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EVALUATION OF CYTOKINE PROFILE DURING MENSTRUAL CYCLE AMONG ADULT FEMALES IN PORT HARCOURT

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ABSTRACT
Menstruation is a natural occurrence among young adult female of reproductive age with evidence of vagirnal bleeding which is regulated by hormones and end result is usually pregnancy. This was a longitudinal study carried out with the aim of evaluating cytokines levels at different phases of menstrual cycle in dysmenorrheic young adult females within the age range of 18-30 years attending University of Port Harcourt, Choba, River State, Nigeria. A total of sixty (60) apparently healthy menstruating females participated of which thirty (30) were dysmenorrhea and thirty (30) control eumenorrhea females. Three milliliters of whole blood was drawn from each subject using standard veinpuncture technique and was allowed to clot and serum was separated for the analysis using enzyme linked immunosorbent assay (ELISA) method, at follicular, proliferative, and secretary phases. The data obtained were analysed using Statistical Package for Social Science (SPSS) version 20. Comparison of mean for the parameters of the three phases was done using ANOVA, IL-6, TNFα and IL-10 were significantly varied ($F=33.22$, $P<0.01$, $F=29.96$, $P=0.02$, $F=58.06$, $P=0.01$) respectively. To identify the phase that had significant mean difference, Tukey's Post Hoc test was used. IL-6, TNF, and IL-10 ($p=0.01$) had significant mean difference between the three phases where as IL-1B show no difference ($P=0.4$). Using Spearson's correlation coefficient, IL-6 was positively correlate with TNFα ($r=0.578$ $p=0.001$) at the secretary phases. The finding of this study recorded a significant variation in the three phases of the cycle of study with higher levels of the pro-inflammatory cytokines IL-6 at follicular phase, TNFα at proliferative phase and anti inflammatory cytokine IL-10 at secretary phase and there was no statistically significant difference in IL-1beta when compared across the phases. However, the cytokines profiled were significantly higher in dysmenorrhea females than control group. This study indicated that the variations of cytokines during menstrual phases could cause hypercontractility resulting in painful menstruation (dysmenorrhea) and could also be implicated in autoimmune disease and decidualization of the uterus. It is therefore recommended that menstrual dysfunction should be an important topic for global reproductive health campaigns; also females should be educated on cause, implication, and importance of timely management of menstrual disorder.

KEYWORDS: Menstrual cycle, follicular, proliferative secretary female, dysmenorrhea, cytokines, Nigeria.

INTRODUCTION
Menstrual cycle is the regular natural phenomenon that occurs in the female reproductive system specifically the uterus, ovaries and the endomentrium that makes pregnancy possible.[1] The uterine cycle is divided into three phases,[1,2] menstruation phase, proliferative phase and secretary phase.[2,3]

The first menstruation usually begins between twelve and fifteen years of age and typical length of time between the first day of one menstruation and the first day of the next is 21 to 35 days in adult female, an average of 28 days.[4] Bleeding usually lasts up to 2 to 7 days.[5,6] and the final stage of the menstrual cycle in female is the maturation of the ovum and the preparation of the female organ to support fertilization and fetal development.[7]

Dysmenorrheal is one of the most common gynecological complaints and is characterized by pain, cramping, and backache occurring during menses in young adult women. It affects up to 50% of women at some point in their reproductive life resulting in a significant socio-economic impact. It is thought to be caused by an exaggerated response to physiological processes at the time of menstruation and there is some evidence that women with dysmenorrheal experience uterine hyper contractility in the premenstrual phase. Analysis of peripheral blood from dysmenorrhea women revealed excessive synthesis and concentrations of

oxytocin (OT), Tumor necrotic factor alpha (TNFα) and IL-6, These mediators could increase uterine contractility there by resulting to inflammation this stimulate the release and migration of immune cytokines to prevent bacteria invasion.

