Downregulative Effects of Nitric Oxide on Oocyte Fertilization and Embryo Development: Possible Roles of Nitric Oxide in the Pathogenesis of Endometriosis-associated Infertility

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Key Words

Nitric oxide • Endometriosis • Peritoneal fluids • IVF-ET

Abstract

Aims: To investigate the effects of elevated nitric oxide (NO) levels in peritoneal fluids (PF) on oocyte fertilization and pre-implantation embryo development, and the relation of those effects to endometriosis-associated infertility. **Methods:** PF from women undergoing laparoscopy for infertility of minor endometriosis, tubal blockage and operation for tubal ligation was aspirated at the pouch of the cul-de-sac during surgery. Oocytes and embryos of adult ICR mice were cultured *in vitro* with or without endometriotic PF. The fertilization rate of oocyte and the cleavage rate of 2-cell embryos were examined. Also, the clinical indexes of IVF-ET of women with minor endometriosis and tubal infertility were analyzed. **Results:** Oocyte fertilization rate of endometriotic women with IVF-ET treatment was significantly lower than that of tubal block women. The dose-related adverse effects of endometriotic PF and SNP (NO donor) in culture medium on oocyte fertilization and embryos development were confirmed. **Conclusion:** Increased NO levels in PF play an important role in

mediating the effects of endometriotic PF on oocyte fertilization and embryo development. IVF might serve as an alternative treatment for endometriosis-associated infertility.

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Introduction

Endometriosis is tightly linked to infertility, which is manifested at very early stages of endometriosis [1]. It is easily understandable that moderate to severe [2] endometriosis results in anatomical destruction and distortion in the peritoneal cavity causing infertility. However, minimal to mild endometriosis [2] was often observed with patent fallopian tubes and normal ovaries. The mechanisms underlying reproductive failure are subtle and remain controversial.

Peritoneal fluid (PF) is a self-contained micro-environment with special products and hormones in concentrations that are drastically different from those in serum. The role of PF components in the pathophysiology of endometriosis is undoubtedly important. PF of women with endometriosis contains elevated amounts of macrophages [3], cytokines [4-6] growth factors [7] and angiogenic factors [8]. The reproductive organs are

bathed in PF and pelvic alteration on the production of such factors may be accused for problem on ovum maturation, fertilization, embryo development and implantation [9].

Nitric oxide (NO), a multifunctional free radical, which is involved in both inflammation and neoangiogenesis has already showed to be one of the most important factors in PF of women with minimal to minor endometriosis [5, 10]. Low levels of NO are reported as an important factor in ovarian function and implantation and cause relaxation of oviduct musculature [11]. High levels of NO have deleterious effects on sperm motility, are toxic to embryos and inhibit implantation [12]. Higher amounts of NO and NO synthase are detected in the eutopic and ectopic endometrium of women with endometriosis [5, 13]. In our previous study, we demonstrated significantly increased NO levels in PF of patients with minimal to mild endometriosis [14]. However, the mechanism of the effects of elevated NO level in PF of patients with endometriosis is still not very clear.

In our present study, the effects of endometriotic PF and SNP, a NO donor, in culture medium on oocyte fertilization and pre-implantation embryos were investigated. Also, the outcomes of IVF treatment for infertility of these patients were monitored and compared with the IVF outcome of tubal infertility as control. We intend to clarify that the alteration of NO levels in PF and the direct effects of NO on oocyte fertilization and embryo development might partly explain the minimal to mild endometriosis associated infertility.

Materials and Methods

The study was conducted at the Women’s Hospital, School of Medicine, Zhejiang University. Our study was approved by the Institutional Review Board (IRB) of School of Medicine, Zhejiang University prior to any clinical and laboratory practices. All patients were consented by the investigator and signed a consent form. One hundred and eighty-four women undergoing laparoscopy for infertility and twenty women undergoing operation for tubal ligation were included in this study. All women were patients in Department of Reproductive Endocrinology, School of Medicine, Zhejiang University. None of these women received any hormonal treatment during the three months preceding laparoscopy. The surgery was performed during a proliferative phase of the menstrual cycle. All women included had a regular 28-32 day menstrual cycle. Eighty-four infertile women with minimal endometriosis (n=44) or mild endometriosis (n=40), which was confirmed using histological methods and classified by the revised American Fertility Society classification [2], participated

