Oxidative stress is increased in women with epilepsy: Is it a potential mechanism of anti-epileptic drug-induced teratogenesis?

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Abstract

Context: Oxidative stress can be a final common pathway for AED-induced teratogenesis. **Aims:** To compare the oxidative stress of women with epilepsy (WWE) and unfavorable pregnancy outcome (fetal malformation or spontaneous abortion - group EM) with that of WWE with normal pregnancy outcome (group ENM) and healthy women with normal pregnancy outcome (group C). **Materials and Methods:** We identified WWE under group EM (n = 43) and group ENM (n = 22) from the Kerala Registry of Epilepsy and Pregnancy (KREP). Group C was constituted of healthy volunteers (N = 20). Oxidative stress was assessed by estimating serum levels of malondialdehyde (MDA) and isoprostane (ISP). The antioxidant profile was evaluated as activity of superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), total antioxidant status (TAO), and glutathione (GSH) content. **Results:** The MDA and ISP levels for group EM (3.46 + 0.82 and 17.77 + 3.0) were higher than that of group ENM (3.07 + 1.02 and 14.0 + 5.3), and both were significantly higher than that of group C (2.42 + 0.51 and 10.77 + 4.1). Their levels of SOD (146.82 + 42.64 vs. 175.81 + 42.61) and GSH (0.98 + 0.98 vs. 1.55 + 1.3) were significantly lower than those of controls. No significant changes were seen in TAO and GR. WWE on polytherapy showed significant increase in MDA when compared to monotherapy group. **Conclusion:** WWE (group EM and ENM) had higher oxidative stress and reduced antioxidant activity. The subgroup of WWE with unfavorable pregnancy outcome (group EM) had higher oxidative stress. Excess oxidative stress can be a final common pathway, by which AEDs exert teratogenic effects.

Key Words

Anti-epileptic drugs, antioxidant, epilepsy, fetal malformation, oxidative stress, teratogenesis

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Introduction

Epilepsy affects an estimated 5 million people in India, and approximately 46% of them are women.[1] They have increased risk of unfavorable pregnancy outcome in terms of fetal loss or Major Congenital Malformations (MCM) in the offsprings. Risk of spontaneous abortion was twice that of control group in the patients followed up in the Kerala Registry of Epilepsy and Pregnancy (KREP).[2] The incidence of MCM in the Kerala Registry of Epilepsy and pregnancy[3] was around 10% while the overall incidence in a large meta-analysis was only 7% (95% confidence interval 5.65-8.4%).[4] The excess risk of MCM in WWE is attributable to antenatal exposure to anti-epileptic drugs (AEDs) as this risk is negligible in babies of women with untreated epilepsy.[5,6] Most WWE need to continue AEDs during pregnancy in order to remain seizure-free. If they remain untreated during pregnancy, seizures may aggravate, thereby increasing the risk of complications to the mother and child.

The increased risk of spontaneous abortion or fetal loss in later part of pregnancy may be related to exposure to anti-epileptic drugs. Severe malformations in the fetus or other metabolic changes can lead to early spontaneous abortion.[7]

AEDs that are currently in clinical use vary widely in their biochemical characteristics, molecular structure, mechanism of action, and metabolic pathways, yet there is considerable overlap in the type of MCM that they induce in fetuses. The commonly identified malformations include cardiac defects, neural tube defects, orofacial malformations, oral clefts, and genitourinary defects including hypospadias. Data from the registries and other studies have shown that all AEDs can

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potentially cause any of these defects.[8] Due to the considerable overlap between embryopathy related to different AEDs, a term "fetal anti-epileptic drug syndrome" can be noted.[9] It is possible that there is a final common pathway, by which these molecules that differ so widely contribute to similar malformations.[9,10] Despite the widespread use of AEDs and knowledge of their teratogenicity, the precise mechanism, by which the AEDs mediate malformations in fetus, is uncertain. The list of suggested mechanisms for AED teratogenicity is long and diverse. AEDs, during their metabolism, can generate reactive metabolites that elicit systemic toxicity by bonding to proteins and other macromolecules.[11-14] AEDs increase oxidative stress, and excess oxidative stress may be one of the mechanisms that contribute to teratogenicity.[15-17]

