ANIMAL EXPERIMENTATION

Effects of Hydrosalpingeal Fluid on Murine Embryo Development and Implantation

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Purpose: Our purpose was to evaluate the effects of various concentrations of hydrosalpingeal fluid (HSF) on the preimplantation development and implantation of murine embryos.

Methods: One-cell mouse embryos were cultured in KSOM culture medium with 0.1, 1.0, 10, or 50% HSF, without and with lactate supplementation. Late-stage embryos were transferred into the uteri of pseudopregnant CD-1 females to determine implantation rates. The embryo transfer technique used was developed by our group and its effectiveness was evaluated during this experiment.

Results: Blastocyst development in the 0.1, 1.0, 10, and 50% group was 45, 55.0, 12.5, and 17.5%, respectively, with lactate supplementation, and 35.0, 52.5, 12.5, and 5.0%, respectively, without lactate supplementation, while in the KSOM (control) group it was 63.8%. Blastocyst development was reduced compared to controls in the 10% HSF and 50% HSF groups. Implantation rates for the 0.1 and 1.0% groups with lactate supplementation were 43.0 and 25.0%, respectively, and those with lactate supplementation were 50.6 and 61.8%, respectively, while in the KSOM group the implantation rate was 65.5%. None of the implantation rates were significantly different.

Conclusions: Hydrosalpingeal fluid has a concentration-dependent inhibitory effect on in vitro murine embryo development, but it has minimal effects on implantation rates.

KEY WORDS: infertility; hydrosalpinges; mouse embryo; lactate; implantation.

INTRODUCTION

The clinical impact of hydrosalpinges on in vitro fertilization (IVF) results has been evaluated by a number of groups (1–8). Shelton *et al.* (1996) and others have shown an improvement in IVF–embryo transfer (ET) success following surgical removal of the hydrosalpinges (3,9–13). Accordingly, in women undergoing IVF, prophylactic removal of the hydrosalpinges has been recommended.

Several mechanisms have been proposed to account for its negative effect on the IVF-ET process. Since hydrosalpinges represent a pathological milieu within the fallopian tube, exposure of the embryo to this fluid may have a direct embryo-toxic effect on its development. Most of the support for this theory is based on experiments demonstrating the inhibitory effect of hydrosalpingeal fluid (HSF) on the in vitro development of murine embryos (14–16). Murray *et al.* (1997) found that supplementation of HSF with either lactate or pyruvate attenuates its inhibitory effect on preimplantation development (17). Therefore they attributed a component of the toxicity to a deficiency of one or more energy sources.

Because the effects of HSF on implantation remains controversial we designed a study to determine the effects of serial dilutions of HSF on preimplantation development and subsequent implantation success. Since supplementation with lactate has been suggested to attenuate the embryotoxic effect of HSF on preimplantation embryo development, this was also investigated in the present study.

The second objective of our experiment was to develop a new method of murine embryo transfer. The traditional method of ET in the mouse was first

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described by McLaren and Michie in 1956 and is the method most widely described in modern mouse embryology textbooks (18). This method isolates the uterus through an incision on the animal's dorsum. Embryos are then transferred to the uterine horn using a glass micropipette. In several respects this method has inadequacies and may cause unnecessary morbidity in the mouse. In our experience, this approach produces significant damage to the tissues involved and often elicits excessive bleeding. Unless the dissection is repeated on the contralateral side, access is also limited to a single horn. The glass transfer pipette is rigid and, combined with the mouth pipette, is awkward to use during such a microsurgical procedure. A simple transfer device adopted at our institution utilizes a plastic intravenous catheter to replace the glass pipette and a 1-ml syringe to replace the mouth pipette. This combination is compact, simple to use, and sensitive to the small volumes transferred. The uterus is isolated through a small midline incision. We find that this approach requires less soft tissue dissection and provides better exposure. In addition, the laparotomy incision gives access to both uteri, allowing the transfer of embryos to both horns. Our technique was successfully performed on all 19 pseudopregnant CD-1 mice used for this experiment.

MATERIALS AND METHODS

Approval to perform this study was obtained from the animal ethics committee of McGill University.

