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Research Article

INFLAMMATORY CYTOKINE RESPONSE IN DENGUE INFECTED FEMALE OF REPRODUCTIVE AGE: INSIGHT WITH REFERENCE TO A PREGNANT CASE

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ABSTRACT

Dengue treatment and management is challenging during the epidemic especially among the risk groups and to mitigate the risks becomes even more complicated in absence of specific medicine and vaccines. Till date, there are no specific research endeavours in Nepal focusing on pregnant women with dengue and there is evident gap in research concerning the treatment of pregnant women due to overlapping clinical and hematological manifestations with physiological changes of gestation. The study comprises clinically diagnosed one dengue positive pregnant female, five female patients with dengue in Sukraraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu and five healthy volunteers of same reproductive age. The study investigates six different inflammatory cytokines; IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF levels in plasma and were quantified using Cytometric Bead Array. The Cytokine profiles and blood parameters were statistically analyzed using confidence intervals of 93.75% as permitted by sample size and visualized with box and whisker plot with a highlighted pregnant case. The study shows IL-6 and IL-10 levels are elevated on dengue cases as compared to healthy while TNF and IL-12p70 levels are elevated exceeding the upper limit of the acute dengue cases in the pregnant subject, underscoring heightened inflammatory activity during synchronous pregnancy and dengue infection. There were significant reductions in hemoglobin and leukocyte counts in the pregnant dengue case compared to non-pregnant. These findings suggest cytokine modulations occur during pregnancy and underscores the importance of these patterns provide insight into targeted therapeutic and diagnostic strategies to mitigate the risks for vulnerable population.

Keywords: Dengue, inflammatory cytokines, pregnant, hematological, Flowcytometry

INTRODUCTION

Dengue has a wide spectrum of clinical presentations thus its diagnosis and management are complex. Prompt and precise diagnosis plays a pivotal role in mitigating mortality rates, especially during the epidemic period when the treatment decision should be prioritized based on the severity, risk groups and resources limitation (Kaur & Kaur, 2014; Rajapakse *et al.*, 2017). Although dengue primarily spread through horizontal transmission, this systemic viral infection can also be transmitted vertically (Kaur *et al.*, 2014). There is higher risk for severe disease in neonate or young child, elderly, pregnant, obese, female sex, high body mass index, viral load, genetic polymorphism (vitamin D receptor and FcγR) and previous infection with any DENV serotypes (Villamor *et al.*, 2017). DENV was first detected in Nepal in 2004 and has become endemic since 2006 with major outbreaks in every three years since 2010 with 54,784 cases in 2022 as compared to 17992 cases in 2019 (EDCD, 2024; Rauniyar *et al.*, 2023). Our preliminary data suggest that DENV1 and DENV3 were though co-dominant in 2022 as opposed to dominance of DENV-2 in 2019, all of these 3 serotypes are circulating in Nepal (Napit *et al.*, (2024). The DENV associated inflammatory response and its impact on dengue disease severity is poorly understood (Khanam *et al.*, 2022). It is hypothesized that in response to DENV infection the primary phagocytes (monocytes, mast cells, and neutrophils) are primarily responsible for overproduction of systemic inflammatory proteins (a cytokine storm) which can damage the blood vessel endothelial cells, eventually leading to vascular permeability.

The haemoglobin level of female is comparatively low than male and while during pregnancy, physiological measures like hemodilution can conceal signs of thrombocytopenia, leucopenia, and hemoconcentration that are indicative of dengue thereby masking the presence of the disease (Paixao *et al.*, 2018). Previous studies have shown that levels of cytokines such as TNFα, IL-1β, IL-6, IL-10, IL-12p70 and chemokines such as the C-X-C motif chemokine ligand 8/Interleukin-8 (CXCL8/IL-8) are elevated in DENV infection (Brat *et al.*, 2005; Chen *et al.*, 2006; Hober *et al.*, 1993). Similarly, TNFα and IL-1β have been shown to