Oxidative stress is defined as the imbalance between FR damage and antioxidant protection. FRs are unstable atoms or molecules carrying odd number of electrons at outer atomic or molecular orbitals. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecules, thereby causing a cascade of reactions that ultimately result in cellular damage.[18] Malondialdehyde (MDA) and isoprostanes (ISP) are products of FR attack on lipids. Antioxidants act for scavenging FR.[19] Under normal conditions, FRs are quenched as soon as they are formed by the powerful antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione (GSH). Oxidative stress may be a consequence of reduced efficiency of these antioxidants or due to excess FR production. Anti-epileptic drugs in therapeutic dosage have been known to unfavorably alter the redox balance in experimental models and in human beings.[20,21]

Several previous studies have demonstrated that persons with epilepsy and using anti-epileptic drugs have increased oxidative stress.[7,22-24] The results of a study showed that the serum lipid peroxidation level was higher in epileptic patients treated with valproic acid and presented a linear relationship with drug plasma levels.[25] Another study showed that total peroxide levels, which are a marker of oxidative stress, were elevated in PB-treated epileptic children.[20] We hypothesized that there is excess oxidative stress in WWE who are using AEDs, and there is a correlation between MCM in infants and maternal oxidative stress. The objective of this study was to correlate the oxidative stress in WWE with fetal outcome (abortions or malformations).

Materials and Methods

This study was carried out in the Kerala Registry of Epilepsy and Pregnancy in Sree Chitra Tirunal Institute for Medical Sciences and Technology, which is one of the tertiary care epilepsy centers in South India. This pregnancy registry has over 1500 registrations and had been operational since 1998. The study has the approval of the institutional ethics committee, and an informed consent was obtained from all registrants and control subjects.

Selection criteria and recruitment

We invited all WWE who had an unfavorable pregnancy outcome – spontaneous abortion or MCM in the baby – to participate in this study. Out of 62 women with unfavorable pregnancy outcome in the KREP (in past five years), 43 participated in this study (group EM). We also invited age- and parity-matched WWE (n = 22) in the registry with normal pregnancy outcome (group ENM). The control arm (group C) consisted age, parity, and socio-economic class-matched healthy women without epilepsy or exposure to anti-epileptic drugs (n = 20). We excluded from this study those women with other concomitant disorders and who were taking antioxidants or other medications. All women in the epilepsy group (group EM and group ENM) were taking AEDs. Clinical and demographic details of the WWE were extracted from their medical records and by personal interview. [Table 1] A Dietary questionnaire was filled up to assess their dietary intake of antioxidants and other nutrients. Blood samples were drawn from the subjects 3 – 36 months after delivery. No subject had a seizure in the previous seven days of blood sampling. (In this retrospective study, in order to get reasonable number of subjects with fetal malformation/abortion, we had to recall patients who had delivered earlier also.) This interval between pregnancy outcome and blood sampling would have lead to some variation in the redox status. Nevertheless, most women were continuing on same AEDs that they were using during pregnancy and did not have any change in their epilepsy activity during this period. Blood samples (5 ml venous blood) were drawn by venous puncture from all subjects. Serum separated from 4.5 ml of blood was used for the estimation of total antioxidant status (TAO), MDA, GR, GSH, and CAT erythrocytes separated from 0.5 ml of fresh blood anti-coagulated with EDTA was used for estimating SOD, and EDTA plasma was used for estimation of ISP.

