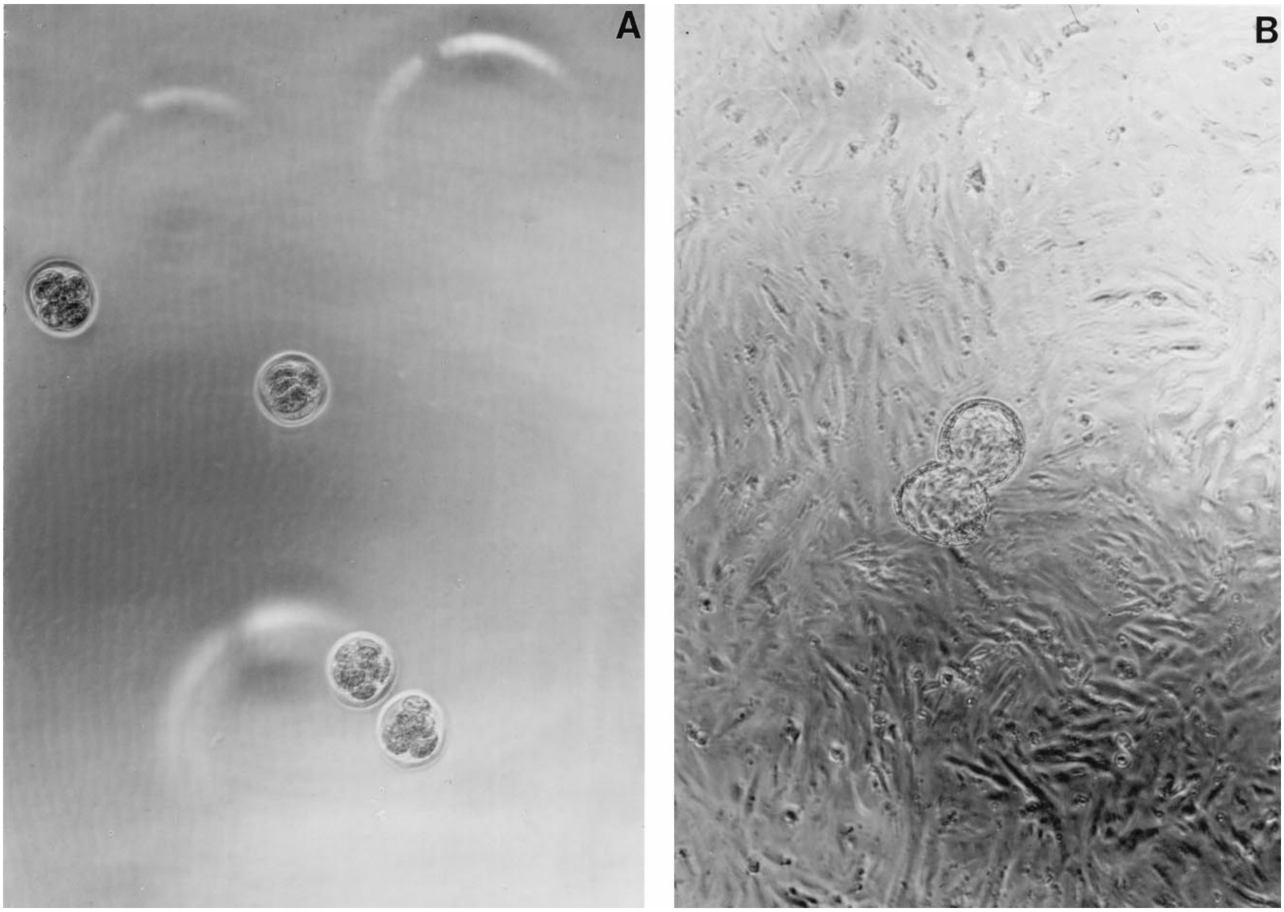
For each of the developmental stages analyzed, we demonstrated that only in concentrations of at least 50% is hydrosalpinx fluid toxic to a developing embryo in a model without added endometrial cells. In our coculture model, which we believe better depicts the in vivo environment, toxicity was not exhibited until a minimum concentration of 75% was used. This suggests that a large amount of refluxed hydrosalpinx fluid is required to yield a significant embryopathic toxic effect.

Unlike the findings of Mukherjee et al. (8), our findings do not support the contention that subclinical reflux of hydrosalpinx fluid is detrimental. In concentrations of hydrosalpinx fluid of up to 50%, using the coculture model that we believe more closely simulates in vivo conditions, we were unable to demonstrate a significant toxic effect. From this model, it appears that large amounts of hydrosalpinx fluid are necessary before negative effects are noted.