Cytokines are glycoprotein messengers that have a broad array of functions.[8] that are important in cell signaling.[9] their release has an effect on the behavior of cells around them (pleiotropic).[10] They act as immunomodulating agents (redundant actions),[11,12]

Cytokines play important role throughout menstrual cycle and implantation of embryo also contribute to the defense of endometrial mucosal epithelium they are crucial for defense against infections and in other immune responses.[13] However, they can become dysregulated and pathological in inflammation, trauma and sepsis.[13] It was discovered that adverse effects of cytokines have been linked to many diseases and conditions ranging from schizophrenia, major depression,[14] auto-immune and Alzheimer's disease to cancer.[15] Excessive secretion of cytokines has been discovered to trigger a dangerous syndrome 'cytokine storm' that could cause severe adverse bleeding and dysmenorrheal.[16]

MATERIALS AND METHODS

Study Design

This was a longitudinal study, performed on60 apparently healthy females of reproductive age, between March 2018 and April 2019.

Study Area

The study was done in River State at the South South zone of Nigeria with a population of 5,198716 according to 2006 census report and is located at coordinates, $4^0$55'N, $6^0$50'E. River state is bounded by the South Atlantic Ocean, the North by Imo, Abia and Anambra states, to the East by AkwaIbom state and to the West by Bayelsa and Delta states. This study was carried out in the University of Port Harcourt, a major tertiary institution, River state between Latitude $4^0$53'58''N and Longitude $6^0$55'43''E.

Study Population

The participants recruited into this study were 60 apparently healthy females in their menstrual cycle, studying in the University of Port-Harcourt.A standardized questionnaire was filled by each subject to obtained social –demographic information.

Inclusion Criteria

The subjects who participated in this study were between age 18 to 30 years and were.

Stable, healthy, no chronic illness, not on any pain killer drugs, not on special medication menstruating, never been hospitalized due to menstruation, did not just had an absorption or miscarriage, dysmenorrheal.

Exclusion Criteria

Subjects not within the age range, young adult females who are on special medications during the menstrual cycle, subjects on birth control pills, subjects with irregular menstrual cycle, subject on hormone therapy, subjects who had an abortion prior to collection of samples, adult female with history of prolonged drug intake, subject having gynecological complications.

Sample Size Determination

G* power version 2.0.10 was used to calculate the sample size with parameters such as error of probability at 0.05, power (1-β error) at 0.95 (95%) and effect size of 0.5. This yielded sample size of thirty (30) adult menstruating dysmenorrheic females and thirty (30) adult menstruating eumenorrhea females as control apparently healthy females giving a total sum of sixty (60) participants.

Informed Consent

Inform consent was obtained from the subjects verbally and through questionnaire.

Ethical Consideration

Ethical clearance was obtained from the ethical committee of University of Port-Harcourt Teaching Hospital and permission granted by the hostel coordinators.

Sample Collection

Applying the standard vein puncture technique, three millimeters of whole blood was collected from each subject at ambient temperature the blood was allowed to clot and serum was obtained for cytokines assays.

Laboratory Analysis

Determination of Interluekin-6 (IL-6)

Method: Sandwich Enzyme-Linked Immunosorbent Assay (S-ELISA) was used. Commercially available kits specific for research purpose from Elabscience Biotechnology Inc. Catalog No:E-EL-HO102, Lot No: Lot: 4J2D79UQ, expiring date on 2019/07/14 was used according to the manufacturer directions and specifications (Elabscience.com).

Determination of Tumor necrosis factor alpha (TNFα)

Method: Sandwich Enzyme-Linked Immunosorbent Assay (S-ELISA) was employed. Commercially available kits specific for research purpose from Elabscience Biotechnology Inc. Catalog No: Catalog No: E-ELHO109. Lot No: LotA517E647, expiring date on 2019/7/14, was used according to the manufacturer directions and specifications. (Elabscience.com).

Determination of Interluekin-1beta (IL-1β)

Method: Sandwich Enzyme-Linked Immunosorbent Assay (S-ELISA) was employed. Commercially available kits specific for research purpose from ElabscienceBiotechnology Inc. Catalog No:E-EL-

HO149:Lot: 4J2D79UQ, expiring date on 2019/07/14 was used according to the manufacturer directions and specifications (Elabscience.com).

Determination of Interluekin-10 (IL-10)

Method: Sandwich Enzyme-Linked Immuenosorbent Assay (S-ELISA) was used. Commercially available kits specific for research purpose from Elabscience Biotechnology Inc. Catalog No:E-EL-HO103 Lot No: Lot: BMNZ6CQA, expiring date on 2019/07/14 was used according to the manufacturer directions and specifications (Elabscience.com).

