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ENDOMETRIOSIS;

INSINUATIONS OF UPSTREAM PROPHETIC VARIABLES AND THEIR INTERPLAY TO DEVELOP ENDOMETRIOSIS IN YOUNG FEMALES

1. PhD
Professor
Institute of Molecular Biology and Biotechnology (IMBB),
The University of Lahore-Pakistan.
2. M. Phil, PhD
Associate Professor
Department of Physiology,
Rahbar Medical and Dental College,
Lahore-Pakistan.
3. M.Phil (Physiology)
Assistant Professor
Department of Physiology,
Shalamar Medical and Dental College, Lahore-Pakistan.
4. M.Phil (Biochemistry)
Research Associate
Institute of Molecular Biology and Biotechnology (IMBB),
The University of Lahore-Pakistan.
5. M.Phil (Biochemistry)
Research Associate
Institute of Molecular Biology and Biotechnology (IMBB),
The University of Lahore-Pakistan.
6. M.Phil (Biochemistry)
Research Associate
Institute of Molecular Biology and Biotechnology (IMBB),
The University of Lahore-Pakistan.

Correspondence Address:
Arif Malik
Institute of Molecular Biology and Biotechnology (IMBB),
The University of Lahore-Pakistan.
arifuaf@yahoo.com

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Arif Malik[1], Iram Qamar[2], Uzma Jamil[3], Gulshan Parveen[4], Sulayman Waquar[5], Hassan Shafique[6]

ABSTRACT…. Objectives: To evaluate the role of endometriosis leading to infertility in working females. **Data Source:** Data was collected and screened from Jinnah Hospital Lahore. **Design and Study:** Comparative cross sectional study was performed. **Setting:** Study was carried out in the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore. **Period:** Present study was carried out over a period of two years from 14-11-2014 to 15-11-2016. **Material and Methods:** Hundred (n=100) patients of endometriosis and hundred (n=100) controls were added in the current study. Subjects were analyzed for the levels of MDA, SOD, CAT, GSH, ILs, MMPs, Vit-D, PGE-2, Estradiol, COX-2 and various hormones with the help of their respective spectrophotometric and kit protocols. **Results:** Levels of MDA, IL-8, MMP-2, TNF-α and Estradiol were significantly higherin patients of endometriosis (2.99±0.16 nmol/ml, 19.65±3.26 pg/ml, 563.23±48.2 ng/ml, 31.29±4.25 pg/ml and 47.16±4.28 pg/ml) vs. healthy females (0.88±0.089 nmol/ml, 10.26±1.99pg/ml, 299.35±54.4 ng/ml, 21.26±3.26pg/ml and 22.28±4.16pg/ml), whereas levels of SOD, CAT and GSH remained decreased significantly in patients infected by endometriosis (652.23±42.6, 1226.06±108.4 and 6.28±0.42 respectively). Levels of testosterone and 17β-HSD-II were also significantly decreased females with endometriosis (15.23±1.99 ng/dL and 0.12±0.04 pg/ml respectively). **Conclusion:** The current study indicates role of oxidative stress and vitamin-D in females with endometriosis. Lower levels of vitamin-D and increased oxidative stress is responsible for release of induced factors i.e., PGE-2 and COX-2. These factors play their role in activation of signaling cascades involved in progression of endometriosis. Hence, vitamin-D and antioxidant supplementation may have a beneficial role in the disease management.

Key words: Melondialdehyde **(**MDA), Vitamin-D, Matrix Metalloproteinases (MMPs), Interleukins (ILs), Testosterone.

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INTRODUCTION

Endometriosis is benign, estrogen dependent progressive female reproductive disorder. Endometriosis is extremely variable relative to age, progression, manner of presentation, variety of symptoms, anatomical locations and probability of recurrence. Estrogen has significant role inneurological, skeletal and cardiovascular systems due to its unique physiological properties.[1] Endometriosis can be characteristically symptomized as re-occurrence of endometrial glands, endometrium and uterine musculature. Histologically in endometriosis, endometrial and stromal cells both are present outside the uterus.[2] Anatomical areas usually affected by endometriosis are surface of the ovaries and pelvic peritoneum that leads to pelvic inflammation, adhesions, chronic pain and infertility. Reactive oxygen species (ROS) level increases under the influence of lipid peroxidation, thus it plays pivotal role in DNA damage especially in peritoneal cells. Females with endometriosis show excess of iron in peritoneal cavity dueto retrograde menstruation. In retrograde menstruation there is increased hemolysis and RBC breakdown that is responsible for increased redox reactions.

