ORIGINAL ARTICLE

Cytokine and Inflammatory Markers in Pregnant Post COVID-19 Survivors

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ABSTRACT

Background: *Post covid syndrome is a cluster of health problems following primary COVID-19 infection. COVID-19 is accompanied with cytokine storm which causes unfavorable gestation events.* ***Objective:*** *We aimed to assess inflammatory cytokine interleukin 6 (IL6), interleukin 8 (IL8) and C-reactive protein (CRP) during early recovery period from SARS-CoV-2 infection.* ***Methodology:*** *It is a case control study. It included 400 pregnant women. Case group included 200 women with singleton pregnancies tested positive by nasal swab for SARS-CoV-2 and blood samples withdrawn within 4–6 weeks after the last negative for SARS-CoV-2 swab. Control group consisted of age matched pregnant women not infected with COVID-19. Measurement of CRP, IL8, and IL6 was done using enzyme-linked immunosorbent assay (ElISA).* ***Results:*** *Higher levels of inflammatory cytokines were notable among case group versus controls mean±S.D for IL6 was (4.90±1.65) among case group vs. (5.30±1.91) among control group, confidence interval (CI); (-.751-.048). As for IL8 Median (min-max) among case group versus controls = 30 (30-250) vs. 30 (30-450), p<0.05 respectively. No statically different noticed in CRP levels; mean±S.D (3.0±0.6) among case group vs. (3.1 ± 0.9) among control group, CI: (-.282-.044), P=0.153. Statistically significant differences was observed between studied groups regarding haemoglobin level, platelet and white blood cell count (p=0.002, 0.00, and 0.01) respectively.* ***Conclusion:*** *Pregnant women in post recovery phase of COVID-19 possessed higher levels of inflammatory cytokines (IL6 & IL8) in comparison to non-infected pregnant women. Potentially having an effect on of some health parameters and affecting pregnancy outcomes.*

INTRODUCTION

Until now it is not fully comprehended the specific reasons of extended post COVID period. There are several theories, such as chronic tissue and blood vessel damage, blood coagulation issues, neurological disorders and autoimmune[1]. Long-term COVID is diagnosed by ruling out other illnesses and relying on (proven) COVID-19 infection or manifestations. It was estimated that the incidence of Post- COVID in 2024 will be roughly 1% in infants and 6–7% in adulthoods[2].In 2024, the Centers for Disease Control and Prevention estimates that "Post-COVID Conditions" will start to appear within four weeks after acute COVID-19, to highlight the significance of initial clinical evaluation along with therapeutic care in the first four to twelve weeks after acute COVID-19" [3].

The drastic unfavorable effects on human well-being as a result of infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was highly observed during coronavirus pandemic in 2019 (COVID-19), it had been an enormous strain on healthcare organizations since then. Non-vaccinated individuals have varying degrees of symptoms fluctuating from mild symptoms such as low grade fever to fatal multi-organ failure[4]. Some groups are particularly highly susceptible to viral infections more than others for instance pregnant females. Even though COVID-19 symptoms is monitored and controlled if it appeared during gestation period, pregnant females still have increased probability for mortality than non-pregnant equivalents[5]. Biological changes occurring in immunological, and endocrine systems during pregnancy affect how the body normally react to viral infections[6] [7] . Pregnant females immune system act as an anti-inflammatory defense system in an attempt to protect the fetus from acquired viral infection[8]. However, some times the body fails to protect the fetus from severe immunological response (Cytokine Storm) against viral infection[9]. Cytokine storms accompanying SARS-CoV-2 infection are usually associated with poor health outcomes [10].

