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RESEARCH ARTICLE

Doppler Study and Cell Free DNA Biomarkers by using PCR in Hypertensive and Diabetic Pregnant Iraqi Women

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Abstract

Doppler ultrasonography has been used to assess both the fetal and placental circulation, to facilitate the diagnosis and monitoring of important conditions. Circulating cell-free (cf-DNA) in plasma is considered a novel biomarker with promising clinical applications. This biomarker was reported in prenatal diagnosis and in adverse pregnancy outcomes**.** Hypertensive disorders of pregnancy and both type 1 diabetes and type 2 diabetes in pregnancy represent a significant cause of maternal and perinatal morbidity and mortality. The aim of the study is to relate the Doppler study of the umbilical cord and uterine artery to the presence of different primers in hypertensive and diabetic pregnant women. Ninety women had been enrolled in this study. Thirty (33%) of them with diabetes mellitus and the other 60 (67%) women were with hypertension. The mean age of study sample was (29.7 ±6.33 years) . Careful history was taken from all women. Uterine blood flow for women below 28 weak gestational age, and umbilical artery blood flow for 28 weak pregnancies and more was done by Doppler Ultrasound. Blood samples were taken from all women and cf-DNA was measured using PCR technique. There is significant association ($P \leq 0.05$) between Autism primers 3 with the Doppler study in hypertensive pregnant women. Also there is significant association between Sirti primers 3, 4 with the Doppler study in diabetic pregnant women, and there is significant association between Trisomy primers 3 with the Doppler study in hypertensive pregnant women. There is no difference between the negative and positive results in relation to Doppler study regarding hypertensive and diabetic pregnant women except for the above primers.

Keywords: *Doppler study, Cf-DNA biomarkers, PCR, Hypertension, Diabetes.*

Introduction

Hypertensive disorders of pregnancy, a general term that includes preexisting and gestational hypertension, preeclampsia, and eclampsia, complicate up to 10% of pregnancies and represent a significant cause of maternal and perinatal morbidity and mortality [1]. The definition of hypertension in pregnancy has not always been standardized, but following the "National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy" recommendation is currently a systolic blood pressure (SBP) ⩾ 140 mmHg and/or a diastolic blood pressure (DBP) ⩾ 90 mmHg [2]. The diagnosis generally requires two separate measurements [3]. The prevalence of diabetes in pregnancy has been increasing in the world. The majority is gestational diabetes mellitus (GDM) with the remainder primarily preexisting type 1 diabetes and type 2diabetes. The rise in GDM and type 2diabetes in parallel with obesity both in United States and worldwide is of particular concern. Both type 1 diabetes and type 2 diabetes in pregnancy confer significantly greater maternal and fetal risk than GDM, with some differences according to type of diabetes. In general, specific risks of uncontrolled diabetes in pregnancy include spontaneous abortion, fetal anomalies, preeclampsia, fetal demise, macrosomia, neonatal hypoglycemia, and neonatal hyperbilirubinemia, among others. In addition, diabetes in pregnancy may increase the risk of obesity and type 2 diabetes in offspring later in life [4].Diabetes is defined as having fasting plasma glucose >126 mg/dl, spontaneous glucose level >200 mg/dl or

HbA1c > 6.5%, and for GDM it appears before 20 weeks of gestation [5].Circulating cell-free nucleic acids in plasma and serum are considered novel biomarkers with promising clinical applications in different medical conditions [6]. These biomarkers were reported in various aspects of obstetrics (especially in prenatal diagnosis) and in adverse pregnancy outcomes [7]. In serum samples, the vast amount of total cf-DNA is derived from the demise of maternal leucocytes [8].

Placental or fetal cf-DNA (cfp-DNA) makes up for a small fraction (≈5%) of the total cf-DNA pool in maternal plasma, and is even lower in serum samples, as it is diluted by the significant increase in maternal leucocyte derived material [9].The cfp-DNA can be detected in maternal circulation as early as the fifth or sixth weeks of gestation [10]. Its concentrations increase steadily with advancing gestational age.

The small fragments of cfp-DNA predominantly originate from trophoblast cells; [11, 12] nevertheless; the quantification of circulating cfp-DNA in maternal circulation requires the utilization of complex methods for DNA extraction, and real-time-polymerase chain reaction (rt-PCR) [13]. Conditions that affect the placenta can directly impact its concentrations of cell-free fetal DNA in the maternal circulation, as its release is closely related to placental morphogenesis [14].