Enhance oxidative stress and inflammatory cytokines in subjects lead to inflammation and progression of disease. In inflamed peritoneal cavity there is recruitment of immune cells, uptake

of cytokines and interleukins which collectively causes oxidation of enzymes in endometrial and stromal cells. In equilibrium in the reactive oxygen species (ROS) and antioxidants i.e., SOD, CAT, GSH etc. leads to proliferation of endometrial cells. Endometriosis being an autoimmune disorder, probably cause defect in immune system of subjects. Women suffering from endometriosis have lower levels of immune and natural killer cells.[3] Because of defective immune system the menstrual debris is not completely removed form peritoneal cavity and contributes in the progression of endometriosis. Immune cells secrete certain growth factors and cytokines that further promotes angiogenesis in endometric lesions. The locally produced cytokines and growth factors act as regulators of MMP's.[4] In endometriosis the role of MMP's are very significant, number of studies show higher concentrations of MMPs i.e., MMP-2 in serum and peritoneal fluid of patients. MMP-2 stimulates prostaglandin E2 in both in vivo and in vitro studies. Then PGE2 is involved in enhancing the formation and functions of the both pro and active form of MMP-2. Pro-angiogenic factors like IL-8, hepatocyte growth factor (HGF) and prostaglandin E2 were reported higher in patients with endometriosis.[5] Levels of induced factors i.e., PGE-2 and COX-2 may increase due to the deficiency of vitamin D under the stress conditions.[6] Increased levels of these induced factors lead to the activation of several signaling cascades like Akt-pathway that is involved in the progression of endometriosis.

Increased levels of arachidonic acid and lipid peroxidation regulate cytokines. Furthermore, reduced levels of vitamin D are also responsible for the invasion of the pro-inflammatory cytokines IL-8 and tumor necrosis factor-α (TNF-α).[7] Reduced levels of vitamin D disrupts normal conversion of 17β-HSD-II into estradiol-17 βwhich is a less potent form.[8] Therefore, 17β-HSD accumulates in patients and is responsible for the disease progression.

MATERIAL AND METHODS

Hundred (n=100) working females with endometriosis and hundred (n=100) healthy females were added in the current study. Patients were screened at Jinnah Hospital Lahore. All of the performed variables were approved by the Institutional Review Board (IRB), The University of Lahore. Female patients of age 20-50 years with clinically confirmed endometriosis were selected. Whereas, any subject with the history of taking drugs such as medicine, cigarette and alcohol were excluded out of the current study. Five milliliter (ml) blood was drawn and serum was separated with the help of centrifugationat 4000 rpm for their future assay.

Biochemical Analysis

Levels of MDA were estimated by the help of spectrophotometric method explained by Ohkawa.[9] For determining the levels of MDA 200 µl of serum sample was taken in test tube and added with 200 µl SDS, 1.5 ml of 20% acetic acid having pH of 3.5 and 1.5 ml of TBA incubated and measured its absorbance at 532nm. Antioxidants such as SOD, CAT and GSH were also measured by their respective spectrophotometric methods.[10-12] Levels of Interleukins (ILs), Matrix metalloproteinases (MMPs), Tumor necrosis factor-alpha (TNF-α), PGE-2, vitamin-D, estradiol, COX-2 and hormones i.e., cortisol and testosterone were estimated with the help of their commercially available ELISA kits by Abcam. Statistical analysis was executed through SPSS Version 17.0 performed tests were independent T-test and Pearson correlation. Results were expressed in form of Mean±SD where ($p<0.05$) shows significant results.

