ISSN 2413-0516

Estrogen-degrading bacteria in women with premature ovarian failure

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(*Submitted: 29 May 2016 – Revised version received: 7 June 2016 – Accepted: 10 June 2016 – Published online: 26 September 2016*)

Objectives Estimation of the level of follicle stimulation hormone (FSH), luteinizing hormone (LH), and estrogen hormone, isolation of bacteria from premature ovarian failure (POF) women, detection of the ability of these bacteria to degrade the estrogen hormone using HPLC in aerobic and anaerobic condition.

Methods In this study, 60 women who suffered from POF; and have menopause at least 8 months after the last period, whose ages range between 20 and 39 years, venous blood samples and high vaginal swabs were collected from these patients who are admitted to the Children and Maternity Hospital and Al-Hilla Teaching Hospital in Hilla city/Iraq from the period of February to July 2015. Also, 40 women with no history of menopause with the age range approximately matched to that of patients as control group. These women were subjected to investigate FSH, LH, and estrogen hormone. Different types of bacteria isolated from the patients vagina were examined for their ability to lyse estrogen *in vitro* by using HPLC technique.

Results FSH and LH hormones showed a significant increase ($P < 0.05$) in the concentration of these hormones in blood of patients when compared to healthy control women. While there is a significant decreasing in the concentration of EST hormone in these women as compared to healthy control. Out of 60 women patients with POF only 42 patients showed positive bacterial growth, while 18 patients show no growth. The bacterial isolates show a ability to degrade estradiol in aerobic and anaerobic condition. And the results demonstrating the ability of vaginal bacteria to exhaust the estrogen efficiently and that may affect the systemic estrogen levels in these women.

Conclusion FSH and LH used as diagnostic tools for detection of premature ovarian failure. Different bacterial types isolated from patients vagina show an efficient degradation of estrogen.

Keywords POF, FSH, LH, EST. EST- degrading bacteria

Introduction

Premature ovarian failure (POF) is an early ovarian malfunction different from normal menopause, which disturbs the production of follicles, resulting in amenorrhea under the age of 40 in 1–3% of reproductive age women.[1] Affected women show menstrual problems followed by an elevated level of gonadotropins, such as follicle stimulating hormone (FSH) ≥ 40 IU/L and hypoestrogenism for an average four months, measuring serum FSH is a routine diagnosis procedure for the disease.[2,3]

Intestinal microbial richness and functions, influence levels of estrogens via enterohepatic circulation; thus, the gut microbial community likely affects the risk for estrogen-related conditions.[4] Aerobic and anaerobic estrogen-degrading microorganisms are phylogenetically diverse; they are mainly isolated from different environment; estrogens can be degraded via growth-linked and non-growth-linked reactions, as well as through abiotic degradation in the presence of selective microorganisms.[5]

Sex steroid hormones play an important physiological role in reproductive and non-reproductive tissues including the immune cells, these hormones exhibit their functions by binding to their specific intracellular receptors, that act as either ligand-dependent transcription factors, or membrane receptors that stimulate several signal transduction pathways.[6]

Development, reproduction, cell proliferation, inflammation, metabolism, differentiation, apoptosis, homeostasis, and brain function a number of physiological roles that these hormones played.[7]

Steroid hormones consisting of estrogens, progestagens, mineralocorticoids, and androgens glucocorticoids; these are powerful signal molecules that regulate a host of organismal functions; among them, estrogens are responsible for the development of female secondary sex characteristics.[8] Interestingly, these steroid hormones also participate in the communication between microorganisms and their mammalian hosts; this type of communication is commonly called "interkingdom signaling", and can be used by microbial pathogens to activate their virulence factors and, control the course and outcome of infection.[9]

Natural estrogens are a group of steroid components named for their importance in the estrous cycle of humans and animals. Three major naturally occurring estrogens are called estrone (E1), 17- estradiol (E2) and estriol (E3), which are synthesized from pregnenolone, these are excreted from the humans and cattle in the urine.[10] And some of these natural estrogens are discharged into environments without processing, it is estimated that, estrogen discharge is increasing in the urban areas.[11] Estrogens are often used as contraceptives, especially synthetic estrogens. Thus, large amounts of estrogens are constantly excreted into the environment from humans; these hormones are chemically very stable in the environment and that is because of the aromatic ring in its structure.[8]

