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Placental anti-oxidant gene polymorphisms, enzyme activity, and oxidative stress in preeclampsia

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Abstract

The etiology and pathophysiology of preeclampsia are not fully understood. However, oxidative stress has been strongly linked to the occurrence of this multi-system disease. This has led to many theories of the pathogenesis of preeclampsia involving placental oxidative stress. In this study, we hypothesized that polymorphisms of anti-oxidant genes in the placental tissue contributed to susceptibility to preeclampsia. Polymorphisms in copper/zinc superoxide dismutase (*CuZn-SOD*), manganese superoxide dismutase (*MnSOD*), glutathione-*s*-transferase M1 (*GSTM1*), and glutathione-*s*-transferase T1 (*GSTT1*) in the umbilical cord tissue were assayed by polymerase chain reaction (PCR) in 23 nulliparous preeclampsia cases and 32 nulliparous normotensive controls. Corresponding enzyme activity levels and an oxidative stress biomarker (8-isoprostane) of the placental tissue were also measured. In addition, maternal plasma 8-isoprostane levels were also determined. Our results showed that no significant differences in polymorphism frequency of the tested genes, enzyme activity levels or 8-isoprostane levels in the placental tissue were detected between the cases and controls. However, maternal plasma 8-isoprostane level was significantly higher in the cases than in the controls (105.8 vs. 27.9 pg/ml, $p = 0.03$). In conclusion, our study showed that polymorphisms of *CuZn-SOD*, *MnSOD*, *GSTM1* and *GSTT1* in the placental tissue were not associated with preeclampsia.

Introduction

Preeclampsia is a potentially life-threatening disease that occurs exclusively in pregnant women during late gestation (>20 weeks). The two hallmark symptoms are hypertension and proteinuria, complicating at least 5% of pregnancies and are usually resolved upon delivery of the placenta. Preeclampsia has been closely associated with oxidative stress in a number of studies [1–4]. The mechanism of oxidative stress associated hypertension has been continuously evolving with the popular theme of endothelial dysfunction [5,6].

The etiology of oxidative stress in preeclamptic women has therefore been the focus of extensive research. Genetic, environmental, and immunological factors have been considered. Furthermore, it is clear that the placenta plays the pivotal role in the development of oxidative stress in preeclampsia [7]. The purpose of this study was to investigate polymorphisms in anti-

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oxidant genes in the placenta as a possible risk factor of preeclampsia via a predisposition to oxidative stress. Several major anti-oxidant genes were selected: copper/zinc superoxide dismutase (*CuZn-SOD*), manganese superoxide dismutase (*MnSOD*), glutathione-s-transferase M1 (*GSTM1*), and glutathione-s-transferase T1 (*GSTT1*). Previous studies have compared anti-oxidant enzyme levels and oxidative stress biomarkers in pregnant women with and without preeclampsia [8–10] or linked anti-oxidant gene polymorphisms to preeclampsia [11,12], but never both. Nor have previous studies examined placental genes, but rather that of the mother. The current study examines the association between fetal, rather than maternal polymorphisms of anti-oxidant genes and preeclampsia. We further corroborated the levels of anti-oxidant enzyme activity and oxidative stress with the genetic polymorphisms.

Material and Methods

Study Population

A total of 27 preeclamptic cases and 34 controls were recruited for a case-control study. The subjects were patients at the Louisiana State University Health Sciences Center at Shreveport, LA. Preeclampsia was diagnosed on admission to the labor/delivery unit and confirmed after screening for inclusion and exclusion criteria. The inclusion criteria for the cases consisted of singleton, nulliparous, normotensive before 20 wks gestation, blood pressure $\geq 140/90$ mmHg 6 hours apart, proteinuria of 2+ on dipstick analysis. Exclusion criteria consisted of conceptions through any fertility treatment, chronic hypertension, preexisting or gestational diabetes mellitus, and chronic renal disease. All the inclusion and exclusion criteria were applied to the controls except that blood pressure never exceeded 140/90 mmHg and proteinuria never occurred during pregnancy. All subjects were interviewed in person. Information on maternal demographic characteristics and family history was collected. Placentas were obtained upon delivery and villous tissue was isolated by sterile dissection. Placental pieces were then washed thoroughly with phosphate buffer saline (PBS) to remove blood from the intervillous space and then snap frozen in liquid nitrogen for storage at -70°C . A 0.5–1.0 inch segment of the umbilical cord was also cut and collected. The cord segment was also then washed thoroughly with PBS and snap frozen in liquid nitrogen for storage at -70°C . Since the umbilical cord tissue was not available in 4 cases and 2 controls, the final study sample included 23 cases and 32 controls.

