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Lipid peroxidation and antioxidant status in patients with primary generalized epilepsy.

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ABSTRACT

The production of free radicals has a role in the regulation of biological function, cellular damage, and the pathogenesis of central nervous system conditions. Epilepsy is a highly prevalent serious brain disorder, and oxidative stress is regarded as a possible mechanism involved in epileptogenesis. The aim of this study is to evaluate the status of some antioxidants in epileptic patients and also verify if there any variation in their concentrations and activities after treatment. A total of 25 patients (15 M:10 F) with primary generalized epilepsy and 25(16M:9F) normal controls all aged 18-48yrs were recruited for the study .Ten patients (6M:4F) who were on Phenobarbital treatment for a minimum of one year with no seizure for ≥6 months were considered for the post- treatment studies. Total antioxidant status (TAS), erythrocyte malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione (GSH) were measured using standard methods. Plasma TAS concentration for epileptic subjects before treatment was significantly decreased ($p<0.05$) compared to the controls. However, erythrocyte MDA concentration for epileptic subjects was significantly increased ($p<0.01$) compared to the controls. The erythrocyte GSH-Px activity for epileptic subject was significantly decreased ($p<0.01$) compared to the controls. Similarly, the activities of SOD and CAT for epileptic subjects were significantly decreased ($p<0.05$) compared to the controls. Erythrocyte GSH concentration for epileptic subject was significantly decreased ($p<0.05$) compared to the controls. A signifcant inverse correlation was obtained between MDA and measured antioxidants among the epileptic subjects before treatment. This study revealed that epileptic subjects are in state of oxidative stress, suggesting that free radicals may be implicated in epilepsy. Antioxidant status in blood of epileptic subjects shows improvement after treatment.

Key Words: Lipid peroxidation, antioxidants, epilepsy

INTRODUCTION

Oxidative stress was described as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species were originally considered to be exclusively detrimental to cells, but now it is considered that redox regulation involving ROS is essential for the modulation of

critical cellular functions (mainly in astrocytes and microglia), such as mitogene-activated protein (MAP) kinase cascade activation, ion transport, calcium mobilization, and apoptosis program activation [1]. Oxygen is involved in the oxidation of organic compounds and the production of energy for cell metabolism. However, only a very small amount of consumed oxygen (between 2 and 5%) is reduced, which leaves a variety of highly reactive chemicals known as oxygen-free radicals or ROS, as well as RNS [2]. The production of free radicals is associated with damage caused to cell structures and the pathogenesis of central nervous system (CNS) conditions, such as Parkinson's disease, stroke, dementia, and epilepsy [2, 3]. The CNS is highly sensitive to O&NS due to its high oxygen consumption and the low activity of antioxidant defenses [4].

Epilepsy or more correctly a seizure is most easily defined in physiological terms. It is the occasional, sudden, excessive, rapid and local discharges in grey matter [5]. Generalized seizures involve diffuse regions of the brain simultaneously in a bilaterally symmetric fashion which may result from cellular, biochemical or structural abnormalities that have a widespread distribution. The inhibitory transmitter gamma amino butyric acid(GABA) is thought to be particularly important in a role of keeping the interconnected neurons of the cerebral cortex in a state of relative quiescence [6]. It is likely that both reduction in inhibitory systems and excessive excitation of excitatory neurotransmitters (acetyl choline, glutamate, aspartate) play a part in the genesis of the seizure activity [6].

Epilepsy is one of the most common and serious brain disorders in the world. It affects at least 50million people worldwide. Approximately 100 million people will have at least one epileptic seizure during their lifetime. It causes serious physical, psychological, social, and economic consequences [7]. The median prevalence of lifetime epilepsy for developed countries is 5.8 per 1,000 and 10.3 per 1,000 for developing countries [8].

