

In the name of God

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**Secretion of Prolactin Following Three Dimensional Culture of
Human Endometrial Tissue.**

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Abstract:

Introduction: Endometriosis is the presence of endometrial glandular and stromal cells outside of the uterine cavity. This disease is found in about 10% of women of the reproductive age and in up to 50% of women with infertility. Surgery continues as the first-line treatment to eradicate endometriotic lesions but recurrence of the condition occurs in up to 47% of women.

Materials and Methods: The aim of the present study is to determine prolactin levels in human culture of endometrial fragment. Endometrial biopsy in Premenopausal patient's women referred to Toronto Center for Advanced Reproductive Technology (T.C.A.R.T) for infertility treatment such as uterine myomas or ovarian cyst. Endometrial samples were collected from a total of ten normal ovulating women on cycle days 19-24. The biopsies were obtained from the fundal region of the uterine cavity. Ten tissue fragments were cultured by three dimensional methods for each patient. Supernatant fluid sample was collected from endometrial samples which were cultured in a three-dimensional fibrin matrix.

Results: Level of prolactin in Supernatant fluid of endometrial samples were placed in a three-dimensional fibrin matrix culture system were determined. These data showed Cell proliferation was observed in 91% of the wells. Angiogenesis was observed in 51 wells that showed cell proliferation (56%). The level of Prolactin in the supernatant fluid of wells that showed angiogenesis were increased ($P < 0.05$) compare to supernatant fluid of wells that didn't show angiogenesis.

Conclusion: Prolactin might play an important role in promoting neovascularization and cell proliferation in establishment endometriosis. Prolactin is involved in the regeneration of the endometrium and the growth of endometriosis.

Keywords: Endometriosis; In vitro; Three dimensional tissue culture; Angiogenesis; Prolactin.

Introduction:

Endometriosis is the presence of endometrial glandular and stromal cells outside of the uterine cavity. This disease is found in about 10% of women of the reproductive age and in up to 50% of women with infertility.⁽¹⁾

In these women, quality of life is often negatively affected by chronic pelvic pain, severe dysmenorrhea, and dyspareunia, no medical therapy has been demonstrated to be effective in eradicating the disease or in preventing it without unacceptable side effect^(2,3) Surgery continues as the first-line treatment to eradicate endometriotic lesions but recurrence of the condition occurs in up to 47% of women.⁽³⁾ In primate, endometrium angiogenesis has been demonstrated either during the early proliferative and in the secretory phases.⁽⁴⁾ Recently, the induction of an angiogenic phenotype in human endometriotic cells has been shown *pari passu* by the establishment of their ability to implant.⁽⁵⁾ Endometrial fragments, similarly to tumor metastases, follow two basic steps to generate endometriosis: implantation and acquisition of a new blood supply through angiogenesis.⁽⁶⁾ Retrograde menstruation is a very common physiologic event in cycling women with patent tubes.^(7,8) This latter evidence suggests that retrograde menstruation facilitates transport of endometrial tissue to the peritoneal cavity but that some other factor renders certain women susceptible to the implantation and growth of ectopic endometrium.⁽⁹⁾ Endometrial fragments can proliferate as ectopic lesions in a fibrin matrix, the same mechanisms un-

derlying the predisposition to form adhesions could provide important information to explain the susceptibility of some women to develop endometriosis.⁽⁶⁾ The endometrium is unique among adult tissues because it undergoes intense proliferation, secretion, regression, and regeneration during each menstrual cycle. At menstruation, endothelial cells sprout out from the ruptured spiral arterioles and venules and recruit other cells: pericytes for vessels to become capillaries and smooth muscle cells for the larger blood vessels.⁽⁵⁾ Angiogenesis, the development of new capillaries from pre-existing blood vessels, is a tightly controlled phenomenon and generally does not occur physiologically except in the female reproductive system⁽¹⁰⁻¹²⁾ Angiogenesis is a major step in the establishment of endometriosis, The mechanisms of angiogenesis and the related growth factors are presented and summarized to a model of angiogenesis in endometriosis.⁽¹³⁾ Angiogenesis, defined by the formation of new capillaries from pre-existing blood vessels, involves the interaction of number of tightly regulated molecules including vascular endothelial growth factor (VEGF), which is recognized as a pivotal angiogenic factor.⁽¹⁴⁾ The Vascular endothelial growth factors (VEGF), the angiopoietins 1 and 2 as well as different matrix metalloproteinase are expressed in the endometrium and endometriosis.⁽¹³⁾ VEGF, the most potent direct-acting angiogenic protein known^(15, 16), is a diffusible endothelial cell-specific mitogen and angiogenic factor that also increases vascular permeability, It elicits a pronounced angiogenic response in a variety of *in vivo* models.⁽¹⁷⁻