Genomic Analysis

Genomic DNA from umbilical cord segments was isolated and purified by using the QIAamp DNA isolation kit (QIAGEN Inc. Valencia, CA). We used the cord DNA to represent the placental/fetal DNA without risk of contamination from maternal blood. A PCR method was used for the genotyping of repeated polymorphism as described previously [13,14]. Genomic DNA (100ng) extracted from each placenta was amplified by iCycler (Bio-Red Laboratories, Hercules, CA) using the specific designed primers for *CuZn-SOD*, *MnSOD*, and *GSTM1* and *GSTT1* as previously described [13–17]. Table 1 shows the primer sequence, PCR products, restriction enzymes and fragments after digestion for each tested gene. β -globin gene was used as an internal positive control for *GSTM1* and *GSTT1* [16,18]. Primers used to detect the β -globin gene were 5'-CAACTTCATCCACGTTCCACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3' which yields a 268 bp product. Digested products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. The results were recorded by photosystem and analyzed by 1-D analysis computer software program (Bio-Red Laboratories, Hercules, CA). All samples were repeated once for quality control to ensure coding errors did not occur.

Placental Enzyme Activity Level Analysis

The levels of SOD and GST activities in the placental tissue were determined by colorimetric assays. Both SOD and GST assay kits were purchased from Cayman Chemical, Ann Arbor, MI. Assay procedures and tissue homogenate preparation were performed following the manufacturer's instructions.

For SOD assay, tissue pieces were homogenized with cold 20mM HEPES buffer (pH 7.2) containing 1mM EGTA, 210mM mannitol and 70mM sucrose. The tissue/buffer ratio was 1:5 for both assays. The homogenate was first centrifuged at 1,500×g. To separate CuZnSOD and MnSOD, the 1,500×g supernatant was further centrifuged at 10,000×g for 15 minutes at 4C. The resulting 10,000×g supernatant contains cytosolic CuZnSOD and the pellet contains mitochondrial MnSOD. The pellet was homogenized again with the same buffer. An aliquot of 10µl of tissue homogenate was used for each assay. All samples were tested in duplicate and measured in the same day. The result for both CuZnSOD and MnSOD assay was expressed as unit/gram tissue. Co-efficient of variation was 6.5% for both CuZnSOD and MnSOD assays.

For the GST assay, tissue pieces were homogenized with cold 100mM potassium phosphate buffer (pH 7.0) containing 2mM EDTA. The tissue/buffer ratio was 1:5. The GST assay kit measuring total GST activity (cytosolic and microsomal). The assay was performed immediately after the homogenate was prepared. An aliquot of 20µl of tissue homogenate was assayed in duplicate. All samples were measured in the same day. The result was expressed as nmol/min/gram tissue. Co-efficient of variation for the GST assay was 8.0%.

Placental tissue 8-isoprostane measurement

We used 8-isoprostane as a marker for oxidative stress. Placental tissue levels of 8-isoprostane were determined by 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI). The assay yields total 8-isoprostane levels in the tissue. Tissue samples were homogenized following the manufacturer's instructions in 0.1M phosphate buffer (pH 7.4) containing 1mM EDTA and 10µM indomethacin. The tissue/buffer ratio was 1:5. Assay procedures were performed following the manufacturer's instructions accordingly. The EIA kit includes ELISA buffer, washing buffer, extraction buffer, substrate, standard enzyme conjugate and an antibody-coated plate. The range of the standard curve for 8-isoprostane assay was 0.8 – 500 pg/ml. An aliquot of 50µl of tissue homogenate was assayed in duplicate. All samples were measured in the same day. The result was expressed as ng/gram tissue. Co-efficient of variation for the tissue 8-isoprostane assay was 4.6 %.

Maternal plasma 8-isoprostane measurement

Maternal 8-isoprostane levels were measured in 26 plasma samples, 13 from cases and 13 from controls (not all subjects had blood samples). Plasma 8-isoprostane was purified by C18 Sep-Pak column (Waters Corporation, Milford, MA) following the standard manufacturer's instruction before assay. Briefly, 0.2ml of ethanol containing 0.01% butylated hydroxytoluene (BHT) was added to 0.5ml of plasma sample and mixed well. Then the mixture was applied into a C18 Sep-Pak column with flow rate to 1.0ml per minute and followed by 2.0ml of hexane. The column was activated by 2.0ml of methanol and 2.0ml of UltraPure water. The purified 8-isoprostane was eluted with 2.0ml ethyl acetate containing 1% methanol. The eluted ethyl acetate was then evaporated with a stream of nitrogen. The residue containing free 8-isoprostane was reconstituted with 0.5ml of EIA buffer provided by the 8-isoprostane EIA kit (Cayman, Ann Arbor, MI). An aliquot of 100µl per sample was assayed in duplicate. All samples were assayed in the same plate. The result was expressed as pg/ml. Co-efficient of variation for plasma 8-isoprostane assay was 3.1%.