Statistical Analysis

The statistical analysis was performed using the Graph pad prism 6.1 and statistical package for Social Science (SPSS 20). Results obtained were presented in Graphs and Tables. Normality of data distribution was tested using Kolmogorov Smimov test. Data descriptions were presented as mean ± values of Standard Deviation (SD). P-value of < 0.05 was considered significant at 95% confidence interval (CI). One-way Analysis of Variance (ANOVA) was used for comparison the difference in mean at the phases of the cycle, Turkey Post Hoc test was used to identify the significant different between the phases and Pearson's correlation coefficient was used for the relationship between cytokines phases.

RESULTS

Socio-Demographic Characteristic of Subjects

Sixty (60) subjects were recruited into this study thirty (30) menstruating females were Dysmenorrhea and thirty (30) menstruating females without pains as control. The socio-demographic information that was obtained from all the participants in this study were age, level of education, occupation, heavy flow, never been hospitalization during menstruation, length of cycle, duration of flow and day of ovulation, not on special medications. The participants were predominantly within the age range of 18-30 years, were all females, had secondary and tertiary education, and had not had abortion as at the point of sample collection. Table 1 depicts the breakdown of the socio-demographic characteristics that are peculiar to the test groups. Heavy flow, dysmenorrhea participant were 23(76.7%) and control 6(20.0%). Not hospitalized dysmenorrheic were 2(6.7%) and control 1(3.3%).

Table 1: Socio-Demographic Characteristics Among paticipants.

	Study groups		
Variables	**Dysmenorrhea 30 (50%)**	**Control 30 (50%)**	**Total 60(100%)**
Age category (years)			
18 – 21	12 (40.0)	20 (66.7)	32 (53.3)
22 – 25	11 (36.7)	9 (30.0)	20 (33.3)
26 – 30	7 (23.3)	1 (3.3)	8 (13.4)
Mean ± SD	22.90±3.06	20.43±2.74	21.67 ±2.81
Range = 18 – 29years			
Educational level			
Secondary	3 (10.0)	17 (56.7)	17 (28.3)
Tertiary	27 (90.0)	13 (43.3)	43 (71.7)
Occupational status			
Student	16 (53.3)	29 (96.7)	45 (75.0)
Employed	10 (33.3)	1 (3.3)	11 (18.3)
Unemployed **Heavy flow**	4 (13.3)	0 (0.0)	4 (6.7)
Yes	23 (76.7)	6 (20.0)	13 (21.7)
No	7 (23.3)	24 (80.0)	47 (78.3)
Take medication for menstruation			
Yes	3 (10.0)	1 (3.3)	4 (6.7)
No	27 (90.0)	29 (96.7)	56 (93.3)
Ever been hospitalized due to menstruation			
Yes	2 (6.7)	1 (3.3)	3 (5.0)
No	28 (93.3)	29 (96.7)	57 (95.0)

Legend; Mean difference; ± SD - standard deviation

Table 2 Length of cycle, duration of flow and ovulation day of study population.

Variables	**Mean ± SD**	**Median (range)**
Length of cycle (days)	28.43±2.21	28 (21 – 35)
Duration of flow (days)	5.12±1.32	5 (3 – 9)
Day of ovulation	13.65±2.10	14 (6 – 17)

SD – Standard deviation

Analysis of Variance (ANOVA) Showing IL-6, TNFα, IL-1β, IL-10 Changes at Different Phases

Table 3 shows the possible variations of the mean of all the measured parameters in the three phases of the cycle were compared using one way analysis of variance. IL-6 was significantly varied at follicular phase ($p< 0.05$) and lower at the secretary phase, TNFα was significantly varied at the proliferative phase ($p= 0.02$) and lowest at secretary phase, IL-10 was significantly varied at the secretary phase ($p<0.05$) and lowest at follicular phase and IL-1β shows no statistically significant different throughout the phases ($p= 0.307$).

Table 3: Analysis of Variance (ANOVA) Showing IL-6, TNFα, IL-1β, IL-10 Changes at Different Phases.

