Cytokine Profiling in Clinical Isolates of pregnant and non-pregnant subjects at Hazaribag district of Jharkhand

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Objectives: -The study was undertaken to establish data on the comparative status of cytokine concentration in healthy pregnancy, labour and non-pregnant subjects. We investigated and established the values of IL-1 beta, IL-6, IL-8, TNF-alpha and IL-10 during all phases of human pregnancy, labour as well as in the non-pregnant control state.

Methods: - A cross-sectional assessment of serum IL-1 beta, IL-6, IL-8, TNF-alpha and IL-10 in healthy pregnant women at antenatal visits and labour with corresponding clinical history deciphered through questionnaire.

Results: - Statistically peak value of IL-1 beta has been recorded in 3rd trimester and labour ($p<.0001$) when compared to non-pregnant subject. The study demonstrated significant increase in IL-6 across 2nd trimester, 3rd trimester and labour as compared to non-pregnant subject. A statistically significant peak value of IL-8 was recorded in 3rd trimester and labour as compared to non-pregnant subject ($p<.0001$).TNF –alpha presented a stable production profile at all phases of pregnancy and labour. Statistically elevated values of IL-10 was recorded in 3rd trimester and labour ($p<.0001$).

Conclusion:- As the pregnancy progresses, maternal shifts away from type-2 dominated immune response to an inflammatory response, thus supporting the concept that labour resembles an inflammatory response. Further research is the current demand to establish reference values differentiating physiology from pathology and refining oversimplified concept of immunesupression or Th2 – biased phenomenon of pregnancy. Utilizing this knowledge and further research may be boonful in setting up of therapeutic interventions in the future.

Introduction:-

Cytokines are the proteins produced in an autocrine or paracrine function,bind to specific receptors initiating a cascade of reactions on different targets having beneficial or harmful effects and its imbalance has been accused for many pathological disorders. Microbial infection, certain auto immune diseases have been linked to over or under production of cytokines which are produced in response to various immune stimuli. The naturally occurring immune reaction in an organism is developed during the gestational period where, inspite of the presence of a semi-allogeneic graft, maternal immune response is regulated to support the fetal allograft. The success of an embryo development is allocated to the important involvement of cytokines where some have been designated critical and others deleterious to fetal growth. TH cells are the major producers of cytokines, and the balance of Th1 versus Th2 cytokines describes the welfare of an organism (1,2) Pregnancy is the natural phenomenon of an immune reaction occurring for a determined time course antagonizing the principles of graft rejection. The semi or allogeneic fetal components emerging in the privileged site of uterus, not only evades maternal immune attack but are assisted by the maternal immune system. Cytokine plays an important role in the maintenance of pregnancy, where a successful pregnancy is correlated to the production of Th2 cytokines and fetal rejection to Th1 cytokines. Although the protective role of the Th2 cytokine IL-10 during mid-gestation (3)as well as the deleterious effects of Th1 cytokines IL-2 (4) TNF-alpha (5,6,7) and IFN-gamma (5,8) have been evidenced in mice, other studies documented

that each cytokine explicits a definite pattern of expression each day of pregnancy in mice (9) These data are an indicative of the fact that inspite of the beneficial or harmful effect of a cytokine during a specific time course of the gestational cycle, a determined up or down regulation of these factors must be followed, a pattern on which the success of pregnancy depends. Thus, it is essential to explore the physiological levels of cytokines during the time course of pregnancy, which may eventually have a prognostic role for pregnancy outcomes.

With the advancement of pregnancy, a maternal shift is observed, away from a type 2-biased immune response towards an inflammatory response. During pregnancy, the female immune system encounters an exquisite stabilizing act. On one hand, it must evolve tolerance to paternal antigens to arrest fatal immunologic charges against the fetus. On the other hand, it must sustain the potential to combat infections from a diverse variety of environmental pathogens. Perturbances in this balance may lead to detrimental consequences, including preterm birth and death of fetus. The normal maternal immune response to pregnancy is remarkably a process experiencing changes, in which the maternal pro/anti-inflammatory profile changes at different phases of gestation (10,11,12).Inspite of this, a thorough, longitudinal immunologic profile of normal pregnancy has not been documented. Such evaluation are crucial for understanding the response to specific infectious and immunologic diseases that manifests disproportionately negative outcomes during pregnancy, such as influenza (13) and ulcerative colitis (14). The inflammatory cytokines, 1L-1 beta, IL-6 and IL-8 are intricated in the maintenance of trophoblast in early pregnancy. IL-1 beta has been propounded as the principle cytokine of the inflammatory response (15) which effectively stimulate IL-6 and IL-8 among other cytokines. IL-8 is a chemotactic cytokine moderating the rupture of the membranes and cervical ripening (16) along with IL-6, it plays a crucial role in preterm cervical remodeling (17,18). Serum IL-1 beta, IL-6 and IL-8 seem to play an important role during pregnancy (19,20,21,22) towards term and at delivery (23,24,25) remarkably with increased levels in labour (20,26,16). The current research focusses to evaluate and establish the cytokine profile across trimesters in pregnant subjects and non-pregnant subjects of the participants attending antenatal clinics of Sadar Hospital Hazaribag. To the best of our knowledge, such immune modelling profile have not been investigated in this locality. So, this study is an attempt to establish physiological concentrations of these cytokines at certain time course, where utilizing this knowledge and further research may be useful for therapeutic interventions in the future.

