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Expressions of Growth Differentiation Factor-9 (GDF-9) of Bovine Cumulus-Oocyte Complex in Peritoneal Fluid Culture of Infertile Patients with Endometriosis

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ABSTRACT

*The decrease of GDF-9 production may impair oocyte-granulosa cell communication and lead to failure of fertilization and embryonic development. The objective of this study was to study expression of growth differentiation factor-9 (GDF-9) cumulus-oocyte complex in peritoneal culture of infertile patients with endometriosis. Peritoneal fluid were collected from infertile patients undergoing diagnostic laparoscopy and classified into non-endometriosis, mild endometriosis, and severe endometriosis according to laparoscopic finding. Cumulus-oocyte complex (COC) were aspirated from antral follicle derived from bovine ovary from local abattoirs. COC were then placed in tissue culture medium-199 (TCM-199) and divided into four group of cultere. First group contain TCM-199 as a medium served as control group, while second, third, and four groups medium were added accordingly with 3% of peritoneal fluid of non endometriosis, mild endometriosis, and severe endometriosis. These four groups were incubated in 5% CO_2 at 38°C for 20 hours and COC were eventually fixated in object glasses, stained by immunocytochemistry, and followed by measurement of GDF-9 expression. Statistic analysis using Kruskal Wallis test showed differences of GDF-9 expression in four culture groups. Mann-Whitney test revealed that GDF-9 expression in endometriosis group (mild and severe) were significantly lower compared with control and non-endometriosis group and expression in severe endometriosis group were significantly lower than in mild endometriosis one ($p < 0.05$). In conclusion,GDF-9 expressions of bovine COC cultured in peritoneal fluid of infertile patients with endometriosis were lowered compared with that of control and non-endometriosis group and this decrease was in proportion with severity of endometriosis.**(MOG 2013;21:71-76)***

Keywords: *GDF-9 expression, cumulus-oocyte complex, infertility, endometriosis, peritoneal fluid, culture*

ABSTRAK

*Penurunan produksi GDF-9 dapat mengganggu komunikasi oosit-sel granulosa dan menyebabkan kegagalan fertilisasi dan perkembangan embrio. Penelitian ini bertujuan mempelajari ekspresi growth differentiation factor-9 (GDF-9) kompleks kumulus-oosit pada kultur peritoneum pasien infertil dengan endometriosis. Cairan peritoneal dikumpulkan dari pasien infertil yang menjalani laparoskopi diagnostik dan diklasifikasikan menjadi non-endometriosis, endometriosis ringan dan endometriosis berat sesuai dengan temuan laparoskopi. Cumulus-oocyte complex (COC) diaspirasi dari folikel antral yang berasal dari ovarium sapi dari RPH setempat. COC kemudian ditempatkan dalam jaringan medium kultur-199 (TCM-199) dan dibagi menjadi empat kelompok kultur. Kelompok pertama berisi TCM-199 sebagai medium berfungsi sebagai kelompok kontrol, sedangkan yang media kelompok kedua, ketiga, dan empat ditambahkan sesuai dengan 3% dari cairan peritoneal non endometriosis, endometriosis ringan, dan endometriosis berat. Keempat kelompok diinkubasi dalam 5% CO2 pada 38 derajat C selama 20 jam dan COC akhirnya terpaku difiksasi pada gelas objek, diwarnai secara imunohistokimia, dan diikuti dengan pengukuran ekspresi GDF-9. Analisis statistik menggunakan uji Kruskal Wallis menunjukkan perbedaan ekspresi GDF-9 pada empat kelompok kultur. Uji Mann-Whitney menunjukkan bahwa ekspresi GDF-9 pada kelompok endometriosis (ringan dan berat) secara signifikan lebih rendah dibandingkan dengan kontrol dan kelompok non-endometriosis dan ekspresi dalam kelompok endometriosis berat secara signifikan lebih rendah daripada di kelompok endometriosis ringan ($p < 0,05$). Sebagai simpulan, ekspresi GDF-9 pada sapi COC yang dikultur dalam cairan peritoneal pasien infertil dengan endometriosis lebih rendah daripada kontrol dan kelompok non-endometriosis dan penurunan ini proporsional dengan keparahan endometriosis.**(MOG 2013;21:71-76)***

Kata kunci: *ekspresi GDF-9, cumulus-oocyte complex, infertilitas, endometriosis, cairan peritoneal, kultur*

