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Chorionic Villi Levels of Malondialdehyde, Tumor Necrosis Factor-Alpha and Matrix Metalloprotenase-9 Proteins throughout Gestation in Normal Pregnancy and in Preeclampsia

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Abstract

Introduction and Objective: For over half a century, growing evidence has demonstrated that in pregnancies complicated with pre-eclampsia, plasma and placental concentrations of tumor necrosis factor-alpha (TNF- α) protein is significantly high. In preeclamptic tissues, TNF-α protein has been reported to be associated with lipid peroxidation product, malondialdehyde (MDA). Additionally, in normal human pregnancy, TNF-α protein is reported to induce the synthesis of placental protein matrix metalloproteinase-9 (MMP-9). In the present study, we determined the gestational age specific alterations in these three interconnected constituents: MDA, TNF-α protein and MMP-9 protein, and have additionally compared the placental parameters of normal pregnancy with that of preeclampsia.

Methods: Placental tissues were collected from normal pregnant women who underwent elective termination of pregnancy and from women who delivered at term. Placentas were also collected from women with preeclampsia, as diagnosed by ACOG's criteria. Chorionic villi were isolated from each placenta. Chorionic villi MDA was measured by the Thiobarbituric acid method; TNF-α and MMP-9 protein expressions were determined by enzyme linked immunoassays. For statistical analyses, one way analysis of variance (ANOVA), Independent T Test and Spearman's bivariate correlation were applied. $P< 0.05$ was considered significant.

Results: 212 placentas were analyzed: 179 from uncomplicated pregnancies and 33 from preeclampsia. In normal pregnancy, the three chorionic villi constituents showed gestational age-specific profiles. Suppression of MDA levels were noted with advance in gestational age. MDA levels were 299.79 ± 312.52, 205.71 ± 157.07, and 183.93 ± 141.39 pmol/mg tissue in the first, second and third trimester, respectively. TNF-α protein showed a mid-gestational peak and the protein expression were 36.22 ± 18.59, 55.64 ± 43.66, 29.35 ± 33.61 pg/100 mg tissue in the first, second and third trimester, respectively. For MMP-9 protein, a progressive increase in protein expression was seen with increase in gestational age (20.32 ± 12.64, 22.75 ± 13.35, 28.82 ± 11.12 ng/100 mg tissue for first, second and third trimester, respectively). The chorionic villi MDA levels of the preeclampsia group were comparable to the first trimester group of normal pregnancy. The chorionic villi TNF- α protein levels in preeclampsia paralleled that of the second trimester group, and the chorionic villi MMP-9 protein levels surpassed that of the third trimester values. In the first trimester, TNF-α protein and MMP-9 protein were significantly correlated ($r = 0.337$, $p = 0.013$). A positive correlation was seen between MMP-9 protein and gestational age ($r = 0.501$, $p = 0.000$). Maternal age and maternal systolic blood pressure ($r = 0.296$, $p = 0.03$) were significantly correlated. In the second trimester, TNF-α protein and MDA levels were negatively correlated ($r = -0.284$, $p = 0.019$); and MMP-9

was significantly correlated with both maternal systolic ($r = 0.326$, $p = 0.007$) and diastolic blood pressure ($r = 0.314$, $p = 0.009$). No correlation was seen between the studied biochemical constituents in the third trimesters of normal pregnancy or in preeclampsia. Maternal systolic and diastolic blood pressure showed significant correlation at all trimesters of normal pregnancy and in preeclampsia ($p < 0.0001$).

Conclusion: In normal pregnancy, the findings show that MDA levels and TNF-α protein expression taper off in the third trimester. In preeclampsia, however, the exacerbated state persists. The data imply that the physiological brake (s) that operates as the pregnancy enters the third trimester may perhaps be lost in preeclampsia. Interactions seen between TNF-α protein with MDA or MMP-9 protein indicate a possible regulatory role for the cytokine in normal pregnancy. The findings support the concept that human pregnancy is not a single event. The findings highlight that for a successful pregnancy outcome, the gestational age-specific alterations in the levels of the studied constituents, as well as the specific correlations between the studied biochemical parameters are important for the various pregnancy-related processes to transpire.

Keywords: *Pregnancy-Specific Protein Expressions; Normal Human Pregnancy; Preeclampsia; MDA; TNF-α and MMP-9 Proteins*

Abbreviations

MDA: Malondialdehyde; MMP-9: Matrix Metalloproteinase-9; TNF-α: Tumor Necrosis Factor-Alpha; GAD: Gestational Age in Days; SBP: Systolic Blood Pressure of Woman from whom the Placenta was Collected; DBP: Diastolic Blood Pressure of Woman from whom the Placenta was Collected

Introduction

The development of the placenta is a complex process. Many physiological changes occur during gestation which would be considered pathological in non-pregnant state. At 8 - 10 weeks of gestation, the partial pressure of oxygen in the placental bed is approximately 18 mm Hg. The low oxygen concentration fulfills two important functions; it activates the development of the placenta and simultaneously downregulates mitochondrial oxygen consumption to limit the generation of reactive oxygen species (ROS) [1]. ROS once formed are toxic to cells and cell membranes; but they are helpful signaling molecules with beneficial physiological functions [1-3]. Generation of ROS in human pregnancy is unavoidable because the altered hormonal status brings about an increase in maternal fat depots and causes hyperlipidemia [4]. The abundant presence of membrane phospholipids at the sites where ROS are formed makes them easy targets for lipid peroxidation [1,4]. Lipid peroxidation products are common constituents of the placenta and are present in all three trimesters of normal human pregnancy [5]. When the ROS formed overwhelms the body's ability to control them pathology of pregnancy ensues. Overabundance of ROS has been reported to contribute significantly to the pathogenesis of preeclampsia [1,6-10].

The etiology for preeclampsia is not fully understood and the exact reason for the increase in lipid peroxidation in preeclampsia is still unknown. Investigators have shown that besides the placenta generating a lot of lipid peroxides, the circulating neutrophils in women with preeclampsia also release an excess of oxygen species [11]; and the activated state of the neutrophils is brought about by cytokines, primarily interleukin 1 β (IL-1β), IL-10 and TNF-α [12]. TNF-α is an important cytokine secreted by cytotrophoblasts. It is involved in immunoregulation. It modulates cell growth and differentiation, and also induces the production of ROS. TNF-α regulates the synthesis and degradation of extracellular matrix (ECM); and coordinates in the removal and replacement of senescent ECM components during embryonic development [13-15].

Cytotrophoblasts of human placenta proliferate, migrate and invade the pregnant uterus during placental development. The invasive property of the trophoblasts depends on their ability to secrete proteases, and matrix metalloproteinases (MMPs) are involved in this in-

vasive process [16]. However, the invasive behavior of cytotrophoblasts is limited in time and needs to be mediated by TNF-α [17]. Studies have shown that MMP-9 is instrumental in trophoblast invasion and TNF-α is a regulator of MMP-9 protein [16-18].