Preeclampsia is associated with elevated concentrations of cell-free fetal DNA in maternal circulation [15]. Doppler ultrasonography has used to assess both the fetal and placental circulation, with the aim of facilitating the diagnosis and monitoring of important conditions, such as fetal growth restriction (FGR), fetal anaemia and twin-to-twin transfusion syndrome (TTTS) [16].

More recently, Doppler has been applied to the screening for conditions such as aneuploidy and pre-eclampsia [17]. Commonly used Doppler assessment in obstetrics encompasses that of the umbilical artery (UA), middle cerebral artery (MCA), uterine artery and ductus venosus (DV). More specialized and uncommonly performed Doppler includes that of the umbilical vein (UV), aortic isthmus and atrioventricular valves [18]. The aim of the study to detect if there is association between the Doppler study results and the presence or absence of Autism, Sirti, and Trisomy primers in hypertensive and diabetic pregnant women.

Materials and Methods

Ninety pregnant women enrolled in this study; attended AL-Karkh Hospital for obstetric from January 2018 to February 2019. Women with complicated pregnancy were included in this study Thirty (33%) of them with diabetes mellitus (DM) and the other sixty (67%) women with hypertension (HPT). The mean age of study sample was (29.7 ±6.33).

Pregnant women with normal pregnancy (no complication) were excluded. History was taken from all women including age, job, previous fetal abnormality, age of pregnancy, gravida, parity, abortion, pregnancy complication, drugs, weight, height, the last menstrual period (LMP), the type of previous pregnancy. Medical examination was done by an obstetrician, fundal height, blood pressure.

Ultrasound examination was done for detection of fetal age, any fetal abnormality, placental site, and the amount of amniotic fluid. Uterine blood flow for women below 28 weak gestational age, and umbilical artery blood flow for 28 weak pregnancies and more was done by Doppler Ultrasound manufactured by Philips Health Care Company. Blood samples were collected in tube contain EDTA and stored in (-20C^0).

DNA Extraction and Estimation of its Concentration and Purity

Procedure of DNA extraction was done according to Kit information, that used proteinase K and four buffers in accurate amount that added to the blood sample. The DNA concentration and purity was determined by using nanodorp spectrophotometer. Two μl of DNA sample was added, the software will be automatically calculated. The nucleic acid concentration (ng/ μl) at 260 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA, the accepted ratios of DNA purity must range between (1.8-2.0).

Primer Design

Primers Reconstitution

- Primers of FMR1 (forward F and reverse R), C16 (forward F and reverse R), SEZ6L2

(forward F and reverse R) for detection of Aulism gene were prepared by adding 100µl of (de ionized water) to the powder of the primer in concentration of 100nM to obtain a concentration of 100µM.

The Tables below show the sequence and product size for each Primer

FMR1

Forward	5, – GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT – 3,	900 bp
Reverse	5, – AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA -3,	900 bp

C16

Forward	5,- ACT GCC CCA GCG AAG ATG -3,	500 bp
Reverse	5, -CCG ACC ACC CAG ACC -3,	500 bp

SEZ6L2

Forward	5, –CCT CTC TCT TCC CCA CAA AGG- 3,	1000 bp
Reverse	5, - TGG ACA GCC TGG TTC TCT CT-3,	1000

- Primers of A allel 1 (forward F and reverse R), A allel 2 (forward F and reverse R), G allel (forward F and reverse R), G allel 2 (forward F and reverse R), for detection of SIRTI gene were prepared by adding 100µl of (de ionized water) to the powder of the primer in concentration of 100nM to obtain a concentration of 100µM.

The tables below show the sequence and product size for each Primer

A allel 1

Forward	5,- CCC AGG GTT CAA CAA ATC TAT GTT G-3,	900 bp
Reverse	5, – GCT TCC TAA TCT CCA TTA CGT TGA C-3,	900 bp

A allel 2

Forward	5,– GGT GGT AAA AGG CCT ACA GGA AA-3,	500 bp
Reverse	5,– CCT CCC AGT CAA CGA CTT TAT C -3,	500 bp

G allel 2

Forward	5'-GAG AAG AAA GAA AGG CAT AAT CTC TGC -3'	500 bp
Reverse	5'-GAT CGA GAC CAT CCT GGC TAA G -3'	500 bp

G allel

Forward	5'-GTA GCA GGA ACT ACA GGC CTG -3'	700 bp
Reverse	5'-CTA TCT GCA GAA ATA ATG GCT TTT CTC -3'	700 bp

- Primers of D21S11 (forward F and reverse R), D21S226 (forward F and reverse R), D18S51 (forward F and reverse R), D13S258 (forward F and reverse R), D13S61 (forward F and reverse R) for detection of Trisomy gene were prepared by adding 100µl of(de ionized water) to the powder of the primer in concentration of 100nM to obtain a concentration of 100µM.the primer then were vortex thoroughly .the primer ID number was written on the lid of the vial .primer then stored in the refrigerator at a degree (-20).