Characteristics	Endometriosis group (n=84)	Tubal block group (n=100)	P ^a
Age (yrs)	30.7±3.1	29.8±4.1	NS
Basal serum FSH concentration (mIU/ml)	7.2±2.2	7.1±2.1	NS
Basal serum E2 concentration (pg/ml)	44.8±17.0	57.2±31.2	NS
Total gonadotropin dosage (IU)	1008.9±520.5	905.2±411.1	NS
No. of follicles of 12-16 mm	3.8±2.0	3.5±1.8	NS
No. of dominant follicles of >16mm	1.5±0.6	1.5±0.6	NS
Basal sperm number (10 ⁶ /ml)	55.1±42.3	49.3±40.5	NS
Basal total sperm motility (%)	58.6±13.5	53.5±18.2	NS

Table 1. Characteristics of the patients with IVF-ET treatment. Note: Data are mean ± SD (median), NS=nonsignificant, ^aNon-parametric ANOVA.

in the present study as endometriotic group. One hundred infertile women with tubular block participated in the study as tubal block group. The control group consisted of 20 women containing no evidences of endometriosis and any other pathological condition.

PF was obtained from the posterior cul-de-sac into sterile tubes at the time of laparoscopy or surgery. Patients whose PF was contaminated with blood were excluded from this study. Peritoneal samples were cooled with ice water and centrifuged at 400 x g for 10 min at 4 °C. Then the sample was separated into aliquots and collected and stored at -70 °C until analysis. After thawing, each sample was filtered (0.22-mm filter, Whatman, England) before addition to the culture medium.

All infertile couples initially underwent routine evaluations including medical background check, physical examination, semen analysis and hormonal assessment. Patients diagnosed with endometriosis by laparoscopy for a time period longer than three months underwent standard long protocol IVF treatment. Oocyte retrieval was carried out through transvaginal aspiration 36h after human chorionic gonadotropin (hCG; Pregnyl, Organon, the Netherlands) injection. All indexes were recorded when IVF-ET were conducted (Table 1). The fertilization rate was determined by the total number of oocytes with two pronuclei over the total number of oocytes in each group. The implantation rate calculated as the total number of gestation sacs over the total number of transfer embryos. The pregnancy rate means the total number of clinical pregnancy which was defined as a gestation sac with fetal heartbeat visualized on ultrasound over the number of the patients. The abortion rate here, that is the early abortion rate, refers to the total number of abortion within 12 weeks of gestation age over the number of pregnancy.

Animal care and use in experimental procedures were conducted in accordance with the Institutional Guide of Laboratory Animal established by the Animal Care and Use Committee (ACUC) and approved by ACUC of School of Medicine, Zhejiang University. Female ICR mice (Zhejiang University Animal Center, Hangzhou, China) were housed in 12h/12h light/dark cycle (light on from 0:00 to 12:00) at 25.0 ± 0.5°C and 50-60% of humidity. The mice were synchronized

and super-ovulated by intra-peritoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG, Intervet, USA).

For oocyte fertilization experiment, mice were killed by cervical dislocation 12-14 h after injection of hCG and the oviducts were excised. Cumulus masses were recovered from the dilated ampullae under a dissecting microscope. The collected cumulus masses were either placed in a drop of human tubal fluid (HTF, Irvine Scientific, Irvine, CA), for *in vitro* fertilization. The vas deferens close to epididymis was cut and placed in a drop of 500 μ l HTF under oil. HTF were incubated for 12 h at 37°C in 5% CO₂ prior to use. The sperm were gently taken out using watchmaker's forceps and incubated for 1.5 h at 37°C with 5% CO₂ to be capacitated. The collected cumulus masses were placed in a drop of 1000 μ l HTF under oil and incubated for 1 h at 37°C with 5% CO₂. One hundred μ l of sperms were then added into the drop containing eggs. The sperm concentration was in a range of 1×10^6 to 2×10^6 /ml. The eggs were co-incubated with sperms for 4 h at 37°C with 5% CO₂. Fertilization was determined by the presence of two pronuclei 3-6 h later.

For embryo culture experiment, forty-eight hours after injection of hCG, the mice were bred with proven breeder males and examined the next day to observe whether a copulation plug was present. Mice were euthanized on day 1 and 2-cell embryos were collected by flushing oviducts and/or uteri with modified human tubal fluid (MHTF, Irvine Scientific, Irvine, CA). The embryos were rinsed 3 times with MHTF and then transferred to the culture medium. Embryo cultures were performed in 1 embryo/10 μ l of culture medium and covered with mineral oil in a humidified atmosphere of 5% CO₂ at 37°C. The 2-cell embryos were evaluated daily under a microscope to monitor embryonic cleavage and the number of embryos expressing cleavage to 4-cell embryos was recorded. A cleavage rate was calculated for each group.