Despite decades of research, the pathogenesis and pathophysiology of preeclampsia are still poorly or incompletely understood. We now know that preeclampsia is a 2-staged disorder that originates in the first trimester of pregnancy. Abnormal vascular development of placental blood vessels results in reduced placental perfusion. The second stage of the disease is the maternal response to the condition, characterized by widespread endothelial dysfunction; and the vasoconstrictive state leads to hypertension, proteinuria, and edema [19,20]. It has also become clear that placenta is both necessary and sufficient to cause the disease [10,19,20]. Nonetheless, studies conducted on preeclampsia are mostly carried out with non-placental tissue comparing plasma or serum samples between normal pregnant women with preeclampsia [21,22]. Studies that have included placental tissues compared the first and third trimester placental samples of normal pregnancy with that of preeclampsia, and most did not include the second trimester placental samples [23,24]. Since, pregnancy involves complex changes in physiology throughout all gestational periods, in the present study we determined the gestational age specific alterations in three interconnected constituents, e.g. MDA, TNF-α protein and MMP-9 protein, and have included second trimester placentas of normal pregnancy in the study. We have additionally compared the placental parameters of normal pregnancy with that of preeclampsia.

Methods

The investigative protocol for this cross-sectional study was approved by the Institutional Review Board of the BronxCare Health System. Women were not enrolled. Discarded placental tissue samples after clinical care were collected from normal pregnant women who underwent elective termination of pregnancy at 6 weeks to 23 weeks and 6 days; and from normal women who delivered uncomplicated singleton pregnancies at term, or underwent cesarean sections between 37 to 42 weeks for obstetrical indications. Placentas between 37 to 42 weeks were also collected from women with preeclampsia. The diagnosis of preeclampsia was based on the criteria recommended by ACOG which included: no history of hypertension before pregnancy, onset of hypertension after 20 weeks of gestation with systolic and diastolic pressure > 140/90 mm Hg on at least two occasions four hours apart, and urinary protein > 0.3 g/24 hr. The IRB protocol also allowed the following clinical information to be collected without the identification of patient's name or medical record number: age, gestational age, systolic and diastolic blood pressure and newborn weight. Additionally, for both groups, placentas from women with history of hypertension or pregnancies that were complicated by diabetes, peripheral vascular disease, chronic renal disease, multifetal gestation or major fetal anomalies were excluded.

Placental tissues were collected within 30 minutes of elective termination of pregnancy, vaginal delivery or cesarean delivery. Placentas delivered overnight or when placenta sample collection could not be done within the 30 minutes window, the placentas were not collected. To process the placenta, the membranes were first removed. Each placenta was weighed. A section of the upper right quadrant of each placenta was removed and thoroughly washed in cold normal saline. The section was further dissected into smaller pieces and placed in a petri dish with normal saline. The tissues were dissected under saline to isolate chorionic villi tissue that was not attached to the decidua or to any emerging blood vessels, as described previously in details [25]. Pieces of these isolated chorionic villi tissues were then placed in individual cryovials labelled with placental ID number. The cryovials were placed on ice while the remaining tissues were processed. Cryovials of the same placenta were placed in a plastic bag and were transported to the laboratory on ice. In the laboratory, the cryovials from the same placenta were placed in individual freezer boxes labelled with placenta ID numbers and dates; and stored at -80°C until assay.

Measurements of malondialdehyde (MDA)

Thiobarbituric acid (TBA) assay is the most commonly used method for determination of the MDA in biological fluids [26]. The assay is based on a condensation reaction of two molecules of TBA with one molecule of MDA, in which the reaction rate depends on tempera-

ture, pH and concentration of TBA. Proteins present in chorionic villi homogenate were precipitated with 20% Trichloroacetic acid. The protein-free supernatant was reacted with 0.33% thiobarbituric acid (TBA). The tubes were boiled for 1 hour at 95°C. After the tubes had cooled, the TBA reactive product was extracted with butanol and the intensity of the pink color was read at 532 nm using a Tecan Infinite 200 Pro programmable Microplate Reader. 1,1,3,3-tetraethoxy propane was used as standard. Standards and samples were assayed in duplicates and the MDA levels were expressed as pmol/mg tissue.

Enzyme-linked immunosorbent assays (ELISA)

ELISA was performed using commercially available ELISA kits from R&D Systems for TNF-α and MMP-9 proteins. The analyses were carried out based on the manufacturer's instruction. The optical density was determined using Tecan Infinite 200 Pro Microplate Reader, at 450 nm with correction wavelength set at 570 nm. The computer software of the microplate reader was used to analyze the absorbance data. MDA assay of all 212 chorionic villi samples were assayed first in chronological order the samples were collected. The TNF-α protein assays were carried out next following the same chronological order, followed by MMP-9 protein assays. Standards and samples were assayed in duplicates. Since the volume of the placenta differs between early and late pregnancies the concentrations of the samples were expressed as pmol/mg tissue for MDA, pg/100 mg tissue for TNF-α and ng/100 mg tissue for MMP-9 protein. The inter assay and intra-assay coefficients of variations for all biochemical assays in the study ranged from 4% to 7%.

Statistical analyses

As stated in the Methods Section, the placentas were collected within 30 minutes of the procedure. Placentas delivered overnight or when placenta sample collection could not be done within the 30 minute window, the placentas were not collected. Hence, we collected every sample that was available to us. Moreover, since the study was exploratory in nature we did not include power analysis in the study design. To keep the sample size consistent we have collected 54 placentas from the first trimester, 68 placentas from the second trimester and 57 placentas from the third trimester of normal pregnancy; and 33 placentas were collected to comprise the preeclampsia group.

The data from the study were analyzed using SPSS version 26 software package (IBM Corporation, Armonk, NY). ANOVA and Independent T test were applied to compare differences between groups; and Spearman's rank correlation coefficient was used to evaluate the degree of association between the variables. The inferential statistics was set at $p < 0.05$.

Results

Of the 212 placental samples collected for the study, 179 were from normal pregnancy. Of them, 54 placentas were collected from the first trimester, with gestational age ranging from 6 to 11 weeks; 68 placentas were collected from the second trimester with gestational age ranging from 12 to 23 weeks and 6 days; and 57 placentas were collected from the third trimester with gestational age ranging from 37 to 42 weeks. Additionally, 33 placentas with gestational age ranging from 37 to 40 weeks were collected from women with mild preeclampsia according to ACOG's guidelines. The characteristics of women from whom the placentas were collected are shown in table 1. The data revealed that the age of the women from whom the placentas were collected were comparable between the two study groups. Among normal pregnant women, age did not vary between the trimester groups. ANOVA results showed that gestational age, and systolic and diastolic blood pressure of the mothers from whom the placental were collected, were significantly different between the four studied groups ($p < 0.0001$). Graphical representation of the data pertaining to age, gestational age, systolic and diastolic blood pressure of the study groups are shown in figure 1A-1C, respectively.

