

Article

Effects of peritoneal fluid on preimplantation mouse embryo development and apoptosis *in vitro*



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Abstract

To evaluate the effect of peritoneal fluid (PF) on preimplantation mouse embryo development and apoptosis, PF was obtained from women presenting with ($n = 7$) and without endometriosis ($n = 7$). Mouse embryos were cultured to the blastocyst stage in human tubal fluid medium alone (control) or 10% PF. Embryo development was assessed and the total cell number per embryo was determined by Hoechst 33258 staining. Allocation of inner cell mass and trophoctoderm in blastocysts, and incidence of apoptosis were determined using confocal microscopy. The blastocyst development rate was significantly lower in the presence of 10% PF ($P < 0.01$). Total cell number and trophoctoderm in blastocysts cultured in culture media alone was significantly higher than in the presence of PF ($P = 0.034$ and $P = 0.01$ respectively). A higher incidence of apoptosis was seen in blastocysts cultured in culture media alone, compared with those cultured in the presence of PF ($P = 0.04$). PF decreases the development of early mouse embryos to the blastocyst stage, as well as the incidence of apoptosis in the resulting mouse blastocysts. No difference was seen in the effect of PF from women with and without endometriosis on mouse embryo development and apoptosis.

Keywords: apoptosis, blastocyst, endometriosis, mouse embryo, peritoneal fluid

Introduction

Endometriosis affects approximately 10% of women of reproductive age and is associated with dysmenorrhoea, chronic pelvic pain and infertility. The mechanisms by which endometriosis impairs fertility have not been completely determined and include: a negative effect on oocyte development (Navarro *et al.*, 2003) altered tubal transport (Suginami *et al.*, 1986) and altered peritoneal fluid (PF) cytokine and prostaglandin milieu (Gomez-Torres *et al.*, 2002). Numerous investigators have reported that women with endometriosis have an increased volume of PF, increased concentrations of peritoneal macrophages, cytokines and prostaglandins (Taketani *et al.*, 1992; Gomez-Torres *et al.*, 2002). The toxic

effect of interleukin-1 (IL-1) and IL-6 from PF of women with endometriosis on mouse embryo development has been shown in previous studies (Fakih *et al.*, 1987; Wu *et al.*, 2001). Peritoneal fluid diffusing into the tubal and endometrial environments can affect sperm-egg interaction, influence early embryo development, and reduce endometrial receptivity.

Apoptosis is essential for normal embryogenesis and occurs in blastocysts to eliminate defectively differentiated cells (Parchment, 1993; Stellar, 1995). However, increased apoptosis is detrimental to blastocyst formation and lead to preimplantation embryo arrest (Parchment, 1993). The association between endometriosis and apoptosis in human granulosa cells and endometrial glandular and stromal cells has

been shown in several reports (Toya *et al.*, 2000; Dmowski *et al.*, 2001). A possible effect of PF from endometriosis patients on triggering apoptosis in developing early embryos has not been studied. The purpose of this study was to evaluate the effect of PF from women with and without endometriosis on embryo development, and to determine whether PF induces apoptosis in preimplantation mouse embryos.

Materials and methods

Human subjects

This study was approved by the Cleveland Clinic Institutional Review Board (IRB) and all participating women gave informed consent. PF (volume range: 0.9 – 25 ml) was collected at the time of laparoscopy for infertility or chronic pelvic pain, from women with endometriosis ($n = 7$) by aspiration from the cul-de-sac. Blood contamination was graded on an arbitrary scale of: 0 = no contamination; + = traces; ++ = mild contamination; +++ = severe contamination. Only PF samples that were clear or with mild contamination were included. Endometriosis was diagnosed by visual appearance of the disease and histological confirmation, and staged according to the revised classification of the American Society for Reproductive Medicine (Canis *et al.*, 1997). The distribution of women with endometriosis was: stage I: $n = 1$; stage II: $n = 4$; and stage III: $n = 2$. PF samples obtained from seven women undergoing laparoscopy for benign conditions (absence of chronic pelvic pain or infertility and endometriosis) served as controls. PF samples were kept on ice during transport and centrifuged at 300 g for 10 min. The cell-free supernatant fluid was separated and filtered through a 0.22- μm filter (Millipore, Bedford, MA, USA) and stored frozen in aliquots at -70°C until use.