Many researchers studied cytokine response mainly interleukin 6 (IL6), interleukin 8 (IL8) and C - reactive

protein (CRP) during the infection with COVID-19 and highlighted the possibility of using them as monitoring parameters during COVID-19 infection. Increased levels of IL-6 and IL-8 among COVID-19 patients triggers cascade of coagulation proteins involved in intrinsic and extrinsic coagulation pathways, stimulation of thrombin synthesis through enhancing tissue factor release from mononuclear cells[11 12]. It is advised to have an early assessment and screening for IL-6 is upon hospital entry for COVID-19 infected individuals to evaluate the deteriorating clinical picture and infection progress among COVID-19 patients[13].IL-8 was found significantly elevated among non-survivors of COVID-19 compared to surviving individuals, and the fluctuations of IL-8 levels is associated with the degree of severity related the viral infection[14]. Positive acute phase reactants accompanying inflammation such as C-reactive protein (CRP) act as bio-marker to track the progression of COVID-19[15]. CRP is precisely consistently associated with the presence of uncontrolled inflammation. Sustained high levels of CRP indicates poor prognosis and disease complexity according to previous published report[16].Massive release of immune-mediators among other various inflammatory proteins released against COVID-19 infection enhances deterioration of clinical picture of pregnant women having COVID-19[17],[18]. Some individuals in the post COVID-19 recovery period exhibit some post covid serious medical consequences. Therefore, Post COVID-19 recovery period need more exploring studies. Thus, our study aimed to study cytokines levels (IL6 and IL8) among other inflammatory marker (CRP) during recovery period from SARS-CoV-2 in pregnant women compared to non-infected SARS-CoV-2 pregnant women in the control group.

METHODOLOGY

Study Subjects and ethics statement

It is a case-control study. Women registered in the study joined routine prenatal appointments at Prenatal Diagnosis and Fetal Medicine department from April 2022- October 2023. The study procedure was accepted by Medical Research Ethical Committee in National Research Centre (NRC), Egypt (No: 131210112022). Written informed consent was obtained from all participants.

Sample size Calculation

The sample was calculated using online calculator https://www.calculator.net/sample-size. It calculates the required number of subjects needed for accurate statistical calculation with slandered error 5% and confidence interval of 95% .

The study included 400 women divided into two groups. 5 ml blood samples was withdrawn from all candidates. A case group included women with two hundred pregnant women in their first trimester who tested positive by nasal swab for SARS-CoV-2 then consequently within 4–6 weeks after the last negative for SARS-CoV-2 swab for each participant blood sample was withdrawn. Control group consisted of two hundred age and gestation matched pregnant women, who were not infected with COVID-19 confirmed by (absent symptoms and negative for SARS-CoV-2 swab for exclusion of asymptotic individuals to avoid bias results.

Pregnant women with the recent or chronic infection, common cold, immune abnormalities, history of thrombosis, previous coronavirus vaccination, exposed to infection with coronavirus during the current pregnancy (at the time of the study) were excluded. Age, consanguinity, gestational age (GA), systolic and diastolic blood pressure (SBP and DBP), white blood cell count, hemoglobin level (Hgb), platelet counts (Plt), medical history, and outcomes were recorded for all participants. Blood sample was withdrawn on EDTA tubes.

All blood samples spun at 1000 xg for thirty minutes. Plasma was then separated. Aliquots were put away at − 20 °C till examination. CRP, IL6 and IL8 were then measured by Human Quantikine ELISA kits from R&D Systems (Minneapolis, MN, USA) and steps were followed according to the manufacturer's instructions respectively.

Principal of the ELISA **assays**

Standards and samples were pipetted into pre-coated microplate with monoclonal antibody specific for IL6, IL8 and CRP respectively. Followed by the wash of unbound substances. Enzyme linked polyclonal antibody specific for interleukin 6, 8, CRP were added to the wells then wash of excess unbound enzyme antibody reagent. After incubation period substrate reagent was added to the wells and color is made which is equivalent to the quantity of IL6, IL8 and CRP found in sample. The reaction is stopped and optical density (O.D.) is measured at 450 nm. After subtracting the average zero standard (O.D.), the mean of duplicates values for each standard, control, and sample is noted. Plotting each standard's mean absorbance on the Y axis versus the concentration on the X axis creates a standard curve.