²¹⁾ Endothelial cell survival in newly formed vessels is VEGF-dependent.⁽²²⁾ Prolactin-mediated increase VEGF production and prolactin also possess both angiogenic and antiangiogenic effects.⁽²³⁾ The pituitary hormone PRL (prolactin) is a multifunctional polypeptide which exerts a role on cell proliferation and may also contribute to cell differentiation. PRL is also produced by immune cells and is regarded as a key component of the neuroendocrine-immune loop and as a local regulator of macrophage response.⁽²³⁾ Prolactin, a polypeptide hormone secreted by the acidophilic cells of the anterior pituitary gland, is implicated in diverse arrays of physiological functions such as moregulation, reproduction, growth, and development.⁽²³⁾ PRL synthesis has been demonstrated in extrapituitary tissue, including endothelial, neuronal, and immune cells such as lymphocytes, mononuclear cells, and thymocytes.⁽²³⁾ The intact human PRL molecule, with a molecular mass of 23 kDa, was found to be angiogenic and increases cell proliferation in bovine brain capillary endothelial cells whereas its respective 16 kDa Nterminal fragment is antiangiogenic.⁽²⁴⁾ In previous studies⁽⁶⁾ had showed that in vitro culture of human endometrial tissue in a three-dimensional (3-D) fibrin matrix can proliferate and sprout new vessels and showed stromal cells(Adult stem cells) are capable of proliferation and the generation of new blood. The aim of the present study was to determine whether endothelial cells in a specific three-dimensional (3-D) in vitro fibrin matrix model can release PRL hormone for development of angiogenesis to promotion of endometriosis.

Materials and Methods:

Patients

Endometrial biopsy in Premenopausal patient's women referred to T.C.A.R.T for infertility treatment such as uterine myomas or ovarian cyst. Exclusion criteria included any endometrial abnormality (polyps, hyperplasia, or cancer) and administration of any hormones, GnRH agonist therapy, or intrauterine device (IUD) within last 3 months. The Research Ethics Committee of T.C.A.R.T authorized the use of fragments of human endometrium as described. A written informed consent describing the procedures and aims of the study was obtained from each donor in compliance with regulations concerning the use of human tissues. Endometrial samples were collected from a total of ten normal ovulating women on cycle days19-24 (for each patient 10 samples). The biopsies were obtained from the fundal region of the uterine cavity using an endometrial sampling device (Endocell; Wallach Surgical Devices Inc., Orange, CT). In all patients, accurate menstrual dating was carried out according to the last menstrual period in the early proliferative phase of the cycle and appropriate histological dating of each biopsy confirmed the endometrium as proliferative.

Materials

Cell culture Medium 199 was purchased from GIBCO (Burlington, ON, Canada); cell culture supplements and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Plastics for cell culture were supplied by Falcon (Becton

Dickinson Labware, Franklin Lakes, NJ). Prolactin (DPC, Los Angeles, CA) levels were measured using the Immulite automated immunoassay analyzer (DPC, Los Angeles, CA).

Detection of PRL by ELISA

The PRL level was determined using a PRL immunoassay available from DPC. The frozen supernatant obtained from tissue culture was thawed at room temperature, pipetted on a sample cup designated with a number. An Immulite automated immunoassay analyzer was then used to determine the level of PRL in each sample. The Immulite system utilizes assay specific antibody, or antigen-coated plastic beads as the solid phase, alkaline phosphatase-labelled reagent, and a chemiluminescent substrate. The Immulite system automates the entire assay process. After incubating the beads with the supernatant and the alkaline phosphatase reagent, the reaction mixture was separated from the bead by spinning the test unit at high speed on its vertical axis. The entire fluid content was transferred to a coaxial waste chamber in the test unit. The bead was left with no residual, unbound label. The bound label was then quantitated with a dioxetane substrate that produces light. Light emission was measured by a photomultiplier tube (PMT) and the results were calculated in each sample.