RESULTS

Results of the current study remained statistically differed in both controls and subjects as shown in the Figures-1, 2 and 3. Levels of malondialdehyde (MDA) as shown in Figure-1 (A). In subjects it remained (2.99±0.16 nmol/ml, $p=0.026$) that statistically differed as compared to healthy controls (0.88±0.089 nmol/ml), levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) described in Figure-1 (B), (C) and (D) were decreased significantly ($p=0.000$, 0.018 and 0.033) in females with endometriosis (652.23±42.6 U/g Hb, 1226.06±108.4 U/g Hb

and 6.28±0.42 μmol/l) as compared to healthy controls (845.26±81.6 U/g Hb, 1599.23±124.8 U/g Hb and 9.26±1.24 μmol/l). In Figure-1 (E), (F) and Figure-2 (A) levels of interleukin-8 (IL-8), matrix metalloproteinase-2 (MMP-2) and tumor necrosis factor-alpha (TNF-α) were expressed that shows statistical significant results (p=0.028, 0.000 and 0.027) in subjects they remained (19.65±3.26pg/ml, 563.23±48.2ng/ml and 31.29±4.25pg/ml) as compared to controls (10.26±1.99pg/ml, 299.35±54.4ng/ml and 21.26±3.26pg/ml) respectively.

Figure-2 (B), (C) and (D) shows the level of Prostaglandin-E2 (PGE-2), estradiol and cyclooxigenase-2 (COX-2) were higher in the patients diagnosed with endometriosis. They were measured in subjects (5.26±1.14 ng/ml, 47.16±4.28 pg/ml and 1.88±0.17 pg/ml, p=0.001, 0.010 and 0.002) whereas, it remained (1.19±0.191 ng/ml, 22.28±4.16 pg/ml and 0.52±0.09 pg/ml) in controls. Levels of vitamin D and testosterone as shown in Figure-2 (E) and Figure-3 (A) were measured (16.23±2.55 ng/ml and 15.23±1.99 ng/dl, p=0.014 and 0.011) in patients as compared to controls (30.78±3.09 ng/ml and 21.22±2.23 ng/dl). Cortisol, aromatase and 17β-HSD-I were significantly higher in the subjects (26.35±4.16 μg/dl, 6.59±1.33 ng/ml and 4.99±1.08 pg/ml, p=0.033, 0.018 and 0.016) as compared to controls (10.99±2.56 μg/dl, 2.89±0.55 ng/ml and 1.22±0.35pg/ml) expressed in Figure-2 (F), Figure-3 (B) and (C) respectively.

DISCUSSION

Roles of circulating stress biomarkers, inflammatory cytokines (ILs and TNF-α), antioxidants, hormones and MMPs were indicated in the working women diagnosed with endometriosis. According to a substantial manifestation retrograde menstruation was often linked with the endometriosis. Due to the various elements including environmental contaminants, iron and macrophages that disturbs the balance in between antioxidants and reactive oxygen species (ROS) females with endometriosis.[13] ROS were critically involved in the modulation of reproductive physiological functions and various pathological conditions including endometriosis, ovarian cancer, uterine fibroids and infertility. Nevertheless, the balance between antioxidants and ROS maintain the redox homeostasis in the females within their reproductive years.[14] The oxidative stress occurs due to imbalance of systematic manifestation of ROS and antioxidants defense mechanism in the female reproductive system. According to Sampson's theory, the menstrual reflux is transporting to the endometrial tissue throughout the fallopian tubes in the peritoneal environment. It is a common physiological event in all menstruating females of reproductive age. In endometriotic patients, the excessive amount of iron can be produced by the breakdown of pelvic erythrocytes. The iron cytotoxicity has predominately associated to catalyze the formation of various free radicals that are involved in the impairment of cellular processes, necrosis, apoptosis and cellular dysfunction by lipid peroxidation.[15] According to the present findings, the average level of MDA was significantly higher in endometriotic patients as compared to control subjects due to increase influx of iron and oxidative stress. Similar indications were also present in another set of studies,[16] However, levels of antioxidants enzymes are significantly lower in the peritoneal fluids of women with endometriosis.

The concentration of peritoneal macrophages was significantly raised in women having endometriosis because these are involved in the secretion of inflammatory cytokines, MMPs, growth factors and prostaglandins (PGE2). It has hypothesized that macrophages have potent function in the progression and initiation of endometriosis.[17] According to the study of Wu et al.[18] the increased concentration of PGE2 has observed in the peritoneal environment of females with endometriosis which are generally synthesized by peritoneal endometriotic tissues and macrophages. This work is similar to the current study in which COX-2 is overproduced in endometriotic tissue. Table-I shows the increased concentration of PGE2 which is responsible to enhanced the level of COX-2 in endometriotic patients because of high levels of arachidonic acid and low concentration vitamin D (PGE2 Vs.