Statistical Analysis

We first compared the preeclampsia cases and normotensive controls on maternal characteristics, frequency of genotypes and levels of enzyme activities. Student's *t*-test and chi-square test were used for continuous and categorical variables, respectively. Wilcoxon rank sums test was used for continuous variables with a skewed distribution. To assess the independent association between genotype, levels of enzyme activities and risk of preeclampsia, we used a multiple logistic regression model to control for parental race because race could be related to the frequency of certain variants and incidence of preeclampsia. Finally, we examined the relationship between fetal genotype, enzyme activities and oxidative stress. Multiple linear regressions were used to control for parental race and case-control status. All analyses were done using the Statistical Analysis System (SAS Version 9.1, Cary, NC).

Results

The characteristics of the cases and controls are presented in Table 2. The cases had significantly shorter gestation and lower infant birthweight. The mean gestational age and birthweight were 35.2 weeks and 2346 grams, respectively, in preeclamptic women compared to 39.1 weeks and 3216 grams, respectively, in normotensive women. Among the 23 cases, 14 had severe preeclampsia (blood pressure $\geq 160/110$ mmHg, proteinuria ≥ 2 g/24 h or 2+ or more on dipstick, elevated hepatic enzymes, persistent headache or epigastric pain); 5 were borderline between mild and severe preeclampsia; and 4 were mild preeclampsia.

The genotypic frequencies, enzyme activity level, and biomarker assay among the cases and controls are presented in Table 3. All subjects exhibited the same genotype of *CuZn-SOD* with the exception of one case (Leu-38Val). Variant genotypes of *MnSOD*, *GSTM1* and *GSTT1* were not significantly associated with preeclampsia. Likewise, the cases did not show a significant increase or decrease in enzyme activity levels of *CuZn-SOD*, *MnSOD*, and *GST* in the placental tissue. Placental tissue total 8-isoprostane levels were not different between the cases and controls (1.6 ± 0.8 vs. 1.4 ± 1.0 ng/gram tissue $p = 0.51$). However, the free 8-isoprostane level was significantly higher in plasma samples from women with preeclampsia (105.7 ± 43.5 pg/ml) than from normal (28.2 ± 3.5 pg/ml) pregnancies ($p = 0.03$).

Subjects were also separated based on the genotype of the fetus rather than the mother, as depicted in Table 4. There was no significant difference in enzyme expression of *MnSOD* and *GST* between *MnSOD*, *GSTM1* and *GSTT1* variants. A significant increase in 8-isoprostane was detected in placentas with the *GSTT1* null genotype ($p=0.034$). After adjusting for parental race and case-control status, the association of *GSTT1* null deletion and increased 8-isoprostane was borderline significant ($p=0.049$).

Discussion

Our study did not find any significant association between genetic polymorphisms of fetal/placental *MnSOD*, *GSTM1* and *GSTT1* and preeclampsia. These results are consistent with previous studies [11,12], in which maternal *MnSOD*, *GSTM1* and *GSTT1* were genotyped.

Glutathione-*s*-transferase (GST) is anti-oxidant enzyme from a large family subdivided into alpha, mu, kappa, theta, pi, and sigma classes. Deletion of *GSTP1* has been associated with an increased risk of preeclampsia in a previous study [19]. *GSTM1* and *GSTT1* were selected for this study due to the high prevalence of homozygous deletions of each gene in humans. *GSTM1* is homozygously deleted in an estimated 50% of the population [20,21] while *GSTT1* is deleted in 10–20% of the population [22]. This deletion is significantly more common in blacks [23]. Interestingly, the null deletion of these genes appears to have no effect on corresponding enzyme activity level, which was also reported by others [24].

CuZn-SOD is a cytosolic enzyme that protects against superoxide radicals, which are elevated in the placental tissue from preeclamptic women detected by electron paramagnetic spin trap resonance [25]. CuZn-SOD enzyme levels increase throughout pregnancy but are downregulated in trophoblast cells of the preeclamptic placenta [9,10,26]. Over 50 mutations have been identified and nearly all are associated with an increased risk of familial amyotrophic lateral sclerosis (FALS) [15,27]. In our study, only one subject had a *CuZn-SOD* mutation, which is consistent with the frequency reported previously [15].

