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Original Research Article

Endothelial nitric oxide synthase gene glu298asp polymorphism in preterm neonates with respiratory distress syndrome

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ABSTRACT

Background: Neonatal respiratory distress syndrome (RDS) is a multifactorial disease of preterm influenced by many factors including gene polymorphism. The aim of the present study was to determine the effect of endothelial nitric oxide synthase gene glu298asp polymorphism (rs1799983) in developing and grading of RDS in preterm neonates.
Methods: This study was performed on 65 preterm neonates; 40 with RDS and 25 healthy controls. Genotyping of endothelial nitric oxide synthase gene glu298asp polymorphism was performed by restriction fragment length polymorphism.
Results: There were statistical significant increases in TT, (GT+TT) genotypes and T allele frequencies of rs1799983 among RDS neonates compared to controls. As groups were categorized by gestational age; TT genotype and T allele frequencies were statistically significantly increased in 33-35weeks RDS neonates compared to controls. TT genotype in RDS was associated with RDS grade III, mechanical ventilation need, and increased mortality. TT genotype, T allele, gestational age (<28-32weeks) and birth weight (<1500grams) were predictor factors for RDS in binary logistic regression analysis.
Conclusions: eNOS glu298asp polymorphism could be implicated in RDS pathophysiology and may affect the disease severity and outcome. TT, GT+TT Genotypes and T allele might be predisposing risk factors for RDS in preterms. TT genotype and T allele might be of the predictors of neonatal RDS.

Keywords: Endothelial nitric oxide synthase, Polymorphism, RDS, RFLP, rs1799983

INTRODUCTION

Neonatal respiratory distress syndrome (RDS) is the most common cause of respiratory failure in preterm, occurring as a result of surfactant deficiency and underdeveloped lung anatomy. The pathophysiology of RDS is complicated.[1] In addition to environmental factors; genetic factors may participate in RDS risk among preterm, and term infants. Discovery of risk alleles may be important for prediction and management of RDS risk.[2] Nitric oxide (NO) is a neurotransmitter for nonadrenergic, non-cholinergic neurons decreasing vascular and bronchial tone. NO facilitates coordinated beating of respiratory cilia.[3]

NO is synthesized by L-arginine oxidation to L-citrulline by nitric oxide synthase (NOS). In humans, three NOS isoenzymes are found: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS).[4] eNOS [enzyme classification (EC:1.14.13.39)] is the predominant in

pulmonary circulation. eNOS gene is on chromosome 7q35-36 and has 26 exons.[5] This gene has many allelic variations, but only few are functional. Guanine to thymine single nucleotide polymorphism (SNP) at position 894 at exon 7 leads to substitution from glutamate to aspartate at amino acid position 298 (rs1799983).[6]

This study aimed to determine the effect of rs1799983 in the developing and grading of RDS in preterm neonates.

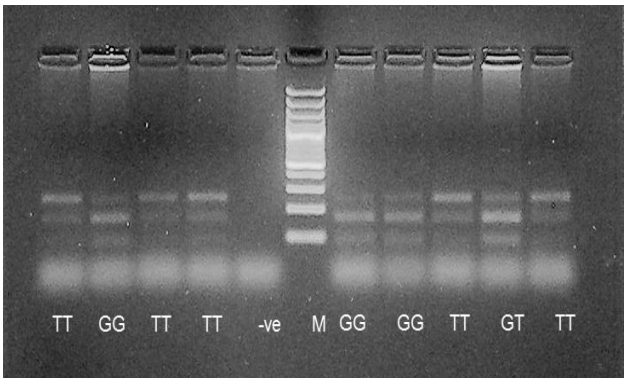
METHODS

This study was performed on 65 neonates: 40 preterm RDS, from Benha University Neonatal Intensive Care Unit and 25 healthy preterm. Informed consents from neonates´ parents were obtained. The study was approved by Ethical Committee of Benha Faculty of Medicine.