directly cause vascular leak both these cytokines along with IL-18, IP-10, IL-8, and MIP-1β are potent inflammatory cytokines produced by many immune cells (Malavige *et al.*, 2020). During the first trimester, cytokines act as vital mediators, balancing the inflammatory and anti-inflammatory responses needed for a healthy pregnancy (Simmons *et al.*, 2012). Pro-inflammatory cytokines such as IL-1β and TNF-α facilitate inflammatory environment necessary for trophoblast infiltration and placental establishment (Haider & Knöfler, 2009), while IL-10 mitigates excessive inflammation, providing protection to the developing foetus (Cheng & Sharma, 2015). Cytokines like IL-8 stimulate the formation of new blood vessels to support embryonic growth (Lane *et al.*, 2002). Although, many inflammatory cytokines, chemokines, lipid mediators and immunological markers are significantly altered in patients with dengue positive population as compared to dengue positive pregnant female population, a substantial correlative data is still too sparce and there is no research in Nepal with regard to the pregnant female. Therefore, during the epidemic, timely diagnosis and intervention can improve the acute and maternal pregnancy phases.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by the Nepal Health Research Council (NHRC Reg. No. 121/2019). The approved written consent read to the patients and were signed by them before collection of the biological specimens for the study. Anonymity of the subjects were maintained.

Study sites and inclusion of study population

A prospective hospital-based study was designed enrolling the clinically diagnosed patients with dengue at Sukraraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu. Only the female subjects of reproductive age group from 18-45 years with or without pregnancy were the study population. Dengue suspected symptoms detected as of the WHO, 2009 dengue fever guideline (Horstick *et al.*, 2014) and/or confirmed referred cases for treatment based on hospital lab report were enrolled. A study cohort of female population comprising 5 dengue positive, 1 dengue positive pregnant and 5 healthy control subjects from Kathmandu district was designed. The samples were collected from May to August 2019 during the outbreak. The epidemiological, demographic and hematological data of the patient were recorded from the case report form (CRF) filled during the counselling of the patients.

Sample collection

Blood sample from peripheral vein were collected for the complete hematological profile in the hospital and plasma was isolated from the 2-5 mL blood collected in separate EDTA vacutainers (BD Vacutainer, USA) from each patient by centrifugation (2500 rpm 10 minutes at ambient temperature). The plasma aliquots (75 µL each for Flow cytometry and Serology) were stored at -20°C in the local hospital. The dengue was confirmed through (Standard Q Dengue Duo) RDT kit in hospital settings by hospital staff. The samples were transported to Central Department of Biotechnology, Tribhuvan University (CDBT-TU), Kirtipur, Kathmandu maintaining cold chain within a week of collection and stored at -80°C freezer until used. All the laboratory activities were performed at CDBT-TU.

Quantification of Cytokines

The levels of inflammatory cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF were determined in the plasma of study subjects using a Cytometric Bead Array (BD Biosciences, USA) Catalogue number 51-9002150 according to the manufacturer's instructions using FACS Calibur-E3318 Flow Cytometer System (BD Biosciences, USA). The acquisition of six different cytokines capture beads with recombinant standards or samples were measured for the intensity of PE fluorescence of each sandwich complex which reveals the concentration of that cytokine after acquiring samples on a flow cytometer (Fig. 2). The cytokines beads standard at different dilution (1:2 to 1:256) were run to get a standard curve that represents the cytokine concentration and then sample cytokine concentration was calculated based in reference to the standard curve concentrations of individual cytokine. The raw data were measured as the relative fluorescence intensity (RFU) and were recorded in excel sheet to be converted to cytokine concentration based on a standard curve generated from the reference concentrations provided in the kit.

Statistical Analysis

In this study, we have independent small samples, and the variables to test are continuous. The non-parametric distribution-free one-sample sign test tool is used in this study. We performed one-sample sign tests on each healthy and dengue infected patient for hematological parameters and cytokines. To infer if the pregnant dengue case has significant increase or decrease values compared to dengue fever, we obtain the confidence intervals for the median based on the binomial distribution. As we have a small sample size, we have provided the 93.75% confidence interval as permitted by the sample size. If any parameter value of the pregnant dengue fever case is not within the confidence interval, we infer it as a significant and outlier case. For the visualization, we have box and whisker plots and marked the plots with clinical values of a pregnant dengue fever case.

