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Effect of Hydrosalpingeal Fluid on the Implantation in-vitro in a Murine Model

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생쥐 배아의 체외배양 시 착상과정에 대한 난관수종액의 영향

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연구목적: 인간의 체외수정 및 배아이식술에서 난관수종을 갖는 환자에서 임신율과 착상률이 감소된다는 보고들이 있지만 이에 대한 명확한 기작은 밝혀지지 않았다. 본 연구에서는 생쥐 배아를 이용한 체외 착상모델에서 인간의 난관수종액 (HSF)이 착상과정에 미치는 영향을 알아보고자 하였다.

연구재료 및 방법: 난관수종액은 난관수종으로 수술을 받은 8명의 환자로부터 채취하였으며, 실험에 사용하기 전까지 냉동고에 보관하였다. 생쥐의 포배기 배아는 2-세포기 배아를 3일 동안 배양하여 그 중 상태가 양호한 포배기 배아만을 선별하여 투명대를 제거한 후 사용하였다. 기본 배양액으로는 Ham's F-10을 사용하였으며, 배양 시 기본 배양액만을 사용한 경우를 group I으로 하였고, 기본 배양액에 0.5% FBS를 첨가한 경우를 group II, 0.5% FBS와 50% HSF를 첨가한 경우를 group III, 100% HSF에 0.5% FBS를 첨가한 경우를 group IV, 100% HSF만을 사용한 경우를 group V로 하였다. 투명대를 제거한 포배기 배아를 각각의 HSF에 대한 5종류의 배양액에서 48시간 동안 배양하였다. 체외 착상 유무는 부착 부위에서 크기가 커진 영양세포들을 관찰하여 판정하였으며, 착상 부위의 표면적은 화상분석기를 이용하여 산출하였다.

결 과: 생쥐 배아의 체외 착상률은 group I, II, III, IV, V에서 각각 0%, 98.9%, 77.5%, 40.4%, 10.0%로 나타났으며, 착상 부위의 평균 표면적은 group II, III, IV, V에서 각각 74,675±25,201 μm^2, 59,024±25,877 μm^2, 45,156±22,654 μm^2, 38,254±17,115 μm^2이었다. 체외 착상률과 착상 부위의 표면적은 HSF의 농도가 증가함에 따라 통계적으로 유의하게 감소하였다 ($p<0.01$).

결 론: 인간의 난관수종액 (HSF)은 생쥐 배아의 체외 착상과 영양배엽세포의 증식을 억제하는 것으로 확인되었으며, 이러한 원인이 난관수종을 갖는 환자에서 임신율이 낮은 것과 밀접한 관련이 있을 것으로 생각된다.

Key Words: Hydrosalpingeal fluid, Murine model, Implantation in-vitro, Outgrowth

Hydrosalpinx affects the clinical outcome of human IVF-ET program in the patients with tubal obstruction.[1~4] It is not established the mechanism of negative impact on the successful

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implantation and pregnancy. Several factors may be involved in the reduction of fertility in the patients with hydrosalpinx. Some investigators have suggested that hydrosalpingeal fluid (HSF) has embryotoxic effects on murine embryogenesis.[5,6] However, our previous study and recent reports have proposed that HSF does not have a toxic effect and have a mild inhibitory effect on the development of mouse embryos.[7~9] This effect may be due to the absence of essential factors, such as energy sources and proteins. It is still controversy on the embryo toxicity of HSF. In addition, HSF may reflux into the uterine cavity and interfere the contact of embryos with endometrial epithelium, which result in inhibition of implantation.[10] Meyer et al[11] demonstrated that inflammatory hydrosalpinx has an adverse effect on endometrial receptivity by alteration of integrin expression in the endometrium. It is necessary to study on the effect of HSF on the implantation process of embryonic side. The aim of this study was to investigate the effect of human HSF on the outgrowth of trophoblasts, implantation in-vitro, in a murine model.

MATERIALS AND METHODS

1. Preparation of hydrosalpingeal fluid and mouse embryos

The hydrosalpingeal fluid (HSF) was sampled from eight patients with hydrosalpinx undergoing salpingoneostomy. Each sample was centrifuged at 3,000 rpm for 10 minutes to remove cell debris and supernatant was collected and frozen at -20℃ before use. Female ICR mice were injected with 7 IU of pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co., St Louis, MO, USA) and 7 IU of human chorionic gonadotropin (hCG, Sigma) 47 hours later. Two cell-stage embryos were obtained by flushing of oviducts at 48 hours after hCG injection, and in-vitro cultured for 3 days to the blastocyst stage in T6 medium containing 0.4% bovine serum albumin (Gibco Life Tehnologies, Gaithersburg, MD. USA). After the culture, expanded blastocysts with good morphology were selected for this study. The zona pellucida of blastocyst was removed by treatment with 0.2% pronase E (Sigma Chemical Co., St Louis, MO, USA) for eliminating the effect of zona pellucida on the attachment and outgrowth of blastocysts in this study.