Oxidative and nitrosative stress are regarded as possible mechanisms in the pathogenesis of epilepsy [9]. Studies have already verified that status epilepticus changes redox potential and decreases the level of ATP, which can lead to a collapse in brain energy production and supply [10]. Liang and Patel have demonstrated oxidative damage to susceptible targets (protein, lipids, and DNA) caused by persistent seizures (status epilepticus) [11]. Several studies (animal models and genetic studies) have demonstrated an increase in mitochondrial O&NS and subsequent cell damage after persistent seizures [11, 12, 13, 14].

The biological effects of free radicals are controlled in vivo by a wide range of antioxidants such as vitamin E, vitamin C, vitamin A, glutathione and antioxidant enzymes including glutathione reductase (GR), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) [15].

Disrupted tissues undergo lipid peroxidation more quickly than healthy ones. The potential toxicity of reactive oxygen species is counteracted by a large number of cytoprotective enzymes and antioxidants which limit the damage caused by such species [16]. The brain and nervous system are particularly prone to lipid peroxidation since the membrane lipids are very rich in polyunsaturated fatty acid chains, and areas of human brain are very rich in iron, which plays an essential role in generating free radical species. The mechanisms of epileptogenesis are not well established. Several studies in the last few years suggested that the body electrolytes, level of some trace elements and membrane lipid peroxidation due to increase in free radicals or decrease in activities of antioxidant defense mechanisms may be causally involved in some forms of epilepsies and also increase the recurrence of seizures [17,18]. Moreover, some antiepileptic drugs may alter free radical scavenging enzyme activities in humans and experimental animals [18, 19]. However, the status of antioxidants and the extent of lipid peroxidation in erythrocytes have not been fully investigated so far in patients with generalized epilepsy. The aim of the study is to evaluate the status of some of the erythrocyte antioxidants in epilepsy, then see if there are variation in their concentrations compared to those with post treatment.

MATERIALS AND METHODS

After the approval of the Ethic committee of the Ladoke Akintola University of Technology teaching hospital Osogbo, Osun state, Nigeria. A total of 25 patients (15 males:10 females) with primary generalized epilepsy aged 18-48yrs were recruited from the Neurology unit of the department of internal medicine of the teaching hospital. All patients were subjected to a detailed clinical examination for assessment of neurological status to make definitive diagnosis. All the patients that were detected to be suffering from primary generalized epilepsy and none on any medication were included in the study.

Ten patients (7 males:3 females) who were on phenobarbital treatment for a minimum of one year and who did not have any clinical epileptic seizure for about six months prior to this study were considered for the post- treatment studies.

The control groups consist of 25 (16males:9females) healthy individuals of the same age group were recruited from general population with the same socio-economic status. Excluded from the study were those with history of any serious physical illness or an organic brain syndrome due to some cause other than epilepsy, with familial history of mental illness, cardiovascular, respiratory, renal, hepatic diseases, diabetic mellitus. smokers, alcohol drinkers and those on antioxidant supplements.

Fasting blood samples was obtained by venepuncture from patients and controls into EDTA bottle which was centrifuged at 2000 g for 15 min, plasma was carefully removed and the erythrocyte pellet was washed three times with equal volumes of saline and centrifuged at 2000 g for 15 min. The washed red blood cells were then haemolyzed in distilled water (1:4, v/v) and by freezing and thawing. The haemolysate was centrifuged and the supernatant and plasma was then stored at -20^{o}C until they were analyzed. Since neuronal oxidative injury processes and underlying dynamic molecular regulatory mechanisms are reflected in peripheral blood cells, we could use red blood cells, platelets, lymphocytes and cultured skin fibroblasts as "window" to the CNS [20].