Chorionic villi biochemical profiles of the two study groups are presented in table 2. ANOVA results revealed significant differences in MDA levels between the four study groups ($p < 0.000$). In the normal pregnancy group, MDA level was highest in the first trimester and the levels were progressively lower in the second and the third trimesters. MDA level in preeclampsia was the highest among the four study groups (Figure 2A). ANOVA results also showed significant differences in TNF-α protein expression between the four study groups

	Groups	N	Mean ± SD	P value
Age (years)	1st Trimester NP	54	26.2 ± 6.7	0.705
	2nd Trimester NP	68	26.0 ± 6.6	
	3rd Trimester NP	57	26.8 ± 5.9	
	Preeclampsia	33	25.2 ± 6.6	
Gestational Age in days	1st Trimester NP	54	57.6 ± 9.0	0.000
	2nd Trimester NP	68	119.9 ± 23.2	
	3rd Trimester NP	57	274.0 ± 8.0	
	Preeclampsia	33	259.4 ± 26.6	
Systolic Blood Pressure (mm Hg)	1st Trimester NP	54	108 ± 11	0.000
	2nd Trimester NP	68	115 ± 15	
	3rd Trimester NP	57	123 ± 15	
	Preeclampsia	33	157 ± 19	
Diastolic Blood Pressure (mm Hg)	1st Trimester NP	54	67 ± 8	0.000
	2nd Trimester NP	68	68 ± 8	
	3rd Trimester NP	57	70 ± 13	
	Preeclampsia	33	92 ± 13	

Table 1: *Clinical characteristics of women from whom the placentas were collected.*

ANOVA results

NP denotes Normal Pregnancy.

First trimester mean gestation age (8 ± 1 weeks).

Second trimester mean gestational age (17 ± 3 weeks).

Third Trimester mean gestational age (39 ± 1 weeks).

Preeclampsia mean gestational age (37 ± 3 weeks).

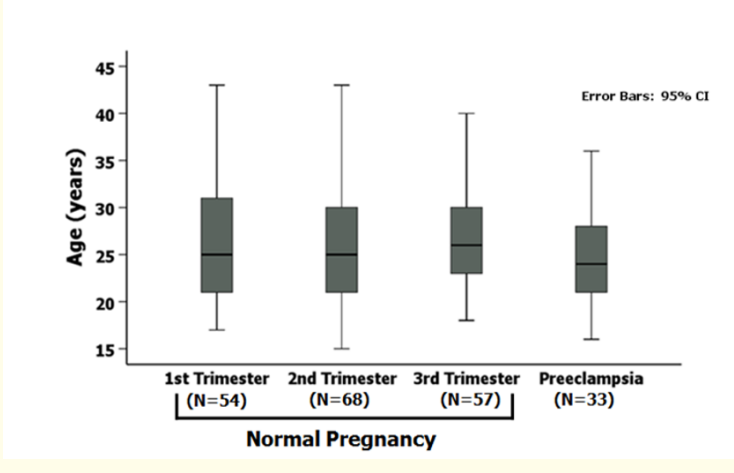

Figure 1A: *Age of the mothers from whom the placentas were collected.*

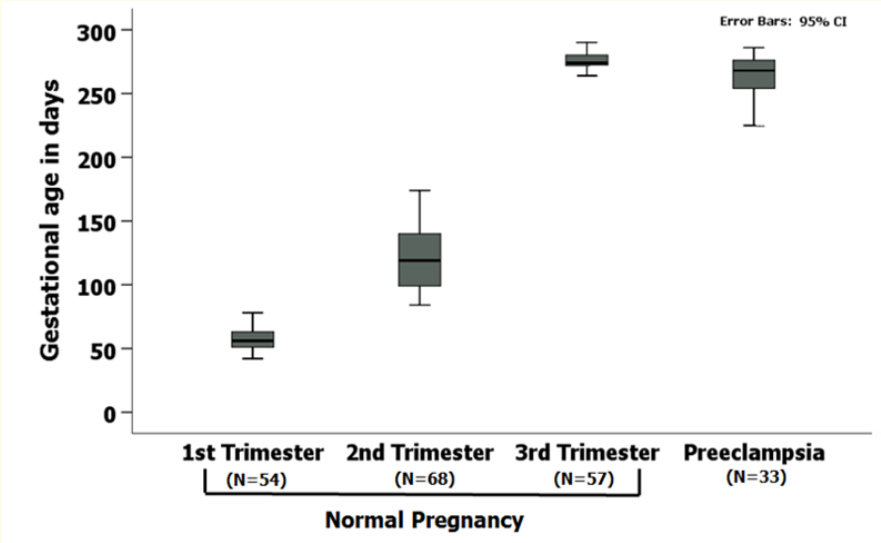

Figure 1B: *Gestational age of the two study groups.*

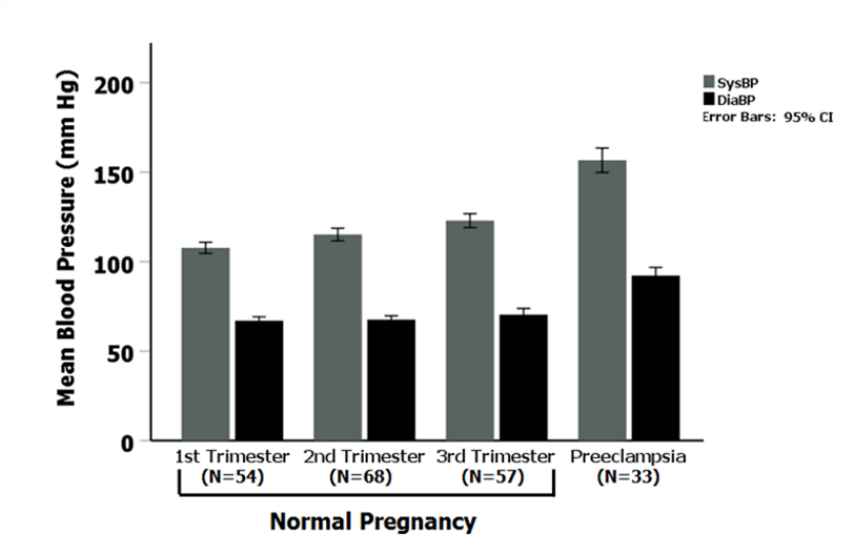

Figure 1C: *Systolic and diastolic blood pressure of the mothers from whom the placentas were collected.*

($p < 0.000$). Chorionic villi TNF-α protein expression in normal pregnancy increased from the first trimester, attained a peak in the second trimester and thereafter, the protein expression declined in the third. Chorionic villi TNF-α protein expression in the preeclampsia group was the highest once again (Table 2 and figure 2A). Chorionic villi MMP-9 protein expression of the four study groups are shown in table 2 as well. ANOVA results revealed significant differences in MMP-9 protein expression between the four groups ($p < 0.000$). The expression pattern of MMP-9 protein showed a progressive increase from the first trimester of normal pregnancy to the third; but was the highest in the preeclampsia group (Figure 2C).

	Groups	N	Mean ± SD	p value
MDA (pmol/mg tissue)	1st Trimester NP	54	299.79 ± 312.52	0.000
	2nd Trimester NP	68	205.71 ± 157.07	
	3rd Trimester NP	57	183.93 ± 141.39	
	Preeclampsia	33	399.33 ± 181.73	
TNF-α (pg/100 mg tissue)	1st Trimester NP	54	36.22 ± 18.59	0.000
	2nd Trimester NP	68	55.64 ± 43.66	
	3rd Trimester NP	57	29.35 ± 33.61	
	Preeclampsia	33	82.34 ± 73.52	
MMP-9 (ng/100 mg tissue)	1st Trimester NP	54	20.32 ± 12.64	0.000
	2nd Trimester NP	68	22.75 ± 13.35	
	3rd Trimester NP	57	28.82 ± 11.12	
	Preeclampsia	33	39.40 ± 21.58	

Table 2: *ANOVA results of chorionic villi biochemical parameters.*

NP denotes normal pregnancy.