	Follicular	Proliferative	Secretary		
Cytokines (pg/mL)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**F**	**p-value**
IL-6	25.42±2.32	23.18±2.49	22.41±3.42	33.422	0.001*
TNF	10.85±2.30	12.54±2.67	9.97±2.19	29.966	0.002*
IL-1 BETA	10.23±0.40	10.30±0.03	10.29±0.05	2.364	0.307
1L-10	37.03±43.93	51.24±37.02	65,12±31.02	58.067	0.001*

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, SD – Standard deviation, F- Friedman.

Multiple Comparisons of Parameters among the phases

Post hoc test was carried out to identify the phases which had significant mean difference. There was no statistically significant mean changes in phases of IL-1β (P=0.4), while IL-6, TNFα and IL-10 had significant mean difference (P=0.01). These are shown in Table 4, 5, 6, 7.

Table 4: Post Hoc Test Showing Multiple Comparisons of IL-6, TNFα.

	Dysmenorrheicgroup		Control group	
Dependent Variable	**Mean Differene**	**p-value**	**Mean Difference**	**p-value**
IL-6(pg.mL) Follicular				
Proliferative	2.47100	0.001*	2.24367	0.001*
Secretory	3.62533	0.001*	3.01133	0.001*
Proliferative				
Follicular	-2.47100	0.001*	-2.24367	0.001*
Secretory	1.15433	0.008*	0.76767	0.062
Secretory				
Follicular	-3.62533	0.001*	-3.01133	0.001*
Proliferative **TNF(pg.mL)**	-1.15433	0.008*	-0.76767	0.062
Follicular				
Proliferative	-2.91133	0.001*	-1.68600	0.001*
Secretory	0.95800	0.072	0.88600	0.031*
Proliferative				
Follicular	2.91133	0.001*	1.68600	0.001*
Secretory	3.86933	0.001*	2.57200	0.001*
Secretory				
Follicular	-0.95800	0.072	-0.88600	0.031*
Proliferative	-3.86933	0.001*	-2.57200	0.001*

*Statistically significant
SD – Standard deviation

Table 5: Post-Hoc test showing multiple comparison of IL-1β at the differen phases.

	Dysmnorrhea group		control group	
IL1-BETA (pg.mL)	**Mean Differene**	**p-value**	**Mean Difference**	**p-value**
Follicular				
Proliferative	-0.06867	0.368	-0.06867	0.368
Secretory	-0.06067	0.422	-0.06067	0.422
Proliferative				
Follicular	0.06867	0.368	0.06867	0.368
Secretory	0.00800	0.103	0.00800	0.103
Secretory				
Follicular	0.06067	0.422	0.06067	0.422
Proliferative	-0.00800	0.103	-0.00800	0.103

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, SD – Standard deviation, F- Friedman.

Table 6: Post-Hoc test showing Multiple Comparison of IL-10 at the Different Phases.

	Dysmenorrheic group		Control group	
IL10 (pg.mL)	**Mean Differene**	**p-value**	**Mean Difference**	**p-value**
Follicular				
Proliferative	13.73533	0.001*	13.88500	0.001*
Secretory	26.92500	0.001*	28.09333	0.001*
Proliferative				
Follicular	-13.73533	0.001*	-13.88500	0.001*
Secretory	13.18967	0.001*	14.20833	0.001*
Secretory				
Follicular	-26.92500	0.001*	-28.09333	0.001*
Proliferative	-13.18967	0.001*	-14.20833	0.001*

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, SD – Standard deviation, F- Friedman.

Correlation of IL-6 and other Cytokines (TNFα, IL-10, IL-1β) at the Follicular Phase

Pearson's correlation coefficient was used to determine the relationship between IL-6 and other cytokines. There was a negative correlation between IL-6 and other cytokines at the follicular phase, as it was observed that as IL-6 is increasing IL-1β is decreasing and as IL-6 is decreasing IL-10 is increasing as showing with the negative sign (r = -0.061, p= 0.751 and r= -0.328, p= 0.077) this mean a negative correlation as seen in Table 7a

Table 7a: Correlation between IL-6 and cytokines (IL-1β, IL-10, TNFα,) at Follicular Phase among subject.