RESULTS

Four hundred singleton pregnancies women were included in the current study (200 cases and 200 controls). Comparison of baseline demographics between cases and controls groups in **Table 1** showed that participants among cases and controls groups were not significantly different concerning maternal age, gestational age, consanguinity, health comorbidities of diabetes, and hypertension. However, cases group had a

significantly higher body mass index (BMI) values compared to controls group mean±SD (25.8±2. 5 kg/m2 vs. 26.3±4.0 kg/m2), p= 0.01. There was a statistically significant differences between groups regarding Hgb levels, Plt and WBC count (p values were 0.002, 0.00, and 0.01, respectively).

On the other hand, comparison of CRP and cytokine levels between cases and controls group revealed that IL-8 **Figure 1** and IL-6 **Figure 2** were significantly increased in post COVID-19 recovered cases with P <0.05 as shown in **Table 2.**

While, CRP levels did not show any statistically significant differences between the two groups, p=0.153. Pearson correlation between cytokine levels and demographic data of our participants was shown in **Table 3.**

According to correlation analysis there was a statistically significant positive correlations of IL-6 levels with different variables (age and platelets count), p = 0.045, and 0.049 respectively as independent predictors of IL-6 levels as shown in **Figure 3.**

Table 1: Demographic data of studied groups.

	CONTROL N=200	CASE N=200		
	Mean ±SD	Mean ±SD	P value	CI (95%)
Age (Years)	25.8± 3.0	26.1 ± 4.0	0.42	(-.993- .416)
BMI (kg/m2)	25.8 ± 2.5	26.3 ± 4.0	0.01	(-.156- 1.156)
GA (Weeks)	5.6 ± 0.4	6 ± 0.2	0.45	(0.338 - 0.462)
Consanguinity % **Negative** **Positive**	 160 (80.0%) 40 (20.0%)	 154 (77.0%) 46 (23.0%)	0.46	(0.741- 1.92)
SBP(mm/Hg)	119± 7.7	120 ± 7.5	0.41	(-0.49- 2.49)
DBP (mm/Hg)	73.9 ± 7.1	74.4 ± 6.0	0.22	(-.792-1.792)
FBS (mg/dL)	78.7± 7.9	80.0± 8.8	.135	(-2.91- .395)
Hgb (g/dl)	12.2± 0.89	12.5± 0.90	.002	(-.462 - .107)
Plt (mm3/ ul)	290.1± 49.3	319.5± 67.3	.000	(17.80- 40.99)
WBC (mm3/ul)	7250± 5019	8412± 2320	0.001	(393.36- 1930.64)

GA: gestational age, **SBP:** systolic blood pressure**; DBP:** diastolic blood pressure**, FBS:** fasting blood sugar**, Hgb:** hemoglobin**, Plt:** platelet**, WBC:** white blood count.**.** P<0.05 is considered significant.

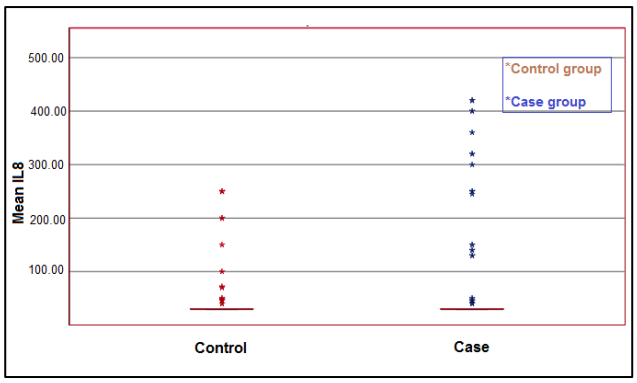

Figure 1. Mean of IL8 between studied groups.

