Attenuated oocyte fertilization and embryo development associated with altered growth factor/signal transduction induced by endometriotic peritoneal fluid

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Objective: To investigate whether the embryotoxic effect of peritoneal fluid (PF) from infertile women with mild endometriosis on mouse oocytes and embryos is associated with changes in embryonic epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and their receptors.

Design: Experimental animal study.

Setting: University-based research laboratory.

Animal(s): Adult ICR mice.

Intervention(s): Peritoneal fluid was obtained from fertile women with no endometriosis (PF-NE) and infertile women with mild endometriosis (PF-E). In vitro fertilization was performed, and mouse two-cell stage embryos were cultured in human tubal fluid medium with or without PF.

Main Outcome Measure(s): Rates of fertilization, cleavage, and blastulation. The embryonic EGF and IGF-I levels in culture medium were analyzed by enzyme-linked immunosorbent assay, and EGF receptor, IGF-I receptor, and phosphorylated extracellular signal-regulated protein kinases (p-ERK) expression was determined by immunofluorescence and confocal microscopy.

Result(s): When oocytes and embryos were cultured in media with PF-E, the fertilization capability of oocytes and the development potential of embryos were decreased. The levels of embryonic EGF, IGF-I, and their receptors were increased. However, p-ERK of the postreceptor signal transduction pathway was down-regulated.

Conclusion(s): Endometriotic PF may attenuate oocyte and embryo development by impairing embryonic growth factor/receptor/signal transduction, resulting in endometriotic infertility. (Fertil Steril® 2010;93:2538–44. ©2010 by American Society for Reproductive Medicine.)

Key Words: Embryo, endometriosis, epidermal growth factor, insulin-like growth factor-I, peritoneal fluid, phosphorylated extracellular signal-regulated protein kinase

Endometriosis and infertility in women are closely associated (1). Usually, there are few or no visible anatomic abnormalities in the peritoneal cavity of women with mild endometriosis. It is known that these women have an increased volume of peritoneal fluid (PF) with more activated inflammatory factors when compared with women who do not have endometriosis (2). Peritoneal fluid can enter the oviductal cavity to influence reproductive processes by modulating the microenvironment. Some studies have demonstrated that the PF of infertile women with endometriosis (PF-E) has an adverse effect on in vitro cleavage of mouse embryos (3) and might be related to endometriosis-associated infertility (4, 5).

However, others have reported no difference in the effects of PF from women with and without endometriosis on mouse embryo development and apoptosis (6–8). Because the findings are rather controversial, further study is needed to clarify the effects of PF-E on oocyte and embryo development and the possible mechanisms (9).

Epidermal growth factor receptor (EGFR) and insulin-like growth factor-I receptor (IGF-IR) signal transduction is important for controlling female reproductive functions in mammals (10–15). Embryonic EGF and IGF-I are receptor-mediated autocrine/paracrine growth factors for embryo development (16–19). Among the EGFR and IGF-IR signaling pathways, mitogen-activated protein kinases (MAPKs) are important signal-transducing enzymes that mediate almost all cellular processes, including cell survival, proliferation, migration, angiogenesis, and inhibition of apoptosis. Phosphorylated extracellular regulated protein kinases (p-ERKs) are active proteins in MAPK signal transduction (20, 21).

Therefore, we hypothesized that PF-E may attenuate the potential of fertilization and embryo development by altering embryo-derived EGF and IGF-I and signal transduction. We investigated the influence of PF from infertile women with endometriosis and fertile women with no endometriosis (PF-NE) on the quality of mouse oocytes and the development of mouse embryos. We also examined the growth factor (EGF and IGF-I) receptor/signal transduction...
pathway to explore the possible mechanism underlying the adverse effects of PF-E on the development of oocytes and embryos.

**MATERIALS AND METHODS**

**Patients and Peritoneal Fluid Collection**

Patients were recruited at the Women’s Hospital of Zhejiang University’s School of Medicine, with the approval of the institutional review board and after obtaining informed consent. The seven fertile women undergoing laparoscopy for infertility had stage I or II mild endometriosis, as scored by the revised classification system of the American Society of Reproductive Medicine (22). The seven fertile women who served as controls were undergoing laparoscopy for myomectomy with no evidence of endometriosis, infection, or other pathologic conditions. None of the women had received hormone treatment for 3 months before recruitment.

All the women were of reproductive age, had a regular menstrual cycle of 28 to 32 days, and underwent surgery during the secretory phase of the cycle. We obtained PF from the posterior cul-de-sac and transferred it into sterile tubes at the time of laparoscopy; the PF was immediately centrifuged for 10 minutes at 2000 × g and 4°C. The supernatant was aliquoted and stored at −70°C until further analysis.