	Dysmenorrheic group		Control group	
	IL-6		IL-6	
Cytokines	**R**	**p-value**	**R**	**p-value**
TNF	0.048	0.799	-0.257	0.170
IL-β	-0.061	0.751	-0.163	0.390
IL-10	-0.328	0.077	-0.150	0.428

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, R- Corelation

Correlation of IL-6 and other Cytokines (TNFα, IL-10, IL-1β) at the Proliferative Phase

It was observed that there was a negative correlation between IL-6 and IL-1β IL-10, as IL-6 IS increasing IL-1β is decreasing and as IL-6 is decreasing IL-10 is increasing as showing with the negative sign (r = -0.075, p= 0.695 and r= -0.224, p= 0.233) respectively, while TNFα is positively correlated (r=0.827, p=0.520) as shown in Table 8.

Table 8: Correlation between IL-6 and (TNFα, IL-1β, IL-10) at proliferative phase.

	Dysmenorrheic group		Control group	
	IL-6		IL-6	
Cytokines	R	p-value	R	p-value
TNF	0.827	0.520	0.124	0.515
IL-β	-0.075	0.695	-0.065	0.732
IL-10	-0.224	0.233	-0.093	0.626

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, R- Corelation

Correlation between IL-6 and its Corresponding Cytokines at Secretary Phase

There was a positive correlation between IL-6 and TNFα (r= 0.578, p= 0.001), this shows as IL-6 is increasing TNFα is also increasing and as IL-6 is decreasing TNFα is decreasing also. The table also shows a negative correction between IL-6 increasing and IL-1β is decreasing as showing (r= -0.106, p= 0.576).

Table 9: Correlation between IL-6 and (TNFα, IL-1β, IL-10) at secretary phase.

	Dysmenorrheic group		Control group	
	IL-6		IL-6	
Cytokines	R	p-value	R	p-value
TNF	0.578	0.001*	-0.152	0.423
IL-β	-0.106	0.576	0.293	0.116
IL-10	0.003	0.988	0.040	0.833

Key *Statistically significant, IL-1β – Interlukin -1 beta, TNFα – Tumor necrosis factor alpha, IL-10 - Interlukin 10, IL-6 Interlukin -6, R- Corelation.