Erythrocyte MDA levels was determined using the method of Draper and Hadley [21] based on the reaction of MDA with thiobarbituric acid (TBA) at 95^{0}C. In the TBA test reaction, MDA and TBA react to form a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2-3 at 95^{o}C for 15 min. The sample was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifigation and an aliquot of supernatant was reacted with 0.67% TBA in a boiling water-bath for 15 min. After cooling, the absorbance was read at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3 tetramethoxypropane). Measurement of TAS in the plasma was performed using a commercial kit from Randox Laboratories (Randox Laboratories Ltd, Diamond Road, Crumlin, Co. Antrim, Ireland) [22]. The assay was calibrated using 6-hydroxy-2, 5, 8- tetra-methylchroman-2-carboxylic acid (trolox). The results were expressed as mmol/L of trolox equivalent. Measurement of erythrocyte GSH-Px (EC# 1.11.1.9) activity was performed using a commercial kit RANSEL from Randox Laboratories (Randox Laboratories Ltd, Diamond Road, Crumlin, Co. Antrim, Ireland). GSH-Px catalyses the oxidation of GSH to glutathione disulphide(GS-SG) by cumene hydroperoxide, in the presence of glutathione reductase and NADPH, GS-SG is immediately converted to GSH with a concomitant oxidation of NADPH to $NADP^{+}$ according to the method of Paglia and Valentine [23]. The concentration of GSH-Px activity is assessed from the decrease in absorption at 340nm and at 37^{0}C using Humalyzer 2000 analyzer. A standard curve was prepared by using the standard provided in the kit, and the value for each sample was read from this curve. SOD (EC. 1.15.1.1) activity was estimated by employing xanthine/xanthine oxidase assay commercial kit RANSOD from Randox Laboratories (Randox Laboratories, Crumlin, Antrim, UK) [24]. The results of SOD activity were normalized to the haemoglobin content in the erythrocyte lysate and expressed as U/gHb. The CAT (EC 1.11.1.6) peroxidative activity was measured by the reaction of formaldehyde produced from methanol with purpald to produce a chromophore according to the method of Johansson and Hakan Borg [25]. Quantitation was carried out by measuring the absorbance at 540 nm and comparing the results with those obtained with formaldehyde calibrators. GSH concentration in erythrocytes was determined in the presence of low-molecular-mass free thiol groups, mercuric salt and sulfanilamide, in a highly acidic medium, biazonian salt was produced. The salt so obtained was conjugated with amine salt, producing a coloured complex, the absorbance of which was measured at 535 nm and calculations were made according to the model curve for GSH and expressed in µmoles GSH/g Hb [26].

Statistical analysis:
All values were expressed as the mean ± standard deviation. Data was analyzed using one-way ANOVA followed by the post-hoc Duncan multiple range test for analysis of bio chemical data using SPSS version 11 (SPSS Inc Chicago, IIinois). Method of correlation analysis (PEARSON) was also used to determine the degree of association between variable of interest.Values were considered statistically significant at $p < 0.05$.

RESULTS

The anthropometric measurements and physical clinical parameters of the subjects and the controls were depicted in Table1. All the studied subjects and the controls were non-obese and normotensive.

The mean plasma TAS concentration of 1.07±0.13mmol/L for pre-treatment epileptic subjects, was significantly decreased ($p<0.05$) compared to 1.96±0.41mmol/L for the controls. However, erythrocyte MDA concentration of 4.99±0.32nmol/gHb for pre-treatment epileptic subjects was significantly increased ($p<0.01$) compared to 2.81±0.29nmol/gHb for the controls. The erythrocyte antioxidant GSH-Px activity of 21.13±3.78U/g Hb for subjects with pre-treatment epilepsy was significantly decreased ($p<0.01$)compared to 36.56±5.13U/g Hb for the controls. Likewise erythrocyte SOD activity of 774.82±72.63U/g Hb for pre-treatmnt epileptic subjects was significantly decreased ($p<0.05$) when compared to 994.58±18.27U/g Hb for the control. Erythrocyte GSH concentration of 4.17±0.5µmol/gHb for pre-treatment epileptic subjects before treatment was significantly decreased ($p<0.05$) compared to 5.10±0.8µmol/gHb for the controls. Similarly, erythrocyte CAT activity of 1170.51±49.6U/g Hb for pre-treatment epileptic subjects before treatment was significantly reduced ($p<0.05$) compared to 1416.12±79.2U/g Hb for the controls. .