RDS was diagnosed according to; respiratory rate>60/m., cyanosis with intercostal and subcostal retractions, typical X-ray. 1 Congenital anomalies, inherited metabolic disorders and sepsis were excluded. Neonates were subjected to history taking, general and local examinations; APGAR score [normal (8-10), mild (6-8), moderate (3-5), severe (0-2)]. Complete blood count and arterial blood gas were performed (ABG for RDS neonates only).

Genotyping of eNOS rs1799983 by restriction fragment length polymorphism (RFLP)

DNA was extracted from 200µl EDTA blood; using Gene JET Whole Blood DNA Purification Mini Kit (Thermo-Fisher Scientific, Germany). Extracted DNA was measured by Nanodrop 2000 (Thermo-Fisher Scientific, Wilmington, USA). Readings were taken at 260 and 280 nm. Pure DNA had OD260/OD280 of 1.7-2.0.[7]



A photo for Gel electrophoresis of eNOS3 glu298asp polymorphism; the homozygous GG genotype has 2 bands (163bp and 85bp), the homozygous TT genotype has only one band (248bp) while the heterozygous GT genotype has 3 bands (248bp, 163bp and 85bp).

Figure 1: Gel electrophoresis for digested PCR products of eNOS glu298asp (rs1799983).

Amplification of DNA was performed using G-storm thermal cycler (United Kingdom), FP; 5′-AAGGCAGGAGACAGTGGATGGA-3′ and RP; 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′.[8] DreamTaq Green PCR Master Mix (25µl), 2.5µl FP, 2.5µl RP, 5µl DNA Template and 15µl nuclease-free H2O were added for each reaction. Thermal cycling: 5m. initial denaturation (94°C), 35 cycles [30s. denaturation (94°C), 1m. annealing (58°C) and 30s. extension (72°C)] and 10m. final extension (72°C).

RFLP was done using BanII restriction enzyme (Thermoscientific, Germany): 10µl PCR products + 2µl BanII + 2µl buffer +18µl nuclease-free water. Mixture incubated at 37◦C (8hrs) then at 65°C (10m.).

The digested DNA was visualized by gel electrophoresis. DNA (10µL) and 100bp ladder (5µL) were separated on 2.5% agarose gel with 0.3ug/ml ethidium bromide. Bands were visualized by Alpha InoTech Gel Documentation System (GG; 163bp and 85bp, TT; 248bp, GT; 248bp, 163bp and 85bp) Figure 1.

Statistical analysis

Healthy controls were in Hardy-Weinberg equilibrium (X^2=0.1, p>0.05) using the Online Encyclopedia for Genetic Epidemiology software (http://www.oege.org/software/ hardy-weinberg.html).

Collected data were tabulated and analyzed using SPSS version 20 software (SPSS Inc, Chicago, ILL Company). Categorical data were presented as numbers and percentages. Quantitative data were expressed as Mean±SD. Chi square test (X^2), Fisher's exact test (FET) and student "t" test were used as tests of significance. Binary logistic regression analysis was used to detect significant predictors of RDS. p<0.05 was considered significant.

RESULTS

Characteristics of studied neonates showed significant decreases in birth weight (p<0.01), gestational age (p<0.05) and platelet count (p<0.01) but significant increases in moderate and severe APGAR score (p<0.01), mortality (p<0.001), maternal DM and preeclampsia (p<0.05 for both) in RDS compared to controls (Table 1).

There were significant increases in TT, GT+TT Genotypes and T allele frequencies among RDS compared to controls (p<0.001, <0.01 and p<0.001 respectively), OR (17.5, 5.1 and 5.3); 95%CI was (3.2-95.2), (1.68-15.4) and (2.4-11.5) respectively (Table 2).

TT Genotype and T allele frequencies were statistically increased in 33-35weeks RDS compared to controls when categorized by gestational age (p<0.001 for both, OR=39.5, 95%CI (1.9-787.7), OR=6.6, 95%CI (2.3-18.8), respectively (Table 3). There were significant

increases in RDS grade III (p<0.001), mechanical ventilation need (p<0.05), and mortality (p<0.05) among TT RDS compared to other genotypes (Table 4). TT genotype, T allele (p<0.001 for both), gestational age <32weeks (p<0.01) and birth weight <1500grams (p<0.05) were possible predictors for RDS (Table 5).