VAR.	MDA	SOD	CAT	GSH	IL-8	MMP-2	TNF-α	PGE-2	Estrad.	COX-2	Vit-D	Cort.	Test.	Aromat.	17-β-HD-I	17-β-HD-II
MDA	1	-0.42**	-0.39*	-0.53	0.62*	**0.66****	0.71**	0.60**	0.51*	0.42*	0.77*	0.47*	-0.37	0.47*	0.34	-0.41
SOD		1	0.32	0.33	-0.56*	-0.59	-0.65**	-0.58	-0.34	-0.48	0.71*	-0.32	0.10	0.19	0.26	0.08
CAT			1	0.59*	-0.84	-0.77	-0.68*	-0.55	-0.15	-0.11	0.49*	-0.14	0.34	0.19	0.33	0.18
GSH				1	-0.67	**-0.75***	-0.49*	-0.38	-0.62	-0.19	-0.39*	0.15	0.11	0.36	0.26	0.23
IL-8					1	0.66*	0.77*	**0.75****	0.65*	0.66*	0.84*	0.46*	0.67	0.16	0.49	0.19
MMP-2						1	0.55*	**0.66****	0.57*	0.75	-0.69	0.19	0.55	0.11	0.33	0.33
TNF-α							1	0.33	0.49	0.72*	-0.76*	0.34	0.14	0.29	0.15	0.56
PGE-2								1	0.65	**0.734****	**-0.82****	0.74	0.26	**0.569****	0.24	**0.36**
Estrad.									1	0.58	0.19	0.33	0.24	0.44	0.45	**-0.68*****
COX-2										1	0.32	0.66	0.11	0.56	0.29	0.18
Vit-D											1	**-0.75****	0.59	0.24	0.18	0.16
Cort.												1	0.34	0.33	0.33	0.36
Test.													1	0.01	0.24	0.12
Aromat.														1	0.16	0.26
17-β-HD-I															1	0.19
17-β-HD-I																1

Table-I. Pearson s' correlation coefficients of prognostic variables in the development of endometriosis
Significant at (<0.05)