Primer sequence for D21S11 was

Forward	5, -TGT ATT AGT CAA TGT TCT CCA G-3,	600 bp
Reverse	5, – ATA TGT GAG TCA ATT CCC CAA G-3,	600 bp

Primer sequence for D21S226 was

Forward	5- ATC AGA TTT CTG AAA ACC AG-3,	600 bp
Reverse	5- TAT TCT TAA AAG TCT TCC CT-3,	600 bp

Primer sequence for D18S51 was

Forward	5, -CAA ACC CGA CTA CCA GCA AC-3,	600 bp
Reverse	5, - GAG CCA TGT TCA TGC CAC TG-3,	600 bp

Primer sequence for D13S258 was

Forward	5, -ACC TGC CAA ATT TTA CCA GG-3,	600 bp
Reverse	5- GAC AGA GAG AGG GAA TAA ACC-3,	600 bp

Primer sequence for D13S61 was

Forward	5- GGC AAC AAG AGC AAA ACT CT-3,	900 bp
Reverse	5, -TAG CCC TCA CCA TGA TTG G-3,	900 bp

Polymerase Chain Reaction (PCR)

The PCR mixture was done in a total volume of 25 µl containing: the DNA extracted primers, and PCR premix.

PCR Premix Kit:

Accupowder ® is a new, powerful, and ready to use PCR reagent optimized for more accurate PCR amplifications supplied by Bioneer Company

Procedure was according to the kit

- The Primers and template DNA were added into the PCR Premix tube.
- Distilled water was put into the PCR Pre Mix tube to 25 µl as total.
- The blue þellet was dissolved entirely and spin down by þipetting. The reaction was maintained at $4C^0$ following the end of amplification and the storage at ($-20C^0$)
- The reaction mixture (8µ)was loaded directly on gel without adding loading dye to analyze the product of PCR

Detection of Selected Autism, Trisomy, and Sirti genes by using Multiplex PCR Technique

Multiplex PCR requires multiple primer sets within the same amplification reaction. This allows determining multiple DNA sequences within the same sample.

For Autism gene all forward and reverse primers of C16 and FMR1 were added in one Gold multiplex PCR Premix tube, the same way all forward and reverse primers of SEZ6L2 were added in another tube.

For SIRTI genes all forward and reverse primers of G allel and G allel 2 were added in one Gold multiplex PCR Premix tube, the same way all forward and reverse primers of A allel 1 and A allel 2 were added in another tube.

PCR reaction tubes were centrifuged by using by vortex to dissolve lypholized master mix. The mixture tubes were placed in the thermal cycler where DNA was amplified

Detection of PCR Products by Agarose Gel Electrophoresis

To detect the size of the amplicons were amplified by multiplex PCR; agarose gel electrophoresis with 2% concentration was used. There after the electrophoresis tank closed with its special lid, and the electric current was matched at 80 volts for 30 minute.

Gel visualized in in gel documentation system/ UV transilluminator revealing bands which were interrupted as primers.

Statistical Analysis

All data were tabulated using Microsoft Excel and statistical analysis was made using SPSS 17.0 .All values were in mean ± SD and using chi-square test where appropriate. A P value of less than 0.05 was considered significant.