Table1: Demographic, clinical and laboratory characteristics of studied neonates.

Variable		RDS (n.=40)	Controls (n.=25)	Test	p
		Mean±SD or n (%)			
Gestational age (weeks)		32.28±2.17	33.92±1.61	X^2=5.87	<0.05*
Birth weight (grams)		1743±328	2035±165	X^2=8.14	<0.01**
Maternal age (years)		26.05±4.91	26.76±4.69	X^2=0.653	>0.05
Gender (♂/♀)		20 (50)/20 (50)	8 (32)/17 (68)	X^2=2.03	>0.05
Mode of delivery	V[a]/CS[b]	10 (25)/30 (75)	5 (20)/20 (80)	FET=0.22	>0.05
Antenatal steroid use		18 (45)/22 (55)	17 (86)/8 (32)	X^2=3.3	>0.05
Gravidity	Primi/multi	16 (40)/24 (60)	14 (56)/11(44)	X^2=1.58	>0.05
Maternal Risk Factor	Diabetes mellitus	13 (32.5)	2 (8.0)	Z=2.28	<0.05*
	Preeclampsia	15 (37.5)	3 (12)	Z=2.24	<0.05*
	PROM[c]	10 (25)	2 (8.0)	Z=1.72	>0.05
APGAR Score	Mild/Moderate/ Severe	14 (35)/10 (25)/10 (25)	4 (16)/0/0	Z=2.72	< 0.01**
Outcome	improved/died	20 (50)/20 (50)	25 (100)/0	X^2=18.06	<0.001**
Total leucocyte count (×10^3cells/mm^3)		15.3±5.55	13.2±2.03	1.84	>0.05
Hemoglobin (grams/dl)		12.2±2.98	12.8±2.00	0.96	>0.05
Platelets (×10^3 cells/mm^3)		154.9±88.20	218.2±51.24	2.91	<0.01**

[a] vaginal delivery, [b] cesarean section, [c] premature rupture of membranes,*significant,**high significant.

Table 2: Genotype and allele frequencies of rs1799983 in studied neonates.

Genotype/Allele	RDS (n.=40)	Controls (n.=25)	Test	p	OR (95%CI)
GG[a]	8 (20)	14 (56)	Reference	-	1
GT[a]	12 (30)	9 (36)	X^2=0.03	>0.05	1.1 (0.36-3.4)
TT[a]	20 (50)	2 (8)	X^2=14.14	<0.001**	17.5 (3.2-95.2)
TT+GT[a]	32(80)	11(44)	X^2=8.9	<0.01**	5.1 (1.68-15.4)
G[a]	28 (35)	37 (74)	Reference	-	1
T[a]	52 (65)	13 (26)	X^2=18.7	<0.001**	5.3 (2.4 -11.5)

[a] n.(%),**high significant.

Table 3: Genotype and allele frequencies of rs1799983 in studied neonates by gestational age.

28-32weeks	Controls (n.=5)	RDS (n.=22)	Z Test	p	OR(95% CI)
GG[a]	1 (20)	2 (9.1)	0.7	-	1
GT[a]	2 (40)	9 (40.9)	0.04	>0.05	2.25 (0.13-38.8)
TT[a]	2 (40)	11 (50)	0.41	>0.05	2.75 (0.16-46.8)
G[a]	4 (40)	13 (29.5)	R	-	1
T[a]	6 (60)	31 (70.5)	FET	>0.05	1.59 (0.38-6.5)
33-35weeks	**Controls (n.=20)**	**RDS (n.=18)**	**Z Test**	**p**	**OR (95% CI)**
GG[a]	13 (65)	6 (33.3)	1.95	-	1
GT[a]	7 (35)	3 (16.7)	1.28	>0.05	0.93 (0.17-4.9)
TT[a]	0 (0)	9 (50)	3.62	<0.001**	39.5 (1.9-787.7)
G[a]	33 (82.5)	15 (41.7)	R	-	1
T[a]	7 (17.5)	21 (58.3)	X^2=13.6	<0.001**	6.6 (2.3-18.8)

[a]n.(%), **high significant.