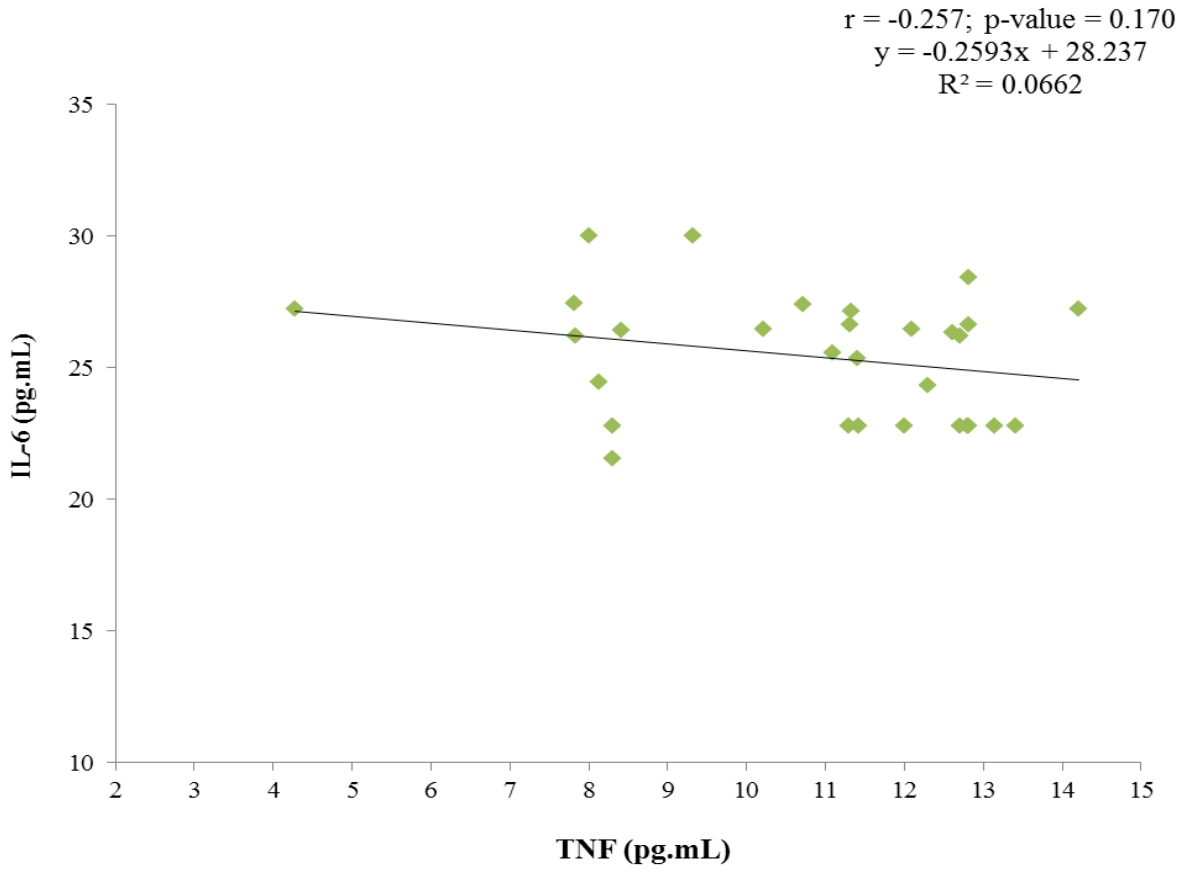

Figure1: Scatter plot showing relationship (correlation) between IL-6 and TNF at the menstruation phase.

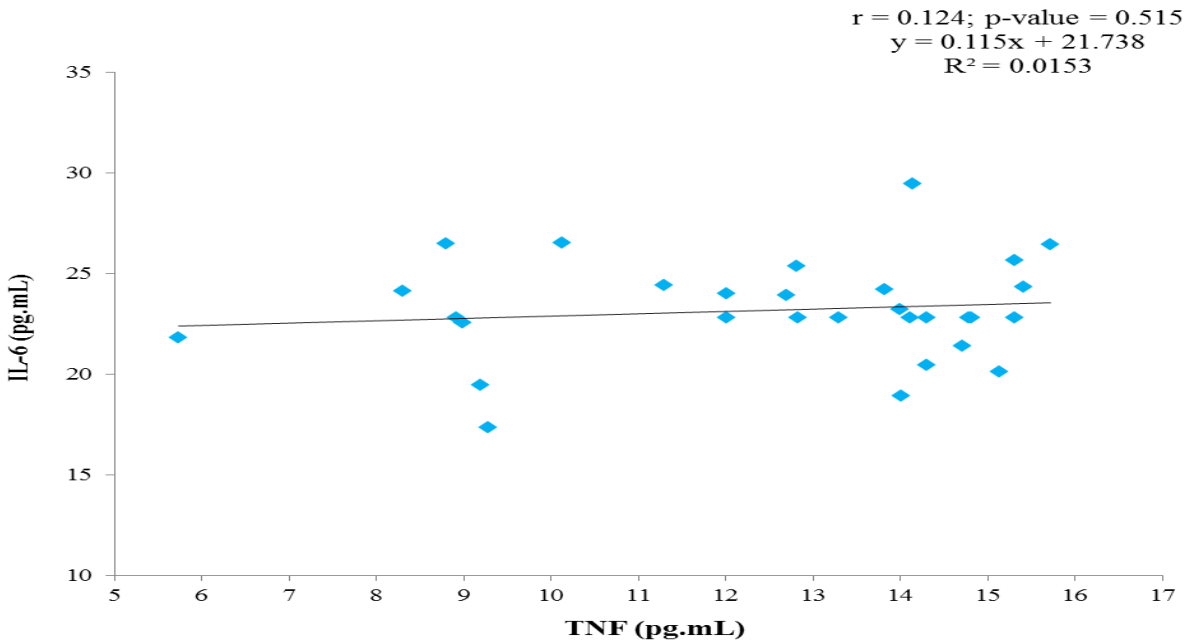

Figure 2: Scatter plot showing relationship (correlation) between IL-6 and TNF at the proliferative phase.