RESULTS

Demographic and clinical manifestation of the dengue patients

The study subjects were of age 26-34 years and all from Bagmati Province with mean age 29.2 ± 3.54 years. All were experiencing continuous fever, myalgia and lethargy while 80% showed symptoms of headache and joint pains and 60% experienced arthralgia, retro-orbital pain and nausea. The single pregnant case was of the age 27-year-old who presented to the Sukraraj Tropical and

Infectious Disease Hospital (STIDH) with all the above clinical signs along with retro-orbital pain.

TLC differentiates healthy, dengue infection and dengue infected pregnant populations

The descriptive statistics applied for healthy individuals (n=5) and acute dengue (n=5), and their comparison with the pregnant female (n=1) on hematological data and analysis as permitted by sample size using a one sample sign test with a confidence interval of 93.75% showed variations. The visualized data highlighted the pregnant case in the box and whisker plot. Based on the analysis, the gradual decrease of TLC discriminated the healthy, dengue infection and dengue infected pregnant population by decrease of the cell numbers at the rate of around half folds. In dengue infected person, the TLC (mean- 3080 ± 976 cells/µL, median -3300 cells/µL) decreased as compared to healthy individuals (mean- 6760 ± 1756 cells/µL, median- 6780) while it was more significantly decreased in case of pregnant female (1800 cells/µL) though lied within the confidence of interval (Table 1; Fig. 1B). The haemoglobin levels were lesser in dengue patient (mean-12.5 ± 0.9 g/dL, median-12.5 g/dL) as compared to healthy individuals (mean 13.5 ± 1.2 g/dL, median-13.7 g/dL) while its level in the pregnant female (6.8 g/dL) lied completely outside the lower limit range of healthy as well as dengue patient expressing it as a distinct hematological parameter among the study cohort (Table 1; Fig. 1A). Similarly, the mean neutrophils level is decreased on infection with dengue (mean- 45.2 ± 17.7%, median- 37%) unlike in dengue positive pregnant female where the neutrophils count is considerably high (67%) (Table 1; Fig. 1C). However, lymphocytes, eosinophils and monocytes mean values are higher on infection 43.6 ± 15.6%, 9.2 ± 1.3%, 2.0±2.6% (median-48%, 10%, 1%) as compared to healthy individuals 40.2 ± 5.7%, 5.6 ± 3.0% and 1.4 ± 1.1% (median- 39.1%, 5. 8%, 1.2%) respectively but their level is lower in the pregnant female though not significant (Table 1; Fig. 1 D, E, F). All the hematological parameters lied within 93.5% confidence interval for pregnant female except the haemoglobin.

Table 1. One-sample sign test for confidence interval and summary statistics of hematological parameters for healthy and dengue fever with reference to the pregnant case

Hematological parameters	Healthy				Dengue				Pregnant DF value
	Lower	Upper	Mean	SD	Lower	Upper	Mean	SD	
TLC	4500	9300	6760	1756	1600	4100	3080	976	1800
Haemoglobin	11.7	14.6	13.5	1.2	11.6	13.8	12.5	0.9	6.8
Neutrophils	45	56	52.8	4.5	29	73	45.2	17.7	67
Lymphocytes	34	48	40.2	5.7	20	60	43.6	15.6	24
Eosinophil	1	9	5.6	3.0	7	10	9.2	1.3	9
Monocytes	0	3	1.4	1.1	0	7	2.0	2.9	1

The units of the hematological parameters: TLC cells/uL, Hemoglobin g/dL, Neutrophils-%, Lymphocytes-%, Eosinophils-%, Monocytes-%.