First trimester mean gestation age (8 + 1 weeks).

Second trimester mean gestational age (17 + 3 weeks).

Third trimester mean gestational age (39 + 1 weeks).

Preeclampsia mean gestational age (37 + 3 weeks).

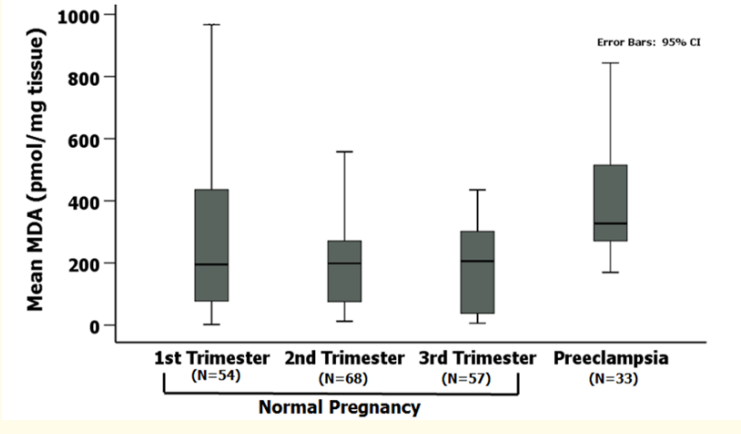

Figure 2A: *Chorionic villi malondialdehyde concentrations in normal pregnancy and preeclampsia.*

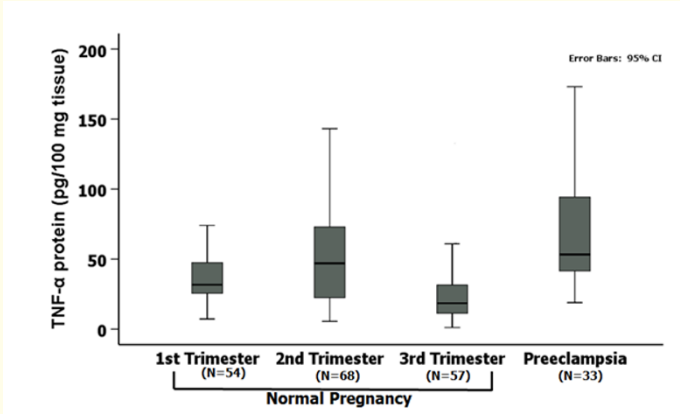

Figure 2B: *Chorionic villi tumor necrosis factor-a protein expression in normal pregnancy and preeclampsia.*

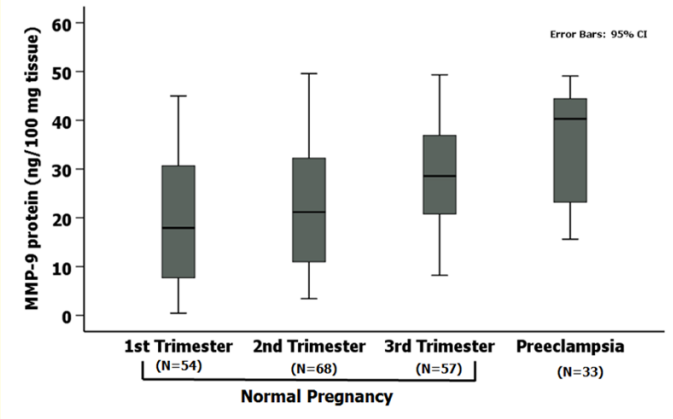

Figure 2C: *Chorionic villi matrix metalloproteinase-9 protein expression in normal pregnancy and preeclampsia.*

Pairwise comparison of the biochemical data of the chorionic villi samples was additionally performed applying the Independent T test, and the results are presented in table 3. The results showed significant differences in the chorionic villi MDA levels between the first and second ($p < 0.047$), and between first and third trimester of normal pregnancy ($p < 0.15$). Chorionic villi MDA levels were comparable between the second and third trimester of normal pregnancy. MDA level in the preeclampsia group was significantly higher than second ($p < 0.0001$) and third trimester ($p < 0.0001$) of normal pregnancy, but was comparable to the first trimester levels. Table 3 also shows that TNF-α protein expression was comparable between the first and third trimester of normal pregnancy but both levels were significantly different from that of the second trimester values ($p < 0.001$). In preeclampsia, chorionic villi TNF-α protein expression was significantly different from that of the first and the third trimester values ($p < 0.0001$), but was analogous to the second trimester levels. Pairwise comparison of chorionic villi MMP-9 protein expressions are also depicted in table 3. The results show that chorionic villi MMP-9 protein

expression between first and second trimester of normal pregnancy was comparable, but in the third trimester the protein expression was significantly higher ($p < 0.006$). Chorionic villi MMP-9 protein expression in preeclampsia was statistically different from that of all normal trimester groups ($p < 0.000$, $p < 0.000$ and $p < 0.012$ for first, second and third trimester, respectively).

Variables	N	MDA (pmol/mg tissue)	TNF-α (pg/100 mg tissue)	MMP-9 (ng/100 mg tissue)
		Mean ± SD	Mean ± SD	Mean ± SD
1st Trimester NP	54	299.79 ± 312.52	36.22 ± 18.59	20.32 ± 12.64
2nd Trimester NP	68	205.71 ± 157.07	55.64 ± 43.66	22.75 ± 13.35
P value		0.047	0.001	NS
1st Trimester NP	54	299.79 ± 312.52	36.22 ± 18.59	20.32 ± 12.64
3rd Trimester NP	57	183.93 ± 141.39	29.35 ± 33.61	28.82 ± 11.12
P value		0.015	NS	0.000
1st Trimester NP	54	299.79 ± 312.52	36.22 ± 18.59	20.32 ± 12.64
Preeclampsia	33	399.33 ± 181.73	82.34 ± 73.52	39.40 ± 21.58
P value		NS	0.001	0.000
2nd Trimester NP	68	205.71 ± 157.07	55.64 ± 43.66	22.75 ± 13.35
3rd Trimester NP	57	183.93 ± 141.39	29.35 ± 33.61	28.82 ± 11.12
P value		NS	0.000	0.006
2nd Trimester NP	68	205.71 ± 157.07	55.64 ± 43.66	22.75 ± 13.35
Preeclampsia	33	399.33 ± 181.73	82.34 ± 73.52	39.40 ± 21.58
P value		0.000	ns	0.000
3rd Trimester NP	57	183.93 ± 141.39	29.35 ± 33.61	28.82 ± 11.12
Preeclampsia	33	399.33 ± 181.73	82.34 ± 73.52	39.40 ± 21.58
P value		0.000	0.000	0.012

Table 3: *Pairwise comparison of biochemical parameters using independent T test.*

The significant differences are highlighted.

NP denotes normal pregnancy.

The gestational age for first trimester (early pregnancy) ranged from 6 to 11 weeks, the gestational age for second trimester (mid pregnancy) ranged from 12 to 23 weeks, plus 6 days, and the gestational age for third trimester (late pregnancy) ranged from 37 to 42 weeks.