2. Preparation of culture media

The stored HSF was thawed and sterilized by filtering (0.22 μm filter, Millipore, Molsheim, France) before use. For each patient, Ham's F-10 without any supplement (group I) and with 0.5% fetal bovine serum (FBS; group II) was used as a negative and positive control medium, respectively. Experimental media for each patient were Ham's F-10 with 0.5% FBS and 50% HSF (group III), 100% HSF with 0.5% FBS (group IV), and 100% HSF only (group V). Five kinds of culture media were prepared 20~30 μl of microdrops, which was covered by mineral oil (Sigma Chemical Co.) in a culture dish (Falcon, NJ, USA). Prior to the culture of the mouse blastocysts, the culture dishes were equilibrated at 37℃ in an atmosphere of 5% CO_2 in air for 6 hours.

3. Assessment of implantation in-vitro and surface areas of outgrowth

Zona-free blastocysts were added to the microdrops of culture media, one blastocyst per microdrop, and cultured for 48 hours. The outgrowth of trophoblasts, i.e. implantation in-vitro, was identified when primary giant trophoblasts were visible around the attachment site of the blastocyst under phase-contrast microscope. To determine the surface area of outgrowth containing contiguous cells, the embryos were photographed, and the areas were measured with an image-analyzing system (Image Analyzer, Vilber Lourmat, France).

4. Statistical analysis

The percentage of outgrowth among the groups was compared using the χ^2-test. The mean area

Table 1. Outgrowth rates of mouse blastocysts after in-vitro culture with the hydrosalpingeal fluid of each patient

No. of patient	Ham's F-10 only	Ham's F-10 + 0.5% FBS*	Ham's F-10 + 0.5% FBS + 50% HSF**	100% HSF + 0.5% FBS	100% HSF only
1	0% (0/15)	100% (15/15)	87% (13/15)	53% (8/15)	0% (0/15)
2	0% (0/6)	100% (8/8)	86% (6/7)	29% (2/7)	0% (0/8)
3	0% (0/14)	100% (14/14)	79% (11/14)	64% (9/14)	0% (0/14)
4	0% (0/7)	100% (8/8)	0% (0/8)	0% (0/8)	0% (0/8)
5	0% (0/13)	100% (13/13)	100% (13/13)	54% (13/7)	69% (9/13)
6	0% (0/8)	100% (8/8)	100% (8/8)	63% (8/5)	0% (0/8)
7	0% (0/14)	100% (14/14)	100% (14/14)	36% (5/14)	0% (0/14)
8	0% (0/10)	90% (9/10)	40% (4/10)	0% (0/10)	0% (0/10)

Data in parentheses are number of outgrowth / number of cultured blastocyst.
* FBS; fetal bovine serum, ** HSF; human hydrosalpingeal fluid.

Table 2. Overall outgrowth rates and mean areas of mouse blastocysts after in-vitro culture with the hydrosalpingeal fluid

	Ham's F-10 only	Ham's F-10 + 0.5% FBS*	Ham's F-10 + 0.5% FBS + 50% HSF**	100% HSF + 0.5% FBS	100% HSF only
Outgrowth rate (n)	0% (0/87)	99% (89/90)^a	78% (69/89)^b	40% (36/89)^c	10% (9/90)^d
Areas of outgrowth (mean $\pm$ SD, μm^2)	Not determined	74,675 $\pm$25,201^a	59,024 $\pm$25,877^b	45,156 $\pm$22,654^c	38,254 $\pm$17,115^d

* FBS; fetal bovine serum, ** HSF; human hydrosalpingeal fluid.
Values within rows with different superscripts are significantly different ($p<0.01$).

of outgrowth was calculated from the individual area of all in each group. The mean area among the groups was analyzed by Students t-test. $p<0.05$ was considered to be statistically significant.