Table 3 shows the correlation coefficient analysis between the levels of antioxidants and the markers of oxidative stress of subjects in primary generalized epilepsy and those subjects with phenobabitone post treatment. A strong significant inverse correlation was obtained between level of MDA vs. GSHPx activity ($r = - 0.4837$; $p = 0.0046$). Similarly, a significant inverse correlation was obtained between the level of MDA vs.TAS ($r = - 0.3573$; $p = 0.0235$), MDA vs. SOD ($r = - 0.3711$; $p = 0.0207$), MDA vs. GSH ($r = - 0.3498$; $p = 0.0265$) and MDA vs. CAT($r = - 0.3919$; $p = 0.0172$).

Figure 1. shows the comparison between measured level of antioxidants (mean±SD) of the pre – treatment epilepsy and post – treatment subjects and the controls.

Table 1. Anthropometric and clinical parameters (mean±SD) of the controls and subjects

	Control (n=25)	Epileptic Pre-treatment (n=25)	p_1 value	Epileptic Post-treatment (n=12)	p_2 value
Age(years)	34.2±7.3	37.1±4.9	ns	35.9±5.6	ns
Height (cm)	170.3±4.6	168.9±5.2	"	171.9±3.7	"
Weight (kg)	67.0± 2.5	67.9±3.1	"	69.2±3.8	"
BMI (Kg/m^2)	23.1±1.1	23.8±0.9	"	23.4±1.5	"
Systolic BP (mmHg)	119.9±2.5	122.0±3.8	"	121.7±2.9	"
Diastolic BP (mmHg)	78.8±1.3	85.3±2.6	"	82.9±2.4	"

ns = non significant
p_1 = when epileptic subjects pre-treatment is compared with control
p_2 = when epileptic subjects post-treatment is compared with control

Table 2. The biochemical parameters (mean±SD) of the controls and subjects

	Control (n=25)	Epileptic Pre-treatment (n=25)	p_1 value	Epileptic Post-treatment (n=12)	p_2 value
Plasma TAS *(mmol/L)*	**1.96±0.41**	**1.07±0.13**	**0.0238***	**1 79±0.15**	**0.0714**
Erythrocyte-MDA *(nmol/ml)*	**2.81±0.29**	**4.99±0.32**	**0.0042****	**3.42±0.31**	**0.0916**
Erythrocyte-GSHPx *(U/g Hb)*	**36.56±5.13**	**21.13±3.78**	**0.0091****	**32.67±3.19**	**0.0887**
Erythrocyte-SOD *(U/g Hb)*	**904.58±81.27**	**774.82±72.63**	**0.0390***	**891.34±43.28**	**0.1071**
Erythrocyte GSH *(µmol /g Hb)*	**5.10±0.8**	**4.17±0.5**	**0.0474***	**4.66±0.6**	**0.0975**
Erythrocyte-CAT *(U/g Hb)*	**1416.12±79.2**	**1170.51±49.6**	**0.0410***	**1307.92±45.31**	**0.1018**

*Significant level at the ($p<0.01$) ** and $p<0.05$ *)*
p_1 = when epileptic subjects pre-treatment is compared with control
p_2 = when epileptic subjects post-treatment is compared with control

Table 3. Correlation coefficient analysis between marker of oxidative stress(MDA) and and antioxidant level

	MDA			
	Pre-Treatment Epileptic		Post Treatment Epileptic	
	"r"-value	p value	"r"-value	p value
TAS	- 0.3573	0.0235	- 0.1082	0.3931
GSH-Px	- 0.4837	0.0046	- 0.2314	0.1803
SOD	- 0.3711	0.0207	- 0.1379	0.3698
GSH	- 0.3498	0.0265	- 0.1705	0.2766
CAT	- 0.3919	0.0172	- 0.1998	0.2139