The gestational age for the preeclampsia group ranged from 37 to 40 weeks.

Spearman's bivariate correlation results of the first trimester normal pregnancy group are shown in table 4. A significant positive correlation was seen between TNF-α and MMP-9 proteins ($r = 0.337$, $p = 0.013$). Correlation between MMP-9 protein and GAD (gestational age in days) was also significant ($r = 0.501$, $p = 0.000$). Maternal age and maternal systolic blood pressure were significantly correlated ($r = 0.296$, $p = 0.03$).

Spearman's bivariate correlation results seen between chorionic villi biochemical parameters in the second trimester of normal pregnancy are shown in table 5. MDA and TNF-α protein showed a significant negative correlation ($r = -0.284$, $p = 0.019$). A significant negative

First Trimester of Normal Pregnancy		
Variables	**Correlation Coefficient (r)**	**P value (2-tailed)**
MDA vs TNF-α	-0.219	0.112
TNF-α vs MMP-9	0.337	0.013
MMP-9 vs GAD	0.501	0.000
Age vs SBP	0.296	0.03

Table 4: *Spearman's bivariate correlation results.*

correlation was also seen between chorionic villi MDA levels and GAD (r = -0.395, p = 0.001); and a significant positive correlation was seen between MDA and age (r = 0.265, p = 0.029). Additionally, MMP-9 protein expression was significantly correlated to maternal systolic (r = 0.326, p = 0.007) and diastolic blood pressure (r = 0.314, p = 0.009). No correlation was seen between the biochemical variables in the third trimester of normal pregnancy, or in preeclampsia. Maternal systolic and diastolic blood pressures were significantly correlated in all four study groups. In the first trimester, second trimester, third trimester and in preeclampsia the correlation results were as follows: r =0.673, p =0.000; r = 0.647, p = 0.000; r =0.673, p =0.000 and r =0.673, p =0.000, respectively.

Second Trimester of Normal Pregnancy		
Variables	**Correlation Coefficient (r)**	**P value (2-tailed)**
MDA vs TNF-α	-0.284	0.019
MDA vs GAD	-0.395	0.001
MDA vs Age	0.265	0.029
MMP-9 vs SBP	0.326	0.007
MMP-9 vs DBP	0.314	0.009

Table 5: *Spearman's bivariate correlation results.*

Discussion and Conclusion

The clinical characteristics of the women from whom the placentas were collected are shown in table 1 and figure 1A-1C. The age of the mothers from whom the placentas were taken ranged from 17 to 43 years and 64% of the women were from Hispanic descent. Table 1 shows age was comparable between the four study groups. Spearman's bivariate correlation results further showed that in the first trimester of normal pregnancy with gestational age between 6 to 11 weeks, maternal age and the systolic blood pressure of the mothers from whom the placentas were taken were significantly correlated (r = 0.296, p = 0.03). The maternal age of the first trimester group that ranged from 17 to 43 years demonstrate that vascular compliance and hemodynamic adaptations vary with age.

In the present study, we have determined the chorionic villi MDA levels throughout gestation in normal pregnancy and have compared the levels with that of preeclampsia. The data are shown in table 2. To our knowledge MDA levels of trophoblast cells and the placentas were reported earlier [10,24], but there are no studies in the literature that determined placental lipid peroxides levels throughout normal human gestation; except for the one that we have previously reported [5]. In the previous study, placental MDA levels and total antioxidant capacity throughout normal human gestation were determined, but placental MDA levels of a preeclampsia group was not included. The current MDA data reveal a gradual suppression of lipid peroxidation as pregnancy advanced (Table 2 and figure 2). Decrease in placental

MDA levels with an advance in gestational age was also reported by other investigators who have compared first and third trimester placental samples [24,27] omitting the second trimester group. In the present study, the chorionic villi MDA levels of the preeclampsia group were comparable to the levels of the first trimester of normal pregnancy; but were significantly higher than that of the second or third trimester (Table 3 and figure 2A). Similar to our chorionic villi MDA results, in pregnancies complicated with preeclampsia, plasma or serum levels of MDA or other lipid peroxidation products were reported to be higher compared to normal pregnancies [6,7,9,10,21,22,27]; while the levels of several antioxidants were reported to be lower in preeclampsia [8,9,21,22,27,28]. Based on the findings several investigators have suggested that free radical mediated cytotoxicity, and imbalance between lipid peroxide and antioxidants may contribute to endothelial cell damage as seen in preeclampsia [8,21,27,28].

In women with preeclampsia, an excess of lipid peroxidation products can also be generated by circulating neutrophils [11] when the neutrophils are activated by cytokines, primarily interleukin 1 β (IL-1β), IL-10 and TNF-α [12]. Of the various cytokines, the changes in TNF-α protein expression have been well researched in human pregnancy [14,15,29], but the baseline data of TNF-α protein expression pattern throughout normal human gestation is still lacking. In the present study, chorionic villi TNF-α protein expression throughout gestation was analyzed and the results are shown in table 2 and figure 2B. The identification of TNF-α protein in all chorionic villi samples that were analyzed, confirmed that trophoblast cells were the source of this protein, as was previously demonstrated by Hunt JS., *et al* [29]. The present study additionally revealed a gestational age-specific pattern in TNF-α protein expression in normal pregnancy. A marked increase in TNF-α protein expression was seen from the first trimester to the second ($p < 0.025$, Table 3), but beyond the second trimester, the protein expression sharply declined in the third ($p < 0.0001$, Table 3). The peak in TNF-α protein expression seen in the second trimester of normal pregnancy could be explained as follows.

The early pregnancy is a period when a hypoxic environment prevails [1,30], brought about by trophoblasts plugging the uterine spiral arteries [1,30]. For normal pregnancy to advance efficiently beyond the first trimester, a transition from this hypoxic environment to a normoxic environment is required, and during the switch, a hypoxia-reoxygenation environment is manifested. In an *in vitro* study it was shown that placental explants when subjected to hypoxia-reoxygenation secrete significantly higher amounts of TNF-α protein compared to control tissues that were maintained under hypoxia alone. When the placental explants were, however, reoxygenated with either 5% or 21% oxygen, the rise in TNF-α protein was comparable; confirming that the rise in TNF-α protein was a hypoxia-reoxygenation effect and was not due to hyperoxic insult [31]. The peak in TNF-α protein expression seen in the second trimester of normal pregnancy in the present study we suggest, could be an outcome of a similar hypoxia-reoxygenation phase, when normoxic environment in the placenta was restored following the removal of the trophoblast plugs that blocked the uterine spiral arteries. In the present study, the significantly higher expression of TNF-α protein of the second trimester showed a 47% drop in protein expression in the third trimester of normal pregnancy. The gestational age of the third trimester and the preeclampsia group was comparable, yet the drop seen in chorionic villi TNF-α protein expression in normal pregnancy (Table 3 and figure 2B) did not occur in preeclampsia. Rather, the chorionic villi TNF-α protein expression in preeclampsia continued to remain high, being similar to the second trimester levels (Table 3 and figure 2B), while being significantly higher than that of the third trimester ($p < 0.0001$). This finding underscores a significant difference in chorionic villi TNF-α protein expression between normal pregnancy and preeclampsia. We speculate that perhaps the break (s) that operates in normal pregnancy to lower the TNF-α protein expression in the third trimester may be lost in preeclampsia.