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INTRODUCTION

Endometriosis is defined as the presence of endometrial-like tissue outside the uterine cavity which induces chronic inflammatory reaction.[1] Women with endometriosis typically present with pelvic pain, infertility or an adnexal mass. Infertility is one of clinical manifestation showed by the difference in fecundity, defined as the probability of a woman achieving a live birth for any given month. In normal couples, fecundity is in the range of 0.15 to 0.20 per month. In untreated woman with endometriosis and infertility, monthly fecundity is 0.02 to 0.10. Early studies suggested that 25-50% of infertile women have endometriosis and that 30-50% of women with endometriosis are infertile.[2,3]

An association between endometriosis and infertility has repeatedly been reported in the literature, but an absolute cause-effect relationship has not yet been confirmed. Several theories has been proposed to identify the pathomechanism of infertility in endometriosis. Severe endometriosis is associated with pelvic adhesions leading to a possible mechanic or anatomic disturbance of fertility, on the other hand the mechanism of infertility associated with minimal or mild endometriosis without such adhesion as well as the negative impact of all stages of the disease on infertility is poorly understood. Many possibilities have been suggested, ranging from abnormal folliculogenesisto impaired endometrium receptivity.[2,4,5]

Peritoneal fluid, a biologic fluid present in the abdominal cavity, has been a focus of research on endometriosis because of the extent of information it potentially carries about the disease. The proximity of peritoneal fluid to endometriotic lesions shows the milieu in which the immune mediators associated with the local inflammation of endometriosis can be studied. It has been suggested that such alterations in cytokines and growth factors interfere with folliculogenesis, ovulation, and fertilization.[2,6]

Peritoneal fluid contains a variety of free floating-cells, including macrophages, mesothelial cells, lymphocytes, eosinophils, and mast cells.[7] The peritoneal fluid of women with endometriosis have confirmed an increased number, concentration, and activation of macrophages which may induce proliferation of cells involved in inflammation through secretion of factors such as IL-1, IL-6, and TNF-α.[7] Other studies similarly found that levels of cytokines, such as IL-6, IL-8, and TNF-α increased in the peritoneal fluid of women with endometriosis.[8] Cytokines, which are produced by many cell types in peritoneal fluid, play a diverse role as toxic effect in constructing the peritoneal environment that induces the development and progression of endometriosis and endometriosis-associated infertility.[9]

Peritoneal fluid bathed the ovaries, hypothetically the inflammatory components in peritoneal fluid maight diffuse into the ovarian follicles, or by paracrine mechanismsimpair the granulosa cell function, oocyte maturation, and folliculogenesis.[10] Folliculogenesis is growth and development process of ovarian follicle consist of oocyte, granulosa, and theca cells that might result in mature and fertilizable oocyte. Women with endometriosis were reported having higher granulosa cell apoptosis rate and a lower percentage of G2/M phase granulosa cells compared with other group of infertile women.This result strongly suggest that the cytokines produced in endometriosis women may be responsible for the disturbance of the cell cycle in the granulosa cells as in other cells and in turn have pathogenic effect on folliculogenesis.[11]

Previous study postulated that apoptosis of granulosa cells caused disturbance in oocyte growth and maturation and associated with decreased grrowth differentiation factor-9 (GDF-9) production.[12] Oocyte-derived GDF-9 is obligatory for normal folliculogenesis and female fertility. Mouse GDF-9 can bind to receptors on granulosa cells and plays multifunctional roles in oocyte-granulosa cell communication and regulation of follicular differentiation and function.[13] The presence of GDF-9 in follicular fluid of preovulatory follicle was confirmedand if it was compared with the level in women with no endometriosis, GDF-9 level in the follicular fluid of women with severe endometriosis was lower. This might impair folliculogenesis leading to reduced oocyte quality.[12]

Exposure of cryopreserved mouse oocytes to the peritoneal fluid from women with endometriosis has resulted in a higher frequency of abnormal meiotic spindle of cytoskeleton and chromosomal misalignment. Mouse oocytes incubated in peritoneal fluid of endometriotic patients also showed significantly DNA damage compared with control and the extent of DNA damage depends on the duration the oocytes exposed to the peritoneal fluid.[14,15]

Based on several studies, it is proposed that pro-inflammatory factors in peritoneal fluid of women with endometriosis may diffuse and impact autocrine-paracrine communication of ovarian folllicles causing cell-cycle alteration and an increased apoptosis in granulosa cells resulting in disturbance of oocyte growth and maturation that could decrease GDF-9 production. It may impair oocyte-granulosa cell communication and lead to failure of fertilization and embryonic development.