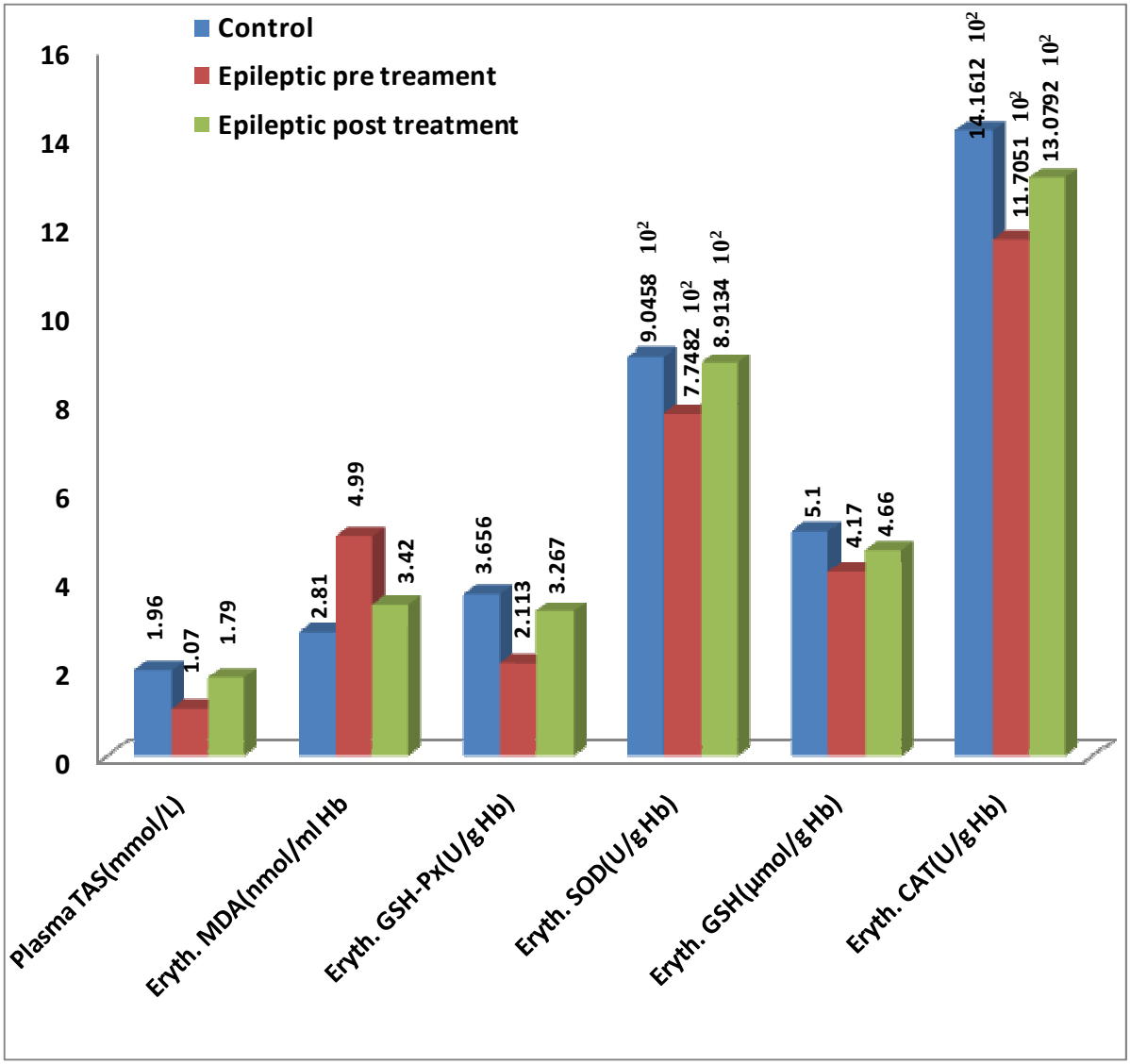

Figure 1. shows the comparison between measured level of antioxidants (mean±SD) of the pre – treatment epilepsy and post – treatment subjects and the controls.

DISCUSSION

Brain has relatively high levels of unsaturated fatty acids that are particularly good substrates for peroxidation reactions, while endogenous defense mechanisms are poor in brain. Free radicals in addition to contributing to the neuronal injury in cerebral ischemia and hemorrhage may be involved in neuronal degenerations.

In epileptic seizures, excitatory amino acid receptor activation by glutamate or N-methyl, D- aspartic acid (NMDA) has been known to accompany generation of reactive oxygen species [27].