MnSOD exhibits similar function to CuZn-SOD, but is targeted to the mitochondria in order to degrade the large amount of superoxide radicals produced during oxidative phosphorylation. Fewer polymorphisms in *MnSOD* have been identified than in *CuZn-SOD*, but are linked to increased oxidative damage [18]. A unique Ala-9Val variant has been identified that alters the targeting of the enzyme to the mitochondria [28,29]. Interestingly, population studies have shown that the heterozygote of the SNP to be the most common genotype in all populations surveyed [30]. The effects of the Ala-9Val are not fully understood and therefore it is difficult to hypothesize as to the effects of the variant on the risk of preeclampsia.

A previous study did show that CuZnSOD activity was decreased in trophoblasts isolated from preeclamptic placentas compared to that from normal placentas [10]. In the present study, we did not find any significant difference between the cases and controls with regard to *CuZn-SOD* and *MnSOD* enzyme activity level in the placental tissue. The lack of difference could be due to the whole villous tissue that was used in this study, since snap frozen tissue contained trophoblasts, stromal tissue, villous core vessels and blood.

In this study, we also measured 8-isoprostane levels in the placental tissue and in the maternal plasma samples. Isoprostanes are produced non-enzymatically by the random oxidation of tissue phospholipids by oxygen radicals. 8-isoprostane is a prostaglandin-derived byproduct formed from superoxide independent of cyclooxygenase [33,34] and is an effective index of oxidative stress [35]. Our results showed that the tissue 8-isoprostane levels were not significantly different between the cases and controls. However, maternal plasma free 8-isoprostane levels were significantly higher in women with preeclampsia than in normal pregnant controls, which is consistent with the previously published work by Harsem et al [36]. Furthermore, Staff et al determined both free and total levels of 8-isoprostane in the placental and the decidua tissues [37]. Their results showed that neither free nor total 8-isoprostane levels were different between normal and preeclamptic placental tissues, which is consistent with our findings, although the decidua tissue free 8-isoprostane levels were higher in the preeclamptic group [37]. The data from our study and the others suggest that free, but not total, 8-isoprostane levels may be a better indicator of increased oxidative stress in biological samples.

In summary, we did not find any correlation of polymorphisms of fetal antioxidant genes in the placental tissue with the susceptibility to preeclampsia. There was no difference between homozygosis of fetal *MnSOD* gene or null deletion of fetal *GSTM1* gene with 8-isoprostane levels in the placental tissues from normal and preeclamptic pregnancies. However, our data did show a significant association between the fetal *GSTT1* null deletion and the increased placental tissue 8-isoprostane levels. This association warrants further investigation.

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Primers and products of polymorphism superoxide dismutase and glutathione-s-transferase genes

Table 1

Genes	Geno Site	Primer Sequence	Restriction Enzyme	PCR Products(bp)	Restriction fragments(bp)
MnSOD ^A	MTS ^C	5'-ACC AGC AGG CAG CTG GCG CCG G-3'	a. <i>Cac8</i> I (GTT>GCT)	107	93, 87 (Ala-9Val)
		5'-GGG TTG ATG TGA GGT TCC AG-3'	b. <i>Not</i> I (GTT>GCT)	107	89, 18 (Ala-9Val)
Cu/ZnSOD ^B	Exon 2	5'-ACT CTC TCC AAC TTT GCA CTT-3'	a. <i>Mae</i> III (GGA>AGA)	132	72, 60 (Leu38>Val)
		5'-CCC ACC TGC TGT ATT ATC TCC-3'	b. <i>Hae</i> III (GGC>AGC)	132	83, 49 (Gly41>Ser)
	Exon 4	5'-CAT ATA AGG CAT GTT GGA GAC T-3'	a. <i>Hin</i> PI (CGC>CGC)	214	192, 22 (Gly85>Arg)
		5'-TCT TAG AAT TCG CGA CTA ACA ATC-3'	b. <i>Mbo</i> II (GAA>GGA)	214	88, 68 (Glu100>Gly)
GSTM1 ^D		5'-CTGCCCTACTTGATTGATGGG-3'		273	
GSTT1 ^D		5'-CTGGATTGTAGCAGATCATGC-3'			
		5'-TTCTTACTTGTCCTCACATCTC-3'		480	
		5'-TCACCCGGATCATGGCCAGCA-3'			

^AMnSOD: manganese superoxide dismutase. Primers sequence was selected based on ref. 13

^BCu/ZnSOD: copper/zinc superoxide dismutase. Primers sequence was selected based on ref. 15.