This study was designed to investigate GDF-9 expression of COC incubated in peritoneal fluid of infertile endometriotic patients. We use bovine COC due to ethical issue and similarity of bovine folliculogenesis and that of human.

MATERIALS AND METHODS

This study design is true experimental.Peritoneal fluid were collected from infertile patients undergoing diagnostic laparoscoy that met inclusion and exclusion criteria in Graha Amerta Fertility Clinic, Surabaya. Based on laparoscopic finding, the peritoneal fluid were classified as non-endometriotis, mild endometriosis (r-AFS I-II), and severe endometriosis (r-AFS III-IV). Bovine ovaries were collected from local abattoirs and transported to the laboratory in warm saline. COCs were aspirated from antral follicles (3 to 8 mm diameter) using an 18-gauge needle and a 10 ml syringe containing 2 ml aspiration medium (phosphat buffer saline, 3% bovine serum albumin, and gentamycin). The basic medium was tissue culture medium-199 (TCM-199) supplemented by FSH and LH 50 mIU/ml. Collected Cocs were then divided into four groups. First group cultured in basic medium served as control group. Second, third, and fourth group were cultured in basic medium added with 3% peritoneal fluid of non-endometriosis (NE group), mild endometriosis (ME group), and severe endometriosis (SE group), accordingly. SE and ME group were then eventually classified as endometriosis group (E group). Complexes were incubated at 38°C with 5% CO2 in humidified air for 20 hours. Eventually, COCs were fixated in glass object and stained by immunocytochemistry. GDF-9 expressions were evaluated semiquantitatively by scoring of 0-1-2-3 under a microscope (Olympus) at 400xbased on modified-Yamashita methods.

RESULTS

In this study, the number of COCs in each group before immuocytochemistry staining were 24 (total: 96). When being processed, some COCs were loss and the remaining number were listed as in table 1.

The highest expressions of GDF-9 were seen in the control group. These expression were decreased in the non-endometriosis (NE) group and more declining in mild-endometriosis (ME) group. The lowest expression of GDF-9 were found in severe endometrriosis (SE) group, in which there were no expression at all. Statistical analysis using Kruskal-Wallis test showed that there was a significant differences among these four groups ($p<0.0001$).

Table 1. GDF-9 expression of COCs cultured in four groups

Group	N	Median	Minimum	Maximum
Control	13	3.00	3.00	3.00
NE	11	2.00	2.00	3.00
ME	14	0.50	0.00	1.00
SE	13	0.00	0.00	0.00
Total	50	1.00	0.00	3.00

Table 2. GDF-9 expressions of COCs in control, non endometriosis (NE), and Endometriosis (E) group

Group	N	Median	Minimum	Maksimum
Control	12	3.00	3.00	3.00
NE	11	2.00	2.00	3.00
E	27	0.00	0.00	1.00
Total	50	1.34	0.00	3.00

If the mild and severe endometriosis (ME and SE) groups were put together as an Endometriosis (E) group, the expression of GDF-9 were lower compared with control and non-endometriosis group. There was also statistically significant differences among these three groups when tested with Kruskal-Wallis ($p<0.0001$).

Table 3 revealed that expression of GDF-9 in endometriosis (E) group were lowered than in control group and this result were statistically significant by Mann-Whitney test ($P<0.0001$).

If expression of GDF-9 were compared between mild and severe endometriosis (ME and SE) group, it was clearly seen that there was a significant lower expression in severe endometriosis group than that of mild endometriosis ($p=0.004$).

Table 3. Comparison of GDF-9 expression between control and endometriosis (E) group

GDF-9 Expression	Control (n=12)	E (n=27)	p
			<0.0001
Median	3	0	
Minimum	3	0	
Maximum	3	1	

Table 4. Comparison of GDF-9 expression between non-endometriosis (NE) and endometriosis (E) group

GDF-9 Expression	NE (n=11)	E (n=27)	p
			<0.001
Median	2	0	
Minimum	2	0	
Maximum	3	1	

Expression of GDF-9 in endometriosis (E) group were also lowered compared with non-endometriosis group and were stastistically significant ($p<0.001$).