We estimated MDA and ISP levels as indicators of oxidative stress. The MDA level in serum was quantitated spectrophotometrically by thiobarbituric acid method.[26] We estimated ISP level in the plasma by competitive enzyme immuno assay technique using assay kits from Cayman chemical Company, Ann Arbor, MI, USA. The antioxidant profile was measured by estimating the TAO, GSH content, and activity of antioxidant enzymes SOD, CAT, and GR. TAO, CAT, and GSH content in serum were measured using assay kits of Cayman chemicals by spectrophotometric methods using microplates. The GR activity and SOD activity were determined by spectrophotometric methods with commercial kits marketed by Randox Laboratories Ltd., Ardmore, Crumlin, Co. Antrim, UK. All tests were standardized in our laboratory and were performed in duplicate.

Statistical analysis

Data were transcribed to a spreadsheet and was analyzed statistically with SPSS for windows package. Data were expressed as mean (SD), and statistical comparison between different groups was performed using independent t test.

Results

Altogether, we studied 85 subjects. (Group C 20, ENM 22, EM 43). There were 65 WWE (group ENM 22 and group EM 43). All WWE were on AEDs, 54 monotherapy and 11 on polytherapy. The details of AED therapy are given in Table 1.

The WWE with unfavorable pregnancy outcome (group EM) consisted of 32 WWE with MCM in their babies and 11 with

abortions in the previous pregnancy. The control group consisted of 20 healthy women without epilepsy, matched for age and socio-economic status. The protein and calorie intake for those with epilepsy and control subjects were comparable. None of the control subjects were on any nutritional supplements or antioxidants.

The subgroup of epilepsy who had fetal malformations included 32 WWE (14 with GE and 18 LRE) with mean age 30.5 ± 3.9 years. Their treatment was monotherapy for 25 (CBZ 6, VPA 10, PB 3, LTG 2, PHT 2, TPM 1, OXB 1) and polytherapy for 7 persons. Of the 32 children with MCM, 18 had congenital heart diseases, 5 with central nervous system defects (2 hydrocephalus, *spinabifida*, meningocoele, Craniosynostosis-microcephaly 1 each, 4 renal defects, 2 cleft lip and palate, hypospadias, umbilical hernia and multiple, congenital anomalies 1 each.)

MDA and ISP levels comparison between groups

The mean concentration of MDA and ISP were significantly higher in WWE (EM and ENM combined) when compared to controls. [Table 2] The group EM had higher levels of MDA and ISP than the group ENM, and both groups had significantly higher levels of MDA when compared to group C. [Table 2] We set a threshold of mean + 2SD derived from the group C to identify those with very high levels of MDA. The epilepsy group, particularly those with unfavorable outcome (group EM), had 24 subjects with MDA level more than this threshold. This proportion was statistically significant ($P = 0.001$) when compared with group ENM with 8 subjects and also with group C with none having high value. There was no significant difference in the levels of MDA or ISP between those with GE and LRE. WWE exposed to AEDs, irrespective of the molecule, showed significantly higher levels of MDA when compared to group C. [Table 2] Those who were on polytherapy ($n = 11$) had significantly higher levels of MDA (3.89 ± 0.91) than those who were on monothearpy (3.22 ± 0.87).

Antioxidant profile

The antioxidant profile for the group EM (WWE with unfavorable outcome) was significantly lower than that of group ENM and group C. [Table 3] A significant proportion of subjects in group EM (14%) had very low SOD levels (< mean – 2 SD values from controls), whereas none in other groups had such low SOD levels. There was no significant difference in the antioxidant profile between those with GE and LRE. Those who were on CBZ showed significant reduction in SOD and GSH while those on PHT showed a significant reduction in SOD only. [Table 4] TAO, GR, CAT showed no significant changes in these groups. No significant changes are seen in any of the antioxidant profile in the case of VPA, PB, and CLB. There was no significant difference between these who were on monotherapy and polytherapy with respect to the TAO, SOD, GR, and GSH levels.