Hydrosalpingeal fluid was obtained from four women by aspirating the fluid at the time of laparoscopy. Cultures of the fluid revealed no bacterial growth. Each sample was centrifuged at 3000g for 5 min to remove any cellular components and heat-inactivated in a 55°C water bath for 30 min. The specimens were then stored at -70°C until use.

To obtain embryos, B6D2F₁ female mice were superovulated with intraperitoneal injections of pregnant mares' serum gonadotropin (PMSG), 7.5 IU (Sigma Corp., Mississauga, Ontario, Canada), followed 48 hr later by human chorionic gonadotropin (hCG), 5.0 IU (Sigma Corp.). They were then mated with CD-1 males. Mating was confirmed the next morning (Day 0) by the presence of a vaginal plug. These mice were sacrificed by cervical dislocation and one-cell embryos recovered from the oviducts.

Healthy one-cell embryos were washed through KSOM culture medium (19) containing 0.1% (w/v) bovine serum albumin (Boehringer Mannheim Ltd.,

Germany) and transferred to 5- μ l drops of the appropriate culture medium overlaid with washed and filtered light paraffin oil (Fisher Scientific, Nepean, Ontario, Canada). The embryos were then cultured at 37°C in an atmosphere of 5% CO₂ in air. Culture media included HSF diluted with KSOM to concentrations of 0.1, 1.0, 10, and 50%. An additional series of HSF dilutions was supplemented with 10 mM lactate. Between 10 and 20 embryos were cultured in each drop of medium. The embryos were examined daily under a dissecting microscope and the cleavage stage was recorded. On day 4, the embryos that had reached the blastocyst or morula stage were transferred into the uteri of pseudopregnant CD-1 females as described below.

Each female mouse was first anaesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol. A 1-cm midline suprapubic incision was made on the skin. After entry into the abdominal cavity, the uterus was carefully exteriorized. Under microscopic visualisation, embryos were drawn into a 22-gauge Cathlon IV intravenous catheter using a 1cc plastic syringe. Using the needle packaged with the catheter, a puncture hole was made in one uterine horn near its junction with the tube. The catheter was then inserted and embryos injected. The catheter was then withdrawn slowly, with gentle traction on the uterus to minimize the risk of spillage. The uterine incision was not sutured and the horn was returned to the pelvis. The catheter was then inspected to ensure that all embryos were injected. The same procedure was then performed on the contralateral side with a separate group of embryos. After the transfers were complete, the skin was closed with interrupted 4-0 absorbable sutures.

Four days after the embryo transfer (Day 8) the mice were sacrificed and the number of gestational sacs was recorded. The percentage of embryos that developed to blastocyst stage and the percentage that successfully implanted in each group were compared. Data were analyzed using ANOVA and Tukey test for multiple comparisons.

RESULTS

A concentration-dependent decline in preimplantation development was observed (Table I). Lactate supplementation did not significantly influence the implantation rate at any HSF concentration. None of these paired values were significantly different. Blastocyst formation was significantly reduced compared

Table I. Percentage of One-Cell Embryos Developing to the Blastocyst Stage in Different Concentrations of Hydrosalpingeal Fluid

	Group				
	Control	0.1%	1.0%	10%	50%
No lactate	51/80 (63.8%)	14/40 (35.0%)	21/40 (52.5%)	5/40 (12.5%)*	2/40 (5.0%)*
Lactate	51/80 (63.8%)	18/40 (45.0%)	22/40 (55.0%)	5/40 (12.5%)*	7/40 (17.5%)*

* $P < 0.005$ compared to controls.

** $P < 0.05$ compared to controls.

*** $P < 0.001$ compared to controls.

with controls in the 10% HSF groups with and without lactate and in both 50% HSF groups.

Implantation data are depicted in Table II. Implantation rates for the 0.1 and 1.0% groups without lactate supplementation were 40.7 and 62.1%, and those for the groups with lactate supplementation were 33.3 and 20.0%, respectively, while in the KSOM (control) group the implantation rate was 65.5%. None of the implantation rates were significantly different. No data were obtained for the 10 and 50% groups due to the low numbers of morula and blastocyst embryos on the day of transfer.