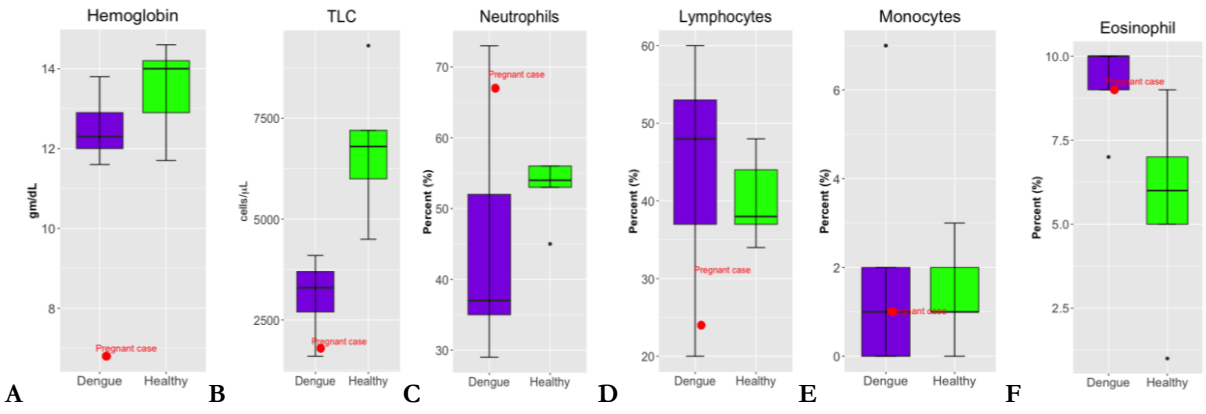

Figure 1. Box and whisker plot of hematological parameters of healthy and dengue cases of female reproductive age group with respect to a dengue positive pregnant female (the red dot plot). The box plot is with median value.

Cytokine expression and analysis as detected by Flow cytometry

The cytokine standards of decreasing concentration acquired beads on gating FL-2 channel vs FL-4 Channel, 1:2 (Fig. 2A) showed that fluorescence intensity is much higher than 1:16 (Fig. 2B) as the fluorescence intensity lied farther from IL-4 channel. When the intensity gradually decreased, the respective cytokine tagged with beads move closer to FL-4 channel with decreased concentration 1:256 (Fig. 2C). The fluorescence intensity of sample (2D) is more spread, clustered, low and closer to FL-4 channel as similar to corresponding cytokine level.