Table 5. Comparison of GDF-9 expression between mild endometriosis (ME) and severe endometriosis (SE) group

GDF-9 Expression	ME (n=14)	SE (n=13)	p
			0.004
Median	0.5	0	
Minimum	0	0	
Maximum	1	0	

DISCUSSION

Peritoneal fluid (PF) has been a focus of research on endometriosis because of its proximity to endometriotic lesions and is the milieu in which the immune mediators associated with the local inflammation of endometriosis can be studied. PF is also the microenvironment in which early reproductive events such as ovulation, ovum retrieval, and sperm-oocyte interaction take place.[2] The pelvic structures including the ovaries are continuously bathed in PF so this fluid may affect extracellular milieu in women with endometriosis which may exert an impact to endometriosis associated-infertility.

Peritoneal fluid of women with endometriosis have confirmed an increased number, concentration, and activation of macrophages which may induce proliferation of cells involved in inflammation through secretion of factors such as IL-1, IL-6, IL-8 and TNF-α. Rich-pro inflammatory factors present in peritoneal fluid of patients with endometriosis may influence cell-cycle in the granulosa cells and in turn have pathogenic effects on folliculogenesis. . Previous study has reported that women with endometriosis have higher granulosa cell apoptosis rate and a lower percentage of G2/M phase granulosa cells compared with other group of infertile women. Higher incidence of apoptotic bodies correlates with a lower quality of oocytes in individual follicles.[11] Cytokines produced in peritoneal fluid of infertile women with endometriosis were suggested to be responsible for these alterations.[9]

Growth differentiation factor 9 (GDF-9) is synthesized in oocytes at all stages of the folliculogenesis process and is obligatory for female fertility. GDF-9 plays mutifunctional roles in oocyte-granulosa cell communication and regulation of follicular differentiation and function.[16] For at least three decades, it has been known that oocyte dependent on granulosa cells and cumulus cells (CC) to provide nutrients and regulatory signals through gap junctions. More recently, a growing body of evidence has shown that GDF-9 as paracrine factors secreted from the oocyte potently regulate follicular somatic cell functions, including proliferation, steroidogenesis, differentiation, apoptosis, and expansion. Expansion of CCs are essential for ovulation, sperm capacitation, and fertilization.[17,18]

Since the oocyte is dependent on CCs for the supply of many regulatory molecules such as CAMP and energy substrates, higher incidence of CCs apoptosis in endometriosis may cause disturbance in oocyte growth and maturation and lead toa decrease in factors secreted by oocyte, GDF-9. This study postulated that cytokine rich peritoneal fluid induce high level of granulosa cells apoptosis that in turn affect oocyte-granulosa cell communication and eventually decrease the secretion of GDF-9.

This study has successfully demonstrated that there wasa significant decrease in GDF-9 expression of bovine COCs cultured in peritoneal fluid of infertile women with endometriosis compared with control and non-endometriosis group and this decline was in proportion with advancing stages of the revised AFS classification (r-AFS) and determine the degree of

disturbance in folliculogenesis in the ovaries of endometriotic patients.

As we know that in severe endometriosis, the number and quality of endometrial cells reflux were greater than in mild-minimal one, hence triggering greater inflammatory response that can result in more apoptosis in granulosa cellsthus a greter deccrease in oocyte secretion of GDF-9. It can be inferred that peritoneal fluid indeed exert a great influence in pathogenesis and pathophysiology of endometriosis, especially infertiliity associated endometriosis. Several theories has been proposed to find the relevance of the peritoneal fluid in endometriosis-associated infertility and this study confirmed that peritoneal fluid, probably by inducing granulosa cell aopotosis,affect oocyte-granulosa cell communication that lead to decrease of GDF-9 production by oocytes.

We also found that GFD-9 expression in non-endometriosis group were lower than that of control group. Unexplained infertility may be caused by increased generation of reactive oxygen species (ROS) in peritoneal cavity. Previous studies revealed that higher concentration of ROS were found in peritoneal fluid in women undergoing laparoscopy for infertility evaluation than in laparoscopic tubal ligation. We proposed that reduce concentrations of antioxydants and increased ROS-lipid peroxidation damage in women with unexplained infertility may impact granulosa cell apoptosis, resulting in decline of GDF-9 secretion.

CONCLUSION

This study found that GDF-9 expressions of bovine COC in peritoneal fluid culture of infertile patients with endometriosis were lowered compared with that of control and non-endometriosis group and this decrease was in proportion with severity of endometriosis. Further research is needed to examine TNF-α expression and granulosa cell apoptotic rate to determine the exact mechanism of decrease in GDF-9 expression. Also advanced research is suggested to measure other variable produced by granulosa cell, kit ligand, to study disturbance of oocyte-granulosa cell communication in infertile patients with endometriosis.

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