DISCUSSION

Since hydrosalpinges usually have a proximal communication with the endometrial cavity, embryos transferred into the uterus following IVF are likely to be exposed to a constant flow of HSF from the tube. It is reasonable to postulate that the HSF may reduce implantation rates in these patients through a direct toxic effect on the embryo. This experiment supports the observations of other authors that HSF has a concentration-dependent inhibitory effect on preimplantation development. Rawe *et al.* (1997) demonstrated toxicity at concentrations of 5, 10, and 20% (16), whereas Mukherjee *et al.* (1996) found that concentrations as low as 1% were toxic (14). Our study demonstrates that one or more biochemical elements are

present in this fluid which retain their toxic properties after serial dilution in a physiological solution.

Hydrosalpingeal fluid has similar sodium, potassium, chloride, and bicarbonate concentrations as human serum but is deficient in calcium, phosphate, glucose, and protein (15,20). Contrary to the results of Murray *et al.* (1997), lactate supplementation did not rescue the embryos from the negative effects of HSF (17). In our study we analyzed each sample separately, whereas in the study by Murray *et al.* (1997) the patient samples were pooled. We found that toxicities at the most dilute concentration varied widely between samples, such that if one of the patient's specimens was particularly toxic, then the pooled sample may reflect the properties of that sample.

Unlike previous murine embryo studies on this subject, we followed the embryos through to implantation. We demonstrated that embryos cultured in HSF can successfully implant in the pseudopregnant uterus from both the morula and the blastocyst stage. This observation would imply that if embryos can mature to later stages in the presence of HSF, then implantation rates should be similar to those of women without hydrosalpinges. This suggests that the failure of mature embryos to implant is endometrial in origin. A recent study by Meyer *et al.* (1997) suggests that chronic exposure of the endometrium to HSF reduces the receptivity of the endometrium through alterations of surface integrins (21). Rawe *et al.* (1997) transferred blastocysts to the uteri of mice along with 10 μ l of HSF and found no difference in implantation rates compared to controls (16). Since the endometrial exposure to HSF was so brief, this model may not adequately mimic the effects of chronic exposure seen in the human state. Human embryos are generally transferred at the four-cell stage; accordingly, it would be difficult to determine if the lower implantation rates are secondary to arrested preimplantation development or alterations of the endometrium. The deleterious effect encountered during the IVF process is likely a

Table II. Implantation Rates of Blastocyst- and Morula-Stage Embryos

	Group		
	Control	0.1%	1.0%
No lactate	19/29 (65.5%)	11/27 (40.7%)	18/29 (62.1%)
Lactate	19/29 (65.5%)	9/27 (33.3%)	6/30 (20.0%)

combination of direct toxic effects on the developing embryo and alterations of endometrial receptivity.

We have found our technique of embryo transfer easy to learn and quick to master. We performed this procedure on a total of 19 pseudopregnant CD-1 mice, with a total implantation rate of 65.5% for morula- and blastocyst-stage embryos cultured in KSOM. No complications were encountered with any of the procedures. Both aspiration and transfer of embryos were simple to perform. Once loaded into the catheter, the embryos are relatively safe from accidental spillage and easily visualized through the transparent catheter. As in the case of the glass pipette, the plastic catheter can be inspected to ensure the absence of embryos following the transfer. Through an anterior incision, the uterus and surrounding organs can be easily identified. In our experience this approach involves less soft tissue dissection compared to the traditional approach and appears to produce less bleeding. Assessment of pain in these mice would be difficult, however, any surgical technique that minimizes tissue damage and bleeding should theoretically reduce morbidity. Embryo transfer success rates in the literature are published as term pregnancy rates and vary between groups but, on average, are about 50%. Although we did not determine term pregnancy rates, our implantation rates are certainly high enough to validate the technique. In general, we feel that this is a simple and effective method for ET in the mouse, and one that has several potential benefits over traditional methods.