Several mechanisms might explain the toxicity exhibited

FIGURE 4

(**A**), Degenerative mouse embryos grown in 75% hydrosalpinx fluid without coculture at 144 hours of development. (**B**), An embryo grown in the same concentration of hydrosalpinx fluid, but with the use of endometrial coculture, resulting in a morphologically normal, hatching blastocyst. Original magnification, ×40.



by hydrosalpinx fluid. One of the obvious potential mechanisms is a purely dilutional effect on essential nutrients. As increasing concentrations of hydrosalpinx fluid in the media are used to grow the embryos, essential nutrients and substrates are diluted, perhaps to a point below a critical level. We therefore undertook similar experiments in which we diluted the media but did not add hydrosalpinx fluid. In the coculture model, when we simulated the dilution effect of a 50% concentration of hydrosalpinx fluid, we were unable to show a significant impact on embryonic development at any of the developmental stages. Thus, although this does not completely discount possible dilution effects, it does appear that simple dilution differences are not the major reason for the toxicity we demonstrated.

A recent study by Murray et al. (9) demonstrated that at a 100% concentration of hydrosalpinx fluid, toxicity is mitigated somewhat by the addition of lactate. However, it is doubtful that the in vivo embryo is ever exposed to nearly 100% hydrosalpinx fluid. We believe that even with very large hydrosalpinges, dilution of essential nutrients is not the primary toxic mechanism of hydrosalpinx fluid on embryonic development.

There are several other possible mechanisms to explain the toxicity of hydrosalpinx fluid. Direct flow of toxins, microorganisms, or microbial products from the dilated tube into the uterus may exert a detrimental effect on the endometrium and/or embryo (4). Irreversible damage to the endometrium during the acute infectious tubal insult also could diminish implantation rates. Further, alteration in endometrial integrin production associated with hydrosalpinges has been proposed (16).

Another potential mechanism of hydrosalpinx fluid–induced toxicity is immunization to the human heat-shock protein 60 kD. The production of antibodies to human heat-shock protein 60 kD is increased in patients with hydrosalpinges (17). Cervical immunity to the chlamydial 60 kD heat-shock protein (Chsp60) has been associated with poor implantation rates, possibly as a result of induction of autoimmunity to the homologous human 60 kD heat-shock protein (17–19). Therefore, in patients with hydrosalpinges, a possibly increased sensitization to the human 60 kD heat-shock protein could explain the toxicity of hydrosalpinx fluid.

The production of proinflammatory cytokines with passage into the endometrial canal may have a deleterious effect on embryonic development and implantation. Interleukin-1 receptor antagonist has been shown to decrease implantation in a mouse model (20). Other potential proinflammatory cytokines that may be detrimental to embryonic development include tumor necrosis factor-α and γ-interferon. We currently are investigating the cytokine profile in hydrosalpinx fluid.

Finally, we have shown that at each developmental stage and for each concentration of hydrosalpinx fluid, the use of human endometrial coculture appears to be beneficial to embryonic development. Since reports in the mid-1960s demonstrated that a higher percentage of mouse preembryos developed and hatched in vitro when they were cultured on a feeder cell line, the use of coculture has been applied successfully to both animal and human IVF programs (21). These helper cell lines appear to enhance the in vitro conditions and provide the necessary factors to allow a variety of species preembryos to overcome their specific in vitro developmental cell blocks. In fact, growth rates and morphology have improved significantly in preembryos that are maintained in coculture systems (21).

A number of studies have evaluated the effect of various somatic cell lines on human preembryo development. We showed that the use of autologous endometrial coculture improves embryonic development in patients with a history of multiple implantation failures (22). Another recent study showed significantly higher rates of blastocyst formation when embryos were cocultured on fallopian tube epithelium compared with conventionally cultured embryos (23).

In a randomized trial, Wiemer et al. (24) found that when bovine oviductal cells were used for coculture, the embryos in coculture had significantly more blastomeres and fewer cytoplasmic fragments than conventionally grown embryos. In addition, a recent prospective randomized trial found that a significantly higher percentage of fertilized oocytes developed to the eight-cell stage when a coculture system was used compared with serum-supplemented medium (25).

Although the literature predominantly suggests improved preembryo development on feeder cell lines, there are some studies that have not demonstrated differences in mean cell numbers per preembryo or blastulation rates on coculture compared with control medium (26, 27). However, the present study indicates that when embryos are subjected to adverse conditions (i.e., hydrosalpinx fluid), the use of endometrial coculture improves embryonic development. Whether this improvement is due to growth factors from these cells or to cell-to-cell contact is not known at this time.

In conclusion, we developed a unique model that we believe mimics the transfer of embryos to a patient with hydrosalpinges. We demonstrated that hydrosalpinx fluid is toxic at high concentrations and showed that this toxicity is not mediated solely by the dilution of essential nutrients. Further, this study highlighted the efficacy of endometrial coculture in helping embryonic development. On the basis of the findings of our study, we do not believe at this point that prophylactic salpingectomy should be offered routinely to patients with hydrosalpinges until a well-designed, prospective, and randomized trial has been conducted.

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