In vitro cultures of human endometrium

Each endometrial biopsy was placed in cold sterile phosphate-buffered saline (PBS) solution containing 2.5 mg/mL amphotericin B and 50 mg/mL gentamycin. It was immediately cut into approxi-

mately 1- mm fragments using fine dissecting forceps and a scalpel. The explants were cleared of residual clots and placed in PBS before their use. Cultures were performed in 24-well culture plates. Each well contained 0.5 mL medium 199 supplemented with 3 mg/mL fibrinogen and mixed with 15 IU thrombin (50 NIH U/mL in 0.15 M NaCl). Each endometrial fragment was quickly placed in the centre of the well after clot formation and covered by an additional 0.5 mL/well of the fibrinogen/thrombin solution, to hold it at the same level between the two clots. After gel formation, 1 mL/well of medium 199 supplemented with 5% heat-inactivated fetal bovine serum, 0.1% ϵ -aminocaproic acid, L-glutamine (2 mM) and antibiotics (streptomycin 50 mg/mL, penicillin 50 IU/mL and amphotericin B 2.5 mg/mL) was added. Explants were cultured at 37°C in a humidified environment of 5% CO₂ in air for 6 weeks. Culture medium was changed every 3 days. Ten tissue fragments were cultured for each patient. Endometrial explants were observed daily and photographed every 3 days using a phase-contrast microscope (Leica DM IRE2, Bensheim, Germany).

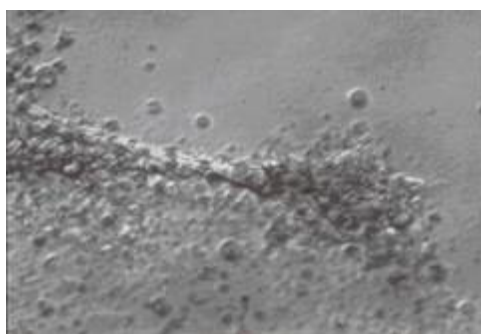
Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed by chi-square using SigmaStat Version 1.0 (SigmaStat Software HighEdit Professional Copyright_ 1993, MicroHelp Inc. and HeilerSoftware GmbH, San Rafael, CA, USA). Comparisons between prolactin level values were made by Student's t-test. A P-value of <0.05 was considered significant.

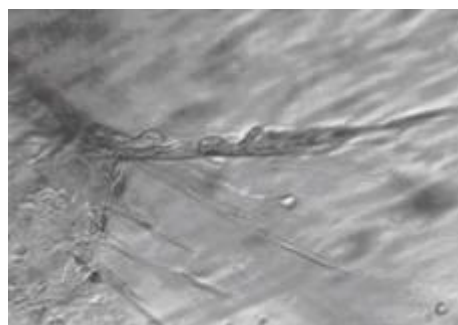
Results:

During the first week of culture, invasion of stromal cells into the fibrin matrix occurred (Fig. 1a). After 2 and 3 weeks, rudimentary capillary-like structures consistent with angiogenesis were observed (Fig. 1b).

Cell proliferation was observed in 91% of the wells and angiogenesis was observed in %51 wells (Fig.2). The level of PRL in the supernatant fluid of wells with angiogenesis were increased ($P < 0.05$) compare to supernatant fluid of wells without angiogenesis (19 ± 0.2 vs. 9 ± 0.1) (Fig.3).



(a)



(b)

Figure 1: Microscopic phase contrast picture of stromal cell invasion into the fibrin matrix during the first week of culture (a), and rudimentary capillary-like structures consistent with angiogenesis after 2-3 weeks in culture (b).