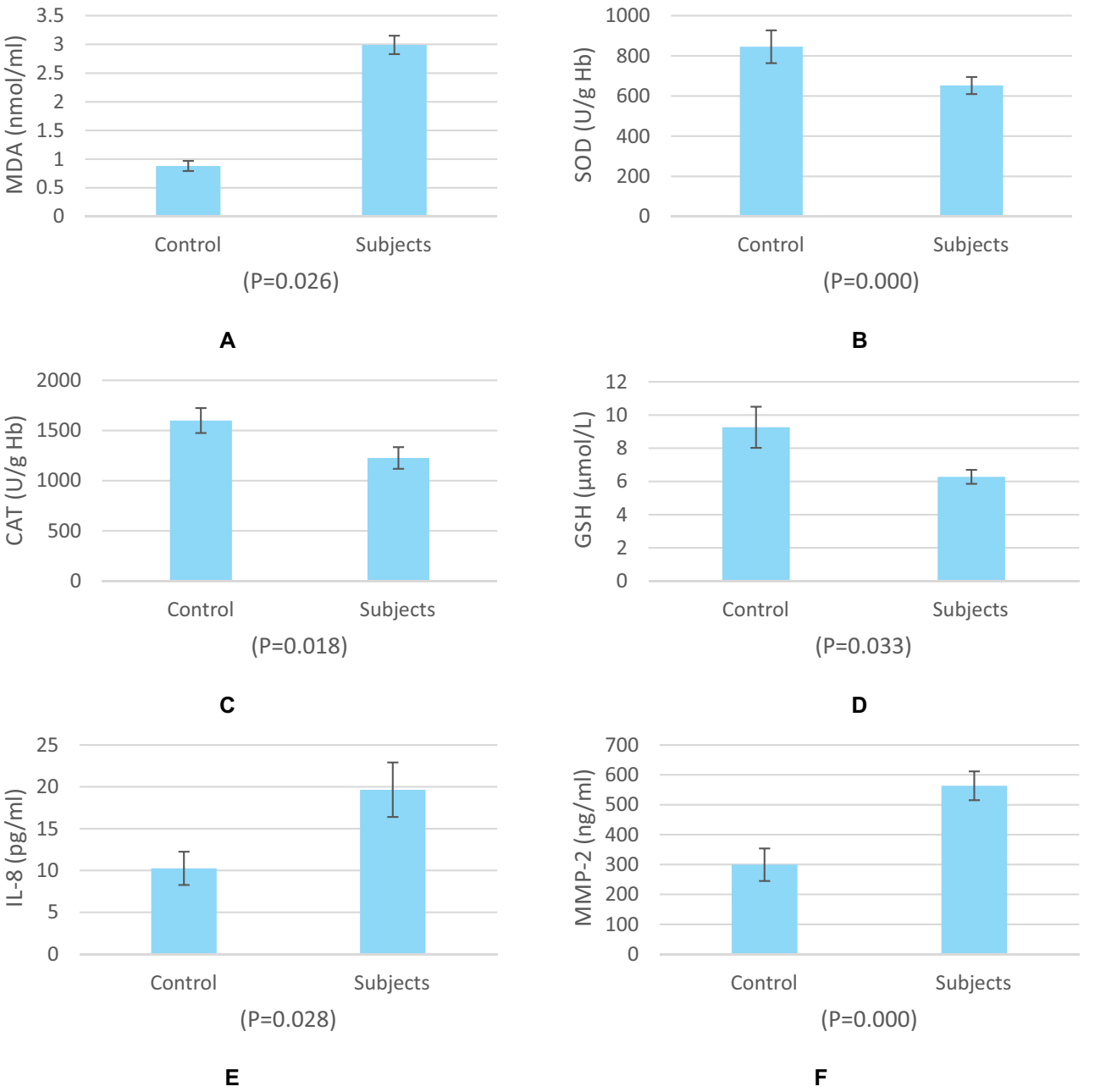

Figure-1. Biochemical variables of control and patients with endometriosis

COX-2, r=0.734**). Various pro-inflammatory cytokines such as IL-8 and TNF-α are contributed to enhance the levels of PGE2 which cause inflammation and endometrial wall degradation in peritoneal cavity. There is significantly positive correlation was established between IL-8 and PGE2 (IL-8 Vs. PGE2, r=0.75*) as illustrated in Table-I. During menstruation, endometrium proliferates rapidly due to the response of estrogen. The endometriosis is estrogen dependent disease which is distinguished as growth of endometrial like tissues outside the wall of uterus.[4] Generally, steroidogenic acute regulatory protein (StAR) and aromatase enzymes both are regulated by PGE2 in stromal cells of females having endometriosis. Mitogenic activity of estrogens is also triggered by overexpression of PGE2 and various growth factors. However,