Mouse embryo culture

Frozen 2-cell mouse embryos were obtained from B6C3F1 mouse crossed with B6D2F1 mouse (Embryotech Laboratories, Inc., Wilmington, MA, USA). A 2-cell mouse embryo culture system was used to evaluate the effects of PF. Cryopreserved embryos (Embryotech Laboratories, Inc., Wilmington, MA, USA) were thawed, and those with a normal morphological appearance were used for the study. The incubation was carried out at 37°C under a humidified atmosphere of 5% CO_2 in air. A total of 221 embryos were randomly allocated into three groups: group I: embryos cultured in human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA, USA) ($n = 72$) served as the control; group II: embryos cultured in HTF supplemented with 10% PF from patients with endometriosis ($n = 69$); and group III: embryos cultured in HTF supplemented with 10% PF from women without endometriosis ($n = 80$). Groups of 9–10 embryos were randomly added to 1 ml of culture medium in centre-well culture dishes. Embryos in all groups were monitored daily at the same time in the morning using Normarski and bright field inverted optics. The number of embryos cleaving to morula and blastocysts (early, expanding, and expanded) were recorded. A blastocyst development rate (BDR) of over 80% was considered satisfactory for internal control.

Evaluation of apoptosis using TUNEL

Individual embryos were stained with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) technique (in-situ cell death detection system; Roche Diagnostic Corporation, Indianapolis, IN, USA). To distinguish apoptosis from necrosis, embryos were stained with propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO, USA) before the TUNEL assay was performed. Embryos were incubated in 20 $\mu\text{g/ml}$ PI in HTF for 10 min. After the embryos were washed in phosphate buffered saline (PBS), they were fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature, or overnight at 4°C . Embryos were washed at least three times in PBS containing 0.3% polyvinylpyrrolidone (PBS/PVP) and permeabilized in 0.5% Triton X-100 on ice for 2 min. The embryos were then washed three times in PBS/PVP and incubated in the TUNEL reaction cocktail at 37°C for 1 h in the dark. The total cell number (TCN) per embryo was determined by staining the embryos with bisbenzimidazole (Hoechst dye 33258; Sigma Chemical Co.). The embryos were washed extensively and mounted with slight coverslip compression in Vectashield anti-bleaching solution (Vector Labs, Burlingame, CA, USA). The slides were sealed with clear nail polish and stored at 4°C in the dark for up to one week until analysis by confocal microscopy (**Figure 1**).

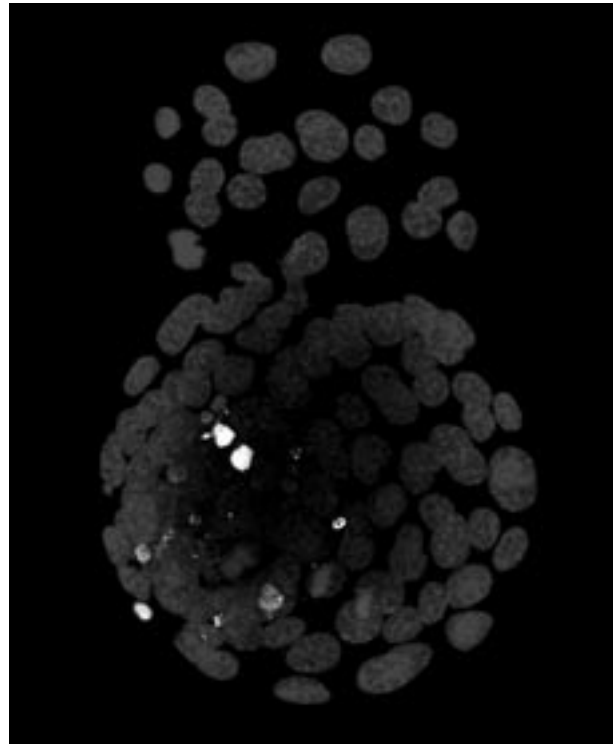


Figure 1. Confocal photomicrograph of hatching blastocyst stained for apoptosis. Embryo is labelled with bisbenzimidazole, TUNEL (fluorescein isothiocyanate) and propidium iodide. All nuclei of the blastocyst are labelled with bisbenzimidazole and apoptotic nuclei with TUNEL. Original magnification $\times 400$.