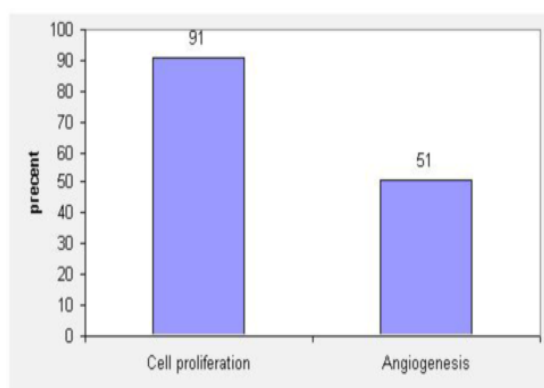


Figure 2: Cell proliferation and angiogenesis in three-dimensional cultured endometrial fragments. Bars indicate the percentage

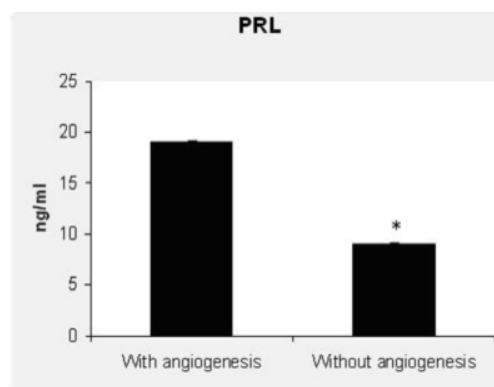


Figure 3: PRL level in supernatant of wells in with angiogenesis and without angiogenesis. Data are expressed as means \pm SE.* Difference from prolactin value in angiogenesis group ($P < 0.05$)

Discussion:

In this report, we describe the expression of PRL in human endometrial tissue in 3D culture and considering proliferation, invasion and angiogenesis are likely important mechanisms in the pathogenesis of endometriosis. This study provides further validation of the 3D culture model of endometriosis. In the fact that ectopic endometrial tissue requires development and activation of a vascular network (angiogenesis) for early proliferation in the pelvis of women is currently well known⁽²⁵⁾, Angiogenesis is dependent on soluble factors released from cells.⁽²⁶⁾ Several peptide growth factors, including FGF-a, FGF-b, PD-ECGF and VEGF, stimulate vascular endothelial cell growth in vitro and angiogenesis in vivo. VEGF was considered essential in uterine angiogenesis.⁽²⁷⁾

PRL-mediated increase VEGF production is dependent on the levels of HO-1 gene expression as evidenced by modulating HO-1 levels by known inducer or inhibitor. Experimental results demonstrated that pre-treatment of macrophages with SnCl₂, an inducer of HO-1, caused a significant increase in PRL-mediated VEGF release.⁽²³⁾

A hormonal alteration of PRL secretion could be the origin of infertility in patients with endometriosis.⁽²⁸⁾ Both endometriosis and abnormal prolactin secretion are important infertility causing factors. While endometriosis has a strong association with infertility, the exact reason for impaired fertility in endometriotic women is still unknown. Several factors,

such as altered prostaglandin secretion, cytokine secretion, luteal phase defect, autoimmune phenomena, and disorder of PRL secretion have been proposed as causes of infertility in patients with endometriosis.⁽²⁹⁻³³⁾ Although there are several clinical and experimental reports suggesting a relationship between endometriosis and abnormal PRL secretion.⁽³⁴⁻³⁷⁾

Reported that the PRL response to TRH was significantly greater in endometriotic patients than in normal women and that the PRL response to TRH before treatment was significantly higher in patients who after danazol treatment showed persistent endometriosis at the second laparoscopy, suggesting a possible prediction of therapeutic results in endometriosis by TRH testing.⁽³⁰⁾ Suggestion that the PRL response to TRH was related with the severity of endometriosis.⁽³⁸⁾ On the other hand hyperprolactinemia may be responsible for the infertility associated with endometriosis, since they observed that some infertile women with endometriosis.⁽³⁹⁾

In the present study we could establish endometriosis in early stage according to facioni method⁽²⁷⁾ and the prolactin level in the supernatant fluid of wells were measured .the PRL in the supernatant fluid of wells with angiogenesis were significant variations with the supernatant of wells without angiogenesis. Suggesting that the endometrium with angiogenesis is more likely to secretion of prolactin implant to establish of endometriosis. It is important to identification in endometrium vessels and it is provided the opportunity to study angiogenesis as a

model for endometriosis. finally, since angiogenesis is very important factor to establishment of endometriosis and the basic factor for angiogenesis is secretion of VEGF, and VEGF will be increase by secretion of prolactin, PRL might play an important role in promoting angiogenesis in establishment of endometriosis. May be if we can control level of prolactin we can control of endometriosis.

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