Results

Table 1: Doppler study in diabetic and hypertensive pregnant women in relation to Autism primers

Disease			APRI1		APRI2		APRI3	
			neg	pos	neg	pos	neg	pos
HPT	Doppler	abnormal	0	6	4	2	2	4
		normal	6	48	41	13	4	50
	Total		6	54	14	46	6	54
	P value		0.389		0.619		0.045*	
Pearson's chi-square, F Fishers' exact test, * Significant at 0.05 level Pos=positive, neg=negative								
			neg	**pos**	**neg**	**pos**	**neg**	**pos**
DM	Doppler	abnormal	0	2	0	2	2	0
		normal	2	26	6	22	6	22
	Total		2	28	6	24	8	22
	P value		0.789 F		0.415		0.646	
Pearson's chi-square, F Fishers' exact test, * Significant at 0.05 level								

Table 2: Doppler study in diabetic and hypertensive pregnant women in relation to Sirti primers

Disease			SPRI1		SPRI2		SPRI3		SPRI4	
			neg	pos	neg	pos	neg	pos	neg	pos
HPT	doppler	abnormal	0	6	2	4	2	4	2	4
		normal	8	46	12	42	14	40	16	38
	Total		8	52	14	46	16	44	18	42
	P value		0.311		0.542		0.697		0.851	
Pearson's chi-square, FFishers' exact test, * Significant at 0.05 level										
Disease			**SPRI1**		**SPRI2**		**SPRI3**		**SPRI4**	
			neg	pos	neg	pos	neg	pos	neg	pos
DM	Doppler	abnormal	0	2	0	2	2	0	2	0
		normal	2	26	6	22	6	22	6	22
	Total		2	28	6	24	8	22	8	22
	P value		0.696		0.464		0.015 *		0.015*	
Pearson's chi-square, FFishers' exact test, * Significant at 0.05 level										

Table 3: Doppler study in diabetic and hypertensive pregnant women in relation to Trisomy primers

Disease			TPRI1		TPRI2		TPRI3		TPRI4		TPRI5	
			neg	pos	neg	pos	neg	pos	neg	pos	neg	pos
HPT	doppler	Abnormal	0	6	0	6	4	2	2	4	2	4
		normal	4	50	4	50	4	50	6	48	10	44
	Total		4	56	4	56	8	52	8	52	12	48
	P value		0.490		0.490		0.001*		0.129		0.389	
Pearson's chi-square, FFishers' exact test, * Significant at 0.05 level												
Disease			**TPRI1**		**TPRI2**		**TPRI3**		**TPRI4**		**TPRI5**	
			neg	pos	neg	pos	neg	pos	neg	pos	neg	pos
DM	doppler	Abnormal	0	2	0	2	0	2	0	2	0	2
		normal	1	27	0	28	6	22	2	26	0	28
	Total		1	29	0	30	6	24	2	28	0	30
	P value		0.75				0.464		0.696			
Pearson's chi-square, FFishers' exact test, * Significant at 0.05 level												

Discussion

Doppler ultrasound evaluates the fetoplacental circulation, the increased resistance to flow in the umbilical and uterine arteries can be detected with Doppler ultrasound that is used as a part of a clinical protocol in surveillance of high risk pregnancies and it is not indicated in low-risk pregnancies [19, 20]. As it is seen in table (1) there is no significant differences between positive and negative values of Autism primer 1and 2 in relation to Doppler study regarding hypertensive women, but there are significant differences between positive and negative reading values of Autism primer 3 to in relation Doppler study regarding hypertensive women and this may be the results of the systemic inflammatory response in hypertensive pregnancies where placenta inflammation plays a central role [21], the hemodynamic changes shown by Doppler velocimetry might be a result of an abnormally increased placenta inflammation secondary to the inflammatory process that accompanies placental ischemia. The inflammatory process can also activate leukocytes, especially granulocytes that can stimulate endothelial cells of the uterine vessels and promote anchoring of inflammatory leukocytes with resulting endothelial cell damage [22]. Accumulating evidence suggests that the immune system and abnormal immune function, including

inflammation, cytokine dysregulation, and anti-brain autoantibodies, influence trajectories of autism, playing a role in its etiology [23, 24]. An abnormal development of the uteroplacental circulation has an impact on placental development and structure leads to fetal growth restriction (FGR). Ultrasound imaging, and in particular color Doppler imaging, has allowed the study of both the umbilicoplacental and uteroplacental circulations from the first trimester of gestation onward [25, 26].

Studies found that low birth weight, prematurity were related to childhood autism [27]. However, in one study the association between low birth weight and ASD was statistically significant only among girls [28].The same table shows that there are no significant differences between positive and negative values of the 1, 2, 3 primers of Autism in relation to Doppler study regarding diabetic women. Table (2) shows that there is no significant differences between positive and negative values of the 1,2,3,4 primers of Sirti gene in relation to Doppler study regarding hypertensive women.

