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UP-REGULATION OF INTERLEUKINS AND THEIR UNDERLYING PART TO DEVELOP ENDOMETRIOSIS IN WOMEN EXPERIENCING RETROGRADE MENSTRUATION

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ABSTRACT

Endometriosis is associated with dyspareunia, dysmenorrhea and often severe pelvic pain as well as considered a chronic inflammatory disease, defined by the presence of endometrial tissue outside the uterine cavity. An important general concept regarding endometriosis is that it is a local pelvic inflammatory process with alteration in immune cell function in peritoneal environment. Previous research has reported increased levels of various cytokines such as TNF-α, IL-1, IL-6, IL-8 in peritoneal fluid of females with endometriosis therefore implicating the role of these cytokines as a potential causative factors to influence the development and progression of endometriosis. Additionally increased expression of cyclooxygenase-2 and cyclooxygenase-2 triggered elevation in prostaglandin-2 level are responsible for the regulation of cell survival, migration and invasion of endometriotic tissue. Defining the etiology of these dysregulated signalling mechanisms at cellular level could be productive to understand the initiation and progression of endometriosis. The aim of present study was to evaluate the levels of inflammatory cytokines in women with endometriosis.

Keywords: Interlukin-1 (IL-1), Interlukin-6 (IL-6), Interlukin-8 (IL-8), Tumor necrosis factor- α (TNF-α), cyclooxygenase-2 (COX-2), prostaglandin-2 (PGE-2)

INTRODUCTION

Endometriosis is considered a chronic inflammatory disease, defined by the presence of endometrial tissue outside the uterine cavity. Among the most common gynaecological disorders, endometriosis affects approximately 5 to 10% of females of reproductive age **[1]**. Endometriosis is associated with dyspareunia, dysmenorrhea and often severe pelvic pain. Additionally, females suffering from endometriosis often experience infertility. Understanding of initiation and progression of endometriosis as well as to determine its molecular etiology is a significant challenge to improve the women's quality of life. Although several studies have pursued to define the potential factors associated with initiation and progression of endometriosis, unluckily the accurate pathogenesis of endometriosis is not clear yet **[2]**. There are numerous hypothesis regarding how the initiation and progression of endometriosis occurs. The most widely accepted explanation for endometriosis involves Sampson's retrograde menstruation theory. Wherein during menstruation, menstrual debris comprising viable endometrial tissue move into the pelvic cavity via fallopian tubes **[3]**. These refluxed endometrial cells then adhere to several tissues including peritoneum, ovary, uterus as well as intestine, invade them subsequently proliferate till they become endometriotic lesions. There is also substantial evidence that genetic predisposition, genital tract abnormalities, imbalance of hormones, inflammatory response, altered immune surveillance and any abnormality in the regulation of endometrial cells are involved in the pathogenesis of endometriosis **[4]**. Survival of endometriotic lesion in pelvic cavity requires the formation of new blood supply **[5]**. Previous studies reveals that sex steroid hormones are likely to play a crucial role in endometrium proliferation additionally the regulation of endometriosis proliferation also depends on some specific growth factors. In response to sex steroid hormones several cytokines and growth factors are produced in endometrium and play very vital role in the regulation of endometrial function **[6]**.

Even though a long history of experimental research and clinical experience, the pathogenesis of endometriosis remains unclear. Recent studies suggest that endometriosis is related with infertility but the mechanism by which endometriosis affects infertility is still controversial but numerous studies reveals that its pathophysiology is associated with an