Confocal microscopy

Images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) using an HCX Plan Apo 63X, 1.32 NA objective at a zoom of 2. The specimen was excited at 364 nm (UV) for bisbenzimidazole, 488 nm for TUNEL fluorescein isothiocyanate (FITC), and 514 nm for propidium iodide. The emitted fluorescence from each of the three probes was detected with three separate photomultiplier detectors whose spectrophotometer slits were set for 400–490, 500–550, and 585–810 nm, respectively. Images were collected sequentially at each level of the specimen to prevent cross talk of the fluorophores and were then collected along the z-axis of the sample with a step size of 1–3 μm . A projection of the three-dimensional stack of images was then created with the Leica software. Each optical section of the blastocyst was analysed for the total number of nuclei and number of TUNEL-labelled nuclei in inner cell mass (ICM) and trophoctoderm (TE). Trophoctoderm nuclei were distinguished from ICM nuclei by a combination of their position and morphology (Brison, 1998). In general, TE nuclei are smaller and more intensely stained, whereas ICM nuclei are larger, rounded, and less intensely stained.

Statistical analysis

The BDR, percentage of embryos developing from two-cell to the blastocyst stage, was calculated for all groups. Statistical analysis was done using the repeated measures analysis of variance for TCN, ICM, TE, and the number of apoptotic blastomeres. Two-tailed tests were used to determine statistical significance at the $P < 0.05$ level. Calculations were performed with SAS version 8.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Embryo development rates

The percentage and number of embryos cleaving to morula and blastocyst in HTF alone and in media supplemented with PF are shown in **Table 1**. The early blastocyst formation rate after 48 h in the three groups was not significantly different, however, a significant difference was found in the percentage of arrested embryos before the morula stage in the presence of 10% PF from patients with and without endometriosis, compared with HTF alone ($P = 0.001$). The overall BDR after 72 h was significantly different between group I and the other two groups (92% versus 59% and 61%, $P < 0.01$). No cytoplasmic fragmentation was observed during the in-vitro culture in all groups.

Blastocyst cell numbers and ICM/TE ratio

The total cell number (TCN) and ICM/TE ratio for the embryos are shown in **Table 2**. The mean \pm standard deviation (SD) of TCN and TE in blastocysts cultured in HTF alone was significantly higher than in the presence of PF ($P = 0.034$ and 0.01 respectively).

Evaluation of apoptosis by TUNEL assay

Positive TUNEL labelling in apoptotic cells was defined as a bright green fluorescence-staining pattern. Positive labelling was observed in the blastocysts of all groups (**Table 3**), but the incidence of apoptosis was significantly higher in group I ($P = 0.04$). Positive labelling could not be identified in embryos that were arrested before morula stage. Apoptosis was first detected at the morula stage in the growth-retarded or arrested embryos. Positive TUNEL labelling was seen in both the ICM and TE, and its occurrence was primarily in the ICM in all groups.

Table 1. Effect of peritoneal fluid on mouse embryo development.

Group	No. 2-cell embryos	No. morulae (%) 48 h	No. early blastocysts (%) 48 h	No. expanding blastocysts (%) 72 h
I	72	37 (53)	34 (47)	66 (92) ^a
II	69	19 (27.5)	30 (43)	41 (59)
III	80	40 (50)	27 (34)	49 (61)

^aSignificantly different from other values within column ($P < 0.05$).

Table 2. Total cell number (TCN) and allocation of inner cell mass (ICM) and trophoectoderm (TE) in blastocysts in three groups.

Group	No. blastocysts	TCN	ICM	TE	ICM/TE
I (n = 72)	66	55.94 ± 6.78 ^a	14.55 ± 2.17	41.8 ± 5.2 ^a	0.35
II (n = 69)	41	50.00 ± 11.06	15.74 ± 4.44	36.8 ± 8.24	0.43
III (n = 80)	49	48.30 ± 12.93	14.04 ± 4.1	33.7 ± 9	0.41

Values are means ± SD.
^aSignificantly different (*P* < 0.05).

Table 3. Apoptosis in blastocysts after culture in human tubal fluid (HTF) alone and HTF + 10% PF. ICM = inner cell mass, TE = trophoectoderm.