The same table shows that there are no significant differences between positive and negative values of the primer 1 and 2 but there are significant differences between positive and negative reading of primers 3 and 4 of Sirti gene in relation to Doppler study regarding diabetic women. Some studies found a significant association between impedance to flow and maternal serum glucose concentration. Furthermore, high impedance e was associated with an increased number of stillbirths and neonatal morbidity.

It was suggested that maternal hyperglycemia causes placental vasoconstriction by impairing prostacyclin production [29]. In another study on singleton pregnancies complicated by maternal diabetes mellitus there was an adverse outcome like delivery before 37 weeks, or fetal risk requiring Cesarean delivery, or fetal growth restriction [30].

The relative risk of adverse outcome because of increased impedance in the umbilical artery, also the left and right uterine arteries [31]. The fetal intrauterine hypoxia environment will enhance the oxidative stress responses in fetus, increase the production of oxygen free radicals and cause oxidizing damage in fetus [32, 33].Diabetes in pregnancy amplify the low-grade inflammation existing in normal pregnancy [34]. Also high blood glucose levels induce oxidative stress and decrease antioxidant defenses, thus leading to increased free radical formation, and these free radicals may damage not only the organs in which they are formed but also the remote organs [35].

Both Inflammation and oxidative stress responses act as crucial part in immune functions and neural development [36, 37]. Oxidative stress has been suggested as a causative agent in human pregnancy-related disorders, such as embryonic resorption, recurrent pregnancy loss, preeclampsia, intra-uterine growth restriction, and fetal death [38].

Development of type II diabetes and intra‐adipose tissue hypoxia and activation of hypoxia inducible factor‐1 (HIF‐1). HIF‐1activation is pivotal to maintain glucose intolerance, insulin resistance and cardiomyopathy. Studies have found that SIRT2 deacetylates HIF‐1□and reduces the capability of HIF‐1□in response to cellular hypoxia [39]. SIRTI activation actually protects neuron cells from apoptosis.

Because during cerebral hypoxia, the levels of ATP and oxygen in neuron cells are lowered, which stimulate both bioenergenic and oxidative stress leading to SIRT1 activation [40]. Table (3) shows that there is no significant differences between positive and negative values of the 1,2,4,5 primers of trisomy gene but primer 3 has significant differences in relation to Doppler study regarding hypertensive women. Fetal vascular malperfusion and maturation defects are more common in trisomy 21 placentas. Abnormal umbilical artery Doppler waveforms are more common in T21 and are associated with maternal vascular malperfusion [41].

Studies show that the placenta of trisomic (18, 13 and 21) fetuses exhibited a significant reduction in small muscular artery count and a small muscular artery to villous ratio. Abnormal umbilical artery (UA) Doppler waveforms correlated strongly with these findings.

Interestingly, there was a wide range in deviation and not all placentas with the same trisomy were uniformly affected [42]. An abnormal UA flow pattern might occur irrespective of fetal growth restriction in fetuses affected by autosomal trisomies. Other Doppler studies in fetuses with abnormal karyotypes revealed conflicting results [43]. Abnormal UA Doppler findings have been associated. With disturbance in placental function of chromosomal abnormal fetuses and the higher rate of fetal growth restriction [44].In contrast to fetuses with trisomy 18 or 13, fetuses affected by trisomy 21 are usually only mildly growth restricted. It is demonstrated that in fetuses with trisomy 21 increased UA indices occurred irrespective of malformations or classical uteroplacental dysfunction.

However, other studies reveal that isolated fetal malformations were not associated with abnormal UA Doppler indices [45] while others [46] described higher rates of malformations among appropriated for gestational age fetuses with abnormal UA Doppler findings compared to ones with normal Doppler. The same table shows that there is no significant differences between positive and negative reading of the 1, 2,3,4,5 primers of trisomy in relation to Doppler study regarding diabetic women

Conclusion

It was concluded that there is no difference between the negative and positive results in relation to Doppler study regarding hypertensive and diabetic pregnant women except for:

Autism primer 3 in hypertensive women

Sirti primers 3, 4 in diabetic pregnant women

Trisomy primer 3 in hypertensive women

Conflict of Interest

There is no important conflict of interest among authors or other research teams.

Source of Funding

The study was done with personal funding without any funding from other institutions or agencies.

Ethical Clearance

The study was approved by the ethical committee of the medical college of university of Baghdad. An informed consent was taken from all patients participating in this study.

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