Also[12] found that, the major one of estrogens for environmental contamination is E2, which is considered about 50 times more potent than E1 and 6 times than E3, and they show that, estrogen excreted from women at a reproductive age (15–59 years old) differs from 5–31 μg per day for E1 and 3–19 μg per day for E2; while during these periods, a woman estrogen excretion is similar to that of a man, with levels about 4–12 μg per day for E1 and 1.5–7 μg per day for E2. Some organisms have the pathways to make use of estrogen compounds as growth substrates and the bacteria, which can degrade estrogens were mainly isolated from different origins.[8]

Under aerobic conditions, steroids can be degraded by different bacterial species of different genera; although bacteria are generally able of growing on estradiol or estrone as a sole source of carbon and energy, only a few degradation

mechanisms have been suggested.[11,13] The oxygen-dependent pathway with its manner of cleavage of the steroid nucleus looks to be a general degradation pathway of steroids and cholesterol in aerobic bacteria.[14]

Another important feature is that, the genes for steroid degradation in some bacterial species are not constitutively expressed, but are induced by their respective steroid substances; moreover, studies of the mechanisms regulating the steroid-inducible gene expression exposed that regulator proteins, binding proteins or intermediate compounds produced in the course of steroid degradation are probable involved in microbial steroid metabolism.[15]

Anoxic environments improve where organic matter is degraded through microbial activity, and oxygen has only limited access, for example, during the denitrification step in wastewater treatment or in sediments of rivers and lakes; steroids that arise in such environments have in common with aliphatic hydrocarbons and monoaromatic compounds a chemical inertness that makes them recalcitrant substrates for bacteria.[14]

Materials and Methods

Clinical samples: This case control study involved women diagnosed with premature ovarian failure, they were referred to Babylon Province and AL-Hilla teaching hospital from the period February to July 2015, all subjects underwent a standard diagnostic work-up to rule out any verifiable cause of POF prior to inclusion into the study.

A. **Patients:** Sixty patients participating in this case control study were related to women who suffered from POF; these women have menopause at least 8 months after the last period, whose ages range between (20–39) years, venous blood samples and high vaginal swabs were collected from these patients under supervision of specialist gynecologist.
B. **Control**: Venous blood samples and high vaginal swabs from 40 women as a control that had a normal menstrual cycle, with no history of menopause with age range approximately matched to that of patients.
C. **Ethical Approval:** The study was done and the samples were collected after getting the agreement of the patients and also gynecologist.

Collection of vaginal swabs: High vaginal swabs were taken from case control women by using cotton tipped swab. Those specimens were collected under the help of advisory to avoid any possible contamination. Swab for culturing should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory or placed in transport media and transformed to the laboratory for culturing. These samples were inoculated on MacConkey agar, nutrient agar, blood agar and chocolate agar plates and incubated aerobically and anaerobically at 37°C for 24–48 hr.

Estimation of FSH, LH and estrogen hormone: All cases of patients and control were subjected to estimate the FSH, LH and estrogen hormone serum level. This assay was achieved according to the method described by the manufacturing company Vidas/Germany.

Microscopic examination and colonial morphology: According to the diagnostic procedures recommended MacFaddin,[16] a single colony was taken from each primary positive culture and its identification depended on the morphological properties (Colony size, shape, color, nature of pigments, translucency, edge, elevation and texture). Bacterial smear stained with Gram stain was used to check the morphological properties of bacterial cells.