Table 1: Comparison of demographic and clinical profile of the three groups under study

Parameter	EM	ENM	Controls
N	43	22	20
Age mean (SD)	31.09 (4.3)	25.45 (3.9)	27.8 (3.2)
Age 95% CI	29.77 to 32.41	23.72 to 27.18	26.3 to 29.30
Diet			
Protein intake (gm/day)	46.11 (14.42)	45.32 (13.17)	47.23 (7.19)
CI	41.67 to 50.55	39.48 to 51.16	43.86 to 50.60
Calorie intake (Cal/day)	1740.98 (695.63)	1552.07 (416.90)	1670.32 (453.07)
CI	1526.9 to 1955.06	1367.23 to 1736.91	1458.28 to 1882.36
Number of persons using folic acid vitamin	5	1	0
Epilepsy classification			
GE (*n*)	20	10	NA
LRE (*n*)	23	12	NA
AED therapy			
Monotherapy (*n*)	35	19	NA
Polytherapy (*n*)	8	3	
Individual AEDs used (*n*) Total (Monotherapy)			
Carbamazepine	19 (12)	9 (7)	NA
Valproate, VPA	13 (12)	9 (8)	NA
Phenobarbitone, PB	8 (3)	3 (2)	NA
Phenytoin, PHT	6 (4)	2 (1)	NA
Lamotrigine, LTG	3 (2)	0	NA
Oxcarbazepine, OXB	1 (1)	1 (1)	NA
Clobazam, CLB	4 (0)	1 (0)	NA
Topiramate, TPM	1 (1)	0	NA
Clonazepam, CLZ	1 (0)	0	NA
Gabapentine, GBP	1 (0)	0	NA

EM = Women with epilepsy for whom babies had malformations, ENM = Women with epilepsy for whom babies who had no congenital malformations, Controls = Healthy women for whom babies did not have any congenital malformations

Discussion

In this study, we compared the WWE (groups with unfavorable outcome and group with normal outcome) and healthy controls (group C) with regard to the blood levels of MDA and isoprostane (markers of oxidative stress) and antioxidant profile (TAO, GSH, SOD, GR, and CAT). The prospective enrolment of WWE in the KREP provided an access to reliable data on AED exposure during pregnancy, epilepsy classification, and precise fetal outcome of the subjects recruited in to this study. Sample size (had enough power) is enough to give any significant deviation among different parameters we have measured.

The key observation in this study is that WWE, particularly those with unfavorable pregnancy outcome (group EM), had high oxidative stress when compared to normal healthy control group. WWE who were on any of the standard AEDs (CBZ, VPA, PHT, and PB) had significantly higher level of MDA or ISP when compared to control group. Further, those who were on polytherapy had higher oxidative stress than those who were on monotherapy. MDA and ISP are reliable markers of the reactive oxygen species, ROS-mediated damage to lipids leading to lipid peroxidation and are good indicators of oxidative stress.[27] The excess oxidative stress observed in WWE is possibly related to the AED usage. Laboratory studies have demonstrated that carbamazepine, phenobarbital, and phenytoin increases oxidative stress/reactive metabolite-responsive gene expression.[28,29] Previous studies have demonstrated that MDA levels are increased in persons with epilepsy exposed to PHT, CBZ, and VPA.[22,30,31] This is the first report that oxidative stress is higher in WWE who had unfavorable pregnancy outcome than WWE who had normal pregnancy outcome and healthy control women. Higher oxidative stress was observed with all the AEDs tested, particularly as polytherapy. It is likely that excess oxidative stress may be a final common pathway for AED-induced teratogenesis, but this hypothesis requires further experimental proof and confirmation.

Table 2: Blood levels (mean ± SD) and 95% Confidence Interval (CI) of MDA and ISP for the Control group (C), WWE (E) and Women with epilepsy no malformation group (ENM) and women with epilepsy and malformation group (EM) and according to the AED exposure.