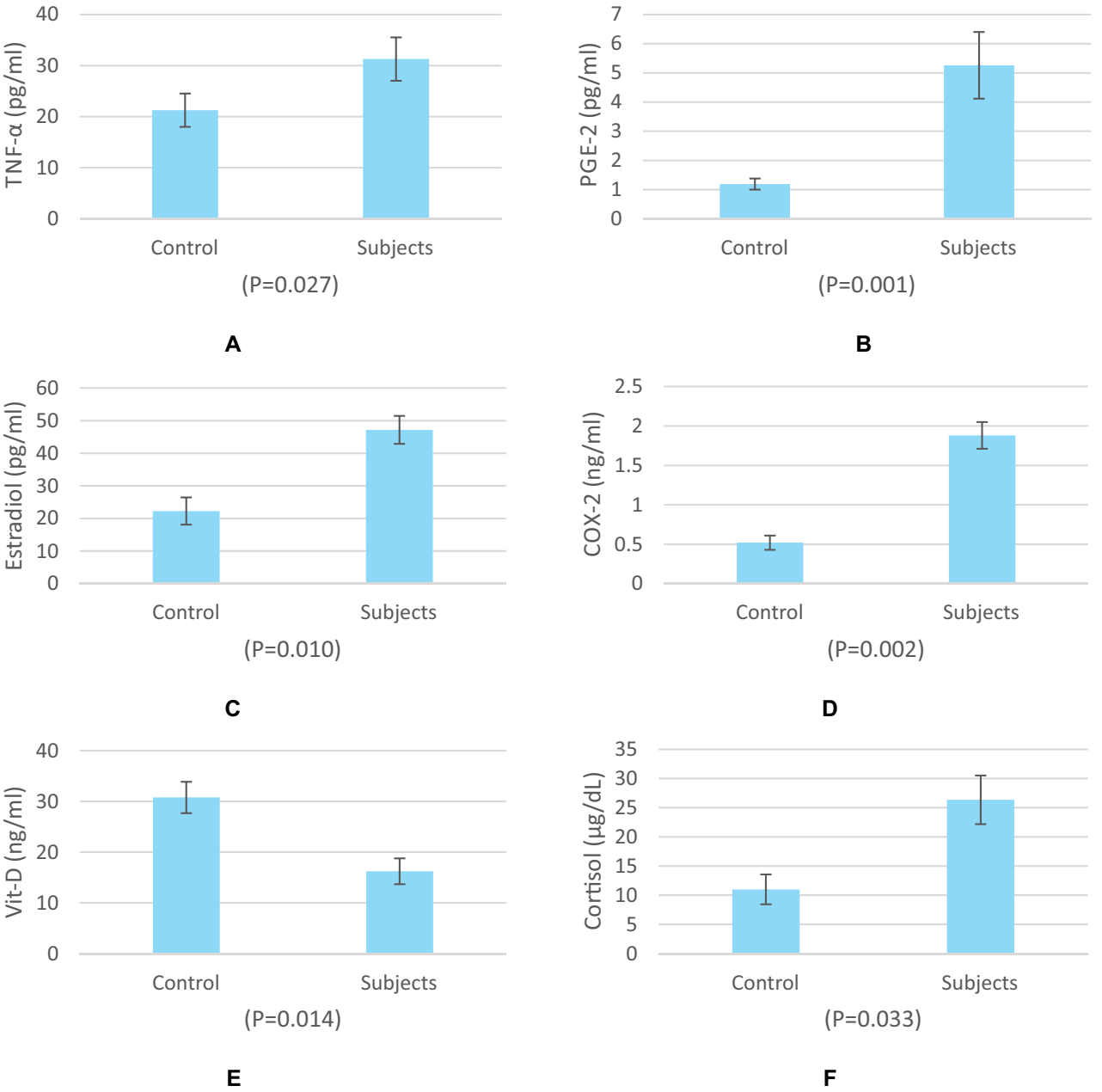

Figure-2. Biochemical variables of control and patients with endometriosis

aromatase is an important enzyme that critically involved in the conversion of androstenedione into estrone (E_1), and testosterone into estradiol (E_2) by 17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD type 1) in ovarian granulosa cells of human. Nevertheless, E_2 can be inactivated by converting into E_1 and this reaction is catalyzed by 17β-Hydroxysteroid dehydrogenase type II (17β-HSD type II) in the eutopic endometrium. In endometriosis, E_2 is not metabolized into E_1 due to the low levels of 17β-HSD type II, thereby leads to enhance the levels of estradiol. Therefore, negative correlation was exist between E2 and 17β-HSD-II in the patients with endometriosis (E2 Vs. 17β-HSD-II, r=-0.68***) as described in Table-I. The increase levels of E_2 may responsible to enhance the formation of PGE2 and growth of endometriotic tissues. Moreover,