Minimal salt medium (MSM): 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.5 g of NaCl, 0.5 g of $(NH_4)_2SO_4$ and 0.15 g of $MgSO.7H_2O$ and trace element were added and they contained, 2 g of $NaHCO_3.10\ H_2O$, 0.3 g $MnSO_4.4H_2O$, 0.2 g $ZnSO_4.7H_2O$, 0.02 g $(NH_4)Mo_7O_2.4H_2O$, 0.1 g $CuSO_4.5H_2O$, 0.5 g $CoCl_2.6H_2O$, 0.05 $CaCl_2.2H_2O$ and 0.5 g $FeSO_4.7H_2O$ dissolved in 1000 ml of distilled water, and then sterilized in an autoclave at 121°C for 15 minutes. After cooling the mixture to 50°C, Estradiol was added to the mixture as the only carbon source in the media in concentration between 20–30 µg/ml. This media used to detection of estrogen-degrading activity.[11]

Estimation of estrogen degradation ability of bacterial isolates by using HPLC: 17 β-estradiol ($C_{18}H_{24}O_2$) was added to the medium as the only carbon source in the media in concentrations between 20–30 µg/ml. Each sample was inoculated directly into the medium. The samples were incubated aerobically and anaerobically at 37°C for 48 hours. And the concentration of estradiol was measured in the containing media using HPLC on a C18 column with gradient elution with Acetontrile at elution rate 1 ml per minute at absorption 210 nm.[17] This analytical technique grounded on the separation of molecules due to the differences in their composition and/or structure; these involve moving of the sample through the system over the stationary phase, the molecules in the sample will have dissimilar interactions and affinities with the stationary phase, that leading to separation of molecules.[18] The sample components that exhibit stronger interactions with the stationary phase will move more slowly through the column, than components with weaker interactions; so different compounds can be separated from each other when they move through the column.[19]

The schematic of an HPLC instrument typically involves a sampler, pumps and a detector; the sampler transports the sample mixture into the mobile phase stream which conveys it into the column; the pumps supply the desired flow and composition of the mobile phase through the column; while the detector creates a signal proportional to the amount of sample component emerging from the column, therefore allowing for quantitative analysis of the sample components.[20]

Statistical analysis: This study used statistical analysis that included the calculation of mean values and percentage. The statistical package for the Social sciences version 18 (SPSS Inc., Chicago, USA) was used for statistical analysis by calculation of *P*-value with 95% confidence interval (95% CI). T-test was used to compare means of (FSH, LH and estrogen hormone) serum level between two groups (women with POF and controls). A P-value ≤ 0.05 was considered as a significant at P-value = 0.001.

Results

POF diagnosed women: From February to July 2015, only 60 patients with POF were included in this study who admitted to the-Children and Maternity Hospital and AL-Hilla teaching hospital for surgery. These women have menopause at least 8 months after the last period, whose ages ranging from (20–39) years. Besides 40 healthy women, that had a normal menstrual cycle, with no history of menopause were also included

as a control group. According to the clinical findings of the gynecologist and according to the hormones levels (FSH, LH and E2) the results of POF patients were scored.

The results showed that, most of women with POF have a high levels of FSH and LH and low level of E2. And the levels of FSH and LH are highly a significant at (P value < 0.05), Figs 1 and 2, respectively. Moreover, the levels of E2 decreased significantly in women with POF as shown in Fig. 3.

High vaginal swabs were collected from the patients and control groups to detect the presence of bacterial infection in these women. Only 42 patients showed positive bacterial growth, while 18 patients show negative results for the bacterial growth Table 1. As compared with a control group who show no growth.

Among the bacteria which isolated from the patients vagina are *Klebsiella pneumonia* (10), *Enterococcus fecalis* (3), *Enterobacter coloaca* (2), *Acinetobacter baumannii* (2), *Escherichia coli* (8), *Proteus vulgaris* (4), *Streptococcus agalactia* (4), *Staphylococcus aureus* (3), *Pseudomonas aeroginosa* (2) *and streptococcus mutans* (4), as shown in Table 2.

And these isolates were examined to show their ability to degrade estrogen *in vitro* by using High Performance Liquid Chromatography (HPLC), where the peak of standard appears at 1.63 min. The results are scored according to the ability of these bacteria to consume estrogen as the sole source of carbon. The results showed that the consumption didn't depend on bacterial species. However, consumption of estradiol at any extent facilitate the growth of bacteria *in vitro* and also the results revealed that the consumption of estradiol increased anaerobically when compared to the aerobic condition as seen in Table 3, and the bacterial isolates show a different peaks and different areas of estradiol derivatives according to the HPLC results.