Group	No. blastocysts	Apoptosis per blastocyst	Apoptosis in ICM ^a	Apoptosis in TE ^a
I	66	5.81 ± 1.11 ^a	2.51 ± 0.75	3.5 ± 1.14 ^a
II	41	2.60 ± 2.73	1.66 ± 1.35	0.9 ± 1.62
III	49	3.18 ± 2.05	1.62 ± 1.14	1.3 ± 1.38

Values are means ± SD.
^aSignificantly different (*P* < 0.05).

Discussion

Peritoneal fluid from women with endometriosis has long been the focus of investigation because of the potential information it provides about the disease. Several studies have demonstrated the toxic effects of PF of mouse embryo development *in vitro* (Morcos *et al.*, 1985; Prough *et al.*, 1990), and there are many reports of the use of 5–10% PF in culture media (Meneses and Pedersen, 1984; Prough *et al.*, 1990; Taketani *et al.*, 1992) but we found similar effect of 5% and 10% PF on BDR and apoptosis in 2-cell mouse embryos (Sharma *et al.*, unpublished). However, apoptosis in preimplantation embryos has not been previously studied as a potential contributory factor in endometriosis-related infertility.

Peritoneal fluid embryotoxicity has been studied in women with and without endometriosis, with conflicting results (Sherif *et al.*, 1987; Prough *et al.*, 1990). Several studies have utilized 5–10% PF in culture media for investigating the effects of PF on embryo development (Prough *et al.*, 1990; Taketani *et al.*, 1992). While some authors did not find any influence of PF from women with endometriosis on the development of 2-cell embryos to the blastocyst stage (Morcos *et al.*, 1985), others reported reduced BDR from 2-cell mouse embryos (Morcos *et al.*, 1985; Prough *et al.*, 1990). This effect was more pronounced when PF from infertile women with endometriosis was used compared with PF of fertile women with endometriosis, or endometriosis-treated patients (Morcos *et al.*, 1985; Martinez-

Roman *et al.*, 1997). These conflicting results may be partly due to differences in PF concentration, differences in strain, or culture medium used. Morcos *et al.* (1985) also observed a high concentration of PF to be toxic in both endometriosis patients and normal controls, suggesting that the toxic factors are not unique to patients with endometriosis. Similar to their findings, the current study also showed a significant decrease in the BDR in the presence of PF from women with and without endometriosis, compared with the HTF medium control group. In fact, higher concentrations of PF reported (Morcos *et al.*, 1985) might not be embryotoxic, but rather a dilution effect of the nutrients necessary for embryo development in the culture medium, which lowers the BDR as well as the TCN. It is also possible that the laparoscopic procedure itself could cause peritoneal inflammation resulting in alterations in cytokine milieu and other unknown embryotoxic factors in the PF.

Apoptosis, or programmed cell death, is a process by which multiple cell types are eliminated during embryogenesis. There are numerous stimuli that trigger apoptosis, including withdrawal of essential growth factors or hormones from the microenvironment. Apoptosis has been proposed as a common phenomenon in rodent blastocysts, and it occurs in a majority of blastocysts that are flushed from the uterus (Hardy, 1997). A critical number of ICM blastomeres are required in the blastocyst for normal post-implantation development, and apoptosis may be responsible for control of this process (Brison and Schulz, 1998). In agreement with other reports (Fouladi-Nashta,

2005), apoptosis was seen primarily in the ICM in all groups, suggesting that ICM is more susceptible to in-vitro conditions than the TE compartment. Peritoneal fluid from endometriosis patients contains various cytokines that are both pro- and anti-apoptotic. PF from endometriosis patients is known to decrease the incidence of apoptosis in endometrial stromal and glandular cells, which in turn contributes to the aetiology or pathogenesis of endometriosis by increasing viability of endometrial cells and facilitating their ectopic survival and implantation (Dmowski *et al.*, 2001). Since there was no significant difference between PF from women with and without endometriosis in the incidence of apoptosis in blastocysts in the present study, it appears that apoptosis in early embryos may not a contributory factor in endometriosis-associated infertility. However, it cannot be ruled out totally that the effects may be evident at earlier or later stages of embryonic development. No relationship was seen between PF volumes, or samples with clear or mild contamination with blood and the above observed effects.