In the present study, erythrocyte MDA levels was increased significantly among primary generalized epileptic subjects compared with the control group, suggesting the generation of free radicals in epilepsy. The increase in erythrocytes MDA level also recorded among epileptic subjects post treatment but not significant suggests that the regular treatment with antiepileptic drugs may have antioxidant effect, thereby decreasing the lipid peroxidation. Our finding was in agreement with a similar study conducted by Sudha et al [28] in which they reported both in vitro lipid peroxidation of RBC and percentage hemolysis in epileptic patients was significantly higher than controls. The nervous system is more susceptible to the damaging effect of oxidative stress, due to the high content of polyunsaturated fatty acids that are susceptible to lipid peroxidation [29].

In the human brain, there is a distinct regional distribution of thio-barbituric-acid (TBA) positive materials in the endogenous pool, with higher levels in the cerebellar vermis and lower levels in the thalamus, cortical regions, substantia nigra, caudate nucleus, pallidum, putamen, thalamus and the pineal gland [30].

GSH-Px is an enzyme that contains single selenocysteine GSH-Px antioxidant properties which allows them to eliminate peroxides as potential substrates for Fenton's reaction. Glutathione peroxidase works together with glutathione tripeptide (GSH), which is present in cells in high (micromolar) concentrations. The substrate for the GSH-Px catalytic reaction is H_2O_2 or organic peroxide ROOH. Glutathione peroxidase catalyzes hydroperoxide reduction using GSH, thus protecting mammalian cells against oxidative damage. In the present study a significant reduction in the erythrocytes activity of GSH-Px and erythrocytes GSH concentration were recorded among the freshly diagnosis primary generalized epileptic subjects before treatment; however, the reduction in the activity of this antioxidant enzymes and GSH level in post treatment epileptic subjects were not significant when both were compared to the controls. This is an indication that deficiency of GSH-Px and GSH may be involve in the pathogenesis epilepsy and that some anticonvulsants therapy may help to restored these antioxidants.

The role of SOD is to protect aerobic cells against $O_2^{\bullet-}$ action. It catalyzes $O_2^{\bullet-}$ dismutation reaction into H_2O_2 and $O_2^{\bullet-}$. There are three known types of SOD: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), and extracellular SOD (ECSOD) [31]. In the present study, the erythrocytes activity of SOD among epileptic subjects before treatment was significantly reduced but for those with post treatment status the activity of SOD was also reduced but not significant, this was in agreement with the finding Nikushkin et al [32] and Surekha et al [33] who reported that SOD activity was about 31% decreased in post treatment epileptic subjects while it was 52% decreased in freshly diagnosed epileptic subjects compared to controls. Catalase is an enzyme that reacts very effectively with H_2O_2 to form water and molecular oxygen and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity. Catalase protects cells against H_2O_2 generated inside them. Although CAT is not essential to some cell types under normal conditions, it has an important role in the acquisition of tolerance to O&NS in cellular adaptive response [34, 35]. In the present study erythrocyte activity of CAT was significantly reduced among freshly diagnosed primary generalized epilepsy; also reduced CAT activity but no significant was recorded among post treatment epileptic subjects. Plasma is in close proximity to the phagocytes and therefore bears the footprints of phagocyte-generated ROS; this also explained why we recorded a significant reduction in the level of plasma TAS among the epileptic subjects before treatment. This finding was in agreement the study of Thanoon [36] who conducted a similar study on epileptic subjects.

CONCLUSION

From the preset study it is evident that epilepsy is linked to oxidative stress as a results of increased free radicals production and defective antioxidant defense. Since neuronal oxidative injury processes and underlying dynamic molecular regulatory mechanisms are reflected in peripheral blood cells, the red blood cells, serve as "window" to the CNS. Our results on post treatment epileptic subjects shows that antiepileptic drugs may exert antioxidant effect

and addition of antioxidants to the conventional drug therapy may enhance further reduction on epileptic activity; also help in returning antioxidant balance to normal status among the epileptic subjects.

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