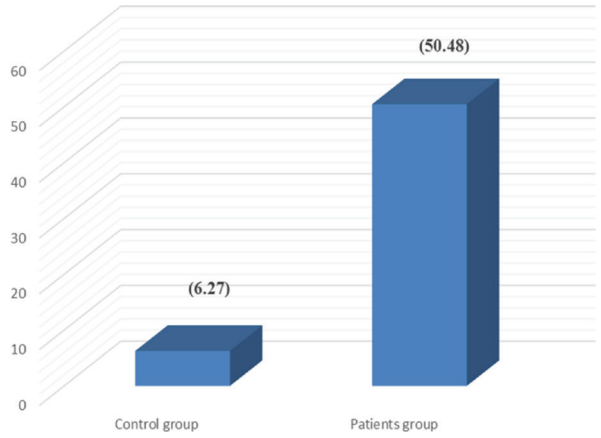

Fig. 1 **The mean and standard deviation (SD) of FSH IU/ml for patients and control groups. *Significant at *P*-value < (0.05). SD for patients ± 30.06; SD for control ± 2.81.**

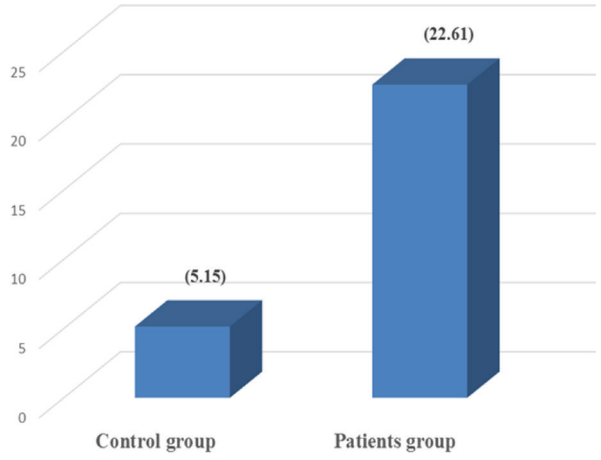

Fig. 2 **The mean and standard deviation (SD) of LH IU/ml for patients and control groups. *Significant at *P*-value < (0.05). SD for patients ± 15.09; SD for control ± 2.47.**

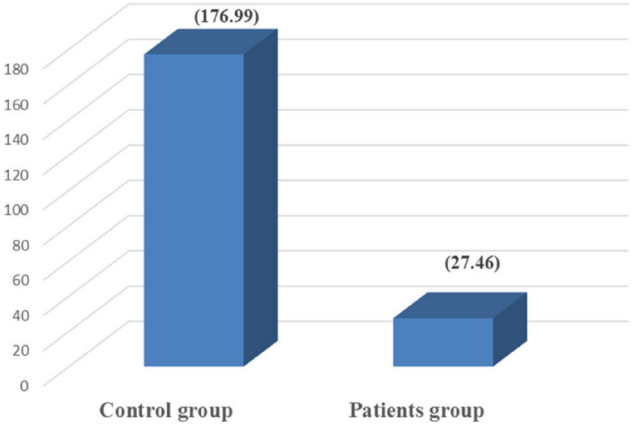

Fig. 3 **The mean and standard deviation (SD) of E2 pg/ml for patients and control groups. * Significant at *P*-value < (0.05). SD for patients ± 3.99; SD for control ± 86.90.**

Table 1. **Number of bacterial isolates in case and control women**

Groups	Positive growth	No growth
Patients (n = 60)	42	18
Control (n = 40)	No pathogenic bacteria	

Table 2. **The types and the number of bacteria isolated from women with POF**

Types of bacteria	Number of isolates
Enterococcus fecalis	3
Enterobacter coloaca	2
Klebsiella pneumoniae	10
Acinetobacter baumannii	2
Escherichia coli	8
Proteus vulgaris	4
Streptococcus agalactia	4
Staphylococcus aureus	3
Pseudomonas aeroginosa	2
Streptococcus mutans	4

Discussion

Hormone investigation (FSH, LH and E2) is necessary and FSH above 40 IU/L and estradiol under 50 pmol/L in women aged below 40 years approve the diagnosis.[21] Along with, both primary and secondary forms of ovarian failure, are biochemically described by low levels of gonadal hormones (like, estrogens) and extraordinary gonadotropins (LH and FSH) (hypergonadotropic amenorrhea); the elevation of FSH is