RESULTS

After 48 hours of culture of mouse blastocysts, outgrowth rates of the five kinds of culture media in each sample were shown in Table 1. There was no outgrowth of trophoblasts in the negative control media (group I), only Ham's F-10 without any supplement. In the positive control medium (group II), Ham's F-10 with 0.5% FBS, the outgrowth of trophoblasts was observed above 90% of blastocysts. According to increase of HSF concentration, the outgrowth rates were decreased in the all samples. In two samples (No. 4 and 8), the outgrowth was completely inhibited in spite of FBS supplementation. One sample (No. 5) of 100% HSF was supported the outgrowth without FBS. Overall outgrowth rates and mean surface area of outgrowth in each of five different media are shown in Table 2. After 48 hours of culture, the outgrowth rates of trophoblasts in group I, II, III, IV and V were 0%, 98.9%, 77.5%, 40.4% and 10.0%, respectively. There were significant ($p< 0.01$) differences among the groups in the outgrowth rates. A sample of image-analysing of the outgrowth area was illustrated in Figure 1. The mean areas of outgrowth have a negative correlation with the HSF concentration. The out-

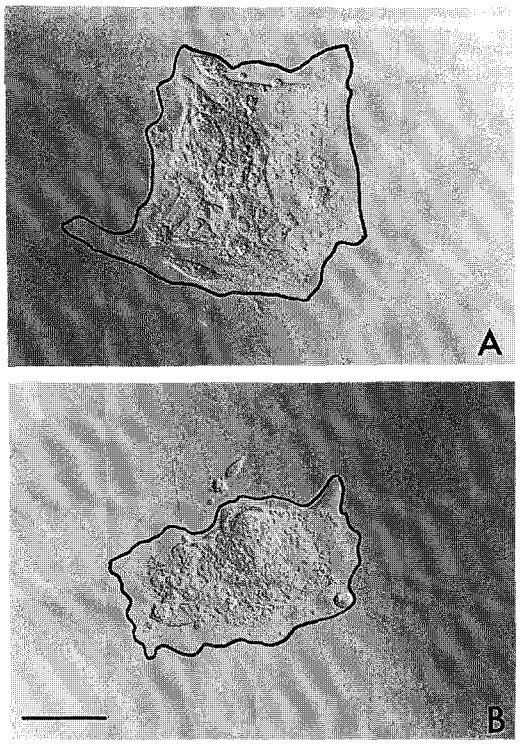

Figure 1. Microphotographs of outgrowth of mouse blastocysts after in-vitro culture for calculating the surface areas of outgrowth. Surface areas of outgrowth is **(A)** 98,725 μm^2 in Ham's F-10 + 0.5% FBS and **(B)** 61,255 μm^2 in Ham's F-10 + 0.5% FBS + 50% HSF. Bar = 100 μm.

growth areas of HSF containing groups were significantly ($p<0.01$) smaller than that of group II (mean $\pm$ SD, 74,675 $\pm$ 25,201 μm^2).

DISCUSSION

In human IVF-ET program, it was reported that pregnancy rates were significantly increased by surgical correction of hydrosalpinx.[12~14] Some investigators proposed that HSF may have a toxic substance which inhibits embryonic development using murine model.[5,6] However, our previous study and recent studies were not detected the toxic effect of HSF.[7~9] About the HSF effect on the implantation, it was noted that a marker of endometrial receptivity, α v β3 integrin expression, was significantly reduced in patients with hydrosalpinges.[11]

Many studies for the implantation process were used the murine model, and the implantation in-vitro was defined as the attachment of primary giant trophoblasts on a culture dish.[15~17] In this study, we used ZP-free blastocysts in order to exclude the affect of hatching on the implantation in-vitro. The intact ZP of blastocysts inhibited the outgrowth of trophoblasts in our pilot study, and it was reported by Cohen and Feldberg[18] that the size and number of zona pellucida openings showed a negative or a positive effect on hatching and trophoblast outgrowth in the mouse embryos. We have determined the minimal concentration of serum supplement for supporting outgrowth of mouse embryos (data not shown), and used 0.5% FBS in this study.

As far as we know, this is the first study about the direct effect of HSF on the embryonic side in the implantation process. Pure HSF, except one sample, did not support the outgrowth of mouse blastocysts. It may be related that HSF has similar sodium, potassium, chloride and bicarbonate concentrations as human serum but is deficient in calcium, phosphate, glucose and protein.[19,20] In the supplementation of FBS, increasing of HSF concentration showed negative correlation of the outgrowth rates and areas. We suggest that HSF have inhibitory factors on the implantation, such as interferon-γ,[21] interleukin-6[22] and/or tumor necrosis factor-α (TNF-α).[23] Recently, the presence of epidermal growth factor and TNF-α in human HSF was reported.[24]

In conclusion, HSF has an inhibitory effect on the outgrowth of trophoblasts, implantation in-vitro, in a murine model. Therefore, we suggest that the HSF may affect the implantation process, and pre-treatment for hydrosalpinx should be needed to increase the pregnancy rate in human IVF-ET program.

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