**Animals**

The care and use procedures for the ICR mice were in accordance with the Institutional Guide for Laboratory Animals established by the Animal Care and Use Committee (ACUC), and were approved by the ACUC of the School of Medicine, Zhejiang University. The mice were housed under a 12/12-hour light/dark cycle at 25°C of Medicine, Zhejiang University. The mice were housed under a 12/12-hour light/dark cycle at 25°C and 50% to 60% humidity, and were fed ad libitum with a standard diet and water.

**Cumulus–Oocyte Complex Collection and Culture**

Eight-week-old mice were synchronized and superovulated. Cumulus–oocyte complexes (COCs) were collected from the oviducts and were placed in basal medium: human tubal fluid medium (HTF; Irvine Scientific, Irvine, CA) with 10% serum substitute supplement (Irvine Scientific).

The COF samples from the seven fertile women without endometriosis were pooled as PF-NE, and the samples from seven infertile women with mild endometriosis as PF-E. In the first experiment, COCs were randomly allocated into three groups: [1] control (basal medium), [2] 10% PF-NE (basal medium with 10% PF-E), and [3] 10% PF-E (basal medium with 10% PF-E). After incubation at 37°C in 5% CO2 for 30 minutes, a portion of the COCs from each of the three groups was collected and treated with 0.1% (80 IU/mL) hyaluronidase (Sigma-Aldrich, St. Louis, MO) in HTF to remove the cumulus. The treated COCs were used to analyze the expression of EGFR and IGF-IR. The remaining samples were transferred into basal medium for in vitro fertilization.

**Sperm Collection, Capacitation, and In Vitro Fertilization**

Spermatocytes were obtained from 10-week-old ICR mice and placed in a 400-μL basal medium drop at 37°C in 5% CO2 for 1.5 hours to be capacitated, as described previously elsewhere (23).

Sperm insemination was realized at a concentration of 1 × 106 sperm/mL. The COCs were inseminated in 100-μL microdrops of sperm suspension in basal medium overlaid with mineral oil for 6 hours at 37°C and 5% CO2. Fertilization was determined by the presence of two pronuclei.

**Embryo Recovery and Culture**

The method of two-cell embryo collection has been described previously elsewhere (24). In the second experiment, the apoptosis rate of embryos cultured in HTF medium alone or HTF medium containing PF-E (10%, 30%, or 50%) for 24 hours was assessed first. Then embryos were randomly allocated into five groups: [1] control: HTF alone, [2] 10% PF-NE: HTF containing 10% PF-NE, [3] 30% PF-NE: HTF containing 30% PF-NE, [4] 10% PF-E: HTF containing 10% PF-E, and [5] 30% PF-E: HTF containing 30% PF-E. Embryo culture was performed with 50 embryos per 500 μL of culture medium and covered with mineral oil at 37°C and 5% CO2.

**RESULTS**

**Rates of Apoptosis, Fertilization, Cleavage, and Blastulation**

After culture for 24 hours, the apoptosis rate of embryos in the control, 10% PF-E, 30% PF-E, and 50% PF-E groups were 15.37%, 36.10%, 55.50%, and 92.79%, respectively. The apoptosis rate of the three PF-E group were all statistically significantly higher than control (Fig. 1A).

In the first experiment, the fertilization rate of COCs cultured in the medium containing 10% PF-E for 30 minutes was statistically significantly lower than found in 10% PF-NE and control groups (see Fig. 1B), but there was no statistically significant difference between the 10% PF-NE group and control (see Fig. 1B).

In the second experiment, the mean two-cell to four-cell cleavage rate was statistically significantly lower in the media
10% PF-E, 30% PF-NE, or 30% PF-E than for the control group (see Fig. 1C), and there was no statistically significant difference between the 10% PF-NE group and control (see Fig. 1C). The mean blastulation rate of embryos in the media containing PF was statistically significantly lower than in the control (see Fig. 1D).

**Concentration of Embryo-derived EGF and IGF-I**
Compared with control, the embryo-derived EGF concentration was statistically significantly higher in media containing 10% PF-E, 30% PF-NE, or 30% PF-E (see Fig. 1E). Also, the levels of EGF among these groups were statistically significantly different (see Fig. 1E). However, there was no statistically significant difference between the 10% PF-NE group and control (see Fig. 1E).

Embryo-derived IGF-I concentration in the medium containing 30% PF-E was statistically significantly higher than in control (see Fig. 1F). No statistically significant differences were found in the other groups.

**Expression of EGFR and IGF-IR in Oocytes**
After COCs were cultured for 30 minutes, oocytes were collected for immunofluorescence analysis.
The mean gray values of EGFR and IGF-IR in the 10% PF-E group were statistically significantly higher than those in the control or 10% PF-NE groups (Fig. 2A, B). On the other hand, the value of EGFR in the 10% PF-NE group was also higher than found in the control group (see Fig. 2A, B). The values of IGF-IR in the control and 10% PF-NE groups were not statistically significantly different (see Fig. 2A, B).