Table 3. **The ability of bacterial isolates to consume β-estradiol as the sole source of carbon by HPLC**

Bacterial types and No.	Mean of conc. of EST ± SD µg/ml in aerobic condition	Mean of conc. of EST ± SD µg/ml in anaerobic condition
K. pneumonia (10)	12.9978 ± 8.17	8.4022 ± 1.08
E. fecalis (2)	15.396 ± 0.035	9.2565 ± 0.39
E. coloaca (2)	12.659 ± 0.78	10.7205 ± 0.08
A. baumannii (2)	11.8457 ± 0.37	8.6575 ± 0.50
E. coli (8)	7.7126 ± 1.10	7.0985 ± 0.90
P. vulgaris (4)	8.3155 ± 0.38	7.9272 ± 0.22
S. agalactia (4)	7.2937 ± 0.33	6.8747 ± 0.56
S. aureus (2)	7.2545 ± 0.34	6.1325 ± 0.08
P. aerogenosa (2)	7.167 ± 0.06	6.570 ± 0.04
S. mutans (4)	10.004 ± 2.29	8.5497 ± 0.41

frequently more marked than that of LH and, an FSH value >30 IU/L is indicative of ovarian failure.[22]

FSH stimulates follicle growth in the ovary and is normally negatively regulated by inhibin and estrogen released from the maturing follicle; in ovarian failure, however, the nonexistence of a maturing follicle results in a lack of negative feedback and raised FSH levels; additionally, they also have low estrogen levels and elevated LH levels characteristic of post-menopausal women.[23] However, there are several reasons for ovarian failure as a result of endocrine dysfunction and these comprise: deficiencies in enzymes essential for steroid hormone synthesis as a result of either genetic abnormalities or autoimmune attack.[24] Mutations in steroid hormone or hormone receptor genes, and targeted damage of endocrine organs including the pituitary, hypothalamus or ovaries by disease processes autoimmune sources, or iatrogenic assault.[25] It is advocated that a decrease in E2 bioactivity could affect ovarian follicle reserve in a number of means; it could supply a less potent negative feedback on the pituitary, leading to increased FSH levels, ovarian stimulation and abnormal folliculogenesis.[26]

The ordinary ovarian activity requires accurate endocrine regulation and hormone signaling, and LH is needed to stimulate ovulation and therefore indirectly offers negative feedback on FSH production, a failure of response could therefore cause infertility and elevated FSH.[25] Several studies show that, there were a decline of FSH levels in POF patients having follicular growth or ovulatory cycles, however, these FSH levels continued higher than the normal FSH levels.[27] Similarly, among premenopausal patients with ovulatory cycles, variability in FSH levels exceeding the normal range has been described, and FSH levels are reliant on underlying follicular activity.[28]

The etiology of bacterial vaginitis is poorly understood and remains a question for discussion, bacterial vaginitis can arise and remit spontaneously or progress into a chronic or recurrent disease.[29] Bacterial vaginitis may sometimes affect women after menopause; the reduction in estrogen levels in perimenopausal and postmenopausal women has been related to an abnormal vaginal flora of 35% and 70%, respectively when compared to the normal flora.[30]

Vaginal *Lactobacilli* have a crucial role in maintaining an environment that restricts the growth of pathogenic microorganisms in the vagina.[31] It has been proposed that, estrogen and *Lactobacillus* are required to achieve an optimal vaginal pH of 4.0 to 4.5.[32] After puberty, under the effect of estrogen, glycogen is deposited in the vaginal epithelial cells, which is metabolized by vaginal epithelial cells to glucose, *Lactobacilli* produce lactic acid from glucose, keeping the vagina at an acidic pH.[33]

However the *Enterobacteriaceae* family is one of efficacious bacterial isolate was capable to grow in the presence of β-estradiol 17. And the intestinal contents can hydrolyze numerous estrogens and estrogen metabolites (EM), these reactions have been attributed to gut luminal bacteria, which greatly effect on systemic estrogen and EM levels.[4] Furthermore[34] found that, *Escherichia* spp. are highly proficient in degradation of estradiol.