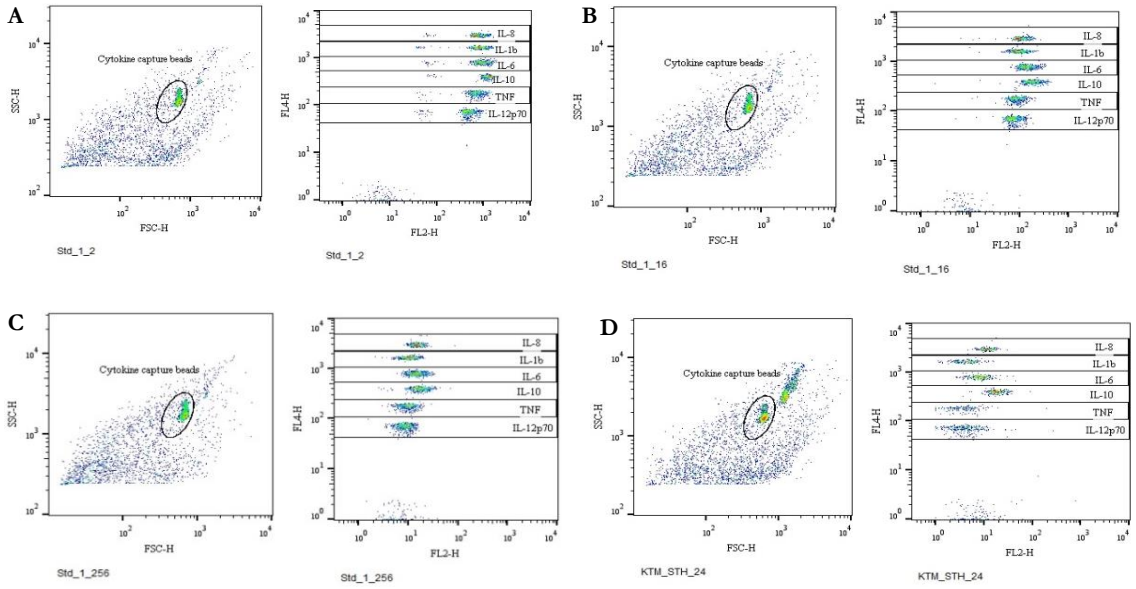

Figure 2 A, B and C. Flow-Jo analysis of the acquisition of data of cytokine standards from 1:2, 1:16, 1:256 dilution as representative plots of dilutions used in experiments (1:2, 1:4, 1:16, and 1:256). D. Test sample acquisition plot of dengue sample as a representative test of acquisition plot of pregnant female and healthy sample.

3.4 IL-6 and IL-10 defined the dengue while IL-12p70, TNF and IL-1β as indicator cytokines for pregnant dengue patient

The six inflammatory cytokines; IL-1 beta, IL-6, IL-8, IL-10, IL-12p70 and TNF in the study cohorts detected the variations among a pregnant, dengue, healthy samples using a one sample sign test with a confidence interval at 93.75% as permitted by sample size and visualized highlighted pregnant case in the box and whisker plots. The IL-6 level (mean-6.9 ± 1.6 pg/mL, median-6.42 pg/mL) was significantly less among healthy females as compared to acute dengue cases (mean-10.5 ± 4.9 pg/mL, median-10.52 pg/mL) with variability in the inflammatory response while for pregnant case it lied near to the median value range (7.4 pg/mL) of healthy group (Table 2; Fig. 3B). IL-10 levels (mean-4.3±0.6 pg/mL, median-4.27 pg/mL) was significantly less with greater stability among healthy females as compared to acute dengue cases (mean-13.6±14.2 pg/mL, median-13.61 pg/mL) while for pregnant it lied way below the median range of dengue patients (Table 2; Fig. 3D). Therefore, the IL-6 and IL-10 could discriminate the dengue infected population and healthy population. IL-8 level was also less (mean-15.2± 1.8 pg/mL, median-14.94 pg/mL) with greater stability among healthy females as compared to acute dengue cases (mean-17.1± 3.0 pg/mL, median-17.1 pg/mL) while for pregnant it lied above the upper whisker of dengue group though below the highest range of a dengue patient (Table 2; Fig. 3C). IL-12p70 was slightly lesser among healthy females (mean-5.1±0.7 pg/mL, median-5.04 pg/mL) as compared to acute dengue cases (mean-6.4±0.9 pg/mL, median-6.39 pg/mL) reflecting variability in the inflammatory response while for pregnant it lied above the upper whisker of dengue group (Table 2; Fig. 3E). TNF also had lesser level (mean-5.9±0.8 pg/mL, median-5.85 pg/mL) with greater stability among healthy females as compared to acute dengue cases (mean-6.6±0.4 pg/mL, median-5.85 pg/mL) reflecting stable inflammatory response while for pregnant it lied above the upper whisker of dengue group (Table 2; Fig. 3F). The confidence interval table showed that the pregnant women values of IL-6, IL-8, and IL-10 cytokine were within the confidence interval of the dengue fever and not significantly different than dengue patients while IL-12p70 and TNF values for the pregnant dengue values are beyond the upper limit of their respective dengue patients and, hence significant (Table 2, Fig. 3). The box and whisker plot, and the one sample sign test showed lesser IL-1β level (mean-9.0 ± 1.0 pg/mL, median-9.32 pg/mL) among healthy females as compared to acute dengue cases (mean-9.0 ± 2.0 pg/mL, median-8.7 pg/mL) while remarkably elevated range in the pregnant positioning its value at the top margin of the upper whisker of dengue group along with the higher range of dengue patients (Table 2; Fig. 3A).