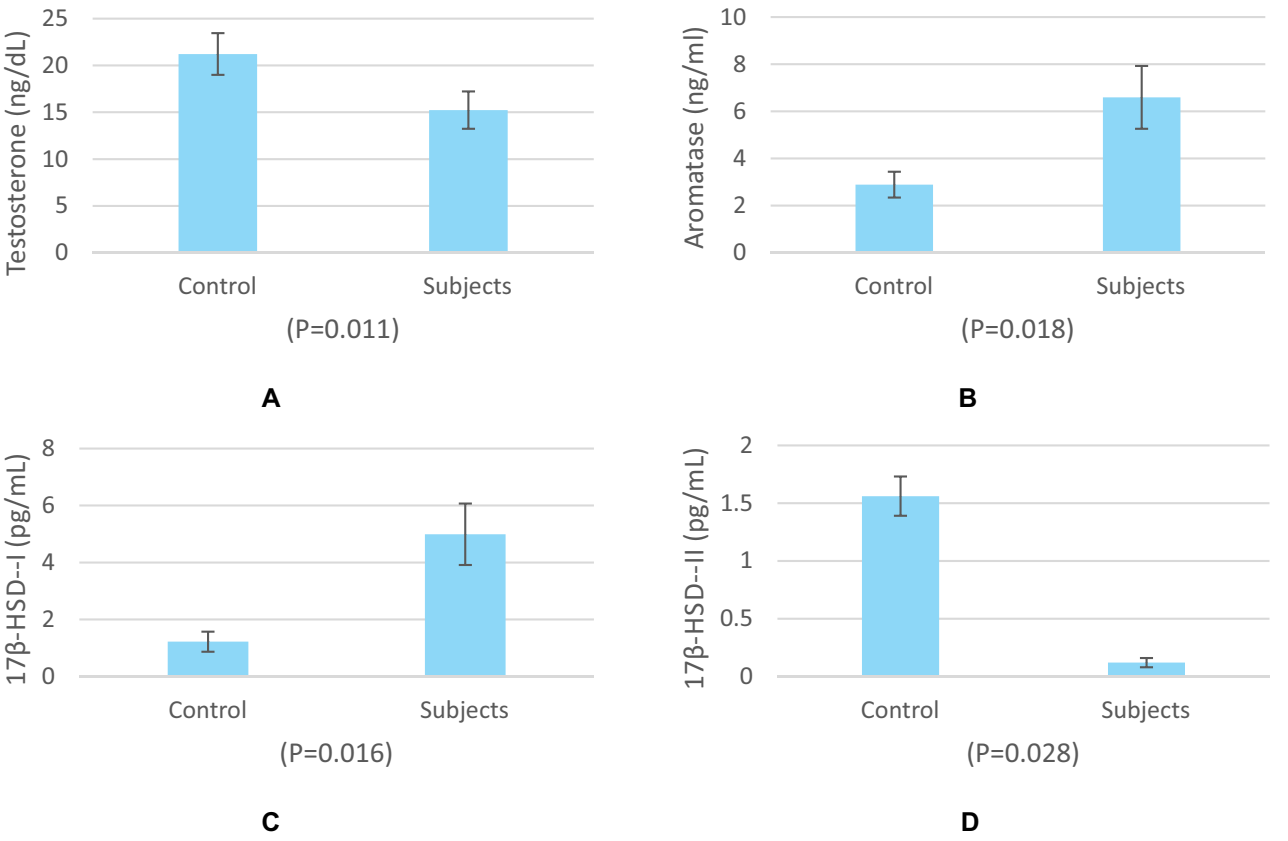

Figure-3. Biocehmical variables of control and patients with endometriosis

MMP-2 manifestation in endometriosis patients was positively correlated with estradiol in the females suffering with endometriosis (MMP-2 Vs. Estradiol, r=0.57*) as shown in Table-I. Moreover, the increase concentration of cortisol in the females having endometriosis can be linked with emotional or physical stress or deficiency of vitamin D that might beinvolve in the progression of this disease. Basically stress stimulates the neurons which produce corticotrophin releasing hormone and resultantly enhance the level of cortisol in endometriotic patients.[19] It has also been reported that patients with endometriosis have reduced cellular immunity as well as low NK activity in peritoneal cavity of females. In chronic stress, the cortisol reduces the vitamin D receptor (VDR) abilities therefore a negative correlation was established between these two variables (CorticolVs. Vitamin D, r=-0.75**).

CONCLUSION

The current study indicates role of oxidative stress and vitamin-D in females with endometriosis. Lower levels of vitamin-D and increased oxidative stress is responsible for release of induced factors i.e., PGE-2 and COX-2. These factors play their role in activation of signaling cascades involved in progression of endometriosis. Hence, vitamin-D and antioxidant supplementation may have a beneficial role in the disease management.

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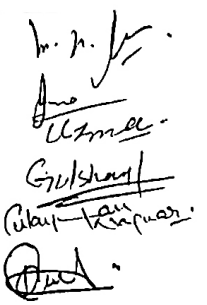
AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Arif Malik	Conceptilization	
2	Iram Qamar	Reading	
3	Uzma Jamil	Reviewing	
4	Gulshan Parveen	Editing, Writing	
5	Sulayman Waquar	Editing, Methodology	
6	Hassan Shafique	Writing, reading	