Materials and Methods:-

A cross sectional study was conducted at Sadar Hospital Hazaribag involving 200 Healthy Pregnant women (90 in 1st Trimester, 45 in 2nd Trimester, 65 in 3rd Trimester) and 100 non-pregnant subjects following institutional review board approval. Participants were enrolled in the study protocol after obtaining their written consent, if the clinical examination was normal. Standardized questionnaires were administered for obtaining history and general status of the study participants.

Criteria for inclusion and Exclusion:-

Inclusion criteria was good health, no history of serious disease, no history of infection and autoimmune diseases. Exclusion criteria were withdrawal of informed consent, auto immune disease, Infectious disease.

Cytokines:-

About 5 ml of venous blood was drawn into sterile glass tubes and centrifuged at 3500 rpm for 10 min at 4˚C, 0.5 ml aliquots of the supernatant were frozen in polypropylene tubes at -70˚C and stored until assay. Commercial enzyme linked immunoassays were used according to the manufacturers recommendations to assess IL-1 beta, IL-6, IL-8, TNF- alpha, IL-10 (BDBioscences kit). Before use, all reagents and samples were brought to room temperature. Frequent freeze thaw cycles were avoided. Measurement of serum cytokine concentrations was done using an ELISA method as specified by the supplier at test and reference wavelengths of 450 nm and 550 nm respectively. All samples were run in duplicates and the mean value was used for analysis.

Statistical analysis:-

Statistical analysis were performed using SPSS Version 21. Data were analysed by one way analysis of variance. Post hoc tukey's test was performed for comparison of differences between means of non-pregnant subject and pregnant subjects across trimesters and labour. All values were expressed as mean±S.D. The mean difference is significant at the 0.0001 level.

Results:-

Cytokine concentrations in the serum are the indicators of pathologic state of an organism and may, in many cases, have a prognostic nature for therapeutic interventions. In this research, we focused our interest to human pregnancy where the rates of fetal rejection extraordinarily increases, we assessed the physiological levels of different cytokines across trimesters in pregnant and non-pregnant subjects.

The objective of this work was to establish the physiological concentration of cytokine in pregnant and non-pregnant subjects. In view of this investigation, we discovered, statistically significant differences between the non-pregnant and pregnant subjects for IL-1 beta (1^{st} trimester vs non-pregnant ($p<0.0001$), 2^{nd} trimester vs non-pregnant ($p<0.0001$), 3^{rd} trimester vs non-pregnant ($p<0.0001$), labour vs non-pregnant ($p<0.0001$). Strikingly significant peak value of IL-1 beta was observed in 3^{rd} trimester and labour when compared to non-pregnant subject.

For IL-6, significant differences between non-pregnant subject and pregnant subjects across trimesters were observed ($p<0.0001$). Strikingly, increased value of IL-6 was observed in 2^{nd} trimester, 3^{rd} trimester and labour as compared to non-pregnant subjects. In respect to IL-8, significant differences between non-pregnant and pregnant subjects were noted in 2^{nd} trimester, 3^{rd} trimester and labour ($p<0.0001$). Statistically peak values were obtained for 3^{rd} trimester and labour as compared to non-pregnant subject ($p<0.0001$). However, no significant correlation was observed when comparing non-pregnant subject to 2^{nd} trimester ($p=0.004$).

Similarly, findings in context to TNF-alpha and IL-10, statistically significant differences between non-pregnant subjects and pregnant subjects in 2^{nd} trimester, 3^{rd} trimester and labour existed ($p<0.0001$). Noteworthy, when comparing non-pregnant subject with pregnancy in the 1^{st} trimester, did not show any significant result ($p=0.870$). TNF-alpha presented a stable production profile at different stages of pregnancy and labour. Statistically significant elevated values of IL-10 was noted in 3^{rd} trimester and labour thus reflecting its counterregulatory role.