**Expression of EGFR and IGF-IR in Embryos**

From the two-cell stage to the morula stage, mean gray values of EGFR and IGF-IR in media containing 30% PF-NE or 30% PF-E were statistically significantly higher than in the other groups.
The expression level of EGFR in the 30% PF-E group was statistically significantly higher than that of the 30% PF-NE group (see Fig. 3A, B; Fig. 4A). There was no statistically significant difference in the expression levels of EGFR or IGF-IR between the 10% PF-NE and control groups (see Fig. 3A, B; Fig. 4A).

Expression of p-ERK in Embryos

After culture for 30 minutes, the mean gray values of ERK and p-ERK expression in two-cell and eight-cell embryos in different media were determined. The expression of ERK was not statistically significantly different in two-cell and eight-cell embryos (see Fig. 3C).

Mean gray values of p-ERK in both two-cell and eight-cell embryos in the 10% PF-E, 30% PF-NE, and 30% PF-E groups were statistically significantly lower than in the control group as well as the 10% PF-NE group (see Fig. 3C; Fig. 4B). There was no statistically significant difference between the 10% PF-NE and control groups (see Fig. 3C; Fig. 4B).
DISCUSSION

We found that the rates of fertilization, cleavage, and blastulation were decreased not only in medium containing PF-E but also in the medium containing PF-NE at the highest concentration. The expression levels of embryonic EGF, IGF-I, and their receptors were increased, while p-ERK, the active protein in MAPK signal transduction coupled to EGF and IGF-I receptors, was down-regulated.

Our study demonstrated that medium containing PF-E induced apoptosis in embryos in a dose-dependent manner. At 50% PF-E, the apoptosis rate reached an extremely high level (around 90%), so we selected culture medium containing 10% or 30% PF for other experiments on embryos. Because oocytes are much more sensitive and vulnerable than embryos, we only cultured oocytes in medium containing 10% PF. Although both PF-E and PF-NE decreased the blastulation rate, the medium containing a low concentration PF-E but not low PF-NE also statistically significantly decreased the cleavage rate and the mean fertilization rate. Thus, we suggest that, besides diluting the elements of HTF, there are harmful agents in PF-E that might induce the high percentage of infertility in women with endometriosis.

It has been confirmed that growth factors such as EGF and IGF-I are important for oocyte and embryo development (25–29). Treatment of embryos in vitro with EFG effectively prevents a decrease in implantation rate (30). Both EGF and IGF-I independently enhance embryo development (31). In addition, EGF and IGF-I can be used as markers of embryo quality (32–34). It is interesting that our study showed that the levels of embryo-derived EGF and IGF-I were statistically significantly up-regulated in the PF-E groups, and that this up-regulation was dose dependent. We also found that, at the same concentration, PF-E had a greater influence than PF-NE. We suggest that PF-E inhibited the development of pre-implantation embryos by impacting the function of embryo-derived factors rather than directly impairing their synthesis and secretion. On the other hand, although growth factors are important for fetal development, their overexpression may also have adverse effects (35). The mechanism involved in this abnormal increase of embryonic growth factors is unclear. Compensatory responses of embryos to a medium containing PF may provide an explanation.

Epidermal growth factor and other members of the EGF superfamily are structurally related peptides that stimulate the intracellular signal transduction pathway by binding to EGF-R (36–38). Activation of the receptor evokes subcellular protein tyrosine phosphorylation, resulting in cell proliferation and differentiation (39–41). Insulin-like growth factor-I activates the signal transduction pathway by binding to IGF-IR. Both EGFR and IGF-IR are coupled to the MAPK-mediated signal transduction pathway (20, 21). Our study showed that medium containing 30% PF statistically significantly increased the expression levels of EGFR and IGF-IR from embryos. The mechanism is unclear but might be due to an up-regulatory response of embryonic growth factor receptors to their increased ligand levels in the abnormal medium containing PF.

An important finding in our study was that the level of p-ERK, which is required to activate MAPKs in the EGFR and IGF-IR signal transduction pathways (20, 21, 42–44), was statistically significantly lower in embryos exposed to medium containing PF. The results suggest that although EGF, IGF-I, and their receptors increased in embryos, the postreceptor signal pathways might be impaired by PF. Furthermore, our results showed that PF-E had the most harmful effect on the growth factor/receptor/signal transduction pathway. In addition, besides growth factors, the transduction pathway is also of great importance in the development of oocytes and embryos. It has been shown that low MAPK expression attenuates oocyte maturation by reducing intracellular calcium release (45). Hence, impairment of the MAPK transduction pathway may play an important role in the adverse effects of PF-E on oocyte and embryo development.

Our study has shown that, in an abnormal microenvironment of medium mixed with PF in the reproductive tract, altered expression of at least some growth factors, such as EGF and IGF-I, and their receptors as well as important enzymes in the postreceptor pathways have an adverse effect on the quality of oocytes and the development of preimplantation embryos. Our results also suggest that this is one possible mechanism underlying endometriotic infertility. Further experiments are needed to clarify which factors in PF-E impair oocyte and embryo development.

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REFERENCES