aberrant immunologic mechanism. An important general concept regarding endometriosis is that it is a local pelvic inflammatory process with alteration in immune cell function in peritoneal environment. A number of studies supports this concept and suggest that there is an increase number of activated macrophages which further stimulate the secretion of various cytokines and growth factors in peritoneal fluid of women suffering from endometriosis **[7]**. In women suffering from endometriosis the peritoneal macrophages in addition to increase in number are highly activated through nuclear factor kappa B (NF-κβ) and induce the release of a number of proinflammatory cytokines as well as promote endometriosis formation via forming an inflammatory milieu **[8, 9]**. Previous research has reported increased levels of various cytokines such as TNF-α, IL-1, IL-6, IL-8 in peritoneal fluid of females with endometriosis therefore implicating the role of these cytokines as a potential causative factors to influence the development and progression of endometriosis **[7]**. Additionally macrophages also stimulate angiogenesis as peritoneal macrophages are well known source of vascular endothelial growth factor which trigger the formation of new blood vessels in endometriotic lesions **[10]**. Production of cytokines is further increased by peritoneal macrophages induced increased expression of NF-kβ and reactive oxygen species production, endometriotic stroma sells and MAPK signalling pathway activation **[11]**. Increasing evidence has stated that in addition to peritoneal factors some cytokines such as interleukin-1β (IL-1 β), IL-6, IL-8, tumor necrosis factor (TNF)-a, vascular endothelial growth factor (VEGF) were elevated in peritoneal fluid of females with endometriosis and have been considered to play critical role in pathophysiology of endometriosis **[12,13]**. Inflammation associated with endometriosis is proposed to trigger lesion growth and develop symptoms of endometriosis **[14]**. For decades the immune factors have been studied the pathogenesis of endometriosis because they both aberrant immune environment and inflammatory status are closely related to each other. There are two different features of immune dysfunction that have been seen in association with endometriosis such as it allows endometrial cells implantation on the peritoneum by impairing the immune rejection of endometrial cells refluxed into the peritoneal cavity as well as stimulate endometriosis development via promoting the abnormal secretion of different molecule

from immune cells **[15]**. In addition to immune cells endometriotic lesions themselves are responsible to cause cytokine secretion that promote the progression of endometriosis. Such as during endometriosis development, endometrial epithelial cells have elevated expression of tumor necrosis factor alpha (TNF-α) than normal endometriotic tissue that stimulate the activation of various signalling pathways including MAPK, p38, JNK, IkB, and PI3K in autocrine manner to trigger endometrial epithelial cell inflammation and invasion therefore esteeming their proliferation **[16]**. Additionally endometriotic epithelial TNF-α also stimulate endometrial stromal cell proliferation by paracrine response through inducing the release of IL-6 and IL-8 in endometrial stromal cell via NF-kβ and AP-1 (Activator protein-1). Hence dysregulation of cytokine signalling network either autocrine or paracrine in endometriotic lesion play a major role to progress endometriosis **[2]**. In addition to TNF-α induced release of IL-6 and IL-8, endometrial lesion can also produce IL-6 and IL-8 **[17]**. IL-6 and IL-8 can promote endometriotic lesion survival by inducing the establishment of T-cell milieus in endometrial lesion as IL-6 is responsible for CD4+T-cell differentiation as well as T lymphocytes infiltration depends upon IL-8 **[18]**. In endometriosis local inflammatory mediators such as TNF-α and IL-1β induce the activation of NF-kβ signalling mechanism which further leads to increased expression of cyclooxigenase-2 (COX-2) that is a rate limiting enzyme in prostaglandin biosynthesis **[19]**. Significantly increased level of COX-2 in peritoneal macrophages are highly associated with prostaglandin level and the severity of endometriosis as raised levels of COX-2 and PGE2 were observed in women with endometriosis **[20]**. Increased expression of COX2 and COX2 triggered elevation in PGE2 level are responsible for the regulation of cell survival, migration and invasion of endometriotic tissue **[21]**. In current study a panel of biomarkers including TNF-α, IL-1β, IL-6, IL-8, VEFG, COX-2 and TGF-β was selected which might be used for diagnosis of endometriosis with clinically adequate specificity and high sensitivity.

MATERIALS AND METHODS

Fifty women with endometriosis participated in current study as a subject and fifty non-endometriosis women served as control, 21-27 year of age with informed consent. Women with confirmed clinical reports of stage IV endometriosis who did not take any medication were screened at Jinnah Hospital Lahore, Pakistan.

Endometriosis women with the history of consuming alcohol, smoking and taking any medication additionally any control/healthy women on medication or with any other disease such as depression, cancer hypertension, diabetes and malnutrition were not included. Current study was carried out in accordance with recommendations of the regulations for experimental work in The University of Lahore, Lahore, Pakistan, "Research and Ethics Committee" at Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan. The protocols for experimental work were approved by the "Research and Ethics Committee" at Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan. 5ml of blood was collected from women with advanced stage endometriosis (n=50) and from controls (n=50), centrifuged at 4000 revolutions per minute. The supernatant was then collected and stored in aliquots at -70°C until analysed.

Biochemical analysis

Interleukins 1, 2 and 6 in sera were measured by enzyme-linked immunosorbent assay (ELISA) using the kits (Quantitave High Sensitivity Human IL-6 kit, R&D systems Inc. USA) following to the manufacturer protocol. Tumor necrosis factor alpha, VEGF and TGF-β were determined by commercially accessible ELISA kit by Abcam. Prostaglandin E2 was measured by the method of human Elisa kit method (Thermo Fisher Scientific). Human COX-2 Elisa kit (ELH-COX-2) was used to measure COX-2 (MyBioSource).