DISCUSSION

NOS3 gene exhibits SNP, variable number tandem repeats, microsatellites, and insertions/deletions.[9] NO produces vasodilatation, inhibition of leukocyte adhesion and platelet aggregation.[10] Also, NO modulates immune-regulation, surfactant maturation or secretion.[11] In this study, we found statistical increases in TT, GT+TT

genotype and T allele among RDS compared to controls. In the contrary, Poggi et al. found that GT+TT is an independent risk factors for bronchopulmonary dysplasia but not RDS.[12] Moreover, Shen et al. observed non-significant increased GG genotype and G allele among RDS neonates in Chinese.[13] However, Sivasli et al. found no association with neonatal RDS among Turkish.[14] Present data demonstrate that T allele is independent risk factor for neonatal RDS. Short-living nitric oxide is cytoprotective antioxidant scavenging reactive oxygen species.[15] It protects against Fenton's reaction which generates highly reactive hydroxyl (OH•) free radicals.[16] T allele is functionally relevant affecting eNOS expression and enzyme activity decreasing NO, leading to oxidant/antioxidant imbalance with limited ability to reduce levels of iron-generated (FeNO) and non-iron-generated oxidative stress.[17,18] NO is also essential for decreased pulmonary vascular resistance after birth.[19] This could be implicated in RDS pathogenesis and delayed lung maturity.[20]

Table 4: Effect of rs1799983 on neonatal RDS severity and outcome.

Variable	GG (n.=8)	GT (n.=12)	TT (n.=20)	Test (FET)	p
Gestational age[a] 28-32weeks	2 (25)	9 (75)	11 (55)	4.6	>0.05
Birth weight[a] <1500 grams	1 (12.5)	4 (33.3)	7 (35)	1.4	>0.05
Antenatal steroid used[a]	4 (50)	5 (41.7)	9 (45)	0.25	1.0
RDS grades[a] I/II/III	8 (100)/0/0	0/12 (100)/0	1 (5)/0/19 (95)	62.3	<0.001**
Mechanical ventilation[a]	1 (12.5)	3 (25)	13 (65)	8.2	<0.05*
Mortality[a]	0 (0)	4 (33.3)	16 (80)	6.6	<0.05*

[a]n.(%), *significant,**high significant.

There were significant increases in TT genotype and T allele in 33-35weeks RDS when neonates were categorized by gestational age. Demirçubuk et al. found increased GG and G allele frequencies in 24-30weeks RDS group.[21] However; Shen et al found significant increases in TG genotype and T allele but in 26-33weeks RDS neonates.[13] eNOS rs1799983 may independently affect gestational age.[12] We suppose that the associated very low birth weight and involvement of other genetic factors could offer additional risk for RDS in lower age (26-33weeks).

Table 5: Binary logistic regression analysis for predictors of RDS.

Variable	OR (95%CI)	p
TT genotype	5.1 (2.6-11.0)	<0.001**
T allele	5.2 (2.3-10.5)	<0.001**
Gestational age (28-32weeks)	4.8 (1.6-14.6)	<0.01**
Birth weight (<1500grams)	3.3 (1.07-13.1)	<0.05*
Platelets (<150×10^{3}cells/mm^3)	2.5 (0.91-24.9)	>0.05

*significant, **high significant

In our study, there was statistical increase in mechanical ventilation need among TT RDS neonates. This disagreed with Sivasli et al. who found that rs1799983 not markedly influence mechanical ventilation.[14]

The statistical significant increase in RDS grade III and mortality among TT genotype in RDS neonates compared to other genotypes may suggest the association of TT genotype with the severity and poor survival of RDS. This was in agreement with McNamara et al., who found that Asp298 is associated with poor survival on studying non-ischemic cardiomyopathy.[22] This work was limited by studying only one SNP (eNOS glu298asp), however, the role of other SNPs and other genes could not be neglected.

CONCLUSION

eNOS glu298asp polymorphism could be implicated in RDS pathophysiology and may affect the disease severity and outcome. TT, GT+TT Genotypes and T allele might be predisposing risk factors for RDS in preterms. TT genotype and T allele might be of the predictors of neonatal RDS.

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