Normal human pregnancy is considered a condition of oxidative stress with transient increase in the production of ROS. Even though ROS are toxic to cells and cell membranes, they are helpful signaling molecules generated by the mitochondrial electron transport chain and by several enzymes that are regulated by growth factors and cytokines [1-3]. ROS are involved in biosynthesis of bioactive lipids (prostaglandins and leukotrienes) and hormones (steroids) [4]. In humans, the oxidative stress is manifested at the maternal-fetal interface from early pregnancy onwards; with ROS participating in a variety of diverse processes e.g. implantation, decidualization, placentation, fetal and placental angiogenesis, and fetal and placental development [32,33], that ultimately result in a successful pregnancy outcome.

Lipid peroxidation is a normal phenomenon that occurs continuously at low levels in all humans [34]. During human pregnancy, the altered hormonal status results in an increase in maternal fat depots and hyperlipidemia [4], and ROS interacts with the polyunsaturated fatty acids to initiate a cascade of reactions known as lipid peroxidation. The end product of this reaction is lipid peroxide, one of which is malondialdehyde. Inherently, an effective defense mechanism exists in humans to counterbalance oxidative stress which includes: endogenously synthesized antioxidants such as superoxide dismutase, glutathione transferase, glutathione peroxidase, catalase albumin, transferrin etc. [6,8-10]. Besides, various antioxidants can also be assimilated from the diet, e.g. vitamin C, E, betacarotene etc. [6,8-10,28] to protect the body from oxidative stress. However, during pregnancy when the ROS formed overwhelms the body's ability to control them, it is then that pathology in pregnancy develops; and overabundance of ROS has been demonstrated as an important contributory factor in the pathogenesis of preeclampsia [6-10].

The findings of the present study additionally emphasize another significant difference between normal pregnancy and preeclampsia. In several *in vitro* studies as compiled by Wang and Walsh [13] an association between TNF-α protein and lipid peroxidation products has been established in preeclampsia. It was shown that TNF-α protein induced the mitochondrial generation of superoxide radicals; while hyperoxia induced the production of TNF-α mRNA and protein. Based on the data, the investigators proposed that a positive feedback loop must exist between TNF-α protein and lipid peroxide in preeclampsia. Unfortunately, in the present study, the correlation results revealed by Spearman's rank correlation analysis between MDA levels and TNF-α protein expression in the preeclampsia was not significant ($r = 0.252$, $p = 0.158$). Nevertheless, Spearman's correlation results in normal pregnancy, showed a negative correlation to exist between MDA levels and TNF-α protein expression. The negative correlation was prevalent in all three trimesters of normal pregnancy ($r = -0.219$, $p = 0.112$ in the first trimester), ($r = -0.284$, $p = 0.019$ in the second trimester) and ($r = -0.140$, $p = 0.298$ in the third trimester); with the negative correlation being statistically significant only in the second trimester. The findings thus highlight that the correlation between MDA level and TNF-α protein in normal pregnancy is distinctly different from that of preeclampsia. We believe that the negative correlation seen is also justified. In early pregnancy, oxygen is a key regulator of human cytotrophoblasts. By plugging the uterine spiral arteries, the cytotrophoblasts specifically create an oxygen gradient, to yield an environment extra conducive for placental and fetal growths [1,30]. As pregnancy advances, the obvious generation of lipid peroxide and the demand for an hypoxic environment no longer prevails, yet the requirement for TNF-α protein still persists, primarily because TNF-α protein continues to actively participates in many of the pregnancy-related processes. For example, TNF-α protein still needs to aid in embryo survival. By inhibiting natural killer (NK) cell-mediated cytotoxicity and NK cell response to fetal antigen, TNF-α provides the embryo with mechanisms to evade the host's immune system [14,15,21,23,35]. It acts as a growth factor that regulates gene expression in the uterus [14]. TNF-α regulates the synthesis and degradation of ECM; induces the activity of MMPs protein [16] and coordinates in the removal and replacement of senescent ECM components during early pregnancy [16,23]. Therefore, the negative association seen between MDA and TNF-α protein in the second trimester of normal pregnancy in this study seems both necessary and justified. This is not the first time that TNF-α protein is seen to function differently *in vitro* vs *in vivo.* It has been reported in angiogenesis studies that TNF-α protein in *in vitro*-cultured endothelial cells can contribute predominantly as an anti-angiogenic protein while *in vivo,* TNF-α participate predominantly as pro-angiogenic [36].

In normal human pregnancy, MMPs are regulators of uterine expansion, placentation and vascular remodeling [37]. In table 3 and figure 2C the chorionic villi MMP-9 protein expression of the study groups are shown. Between first and second trimester of normal pregnancy, MMP-9 protein expression was comparable but in the third trimester, the protein expression was significantly higher; and in preeclampsia, the expression profile exceeded that of the third trimester levels (Table 2 and figure 2C). The prominence of MMP-9 protein in trophoblast invasion was established many years ago when among other MMPs and growth factor regulators; MMP-9 protein was exposed as a prerequisite in the invasion of Matrigel by human trophoblasts [16,17]. The presence of MMP-9 protein in all chorionic villi samples in the first trimester of normal pregnancy (Figure 3) supports the role of MMP-9 in ECM remodeling and trophoblast invasion of the spiral arteries. In the present study, Spearman's correlation results revealed a significant positive correlation between TNF-α protein and MMP-9 protein in the first trimester of normal pregnancy ($r = 0.337$, $p = 0.013$) (Table 4). In an *in vitro* study it was also shown that among other

cytokines and growth factors TNF-α protein specifically induced the synthesis of MMP-9 protein by human cytotrophoblasts [16-18]; and the positive correlation seen between TNF-α and MMP-9 protein in this study upholds the *in vitro* results. Spiral artery remodeling is an important feature of normal human pregnancy which accommodates significant increase in blood flow that is required during that time; and the remodeling process is accomplished by the cooperative actions of TNF-α and MMP-9 proteins [1,15,17,18,37]. The significant positive correlation seen between MMP-9 protein and gestational age in this study (r = 0.501, p = 0.000, Table 4) suggest that MMP-9 protein is instrumental in trophoblast invasion; while the significant positive correlation seen between TNF-α and MMP-9 protein (r = 0.337, p= 0.013, Table 4) lends support to the *in vitro* study results that TNF-α protein regulates the action of MMP-9 protein [18].