	MDA (nmol/ml)	*P*	ISP (pg/ml)	*P*
C (20) Mean (SD)	2.42 (0.51)		10.77 (4.1)	
95% CI	2.18 to 2.66		8.85 to 12.69	
E (65) Mean (SD)	3.33 (0.91)	0.000#	15.90 (4.7)	0.02#
95% CI	3.1 to 3.6		14.7 to 17.1	
ENM (22) Mean (SD)	3.07 (1.02)	<0.05#	14.0 (5.3)	0.108#
95% CI	2.62 to 3.52		11.65 to 16.35	
EM (43) Mean (SD)	3.46 (0.82)	0.000#	17.77 (3.0)	0.008#
95% CI	3.21 to 3.71		16.85 to 18.69	
Valproate (22) Mean (SD)	3.43 (0.96)	<0.05#	-	
95% CI	3.00 to 3.86			
Phenytoin (8) Mean (SD)	3.45 (0.9)	<0.05#	-	
95% CI	2.7 to 4.2			
Phenobarbitone (11) Mean (SD)	3.87 (0.71)	<0.05#	-	
95% CI	3.39 to 4.35			
Carbamazepine (27) Mean (SD)	3.46 (0.88)	<0.05#	-	
95% CI	3.11 to 3.8			
Clobazam (5) Mean (SD)	3.45 (0.95)	<0.05#	-	
95% CI	2.27 to 4.63			

There was considerable variation in the antioxidant profile for subjects under study. The SOD and GSH activity were reduced for those exposed to CBZ, and SOD level alone was reduced for those exposed to PHT. There was no significant change in the levels of other antioxidant enzymes. There is no consistent pattern of antioxidant profile with AED exposure according to different studies. Some of the AEDs are known to increase oxidative stress in human studies.[20,23,31] But, none of these studies have examined the oxidative stress in WWE and unfavorable pregnancy outcome. Our study showed a significant decrease in the activities of SOD and GSH content in WWE. The increase in the oxidative stress can be due to the low activity of antioxidant enzymes and GSH content. In our study, the SOD activity was significantly reduced in WWE exposed to AEDs, thereby leading to excess of superoxide radical. It can be speculated that AEDs have a dual adverse effect; first increasing the oxidative stress and second attenuating the antioxidant mechanisms, both together leading to excess ROS and fetal damage.

There are several limitations in this preliminary study. The small number of patients under the unfavorable outcome group, particularly with respect to the individual AEDs, was a major limitation in this study. Adverse outcome group includes both, abortions and malformations and hence are a heterogeneous population. The trend was comparable for

Table 3: Antioxidant profile of WWE compared to the controls

Group (n)	TAO (mM)	GSH (μM)	SOD (U/ml)	GR (U/l)	CAT (nmol/min/ml)
C (20)	2.43 (0 .92)	1.55 (1.3)	175.81 (42.61)	42.71 (9.08)	2.62 (2.04)
CI	2.00 to 2.86	0.94 to 2.16	155.87 to 195.75	38.46 to 46.96	1.67 to 3.57
E (65)	2.33 (0.68)	0.98 (0.98)*	146.82 (42.65)*	41.46 (10.18)	2.54 (2.81)
CI	2.16 to 2.50	0.74 to 1.22	136.25 to 157.43	38.93 to 43.98	1.84 to 3.24
ENM (22)	2.31 (0.66)	1.21 (1.05)	150.61 (35.97)*	37.6 (15.67)	2.76 (4.03)
CI	2.01 to 2.60	0.74 to 1.68	133.64 to 166.56	30.65 to 44.54	0.97 to 4.55
EM (43)	2.34 (0.70)	0.83 (0.94)*	144.88 (45.97)*	41.71 (9.90)	2.43 (1.96)
CI	2.12 to 2.56	0.54 to 1.12	130.73 to 159.73	38.66 to 44.76	1.83 to 3.03