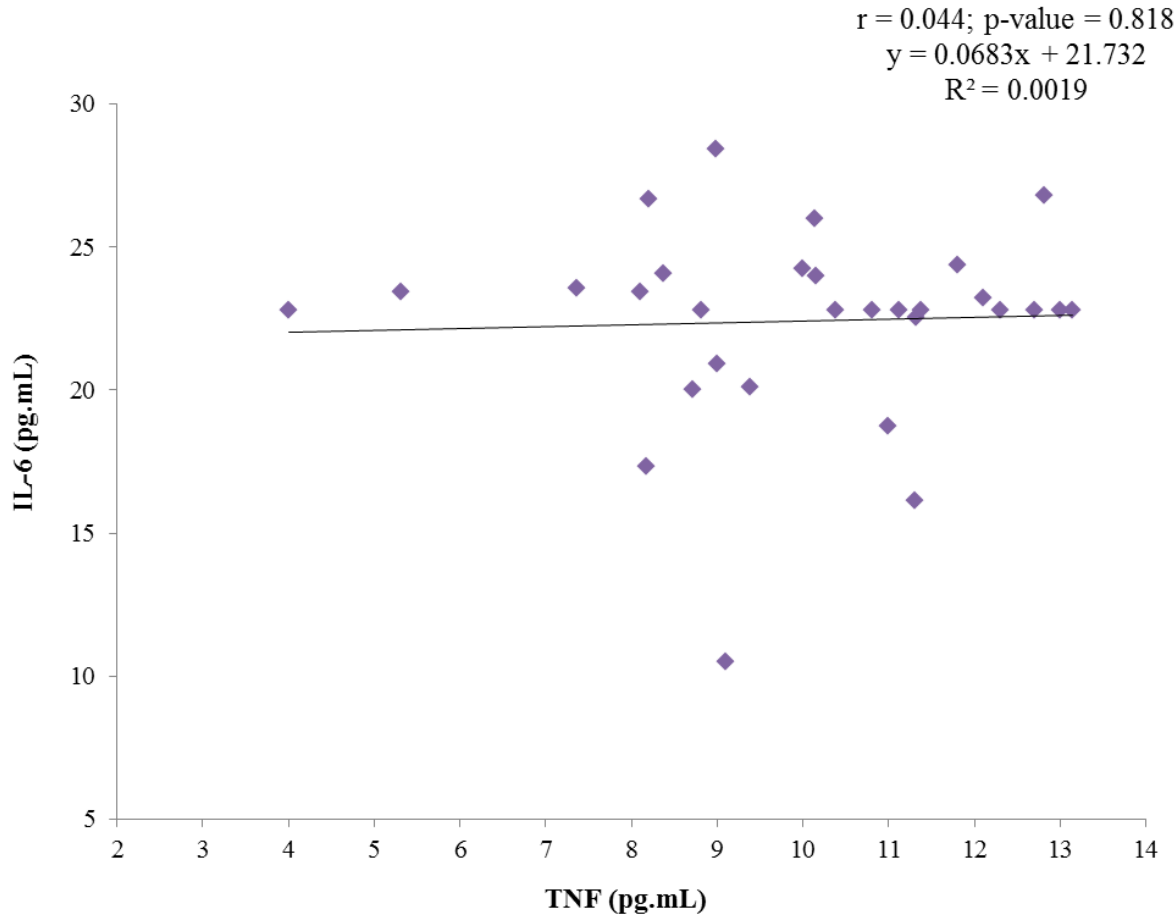

Figure 3: Scatter plot showing Relationship (Correlation) between IL-6 and TNF at the Secretary Phase.

DISCUSSION

Immune cytokines profiles are useful in assessing the state of health in humans and in the determination of pro-inflammatory and anti-inflammatory immune response. It is believed that cytokines are essential biomarker in detecting many diseases such as dysmenorrhea, autoimmune, cardiovascular etc.

This study was set out with the aim of evaluating cytokines at difference phases of menstrual cycle among young adult females experiencing dysmenorrhea in University of Port Harcourt, Nigeria in order to find out if there were variations in these cytokines secreted at different phases (follicular phase, proliferative phase and secretary phase) of uterine cycle among dysmenorrheal group with control that could be useful in identifying some menstrual dysfunctions like dysmenorrhea, and as biomarkers. However, the key findings were obtained from comparison of the parameters at different phases of the uterine cycle. The cytokines assessed and compared were IL6, TNFα, IL-1β, and IL-10.