DISCUSSION

The rainy season in Nepal grips fear with growing concern about the threat of dengue among people. The dengue diseases has been a serious health issue in Nepal with the official cases of 54,784, 51,243 and 34,385 thousand cases in the last three consecutive years from 2022 to 2024 causing deaths of 88, 20 and 13 patients respectively (EDCD, 2024; Rimal *et al.*, 2023). There is debate for the trail and launch of vaccine against dengue in the world and obviously Nepal has as well. It is imperative to understand the immune responses due to dengue infection and there have not yet been any studies on the role of inflammatory cytokines immune response in Nepalese population. A pregnant woman detected with dengue infection among this small sized study population tracked an additional exploration for comparative study of those cytokines in dengue infected pregnancy period with healthy population which is ever the first study in Nepal. Hematological data were also analyzed as a supportive parameter for the study.

Table 2. One-sample sign test for confidence interval and summary statistics of Inflammatory cytokines for healthy and dengue fever with reference to the pregnant case.

Inflammatory Cytokines	Healthy				Dengue				Pregnant DF value
	Lower	Upper	Mean	SD	Lower	Upper	Mean	SD	
IL-1 beta	7	10	9	1	7	12	9	2	11.8
IL-6	4.4	8.7	6.9	1.6	6.5	16.6	10.5	4.9	7.4
IL-8	12	16	15.2	1.8	15	22	17.1	3.0	19.0
IL-10	3	5	4.3	0.6	5	39	13.6	14.2	6.2
IL-12p70	4	6	5.1	0.7	5	7	6.4	0.9	7.5
TNF	5	7	5.9	0.8	6	7	6.6	0.4	8.0

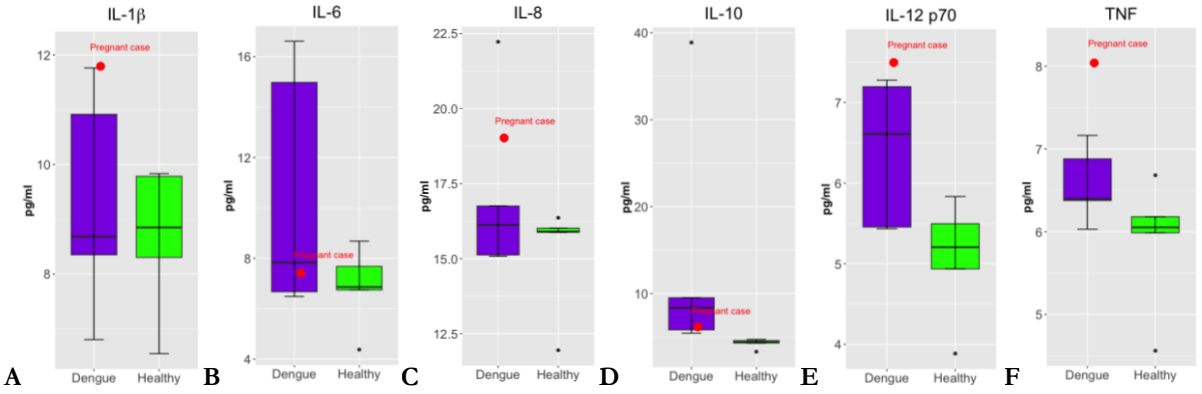

Figure 3. Box and whisker plot of inflammatory cytokine levels of healthy and dengue cases of female reproductive age group. The red dot indicates the level of the cytokines of a dengue positive pregnant female. The box plot is with median value.

Dengue patients report with differences in hematological parameters while compared with healthy people. In coherence to the other studies, leukopenia has been noted in all cases with decrease of TLC to mean value of 3080 cell/μL (Bhattarai *et al.*, 2023), with further sharp significant decrease in pregnant case. A study on maternofetal outcomes during dengue infection in pregnancy highlighted anemia and leukopenia as common hematologic observation, with pregnant women exhibiting more distinct reductions due to the combined effects of pregnancy and dengue infection (Sharma *et al.*, 2016). These findings highlight the compound suppression of the immune system resulting from both pregnancy and dengue infection. The notable reduction in the haemoglobin relates to hemodilution and dengue induced erythropoietic suppression. Eosinophil was found significantly elevated in dengue patients (Abu-Raya *et al.*, 2020), while lymphopenia was observed in some cases. Lymphocytes count is significantly lowered in pregnant dengue cases in consistent with dengue-associated lymphopenia. The virus-induced suppression of lymphocytes contributes to immune dysregulation during infection which becomes more pronounced in pregnancy due to altered immune (Cornish *et al.*, 2020). Pregnancy can amplify inflammatory responses, leading to increased neutrophil counts during infections, including dengue. This immune modulation is a physiological adaptation to protect both mother and fetus (Bert *et al.*, 2021). Monocytes and Eosinophils show minimal or no significant changes across groups. Minimal or no significant changes in these cell populations across different groups have been noted in studies, consistent with findings that dengue infection primarily affects