Discussion:-

The investigation carried out allocates a preliminary normative data for the cytokine profile in pregnant and non-pregnant subjects.It has been propounded that pro-inflammatory cytokines (e.g. IL-6) are important in triggering birth (27,28). Our findings indicate that the 3^{rd} trimester of pregnancy and labour is marked by elevated inflammatory (e.g. IL-1 beta, IL-6, IL-8) cytokine environment. Our results also reflected the peak production of IL-10 during 3^{rd} trimester and labour, an agent known to play a defensiverole during pregnancy (2,29). Above finding corroborates with other reports of systemic immune activation as well as counterregulation at the end of pregnancy (12,30,31,32). Increasingly IL-1 beta level may principally be associated to production from placental tissues (33,34) or mononuclear phagocytes (35,36) in late gestation. Since, our study is a single time point,the exact immunologic mechanisms accountable for this shift cannot be estimated by our current investigation. Furthermore, the changes in the concentrations of the cytokines pinpointed in our investigation could probably be due to changes in peripheral blood cellular composition. Further investigations will be mandatory to validate whether placental tissues, circulating leukocytes, stromal cells, or other sources also accord for the changes indicated in our results. Some studies documented decreasing IL-1 beta levels throughout gestation (37) contradictory to our investigation in which IL-1 beta peak has been observed in 3^{rd} trimester and labour. Our report confirmed increase in IL-1 beta, IL-6, IL-8 notably during 3^{rd} trimester and labour indicating that these cytokines do play a major role during pregnancy (19,20,21,22) towards term and at delivery (23,24,25) remarkably with peak levels in labour (20,26,16). In view of current investigation, our study documents that the inflammatory cytokines IL-1 beta, IL-6 and IL-8

already exists in maternal serum in early pregnancy but undergo little variation subsequently until term. High cytokine concentration (IL-1 beta, IL-6, IL-8) accounted in present research at the 3rd trimester and labour reflects that labour resembles an inflammatory process. Peak value of IL-8 in 3rd trimester and labour observed in our study demonstrates that IL-8 has been proposed as the final common step in prostaglandin and anti progestagen action in parturition (38). The increase in the concentration of IL-6 and IL-8 in preterm (39,40) and term labor (23) is still controversial (41). Some of the earlier studies contradicts our findings, documenting the decrease of TNF-alpha, IL-1beta and IL-6 across trimesters in samples of healthy pregnant women collected during each trimester (37). Individual heterogeneity and lack of longitudinal analysis in our study may be accountable for some differences with prior studies.

Our study corroborates with the report (20) which shows an increase of IL-6 and IL-8 during normal term labour (20). Our current investigations validate the notion that IL-1beta, IL-6, IL-8 do play a role in the maintenance of human pregnancy. They reflect that increased cytokine levels are the function of labor. In respect to TNF-alpha level in our present finding, a balanced cytokine profile has been accorded for the same, an agent accused for pregnancy failure (8,6,7). IL-10, shown to play a protective function during pregnancy (3,29) recorded a peak value in 3rd trimester and labour. One mechanism by which the fetus maintains its immunological advantage in the uterus is to firmly regulate the cytokine concentration at the maternal-fetal interface (42). Although some physiological roles of pro-inflammatory cytokines at the maternal-fetal interface have been explained in association with the growth of the placenta and decidua (43) much of the literature authenticated the concept that excessive production of proinflammatory cytokines such as IL-1 beta, TNF-alpha and IFN-gamma at the maternal fetal interface is detrimental to pregnancy. IL-10 is plausibly an important counterregulator because it down regulates the production of pro-inflammatory cytokines by other cells and a number of studies suggested its production at the maternal-fetal interface (44,45,46). Earlier studies documented that successful pregnancy is predominantly a type 2 immune response-biased phenomenon(47,48,49). In view of current investigation, with the advancement in the stages of pregnancy, a maternal shift away from a type 2-biased immune responses and towards an inflammatory response is observed. Furthermore, anti-inflammatory or type 2 immune response do play a protective role during pregnancy as evidenced by the peak value of IL-10 in 3rd trimester. In view of the collective evidence, further refinement is the need to describe pregnancy in terms of type 2 immune responses, both in the overall description of key cytokines as well as in the time related dynamics.

Our study is limited by lacking the longitudinal analysis of the sample. Our results require further authentication by longitudinal analysis which could better describe individual maternal immune variation.

Cytokine	**Cytokine levels (ng/ml±S.E)**					
	Non Pregnant Women	**1st Trimester**	**2nd Trimester**	**3rd Trimester**	**Labour**	**P-Value**
IL-1 beta	1.66±0.018	1.65±0.017$^{\times}$	1.45±0.015$^{\times}$	1.86±0.023$^{\times}$	1.86±0.0091$^{\times}$	<.0001
IL-6	2.66±0.020	2.32±0.143$^{\times}$	2.84±0.014$^{\times}$	2.85±0.018$^{\times}$	2.82±0.014$^{\times}$	<.0001
IL-8	1.77±0.022	1.75±0.012^{ns}	1.75±0.013$^{\times}$	1.92±0.042$^{\times}$	1.98±0.010$^{\times}$	<.0001
TNF-alpha	0.78±0.013	0.77±0.012^{ns}	0.67±0.037$^{\times}$	0.69±0.015$^{\times}$	0.69±0.008$^{\times}$	<.0001
IL-10	0.78±0.013	0.77±0.013^{ns}	0.76±0.017$^{\times}$	0.89±0.014$^{\times}$	1.008±0.046$^{\times}$	<.0001

$P^{\times}$ Significant when compared to non-pregnant subject.

P^{ns} Non-significant when compared to non-pregnant subject.

Results were significant at $P<0.0001$.

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