C = Group control, E = Group E, ENM = Epilepsy with no unfavorable outcome-group ENM), EM = Epilepsy with unfavorable outcome -group EM *= $P < 0.05$ on t test comparing with group C

Table 4: Comparison of antioxidant profile of WWE according to different drugs

Group	TAO (mM)	SOD (U/ml)	GR (U/l)	CAT (nmol/min/ml)	GSH (μM)
C	2.43 (0.92)	175.81 (42.61)	42.71 (9.08)	2.62 (2.04)	1.55 (1.3)
N = 20					
CI	2.00 to 2.86	155.87 to 195.75	38.46 to 46.96	1.67 to 3.57	0.94 to 2.16
VPA	2.39 (0.83)	152.04 (41.94)	38.01 (7.19)	3.16 (3.99)	1.37 (1.38)
N = 22					
CI	2.02 to 2.76	133.44 to 170.64	41.20 to 34.80	1.39 to 4.93	0.76 to 1.98
PHT	2.13 (0.53)	120.69 (39.3)*	41.62 (10.5)	1.53 (0.81)	0.86 (0.74)
N = 8					
CI	1.69 to 2.57	87.83 to 153.55	32.84 to 50.40	0.85 to 2.21	0.24 to 1.48
PB	2.43 (0.51)	143.73 (52.42)	40.28 (8.19)	2.84 (2.32)	1.15 (0.6)
N = 11					
CI	2.09 to 2.77	108.51 to 178.95	34.78 to 45.78	1.28 to 4.40	0.75 to 1.55
CBZ	2.32 (0.66)	140.81 (36.52)*	42.86 (12.27)	2.39 (2.05)	0.79 (0.69)*
N = 27					
CI	2.06 to 2.58	126.36 to 155.26	38.00 to 47.71	1.58 to 3.20	0.52 to 1.06
CLB	2.14 (0.79)	158.58 (16.59)	44.0 (5.27)	1.58 (1.23)	0.98 (0.95)
N = 5					
CI	1.16 to 3.13	137.98 to 179.18	37.46 to 50.54	0.05 to 3.11	0.20 to 2.16

TAO = Total antioxidant status, SOD = Superoxide dismutase, GR = Glutathione reductase, CAT = Catalase, GSH = Glutathione, VPA = Valproate, PHT = Phenytoin, PB = Phenobarbitone, CBZ = Carbamazepine, CLB = Clobazam *significantly different ($P < 0.05$) from group C (C) group by independent t test

the abortions and malformations groups separately, but the numbers were inadequate to make statistical analysis. Though the different anti-epileptic drugs consumed have different structure, chemical composition etc., they all have same metabolic pathways, which produce intermediate compounds, which increase oxidative stress. Other drugs that have different metabolism in the body, like valproate, also were shown to have increased oxidative stress in other studies. Though the different AEDs have different structure, chemical composition, metabolism, and mechanism of action, it had been observed that the malformations observed with these AEDs share much in common and are often indistinguishable. This suggests that there exists a final common pathway for the metabolism, and the malformations are caused by a common mechanism. The observations in this study demonstrated increased oxidative stress in WWE who were exposed to AEDs, but had not confirmed any causal relationship between oxidative stress and unfavorable outcome. There is a possibly an important link between AED usage and fetal malformations in WWE. This opens a possible opportunity to modify oxidative stress and thereby reduce the risk of malformation by the administration of antioxidants during pregnancy. Further studies are required to ascertain whether reduction of oxidative stress would prove the hypothesis.

Oxidative stress can be an important link between AED usage and fetal malformations in WWE. Oxidative stress in biological systems can be induced by the depletion of antioxidants and /or by an overload of FR. Oxidative stress has been shown to be related to the teratogeniciy of AEDs. Administrations of antioxidants are known to reverse oxidative stress and reduce the risk of malformation.[32-35] This opens an opportunity for modifying oxidative stress and thereby the risk of malformations by the usage of antioxidants.

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