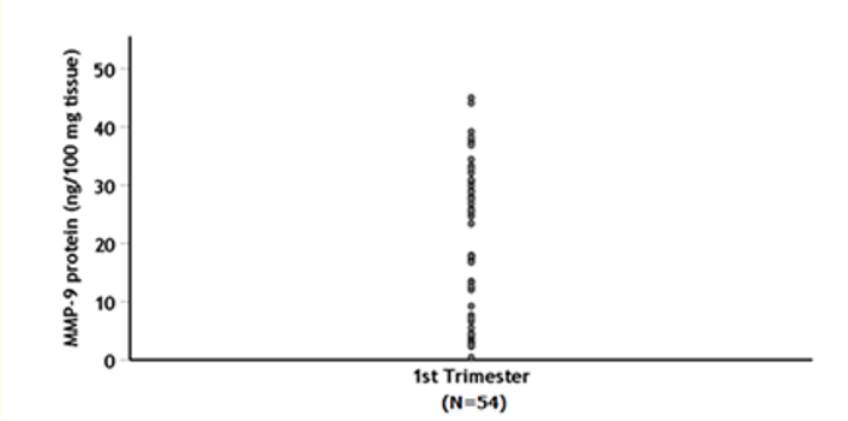

Figure 3: *Chorionic villi matrix metalloproteinase-9 protein expression in the first trimester of normal pregnancy.*

An unexpected finding of the present study was the significant positive correlations seen in the second trimester of normal pregnancy between chorionic villi MMP-9 protein and systolic and diastolic blood pressure (r = 0.326, p = 0.007 and r = 0.314, p = 0.009, respectively) of the mothers from whom the placentas were collected. The findings of the present study thus suggest that MMP-9 protein may have an impact on vascular biology. The impact of MMP-9 protein on human blood pressure has also been evidenced in an Anglo-Scandinavian study [38]. It was demonstrated that treatment of uncontrolled hypertensive patients with antihypertensive drugs for a period of 3 months resulted in significant reduction in both systolic and diastolic blood pressure from their corresponding baseline values, during which time specific reduction in plasma MMP-9 protein was noted as well. The reduction in plasma MMP-9 protein additionally correlated with changes in arterial wall morphology [38].

An important aspect of human pregnancy is that it can be compared to an organ transplant in immunology. Throughout pregnancy, the fetus is contemplated as a semiallogenic graft, capable of initiating an immune response because of the presence of antigens from two histoincomptible individuals. Yet the mother's immune system does not reject the graft. Various immune effectors and molecules participate in the immune microenvironment to establish maternal tolerance toward the semiallogenic fetus. Naïve $CD3^{\pm}CD4^{+}$ T cells are stimulated to differentiate into specific T cells subsets, each with a significant and distinct role in human pregnancy [39,40]. It is believed that Th1 immunity, which is dominant during peri-implantation period, is characterized by the presence of TNF-α protein [40]. In this study, the identification of TNF-α protein in all chorionic villi samples in early normal pregnancy (Figure 4) supports such a role. The third trimester of normal human pregnancy is a time of rapid fetal growth when the immunological profile at the feto-maternal interface is predominantly anti-inflammatory, that promotes the development of the Th2 phase, supported by various hormones [39,40]. The 47% drop in TNF-α

protein expression seen in the study (Table 3 and figure 2B) we suggest, could be a physiological event to facilitate the maternal immune response to switch from pro-inflammatory Th1 phenotype towards anti-inflammatory Th2 phenotype.

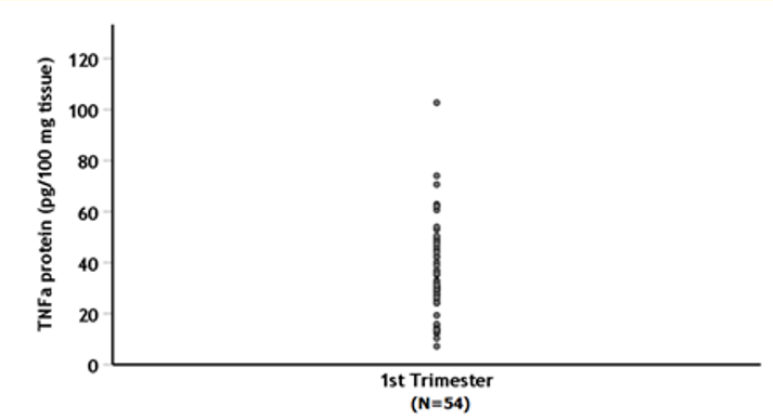

Figure 4: *Chorionic villi tumor necrosis factor-a protein expression in the first trimester of normal pregnancy.*

Successful pregnancy is the result of the combination of signals and responses originating between the mother and the fetal-placental interface. We support the concept of Mor., *et al.* [41] that human pregnancy should not be considered as a single event. In this study we demonstrated that human pregnancy has three different phases which are defined as trimesters; characterized by biological processes that are distinctly gestational age-specific. The findings show that the biochemical profiles of three interconnected constituents in the second trimester of normal pregnancy differ markedly from the profiles of the first and the third trimesters, whereas the levels of the constituents in preeclampsia remain either parallel to the first (in case of MDA levels) or the second trimester group (in case of TNF-α protein expression).

The higher expression of MMP-9 protein in the third trimester of normal pregnancy we hypothesize marks the completion of fetal development phase with initiation of the phase for parturition. The increased levels of chorionic villi MMP-9 protein may facilitate remodeling and weakening of the extracellular matrix by triggering collagen breakdown, that may lead to rupture and disassociation of the fetal membranes, as well as rejection of the placenta [37].

The limitation of our study is that we have not confirmed our data by western blot analysis, polymerase chain reaction or by immunohistochemical methods. We also were unable to investigate the significance if any latent versus activated form of MMP-9 in the context of our findings. We do not currently have the facility to carry out these techniques. Moreover, the latent and active form of MMP-9 protein could not be determined because the ELISA kits to assay them individually are not commercially available. We acknowledge that TNF-α is a cell signaling molecule and the cytokine interacts with many other molecules besides these two. We believe that addition of all these factors would have made the study more complex and any conclusions drawn from the study would have been extremely difficult to interpret. The strength of our study is our relatively larger sample size in each of the trimester groups. Simultaneous expressions of malondialdehyde, TNF-α protein and MMP-9 protein in human chorionic villi tissue throughout gestation in normal human pregnancy have not been reported before. Moreover, in this study, stringent ELISA methods using monoclonal antibodies to human TNF-α and MMP-9 proteins were used as capture antibodies. The ELISA methods used were sensitive that allowed the detection of TNF-α protein as low as 15.6 pg/ml and MMP-9 protein as low as 31.3 pg/ml.

This study is not intended to offer mechanisms in the pathogenesis of preeclampsia. We acknowledge that there are whole hosts of other constituents besides the studied three, which participate in normal human gestation. The study underscores the striking differences that we have observed between normal pregnancy and preeclampsia.

The findings highlight that for a successful pregnancy outcome, the gestational age-specific alterations in the levels of the studied constituents, as well as the specific correlations between the studied constituents seem important for the various pregnancy-related processes to transpire. While the present study does not contribute to the early diagnosis of preeclampsia inflammatory biomarkers can be helpful. Furthermore, the present study substantiates the role of free radical damage in the pathogenesis of the disease. The findings show that in normal pregnancy, MDA levels and TNF-α expression taper off in the third trimester. In preeclampsia, the exacerbated state persists. This implies that the physiological brake(s) that operates as the pregnancy enters the third trimester may perhaps be attenuated.

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Conflict of Interest

The authors declare no conflicts of interest with respect to the research, authorship and/or publication of this article.

Bibliography

1. Burton GJ., *et al*. "Maternal arterial connections to the placental intervillous space during first trimester of human pregnancy: the Boyd collection revisited". *American Journal of Obstetrics and Gynecology* 181 (1999): 718-724.