Since most intestinal bacteria are the main source of bacterial vaginitis so, this will give evidence on the role of intestinal bacteria in degradation of estrogen. So[35] published that, *Pseudomonas* strains display estrogen-metabolizing activity; the ability of the isolated strains to metabolize a mixture of the different estrogens types was observed, and demonstrated that the transformations were involved the oxidation of βE2 into E1.

The fecal microbiome (number and species) was very directly and strongly associated with systemic estrogens level; these associations were robust to different classifications of microbiome diversity, and they held for estradiol, estrone and EM; and the diversity analysis advises that estrogen levels are not associated with any particular cluster or class of that microbiome.[36,37] Moreover, the bacteria can improve many mechanisms to exhaust or to eliminate sex hormones in their benefit, by using them as carbon and energy sources, in principal through their modification or chemical degradation.[6]

Degradation pathways of the natural estrogens in addition to E2 are still questionable; and all these pathways are only valid under aerobic circumstances and the breakup of C-C bonds and the oxidation of quaternary carbon atoms are dependent on oxygen; besides, it is not yet clear how the degradation pathway resembles if oxygen is absent although it was displayed that, steroids can be degraded under anaerobic conditions.[38] So while another bacteria were reported by[39] to be able to completely degrade 20, 000 µg of E2 within 18 hours, *Klebsiella* sp. exhibited a good estrogen degrading activity when it was inoculated in estradiol anaerobically.[17]

The multiplicity of the gut microbiota, could influence systemic estrogen levels through enzymatic and other pathways.[4,40] Besides[41] finding that, intestinal microbial richness and specific taxa may contribute to systemic estrogen levels and associated diseases.

Utmost of POF patients women do not have any symptoms; however, in untreated POF, typical symptoms of estrogen withdrawal may be present, they include irritability, nervousness, restlessness, hot flushes, insomnia, depression, loss of concentration, loss of libido, etc; physical examination may reveal painful bones, thinness of the skin, stiffness or weight gain.[42]

Young women with POF are at an enlarged risk of coronary heart disease, osteoporosis, cardiovascular accidents and depression, and exogenous estrogens have been shown to have advantageous effects on cardiovascular status and bone density, they also have increasing levels of cardioprotective high density lipoproteins and reduction total cholesterol and low density lipoprotein level.[43] Reproductive organs which are highly modified by estrogen, such as ovary, uterus, vagina and

cervix; these changes are consistent with increased estrogen levels in treated females.[44] Nevertheless, the capability and the time intervals in which the tested miscellaneous bacteria which degraded estradiol is more or less acceptable compared to other bacterial isolates previously testified as potential *β*-estradiol-degrading bacteria; such as *Rhodococcus zopfii*, which completely degrade 100 mg/L of β-estradiol plus ethinyl estradiol estrone, and estriol within 24 hours, and they found that, these substance had no estrogenic activity at all and, estrogens are decomposed entirely.[11]

The longer the duration of estrogen deficiency, the more severe are the consequences; hence starting treatment early affords longer-term benefits to the health of women; hormone replacement therapy (HRT) should be used in younger women with POF, except contraindicated, until the age of menopause and then reviewed.[45]

As well[46] found that, hormone replacement therapy use before the age of 60 years results in a 24% decrease in coronary heart disease and 30% reduction in total mortality. From the systemic adverse effects of estrogen insufficiency were minimum estrogenise the vaginal epithelium and loss of ovarian activity, that lead to reduce androgen production by 50%, which can have profound effects on general and sexual wellbeing.[45]

Loss of ovarian function at an early age disturbs bone architecture at the very time when bone buildup is at its maximum due to the estrogen deficiency.[47] Even though early loss of ovarian function has been allied as a risk factor for CV mortality, there are no enough data indicating that these patients are at an increased risk of CV adverse effects from hormonal therapy.[48] So estrogen replacement therapy is the mainstay for treatment of women with POF.[49] The first line therapy is a trial with estradiol replacement with close observing of ovulation; exogenous estrogens could act by sensitizing the granulosa cells to the effect of FSH leading to ovulation; estrogens may act similarly by down regulation of the LH and FSH receptors; they can be counseled until the age of 55 years.[21]

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