From this study we observed that IL-1βacross the study group was higher at proliferative phase, lower at follicular phase however, there was no statistically significant difference with a (p= 0.307) across the study group, this finding was compared with[17] similar research they reported that IL-1β was greatly secreted at menstrual phase than any other cytokines.

In this study IL-6 was significantly higher at the follicular phase followed by proliferative phase and lower at secretary phase across the cycle, however, there was statistically significant difference with a (p= 0.001) comparing with the previous work done in developed countries, like China they observed same results as with our study.[17] IL-6 is said to stimulate B-lymphocytes and T-lymphocytes differentiation enhancing cellular immunity preventing bacteria invation when the endometrium agited and slough. It is also said to possess anti-inflammatory properties.[17,18,19] however, reports of IL-6 levels during the menstrual cycle have been controversial. Whereas.[20] reported higher levels of IL-6 during the follicular phase which concur with our finding in this study.[21] Reported no significant mean difference while.[22] reported that IL-6 was highly decreased in the menstruation phase of the cycle.

In this study there was significantly higher level of TNFα at the proliferative phase across the cycle with (p = 0001). TNFα is a cytokine that induced apoptosis, it is reported that TNFα apoptised the decidual cells in the uterus in preparation of implantation hence the function of the TNFα is consistence with our findings. However comparing this report with.[17] study in apparently healthy women, reported that TNFα was higher at the secretary phase when monocytes was activated.[22,2,24] This is probably due to the fact that at secretary phase the fertilization has taken place and the fertilized ovum requires protection against infectious agents hence the increase in TNFα activity.[24]

In this study it was observed that IL-10 across study group was significantly higher at the secretary phase followed by proliferative phase and lowest at the menstrual phase with a (p= 0.0001) this finding were in agreement with the work done by.[25] The elevated IL-10 at the secretary phase following proliferative phase is in line with the report of.[26,27,28,29] IL-10 is reported to be involved in the maintenance of pregnancy, also thought to be produced at the feta-maternal interface to control fetal-ablating immune responses. However, expression of IL-10 in nonhemopoietic maternal cells of the human uterus has not been classified in detail.[30]

It was observed that IL-6 was positively correlated with TNFα reason being that probably IL-6 could inhibit the action of other cytokines IL-10 and IL-1β.

Our results clearly demonstrated that differential expression of cytokines in peripheral blood across menstrual cycle among dysmenorrhea group occurs throughout in the phases(follicular, proliferative and secretary phases) according to the activity and the state of health at that time of the cycle. The role of cytokine response differs during the cyclical changes of the endometrium. During the secretary phase, pro-inflammatory cytokines (IL-1β and TNF-α) are believed to be involved in endometrial decidualization and in auto-immune disease [31, 32, 33, 34]. IL-10 is involved in feta-maternal development and IL-6 is said to be involved in inflammatory responses and prevent bacteria invasion and multiplication.

CONCLUSION AND RECOMMENDATIONS

Conclusion

The finding of this study recorded a significant variation in the three phases of the menstrual cycle among dysmenorrhea group, with higher levels of pro-inflammatory cytokines IL-6 at follicular phase, TNFα at proliferative phase and anti-inflammatory cytokine IL-10 at secretary phase and accompany by no statistically significant mean difference in IL-1beta, in comparison. The variations of these cytokines during the menstrual phase is believed to stimulate the synthesis and release of prostaglandin and oxytocin that induce uterine hyper contractility, and decrease endometrial blood flow and thereby resulted to menstrual pain (dysmenorrhea) also trigger the secretion and migration of these cytokines. The changes in the levels of these(IL-10, IL-6 and TNF) cytokines produced at different phases of the menstrual cycle is said to cause chronic auto-immune disease in future, evaluations of these cytokines are suggestive useful biomarkers for early detection of dysmenorrhea, autoimmune disease and decidualization of endometrial ablation in pregnancy. Higher variations of cytokines assayed were observed among dysmenorrheic participant than in the control group.

Recommendations

It is recommended that one or more of these cytokines profiled should be incorporated as a routine screening in dysmenorrhea females as this will aid in early detection of autoimmune disease. Also, menstrual dysfunction should be an important topic for global reproductive health campaigns and finally females should be educated on cause, implication, and importance of timely management of menstrual disorder.

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