2. Sies H. "Oxidative stress: a concept in redox biology and medicine". *Redox Biology* 4 (2015): 180-183.

3. Perillo B., *et al*. "ROS in cancer therapy: the bright side of the moon". *Experimental and Molecular Medicine* 52 (2020): 192-203.

4. Harrera E and Ortega-Senovilla H. "Lipid metabolism during pregnancy and its implications for fetal growth". *Current Pharmaceutical Biotechnology* 15.1 (2014): 24-31.

5. Basu J., *et al*. "Placental oxidative status throughout normal gestation in women with uncomplicated pregnancies". *Obstetrics and Gynecology International* (2015): 6.

6. Chiarello DI., *et al*. "Oxidative stress: Normal pregnancy versus preeclampsia". *BBA-Molecular Basis of Disease* 1866.2 (2020): 165354.

7. Mannaerts D., *et al*. "Oxidative stress in healthy pregnancy and preeclampsia is linked to chronic inflammation, iron status and vascular function". *PLoS ONE* 13.9 (2018): e0202919.

8. Bilodeau JF and Hubel CA. "Current concepts in the use of antioxidants". *Journal of Obstetrics and Gynaecology Canada* 25.9 (2003): 742-750.

9. Bayhan G., *et al*. "Significances of changes in lipid peroxides and antioxidant enzyme activities in pregnant women with preeclampsia and eclampsia". *Clinical and Experimental Obstetrics and Gynecology* 27.2 (2000): 142-146.

10. Walsh SW. "Lipid peroxidation in pregnancy". *Hypertension in Pregnancy* 13.1 (1994): 1-32.

11. Crocker IP., *et al*. "Neutrophil function in women with preeclampsia". *BJOG: An International Journal of Obstetrics and Gynaecology* 196 (1999): 822-828.

12. Rinehart BK., *et al*. "Expression of the placental cytokines tumor necrosis factor α, interleukin 1β, and interleukin 10 is increased in preeclampsia". *American Journal of Obstetrics and Gynecology* 181.4 (1999): 915-920.

13. Wang Y and Walsh SW. "TNF-α concentrations and mRNA expression are increased in preeclamptic placentas". *Journal of Reproductive Immunology* 32 (1996): 157-169.

14. Vilcek J and Palombella VJ. "TNF as a growth factor". In: Aggarwal BB and Vilcek J (editions) Tumor necrosis factors: structure function and mechanism of action. Dekker, New York (1992): 269-287.

15. Wride MA and Sanders EJ. "Potential roles for tumor necrosis factorα during embryonic development". *Anatomy and Embryology* 191 (1995): 1-10.

16. Bischof P., *et al*. "Importance of matrix metalloproteinases in human trophoblast invasion". *Early Pregnancy* 1.4 (1995): 263-269.

17. Bischof P., *et al*. "Biochemistry and molecular biology of trophoblast invasion". *Annals of the New York Academy of Sciences* 943 (2001): 157-162.

18. Cohen M., *et al*. "Involvement of MAPK pathway in TNF-α-induced MMP-9 expression in human trophoblastic cells". *Molecular Human Reproduction* 12.4 (2006): 225-232.

19. Redman CW and Sargent IL. "Latest advances in understanding preeclampsia". *Science* 308 (2005): 1592-1594.

20. Roberts JM and Hubel CA. "The two stage model of preeclampsia: Variations on the theme". *Placenta* 30 (2009): S32-S37.

21. Spence T., *et al*. "Maternal serum cytokine concentrations in healthy pregnancy and preeclampsia". *Journal of Pregnancy* 33 (2021): 6649608.

22. Ilhan N., *et al*. "The changes in trace elements, malondialdehyde levels and superoxide dismutase activities in pregnancy with or without preeclampsia". *Clinical Biochemistry* 35 (2002): 393-397.

23. Chen H-L., *et al*. "Tumor necrosis factor alpha mRNA and protein are present in human placenta and uterine cells at early and late stages of gestation". *The American Journal of Pathology* 139 (1991): 327-335.

24. Sekiba K and Yoshioka T. "Changes of lipid peroxidation and superoxide dismutase activity in the human placenta". *American Journal of Obstetrics and Gynecology* 135 (1979): 368-371.

25. Basu J., *et al*. "Influence of placental metalloproteinase -9 protein on the branching architecture of chorionic blood vessels of human placenta". *Gynecology and Reproductive Health* 5.5 (2021): 1-9.

26. Draper HH and Hadley M. "Malondialdehyde determination as index of lipid peroxidation". *Methods in Enzymology* 186 (1990): 421-431.

27. Walsh SW. "Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia". *Seminars in Reproductive Medicine* 16.1 (1998): 93-104.

28. Mikhail MS., *et al*. "Preeclampsia and antioxidant nutrients: decreased plasma levels of reduced ascorbic acid and α-tocopherol, and beta-carotene in women with preeclampsia". *American Journal of Obstetrics and Gynecology* 171 (1994): 150-157.

29. Hunt JS., *et al*. "Tumor necrosis factor: Pivotal components of pregnancy". *Biology of Reproduction* 54 (1996): 554-562.

30. Jauniaux E., *et al*. "Comparison of colour Doppler features and pathological findings in complicated early pregnancy". *Human Reproduction* 9 (1994): 2432-2437.

31. Hung TH., *et al*. "Secretion of Tumor necrosis Factor-α from Hunan Placental Tissues Induced by Hypoxia-Reoxygenation causes endothelial cell activation *in vitro*. A Potential Mediator of Inflammatory Response in Preeclampsia". *The American Journal of Pathology* 164.3 (2004): 1049-1061.

32. Pilar C-F. "Oxidative stress at the maternal-fetal interface". *The Journal of Pediatric Biochemistry* 3.3 (2013): 129-136.

33. Winn VD., *et al*. "Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term". *Endocrine* 148.3 (2006): 1059-1079.

34. Little RE and Gladen BC. "Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature". *Reproductive Toxicology* 13 (1999): 347-352.

35. Lachapelle MH., *et al*. "Embryonic resistance to tumor necrosis factor-α mediated cytotoxicity: a novel mechanism underlying maternal immunological tolerance in the fetal allograft". *Human Reproduction* 8 (1993): 1032-1038.

36. Sainson RC., *et al*. "TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype". *Blood* 111 (2008): 4997-5007.

37. Chen J and Khalil RA. "Matrix metalloproteinases in normal pregnancy and preeclampsia". *Progress in Molecular Biology and Translational Science* 148 (2017): 87-165.

38. Tayebjee MH., *et al*. "Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in hypertension and their relationship to cardiovascular risk and treatment: a substudy of the Anglo-Scandinavian Cardiac Outcomes trial (ASCOT)". *American Journal of Hypertension* 17.9 (2004): 764-769.

39. Van Nieuwenhoven ALV., *et al*. "The immunology of successful pregnancy". *Human Reproduction Update* 9.4 (2003): 347-357.

40. Meyyazhagan A., *et al*. "Cytokine see-saw across pregnancy, its related complexities and consequences". *International Journal of Gynecology and Obstetrics* (2022): 1-10.

41. Mor G., *et al*. "Inflammation and pregnancy: the role of the immune system at the implantation site". *Annals of the New York Academy of Sciences* 1221 (2011): 80-87.

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