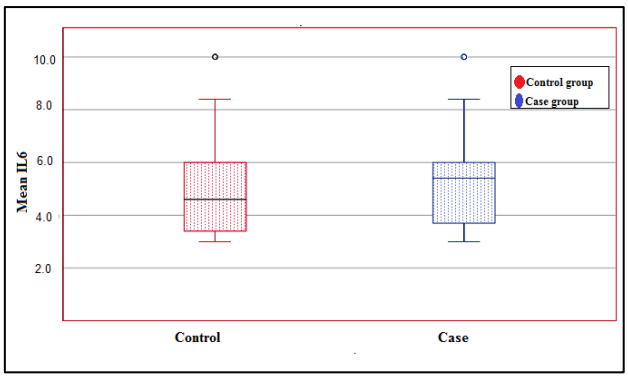

Fig. 2. Mean of IL6 between studied groups.

Table 2: CRP, IL-6, IL-8 levels among studied groups.

	CONTROL N=200		CASE N=200			
	Mean± SD	SE	Mean ± SD	SE	P value	CI (95%)
CRP	3.0 ±0 .6	0.49	3.1 ±0.9	0.06	.153	(-.282-.044)
IL 6	4.90±1.65	0.11	5.30±1.91	0.13	.026	(-.751-.048)
IL8 **Median (Min- Maxi)** **Mann-Whitney**	30 (30-250)		30 (30-450)		0.00	-

SD: standard deviation,**SE** : systemic error, **CI** : confidence interval, **CRP:** C reactive protein, **IL8:** interleukin 8, **IL6:** interleukin 6. **P<0.05 i**s considered significant.

Table 3: Correlation of studied cytokines with clinical parameters

		Age	BMI	GA	Systole	Diastole	FBS	Hgb	platelet	WBC	CRP
IL6	R	.098*	-.020-	-.011-	.015	-.027-	.090	.035	.100*	-.007-	.040
	P value	.049	.694	.827	.765	.590	.071	.479	.047	.897	.421
IL8	R	-.093-	.035	.075	-.016-	-.066-	-.037-	-.036-	.027	.032	.065
	P value	.063	.491	.134	.757	.185	.459	.470	.587	.523	.194

BMI: Body mass index**, GA:** gestational age**, FBS:** fasting blood sugar**, Hgb:** hemoglobin, **WBC:** white blood count, **CRP:** C- reactive protein**, IL8:** interleukin 8**, IL6:** interleukin 6. **P<0.05** is considered significant.

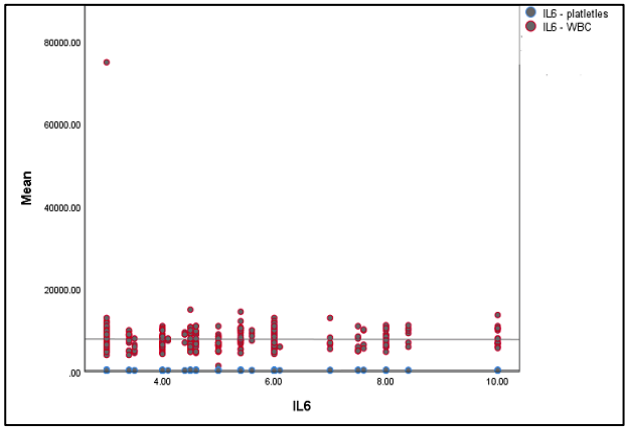

Fig. 3. Pearson Correlation analysis of IL6 with platelets and WBC

On the other hand, no significant correlations between CRP and IL-8 and in dependable variables was observed in the regression model.

Regarding pregnancy outcome, there was a significant difference between pregnancy outcome in pregnancies among women in the case group compared to controls p>0.05. As there was normal pregnancy outcomes 91 % in controls group versus 76 % in case group p>0.05 as shown in **Table 4.** With pregnancy loss being the most unfavorable outcome documented, 13 incidences in case groups versus 3 among control group.

Table 4: Pregnancy outcomes among studied groups.