REFERENCES

- Andersen AN, Yue Z, Meng FJ, Petersen K: Low implantation rate after in-vitro fertilization in patients with hydrosalpinges diagnosed by ultrasonography. *Hum Reprod* 1994;9:1935-1938
- Strandell A, Waldenstrom U, Nilsson L, Hamberger L: Hydrosalpinx reduces in-vitro fertilization/embryo transfer pregnancy rates. *Hum Reprod* 1994;9:861-863
- Vandromme J, Chasse E, Lejeune B, Van Rysselberge M, Delvigne A, Leroy F: Hydrosalpinges in in-vitro fertilization: An unfavourable prognostic feature. *Hum Reprod* 1995;10:576-579
- Fleming C, Hull MGR: Impaired implantation after in vitro fertilisation treatment associated with hydrosalpinx. *Br J Obstet Gynaecol* 1996;103:268-272
- Katz E, Akman MA, Damewood MD, Garcia JE: Deleterious effect of the presence of hydrosalpinx on implantation and pregnancy rates with in vitro fertilization. *Fertil Steril* 1996;66:122-125
- Sharara FI, Scott RT, Marut EL, Queenan JT Jr: In-vitro fertilization outcome in women with hydrosalpinx. *Hum Reprod* 1996;11:526-530
- Akman MA, Garcia JE, Damewood MD, Watts LD, Katz E: Hydrosalpinx affects the implantation of previously cryopreserved embryos. *Hum Reprod* 1996;11:1013-1014
- Blazar AS, Hogan JW, Seifer DB, Frishman GN, Wheeler CA, Haning RV: The impact of hydrosalpinx on successful pregnancy in tubal factor infertility treated by in vitro fertilization. *Fertil Steril* 1997;67:517-520
- Shelton KE, Butler L, Toner JP, Oehninger S, Muasher SJ: Salpingectomy improves the pregnancy rate in in-vitro fertilization patients with hydrosalpinx. *Hum Reprod* 1996;11:523-525
- Kassabji M, Sims J, Butler L, Muasher S: Reduced pregnancy rates with unilateral or bilateral hydrosalpinx after in vitro fertilisation. *Eur J Obstet Gynecol Reprod Biol* 1994;56:129-132
- Levy MJ, Murray D, Sagoskin A: The adverse effect of hydrosalpinges on IVF success rates are reversed equally well by salpingectomy, proximal tubal occlusion and neosalpingostomy. Presented at the meeting of the American Society for Reproductive Medicine 1996. American Society for Reproductive Medicine, Program Supplement, 1996, p S64
- Freeman MR, Whitworth CM, Hill GA: Permanent impairment of embryo development by hydrosalpinges. *Hum Reprod* 1998;13:983-986
- Murray DL, Sagoskin AW, Widra EA, Levy MJ: The adverse effect of hydrosalpinges on in vitro fertilisation pregnancy rates and the benefit of surgical correction. *Fertil Steril* 1998;69:41-45
- Mukherjee T, Copperman AB, McCaffrey C, Cook CA, Bustillo M, Obasaju MF: Hydrosalpinx fluid has embryotoxic effects on murine embryogenesis: A case for prophylactic salpingectomy. *Fertil Steril* 1996;66:851-853
- Beyler SA, James KP, Fritz MA, Meyer WR: Hydrosalpingeal fluid inhibits in-vitro embryonic development in a murine model. *Hum Reprod* 1997;12:2724-2728
- Rawe VJ, Liu J, Shaffer S, Compton MG, Garcia JE, Katz E: Effect of human hydrosalpinx fluid on murine embryo development and implantation. *Fertil Steril* 1997;68:668-670
- Murray CA, Clarke HJ, Tulandi T, Tan SL: Inhibitory effect of human hydrosalpingeal fluid on murine preimplantation embryonic development is significantly reduced by the addition of lactate. *Hum Reprod* 1997;12:2504-2507
- McLaren A, Michie D: Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. I. Factors affecting the implantation and survival of native and transferred eggs. *J Exp Biol* 1956;33:394-416
- Lawitts J, Biggers J: Culture of preimplantation embryos. In *Guide to Techniques in Mouse Development*, PM Wassarman, ML DePamphilis (eds). San Diego, Academic Press, 1993, pp 153-164
- David A, Garcia C, Czernobilsky B: Human hydrosalpinx: Histologic study and chemical composition of fluid. *Am J Obstet Gynecol* 1965;105:400-411
- Meyer WR, Castelbaum AJ, Somkuti S, Sagoskin AW, Doyle M, Harris JE, Lessey BA: Hydrosalpinges adversely affect markers of endometrial receptivity. *Hum Reprod* 1997;12:1393-1398