The oocytes and embryos were randomly allocated into 3 groups individually. The HTF group includes oocytes or embryos cultured in HTF and served as the control. The PF-E group contained one set of oocytes or embryos cultured in HTF supplemented with 10% PF and a second set of oocytes or embryos with 30% PF of women with endometriosis. Lastly, the PF-NE group consisted of one set of oocytes or embryos cultured in a HTF supplemented with 10% PF and a second set of oocytes or embryos with 30% PF of women without endometriosis. Four-well dishes with 3 groups of oocytes or embryos (control group, 10% PF-E group and 10% PF-NE group; or control group, 30% PF-E and 30% PF-NE group) were used in each experiment. The choice for concentration of PF was based on a preliminary study, where the effects of various concentrations of PF on the oocyte fertilization and 2-cell mouse embryo growth were determined. All the experiments were repeated 5 times using the PF randomly selected from 5 women with endometriosis and 5 women without endometriosis.

Sodium nitroprusside (SNP) was used as the NO donor for the NO *in vitro* experiment [15]. Various concentrations of SNP (10^{-7} , 10^{-5} , 10^{-3} M) were prepared using HTF. Oocytes or 2-cell embryos were randomly divided into 4 groups (HTF group, 10^{-7} M group, 10^{-5} M group and 10^{-3} M group). As mentioned above, in one experiment, four-well dishes with 4 groups of

Fig. 1. The parameters of IVF-ET in patients with tubal infertility and endometriosis. T: group with tubal infertility; EM: group of endometriosis. FR: fertilization rate, IR: implantation rate, PR: pregnancy rate; AR: abortion rate. Data are shown as %. ** $p < 0.01$ compared with corresponding control.

oocytes or embryos in one four-well dish were used. The solutions containing SNP were freshly prepared and the experiment was repeated 6 times.

SPSS 16.0 were used for the data analysis. The data of fertilization rate and cleavage rate are expressed as the mean $\pm$ SEM. The Chi-Square test and non-parametric ANOVA were used to estimate statistical differences. Values were determined to be significant when $P < 0.05$.

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Results

Comparison of characteristics of patients with IVF-ET treatment between tubal block group and endometriotic group

In the present study, there are no significant differences between the infertile women with tubal block and with endometriosis in age, basal serum FSH level, total gonadotropin dosage used, number of follicles around 12-16 mm diameter, number of dominant follicles >16 mm (Table 1). Their partners showed no differences in basal sperm number and basal total sperm motility between two groups (Table 1). In the outcomes of IVF, two groups showed no significant differences in implantation rate, pregnancy rate and abortion rate (Fig. 1). However, fertilization rate in endometriotic women was significantly lower than that in tubal block women (Fig. 1).

PF from the endometriotic patients significantly reduced fertilization rate of oocytes and cleavage rate of embryos

Treatment of oocytes with PF from the women with or without endometriosis demonstrated that, compared with control or 30% PF-NE group, 30% PF-E treatment significantly reduced the fertilization rate of oocytes (24.0% vs 56.3% in control and 63.3% in PF-NE, $p < 0.05$). However, 10% PF-E treatment did not result in significant difference in the fertilization rate (63.3%) from control (59.7%) or PF-NE (57.9%) group (Fig. 2A).

On the other hand, we examined the effect of PF on the cleavage rate of embryos. Our results showed that the PF from patients with minimal to mild endometriosis concentration-dependently inhibited the development of mouse 2-cell embryos *in vitro*. The cleavage rates in 10% and 30% PF-E groups were 48.7% and 4.1%, respectively, significantly lower than those in control and PF-NE groups (Fig. 2B).

NO donor SNP dose-dependently reduced the fertilization rate and cleavage rate

Our previous study demonstrated that a high NO level was detected in PF-E [13]. In the present study, we examined whether NO had a harmful effect on oocyte fertilization and embryo cleavage using SNP as a NO donor. The concentrations of SNP used in the present study were 10^{-7} , 10^{-5} and 10^{-3} M. The SNP at 10^{-3} M was not detected to have obvious toxic effect on oocyte or embryo viability in our study. All oocytes and embryos cultured in medium with different concentration of SNP were alive. The fertilization rate of oocytes was significantly reduced when they were cultured in the medium with SNP at different concentrations (Fig. 3A). The inhibition of oocyte fertilization by SNP was in a concentration-dependent manner when oocytes were exposed in the concentrations of SNP from 10^{-5} to 10^{-3} M (Fig. 3A). The cleavage of 2-cell embryos was also significantly delayed when embryos were cultured in the

medium with SNP. It was shown that the inhibition of embryonic development by SNP at different concentrations (10^{-7} , 10^{-5} , 10^{-3} M) (Fig. 3B).