Outcome	Case N=200	Control N=200	P value
-Normal	153(76 %)	183(91%)	
- Poor	47(23.5%)	17(8%)	**< .05**
Pregnancy loss	13	3	
GHT	4	-	
Still birth	4	3	
Neonatal death	3	2	
Preterm	8	5	
Gestational diabetes	1	1	
Low birth weight	6	1	
Cesarean	8	2	

GHT: Gestational hypertension ,**P<0.05** is considered significant.

In our study, after analyzing data of maternal IL6, IL8 levels for all pregnant women in the control group and case group, we found out that maternal IL6, IL8 levels remained sustainably elevated in the maternal blood even after recovery from COVID-19. Whereas there was no statistical significance observed in CRP levels between the studied groups (p>0.05). Also higher level of maternal IL-6 found among case group showed a direct correlation with the age of pregnant females and it was also directly correlated with increased platelet count among cases group. CRP levels showed no statistical significant difference between the cases and controls group in the post covid recovery phase. We also observed that there was no statically significance between case and control group regarding the pregnancy outcome, however its worth to note that pregnancy loss incidences was more among post covid recovered cases than the incidences among control group; 13 pregnancy loses versus only 3 pregnancy losses in the control group.

DISCUSSION

SARS-CoV-2 vertical transmission from mother to fetus for had been a major concern for caregivers and pregnant women. During gestation COVID-19 pregnant women present with a wide range of unfavorable clinical manifestations; abortion, premature birth among other neonatal fatalities. Overstimulation of the immune system and aggressive inflammatory responses in response to SARS-CoV-2 is dangerous as both actions have can cause injury to the fetus in utero, affecting intrauterine fetal maturation processes during early stages of pregnancy [19] [20]. Samples taken from umbilical cord blood from cohorts of gestational females infected with COVID-19 during third trimester showed altered expression for fetal inflammatory cytokine levels for IL-6 and IL-8 and leukocyte cells count [21]. Pregnant women exposed to COVID-19 during pregnancy, COVID-19 can negatively affect fetal central nervous system normal development along with other mental disabilities taking place later during adulthood[22] [23]. After recovery from COVID-19, there what is called post COVID-19 syndrome where the patient still suffers from side effects of COVID-19 exposure which ranges from symptoms like anemia, deep vein thrombosis , chronic unresolved cough and chest infection to severe

deterioration to death incidences[24],[25]. To our knowledge, there is no previous research has measured maternal cytokine levels during the post-COVID period and its impact on pregnancy in Egypt. Therefore, in our study we aimed to assess the levels of inflammatory cytokine (IL6, IL8 and CRP) in early recovery period from SARS-CoV-2 infection among pregnant females in comparison to non-infected pregnant women.

Influence of COVID -19 on the general medical picture during gestation was evaluated by several studies. It was observed that pregnant COVID-19 patients with chronic medical conditions such as high blood pressure or diabetes had a poor general clinical condition in contrast to COVID-19 patients who were not pregnant [26]. Sun and colleagues [23] conducted a study on 120 healthy free controls compared to 60 pregnant females infected with COVID-19. They observed that among COVID-19 pregnancies 71.67% underwent caesarean birth; 21.67% got diabetes and 18.33% got high blood pressure. COVID-19 pregnant females had substantially elevated neutrophil counts and C-reactive protein values. A study documented that pregnant individuals with COVID-19 had a greater chance for preeclampsia as well as higher maternal death rate compared to pregnant individuals without SARS-CoV-2 infections [27].

Similarly, to our findings; Rosen and colleagues[28] found that levels of IL-8 was significantly higher among forty-four positive COVID-19 pregnant females in comparison to twenty-five COVID-19 negative pregnant females in the control group and authors highlighted that elevation titer of cytokine was openly correlated to disease intensity .In a Turkish study, thirty normal pregnant females free from COVID-19 infection and seventy COVID-19 positive pregnant female were incorporated in the study. All are in third trimester of pregnancy, blood concentrations for IL-6 as well as IL-10 tested for all subjects. Authors reported that level of IL-6 was considerably reduced among COVID-19-negative pregnant females (control) in comparison to pregnant females positive for COVID-19 (p=0.01) [2930].