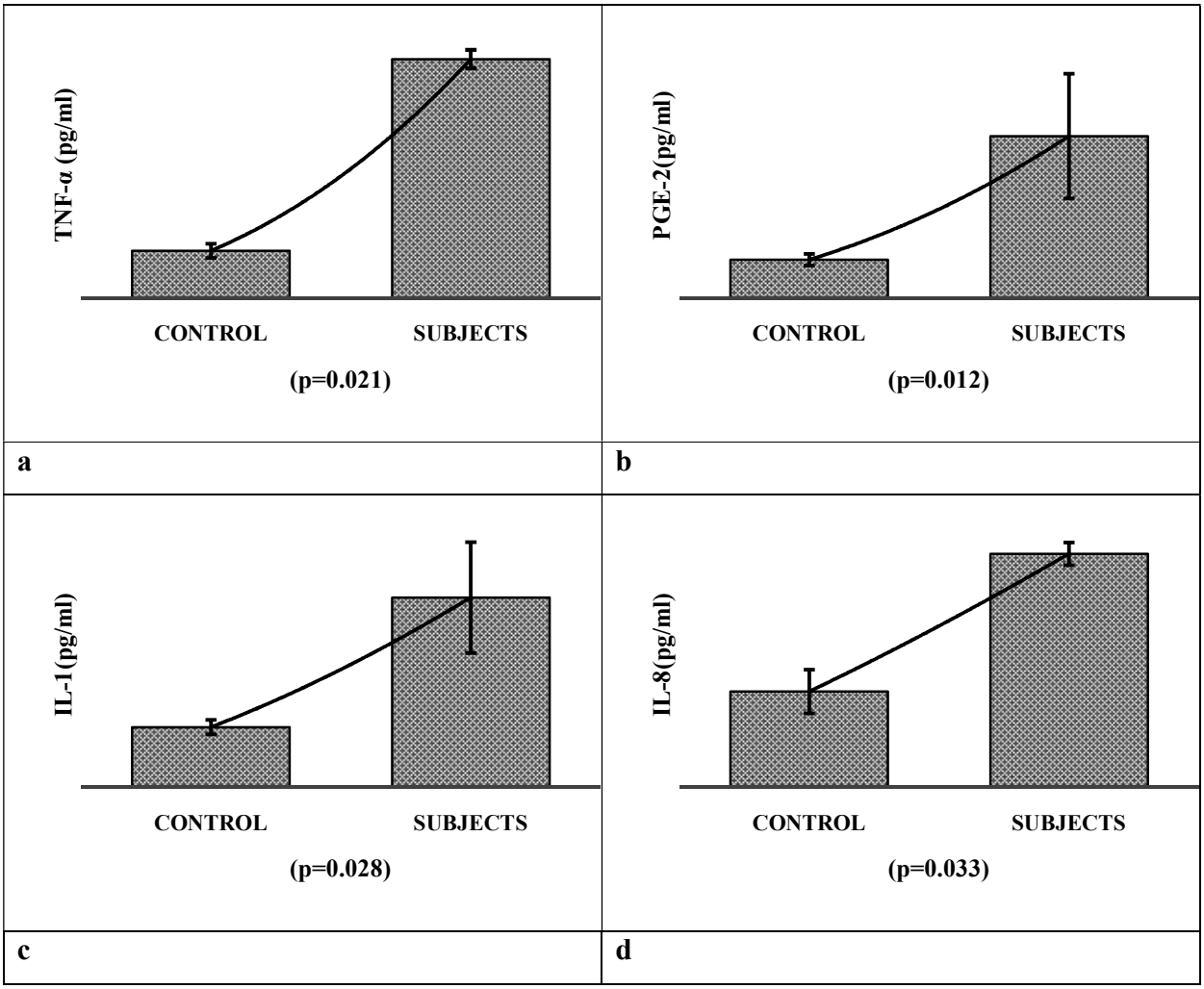
Statistical analysis

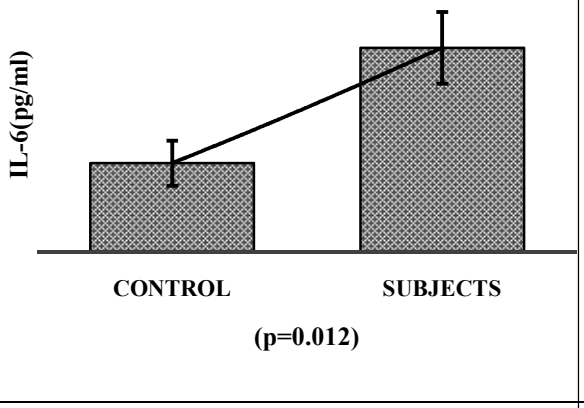
Data was analysed by independent sample t-test by using SPSS Statistics 17.0. The results were expressed as the means ± SD of repeated experiments. The Pearson's correlation coefficient (r) was used to evaluate the correlation between different variables of women with endometriosis. A P-value was estimated by using one way ANOVA and $p<0.05$ was considered to be significant statistically.

RESULTS

One hundred women contained sufficient material for analysis were included in current study. Data on the concentration of different variables are presented in **Table 1**. Eight inflammatory mediators analysed including Tumor Necrosis factor-alpha (TNF-α) (pg/ml), Interleukin-1 (IL-1) (pg/ml), Interleukin-8 (IL-8) (pg/ml), Interleukin- 6 (IL-6) (pg/ml), Prostaglindin-E2 (PGE-2), (pg/ml), COX-2 (ng/ml), TGF-β (ng/ml) and Vascular endothelial growth factor (VEGF) (pg/ml) were detectable in