Discussion

The present study showed that PF from patients with minimal to mild endometriosis significantly reduced the fertilization rate of oocytes and the cleavage rate of embryos. Although a previous investigation by Miller et al. showed that serum from infertile women with endometriosis reduced the fertilization rate and subsequent embryonic development [16], the present study demonstrated direct evidence that PF from the patients with minimal to mild endometriosis impaired oocyte fertilization and embryo development. Compared with serum, PF in culture medium has more direct effects on oocytes and embryos than serum because the reproductive organs are bathed in PF. On the other hand, another research reported that exposure of retrieved human oocytes to endometrioma fluid had no detrimental effect on oocyte fertilization rate or quality and embryo development [17, 18]. The difference between that result and our data may be due to different components in endometrioma fluid and in endometriotic PF.

The present study also demonstrated that, in IVF treated patients, there was a significant difference in fertilization rate but not in the implantation rate, pregnancy rate and abortion rate between minimal to mild endometriosis group and tubal infertility group. The results suggest that endometriotic PF mainly affects fertilization and embryo development before implantation. Because oocytes and early embryos are soaked in PF when they develop, any toxic effect of PF is directly exerted on them during oocyte fertilization and embryo development. However, if subsequent formed embryos could escape from the adverse pelvic environment and be avoided to contact with PF, they would normally develop because the other parameters of IVF such as implantation rate, pregnancy rate and abortion rate reserved and were similar to those in tubal infertility group.

In the present study, we found that the PF from patients with minimal to mild endometriosis has adverse effects on the fertilization of oocyte and development of embryos. 10%, 30% PF-NE or 10% PF-E did not show to have inhibitory effect on fertilization rate. Only 30% PF-E reduced fertilization rate. On the other hand, both PF-NE and PF-E at 10% or 30% concentration reduced cleavage rate. Our results also demonstrated that at the

same concentration, PF-E had more significantly inhibitory effect on cleavage rate compared with corresponding PF-NE group. Our data suggest that PF-E, not PF-NE, may reduce oocyte fertilization, and PF-E may have more toxic effect on embryo development than PF-NE.

The question is which component(s) in endometriotic PF impairs the development and functions of oocytes and embryos. As mentioned above, our previous work has already confirmed that NO levels in the PF of minimal to mild endometriosis patients were significantly higher than those in patients of tubular block and control women without any evidence of pelvic pathological condition [14]. It has been reported NO was an important modulator of female reproductive functions, including folliculogenesis [19], oocyte maturation [20], fertilization [21] and implantation [22]. However, high levels of NO will do damage to sperm [23], oocyte [24] and embryos [17, 25]. Take the biphasic effects of NO on reproduction into consideration, we supposed that increased NO levels in PF might play an important role in the endometriosis-associated infertility. The present results that NO donor SNP dose-dependently reduced the fertilization rate of oocytes and the cleavage rate of zygotes demonstrate direct evidence that high concentration of NO impairs both oocyte and early embryo development. One previous study found that low concentration of SNP (10^{-8} M) had no significant effects on survival and fertilization, but SNP at 10^{-6} M to 10^{-7} M significantly inhibited oocyte survival and fertilization [26]. The effective concentration of SNP is different from our study might due to the cryopreserved oocytes used in that study and fresh oocytes used in the present study.

In this study, the fertilization and cleavage rate decreased by SNP indicated that the quality of oocytes also decreased. These results differ from those shown by Viana et al. [27] in cattle. When 10^{-5} M SNP was added there was an increase in the rate of blastocysts and when 10^{-3} M SNP was added in the culture medium of maturation, the meiotic arrest occurred and decreased membrane integrity of oocytes, probably due to differences in the culture medium and differences between species in sensitivity/ responsiveness to SNP. Moreover, the present study showed that SNP at 10^{-3} M had strong inhibitory effects on oocyte fertilization and embryo development. Francavilla et al revealed that the binding of the sperm to the zona pellucide were blocked by high-concentration of NO-releasing compounds, which might explain our results [28].

In summary, our present results provide the evidence of the relationship of increased NO levels in minimal to

mild endometriotic PF and the endometriosis-associated infertility. The negative effects of high levels of NO in PF on oocytes fertilization and pre-implantation embryos development might be the primary reason. Additional, take the results of our study into consideration, IVF, a process that avoids direct contact of embryos with potentially toxic PF, may be a better selection compared with IUI (intrauterine insemination) for women with minimal to mild endometriosis in clinic.

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