In December 2020, a comprehensive review research gathered evidence from Web of Science, Pubmed, and Embase regarding the (IL-6, IL-8) levels among COVID-19 individuals. After analyzing the combined data, authors' weighted mean difference, and its 95% confidence interval computed. They revealed that overall 61 investigations with 14,136 participants (14,041 individuals with the disease and 95 healthy individuals) high blood levels of (IL-6, IL-8) were observed among COVID-19 cases in comparison to negative healthy individuals. The review also stated that IL-6 and IL-8 was noticed in a higher level among non-survivor individuals in comparison to individuals who were able to survive after Covid-19 infection which is similar to our results[31,32]. Furthermore a study conducted in China stated that blood tests revealed elevated C-reactive protein among women having COVID-19 in comparison to health controls. Premature birth was the most unfavorable outcome among women with confirmed COVID-19 positive test [3334]. In a distinctive study, researchers used deciduous tissues from spontaneous abortions to extract CD56+, uNK cells. All obtained cells had IL-8, IL 6 levels measured. Authors found out that deciduous tissue of spontaneous abortions generated less amounts of IL-6 (P ¼ 0.04, P ¼ 0.01,) along with IL-8 (P ¼ 0.0007, P ¼ 0.002) when compared to healthy fetuses of COVID-19 negative mothers (controls)[35 36] .

In agreement with our finding, Paixão and colleagues [37] done a study included 226 gravid females. Authors documented that CRP does not act as a potential indicator for the adverse pregnancy outcome in cases affected COVID-19 among other studies reached the same conclusion regarding CRP[38,39]. However in contrast to our finding; a study investigated the importance of CRP/albumin ratios in monitoring individuals admitted to critical care in comparison to individuals who hadn't been admitted critical care. Patients hospitalized in critical care units had considerably greater concentrations of CRP-albumin ratio P < 0.01. They concluded that CRP-albumin ratio is an significant biomarker used for prediction and observing pregnant COVID-19 [40].

In a study involving 1,484 COVID-19 subjects, it was proven that high levels of IL-8 was directly linked with reduced surviving rate[41]. Sabharwal and colleagues[42] conducted a study compared cytokine levels among mothers who received the SARS-CoV-2 vaccination while pregnant with unvaccinated mothers during postpartum period. They observed a decrease in cytokine levels among vaccinated women in comparison to unvaccinated group control group.

Limitations

We were incapable to create a third group of pregnant women who got the vaccine against COVID-19 because many women who were planning to get pregnant refused to get the vaccine. This would have enriched the study, to assess if the vaccine affects the level of immune and inflammatory response to infection.

CONCLUSION

In conclusion Post COVID-19 pregnant women possessed higher levels of inflammatory cytokines (IL6 & IL8) in comparison to non-infected pregnant women, potentially having an effect on some health parameters and affecting pregnancy outcomes. It is important to proceed with caution when interpreting these data because there are not many studies on recovery periods following COVID-19. To reduce the possibility of unfavorable outcomes in pregnant women who report with a recent history of COVID-19 it is advised to

regularly track cytokine levels of IL-6 and IL-8 in COVID-19 survivors.

Ethics approval and consent to participate:

All measures followed were in accordance with the ethical standards of Medical Research Ethical Committee in National Research Centre (NRC) and with the Helsinki Declaration of 1975, as revised in 2008. Informed Consent is obtained from all patients included in the study. Ethical approval numbered (131210112022).

Conflicting interests:

The authors declare that they have no competing interests.

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Availability of data:

The data generated throughout the current study are not visibly accessible due [patient‘s privacy] but are obtainable from the corresponding author on reasonable request.

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