100% of the samples. The results of proinflammatory cytokines such as Tumor Necrosis factor-alpha (TNF-α) (pg/ml), Interleukin-1 (IL-1) (pg/ml), Interleukin-8 (IL-8) (pg/ml), Interleukin- 6 (IL-6) (pg/ml) differed significantly (p=0.021, p=0.028, p= 0.033and p=0.012) between subject and controls respectively. The serum concentration of Prostaglindin-E2 (PGE-2) and COX-2 was significantly higher in women with endometriosis (4.89±1.89 pg/ml) and (1.56±0.14 ng/ml) than controls (1.15±0.18 pg/ml) and (0.61±0.05 ng/ml) respectively. Vascular endothelial growth factor levels in the serum was (15.29±2.19 pg/ml) in endometriosis women and (8.59±2.06pg/ml) in control. TGF-β concentration in endometriosis women 74.59±12.29 ng/ml which was significantly (p=0.005) increased than 51.59±6.92 ng/ml in women without endometriosis.





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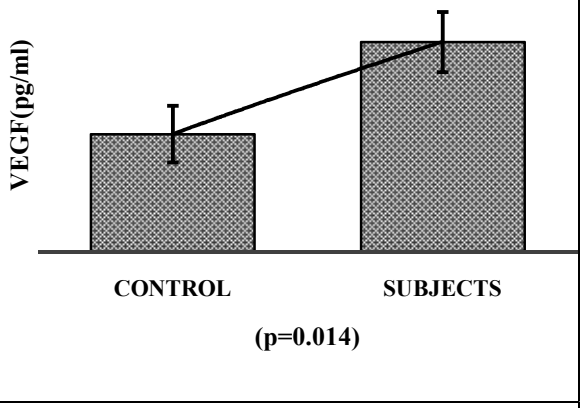

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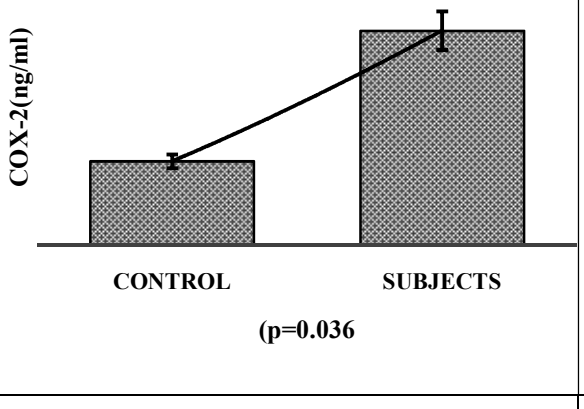

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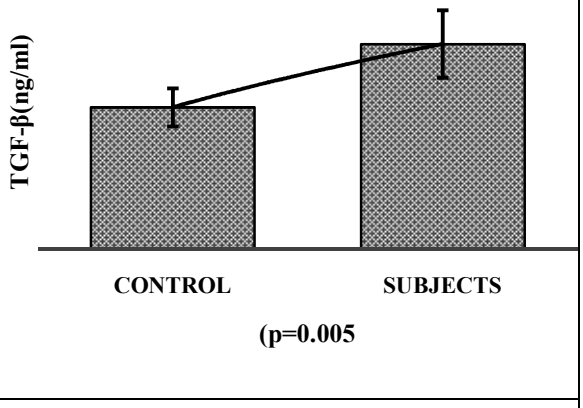