lymphocytes and neutrophils but monocytes, eosinophils, and basophils often remain within normal ranges (Salazar Flórez *et al*., 2024).

All the six different cytokines are elevated during the dengue infection however, IL-10 and IL-6 showed significant difference between healthy and dengue infected female study population. This is the reflective of the immune system's efforts in the recruitment of immune cells and combating viral replication effectively through secondary response. The lower level of IL-6 in pregnant is the reflection of the adaptive response of human during pregnancy that helps to balance immune activation against the infection while minimizing excessive inflammation that could harm fetal development (Prins *et al*., 2012). The elevation of IL-10 is highly significant to counterbalance the increased inflammatory response and cytokine induced damage such as vascular permeability and endothelial dysfunction (Tinsley, 2010). Therefore, pregnancy maintained elevated IL-10 to protect the fetus, but levels remain balanced compared to acute case. In the pregnant dengue patient, IL-1β levels exceeded like those in severe dengue cases, indicating a compound inflammatory response of endothelial activation (Wong *et al*., 2023). The higher value in pregnant cases highlights the dual role of IL-8 in supporting the vascular demands during pregnancy and the response to the infection through heightened neutrophil recruitment during pregnancy. However, excessive levels could pose potential threat of vascular complications (Vilotić *et al*., 2022). TNF plays a significant role in enhancing vascular permeability and coagulation pathways, contributing to severe dengue pathology. Elevated levels in pregnancy could signify compounded risks of endothelial damage and inflammation. There is a complex cytokine landscape interplay between pregnancy and dengue infection. As per our study, on infection with dengue IL-1β, IL-6, IL-8, IL-10 cytokines are modulated to balance inflammation and immune activation while other cytokine TNF and IL-12p70, show pronounced elevation highlighting the need for targeted therapeutic strategies in managing dengue in pregnant women.

This study has limitation of small size population with single case of pregnant subject; however, this kind of work is carried for the first time in Nepal which enrich the immunological data of Nepalese population.

CONCLUSIONS

Nepal is a dengue endemic country and has been severely gripped in its densely populated pocket areas by the disease. We focused on the very specific study cohort of reproductive age group females infected with dengue virus to understand the inflammatory cytokines' immune responses in Nepalese women which is the first kind of research in Nepal. Study revealed that elevation of IL-6 and IL-10 along with leukopenia would be the marker cytokines to define the dengue infection in the reproductive age group female population. Similarly, the pregnant female expresses elevation level of IL-12p70, TNF and IL-1β as indicators along with the decrease of haemoglobin when infected by dengue virus. These findings suggest cytokine modulation play role during the dengue infection and differently in dengue infected pregnancy period of reproductive age group of females. The patterns provide insight for targeted therapeutics and diagnostic strategies to mitigate the risks for vulnerable population. Further ahead, the inflammatory cytokines data are useful while Nepal plans implementation of strategic treatments and/or preventive applications like vaccine.

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AUTHOR CONTRIBUTIONS

RR: Collected the data, drafted the manuscript and performed the experiment, and hospital administrative liaison; BSC: Provided clinical guidance. BM: Performed the statistical analysis and calculation; KDM: Designed, supervised, guided the experiments; IBJ, SCJ, BM, and KDM: Revised the manuscript; PKW and WT: Supported the CBA kit and experimental work on flowcytometry.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

The data that supports this study is with corresponding author Prof. Krishna Das Manandhar and can be made available upon reasonable request.

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