In conclusion, this study has demonstrated that exposing mouse 2-cell embryos to PF in culture reduces the BDR, the cell number in the TE and the blastocyst overall, and reduces apoptosis in the blastocyst. Although the apoptosis was lower in embryos cultured in the presence of PF from endometriosis patients, the difference was not significant. Therefore, apoptosis cannot be attributed to the endometriosis but to the presence of PF in the culture media. Reduced implantation reported in endometriosis patients is unlikely to be associated with the phase of development studied in this group of patients. It is possible that PF in endometriosis patients may compromise fertility by interfering with the final stages of meiosis, fertilization, later embryo development or implantation, possibly with the expression of implantation receptors (Illera *et al.*, 2000). Also, it is unclear whether human and mouse embryos respond similarly to the effects from PF since, unlike in humans, PF cannot easily enter the tubes in mice *in vivo*.

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References

- Brison DR, Schultz RM 1998 Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. *Biology of Reproduction* **59**, 136–144.
- Canis M, Donnez JG, Guzick DS *et al.* 1997 Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertility and Sterility* **67**, 817–821.
- Dmowski WP, Ding J, Shen J *et al.* 2001 Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. *Human Reproduction* **16**, 1802–1808.
- Fakih H, Baggett B, Holtz G *et al.* 1987 Interleukin-1: a possible role in infertility associated with endometriosis. *Fertility and Sterility* **47**, 213–217.
- Fouladi-Nashta AA, Alberio R, Kafi M *et al.* 2005 Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. *Reproductive BioMedicine Online* **10**, 497–502.
- Gomez-Torres MJ, Acien P, Campos A *et al.* 2002 Embryotoxicity of peritoneal fluid in women with endometriosis. Its relation with cytokines and lymphocyte populations. *Human Reproduction* **17**, 777–781.

- Hardy K 1997 Cell death in the mammalian blastocyst. *Molecular Human Reproduction* **3**, 919–925.
- Illera MJ, Juan L, Stewart CL *et al.* 2000 Effect of peritoneal fluid from women with endometriosis on implantation in the mouse model. *Fertility and Sterility* **74**, 41–48.
- Martinez-Roman S, Balasch J, Creus M *et al.* 1997 Immunological factors in endometriosis-associated reproductive failure: studies in fertile and infertile women with and without endometriosis. *Human Reproduction* **12**, 1794–1799.
- Morcros RN, Gibbons WE, Findley WE 1985 Effect of peritoneal fluid on in-vitro cleavage of 2-cell mouse embryos: possible role in infertility associated with endometriosis. *Fertility and Sterility* **44**, 678–683.
- Navarro J, Garrido N, Remohi J *et al.* 2003 How does endometriosis affect infertility? *Obstetrics and Gynecology Clinics of North America* **30**, 181–192.
- Parchment RE 1993 The implications of a unified theory of programmed cell death, polyamines, oxyradicals and histogenesis in the embryo. *International Journal of Developmental Biology* **37**, 75–83.
- Prough SG, Aksel S, Gilmore SM *et al.* 1990 Peritoneal fluid fractions from patients with endometriosis do not promote two-cell mouse embryo growth. *Fertility and Sterility* **54**, 927–930.
- Sherif GA, Chad IF, Amin UH 1987 Local peritoneal factors: their role in infertility associated with endometriosis. *American Journal of Reproduction and Gynecology* **157**, 1207–1214.
- Stellar H 1995 Mechanisms and genes of cellular suicide. *Science* **267**, 1445–1449.
- Suginami H, Yano K, Watanabe K *et al.* 1986 A factor inhibiting ovum capture by the oviductal fimbriae present in endometriosis peritoneal fluid. *Fertility Sterility* **46**, 1140–1146.
- Taketani Y, Kuo TM, Mizuno M 1992 Comparison of cytokine levels and embryo toxicity in peritoneal fluid in infertile women with untreated or treated endometriosis. *American Journal of Obstetrics and Gynecology* **167**, 265–270.
- Toya M, Saito H, Ohta N *et al.* 2000 Moderate and severe endometriosis associated with alterations in the cell cycle of granulosa cells in patients undergoing in-vitro fertilization and embryo transfer. *Fertility and Sterility* **73**, 344–350.
- Wu MY, Chen SU, Chao KH *et al.* 2001 Mouse embryo toxicity of IL-6 in peritoneal fluid from women with or without endometriosis. *Acta Obstetrica et Gynecologica Scandinavica* **80**, 7–11.

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