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DISCUSSION

The results of current study showed a significant difference in cytokine levels between women with endometriosis and control group (healthy women without endometriosis). Previous reports stated that IL-1β concentration was increased in women with endometriosis. IL-1β paly very critical role in the pathogenesis of endometriosis via stimulating growth, adhesion, formation of new vessels and invasion of endometrial fragments outside the uterus **[22]**. These observations are in line with our results as in current study increased level of IL-1β was analysed in patients of endometriosis as compared to normal females and suggested the role of IL-1β in the development and progression of endometriosis. In a study by Zhang *et al.,* the increase levels of IL-8 and VEGF were shown for women with endometriosis than healthy controls suggesting the role of IL-8 and VEGF in the

pathogenesis of endometriosis which may stimulate endometrial cell implantation, peritoneal epithelium neoangiogenesis and the development and progression of endometriosis **[23]**. It has been suggested that increased levels of IL-8 and VEGF for endometriosis women are associated with increased risk of endometriosis. Present study found a significant increase in the level of IL-8 and VEGF for women with endometriosis and implied that IL-8 and VEGF may play an important role in the pathogenesis of endometriosis. IL-6 is a multi-functional cytokine that promote the occurrence and development of endometriosis via cytokine network serving as a proinflammatory molecule as well as by altering immune cell function **[16]**. To facilitate endometrial survival activated macrophages induce the release of IL-6 which play major role to cause endometriosis **[24]**. Present study shows that the level of IL-6 in endometriosis females is significantly increased as compared to healthy controls. The result of current study reveals that peritoneal macrophages are the main source of IL-6 production and the main target cell for IL-6 that can influence the pathogenesis of endometriosis.

TNF-α is another proinflammatory cytokine which is secreted by activated peritoneal macrophages and promote the growth of new blood vessels. In addition to that it also stimulate endometrial stromal cell proliferation thus contribute as an angiogenic factor to cause angiogenesis of endometriosis **[25]**. In current study the level of TNF-α have been shown to be increased in women with endometriosis. This finding support the hypothesis of previous studies that increased level of TNF-α in women with endometriosis might be involved in the pathogenesis of endometriosis. Regulation of a variety of cell function such as proliferation, adhesion migration, invasion, differentiation, apoptosis, angiogenesis and immune function depends upon transforming growth factor beta (TGF-β). In previous studies increased expression of TGF-β was observed in women with endometriosis and implicated the increased expression of TGF-β with endometrial lesion development. Increased expression of TGF-β in endometriosis environment might be involved in the pathophysiology of endometriosis by inducing alteration in cell metabolism, enhancing cell invasion and initiating new blood vessel formation **[26]**. In current study the increased level of TGF-β was recorded in patients as compared to control one. COX-2 have been shown to play an important role in initiation and progression of endometriosis as

well as in previous studies elevation in the expression of COX-2 was described in endometrial tissue of women with endometriosis which contribute to elevated concentration of PGE2 which further contribute in the development of endometriosis. In endometrial cells proinflammatory cytokines induce the increased expression of COX-2 that will lead increased cell proliferation, migration, angiogenesis, endometriosis related pain and infertility as well as inhibit cell apoptosis which further promote the development of endometriosis **[27-33]**. Current study demonstrate the increased level of COX-2 in patients with endometriosis than normal one. Previous studies reveals that increased expression of COX-2 stimulate the release of proangiogenic factor such as VEGF that is required for the formation of new blood vessels in endometrial cell thus supporting the role of COX-2 in the pathogenesis of endometriosis. In current study we have discussed how cytokine dysregulation are associated with increased risk of endometriosis **[34-38]**. Cytokine network dysregulation may predispose the females to develop endometriosis while it leftovers to be determined what causes this cytokine dysregulation to cause endometriosis. Furthermore additional research is needed on functional correlation between cytokines and disease severity.

CONCLUSION

In conclusion, present study shows that several inflammatory cytokines produced by different cell types in peritoneal fluid might contribute in developing the pathogenesis of endometriosis. Inhibition of these cytokine function via using certain chemical agents can be helpful to treat endometriosis.

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CONFLICT OF INTEREST

Authors declare no conflict of interests.

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