^CMTS: Mitochondrial targeting sequence

^DGST: glutathione-s-transferase, M1 and T1. Primers sequence was selected based on ref. 16,17.

Table 2
Basic Characteristics of Preeclampsia Cases and Normotensive Controls

	Case (N=23)	Control (N=32)	P-value ^A
Age (Mean ± SD, years)	22.0 ± 4.8	20.0 ± 2.7	0.08
Gestational Age at Delivery (Mean ± SD, weeks)	35.2 ± 4.3	39.1 ± 1.5	0.0003
Birthweight (Mean ± SD, range, weeks)	2346 ± 803 (840, 3317)	3216 ± 459 (2410, 4224)	0.0007
Body Mass Index (kg/m ²):			0.42
<25	9 (40.9%)	18 (58.1%)	
25–30	6 (27.3%)	4 (12.9%)	
>30	7 (31.8%)	9 (29.0%)	
Maternal Race:			0.50
White	6 (26.1%)	5 (15.6%)	
Black or Native American	17 (73.9%)	27 (84.4%)	
Paternal Race:			0.14
White	6 (26.1%)	3 (9.4%)	
Black	17 (73.9%)	29 (90.6%)	
Mode of Delivery			0.56
Caesarean delivery without labor	7 (30.4%)	7 (23.3%)	
Vaginal or emergent caesarean delivery	16 (69.6%)	23 (76.7%)	
Gravidity:			0.69
Primigravid	21 (91.3%)	27 (84.4%)	
Multigravid	2 (8.7%)	5 (15.6%)	

^A Student's *t*-test and chi-square test were used for continuous and categorical variables, respectively.

Table 3
Genotypic frequencies, enzyme levels, and oxidative stress biomarker in preeclampsia cases and normotensive controls

Genotypes of the umbilical cord	Case (N = 23)	Control (N = 32)	P-value ^B
MnSOD ^A ; Val/Ala and Ala/Ala Val/Val	17 (73.9%) 6 (26.0%)	24 (75.0%) 8 (25.0%)	1.00
GSTM1 ^A ; Present	17 (73.9%) 6 (26.0%)	26 (81.2%) 6 (18.8%)	0.53
Null deletion GSTT1 ^A ; Present	19 (82.6%) 4 (17.4%)	25 (78.1%) 7 (21.9%)	0.75
Enzyme levels of the placental tissue: CuZn-SOD (unit/gram tissue) ^A MnSOD (unit/gram tissue) GST (nmol/min/gram tissue) ^A 8-Isoprostane level Placental tissue (ng/gram tissue) Maternal plasma (pg/ml)	1.6 ± 1.8 1.0 ± 0.4 628 ± 276 1.6 ± 0.8 105.7 ± 43.5	1.8 ± 1.3 1.1 ± 0.3 670 ± 163 1.4 ± 1.0 28.2 ± 3.5	0.79 0.57 0.52 0.51 0.03

^A MnSOD: Manganese Superoxide Dismutase, GSTM: Glutathione s-transferase M, GSTT: Glutathione s-transferase T, Cu-Zn SOD: Copper-Zinc Superoxide Dismutase, GST: Glutathione s-transferase.

^B Chi-square test and Student's *t*-test were used for categorical and continuous variables, respectively.

Table 4
Enzyme levels and oxidative stress biomarker by genotype

Enzyme/Biomarker	Genotype		P-value ^A
MnSOD 8-isoprostane	Manganese Superoxide Dismutase (<i>MnSOD</i>)		
	Val/Ala and Ala/Ala (N=41);	Val/Val (N=14);	
	1.1 ± 0.3 1.4 ± 0.9	0.9 ± 0.4 1.8 ± 0.9	0.278 0.227
GST 8-isoprostane	Glutathione s-transferase M1 (<i>GSTM1</i>)		
	Present (N=43);	Null deletion(N=12);	
	671 ± 226 1.4 ± 0.9	586 ± 183 1.9 ± 0.9	0.197 0.143
GST 8-isoprostane	Glutathione s-transferase T1 (<i>GSTT1</i>)		
	Present (N=44);	Null deletion(N=11);	
	647 ± 233 1.4 ± 0.9	670 ± 153 2.0 ± 0.8	0.705 0.034

^A Student's t-test was used.