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Original Article

Prognostic Significance of Plasma Homocysteine and Malondialdehyde in Patients with Acute Ischemic Stroke

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ABSTRACT

Introduction: Ischemic strokes occur as a result of an obstruction within a blood vessel supplying blood to the brain. Ischemic stroke comprises 85% of all stroke cases. Stroke is the third major cause of mortality worldwide and an important cause of long term disability contributing to major economic burden in most of the countries. **Objectives:** The purpose of the study was to estimate the biochemical markers of oxidative stress and lipid peroxidation (homocysteine and malondialdehyde) in acute ischemic stroke patients and compare them with controls and also to correlate these biochemical parameters with the degree of neurological impairment (stroke severity) and outcome. **Materials & Methods:** The present study was conducted on 45 acute ischemic stroke patients and 80 age and sex matched controls. The first blood sample was taken within 72 hours of onset of stroke and second sample was taken 7 days following the first sampling. Plasma homocysteine and malondialdehyde (MDA) levels were estimated in patients and controls. The biochemical parameters were also correlated with stroke severity (NIHSS score- National Institute of health stroke scale score), short term outcome after 7 days and long term outcome at 6 months using modified Rankin scale (MRS) and Barthel Index. **Results:** In the present study, level of homocysteine was higher on both day 1 and day 7 when compared to controls. But there was no significant correlation between the levels of homocysteine and severity of stroke, nor did it correlate with the outcome in terms of recurrence of stroke/TIA or death, disability and functional independence. MDA levels on the day of admission were higher than controls and the levels correlated with NIHSS score. However MDA levels were not increased in patients who had recurrence of stroke or who expired within 6 months compared to those who survived and did not have any further vascular events. In the present study, the levels of MDA levels on the day of admission as well as after 7 days predicted the disability of the patients (assessed by Modified Rankin Scale) on 7th day and after 6 months. But the levels did not predict functional outcome [Barthel Index] of stroke patients after 6 months. **Conclusion:** MDA and homocysteine levels rise in the acute phase following ischemic stroke. The levels of MDA on the day of admission and after 7 days correlate positively with the severity of stroke as measured by NIHSS score.MDA on the day of admission correlate with the short term prognosis(around one week) of the patients (measured by MRS and BI). Homocysteine levels do not have any correlation with the outcome of the patients. **Key Words:** Homocysteine, malondialdehyde, acute ischemic stroke, plasma, Barthel Index, Modified Rankin Scale

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1. INTRODUCTION

In India stroke contributes to 2% of all hospital cases and 20% of all neurological admissions[1].Cerebral ischemia leading to infarction may be global, wherein low cerebral blood flow due to cardiac arrest or severe hypotension is maintained for longer duration or localized, when one of the vascular territories is affected either due to thrombosis and embolus[2].

During acute ischemia oxidative stress has been shown to be an important factor which goes hand in hand with inflammatory mechanisms in precipitating as well as elaborating the neuronal injury[3]. Since neuronal lipids are rich in polyunsaturated fatty acids and sparse in glutathione peroxidase, the neurons are more prone to lipid peroxidation[3]. Malondialdehyde (MDA) is formed as a result of lipid peroxidation and has been studied in acute brain ischemia.

Homocysteine (Hcy), an amino acid and a novel marker of atherosclerosis has been found to increase oxidative stress by generating superoxide and hydroxyl radical, while itself undergoing oxidation[4]. Elevated homocysteine has been shown to be an independent risk factor of ischemic stroke[5].

1.1 Homocysteine in acute ischemic stroke

The exact pathogenic mechanism of ischemic cell injury and apoptosis is still a subject of research and new mechanisms are evolving with time course. One of the proposed risk factors of acute thrombotic stroke is hyperhomocysteinemia[6]. Studies have been conducted showing the detrimental effects of homocysteine on blood vessels as well as on tissues[6, 7]. This recently focused risk factor might also contribute to the tissue injury in penumbral zone of ischemia and lead to inclusion of penumbra into ischemic core thus enhancing the brain damage due to ischemia.

Homocysteine is a thiol amino acid formed by demethylation of methionine[7]. Normal plasma level of homocysteine is < 15 µmol/L[7]. Homocysteine exists in plasma as homocysteine, oxidized disulphide homocystine and homocysteine cysteine mixed disulphide. Total homocysteine comprises of all these three forms.

1.1.1 Pathogenesis of hyperhomocysteinemia

The most common cause of hyperhomocysteinemia is defective or deficient cystathionine β synthase, a pyridoxal phosphate dependent enzyme; hence administration of vitamin B6 has therapeutic effects in some patients with hyperhomocysteinemia[8]. Some of the treatment protocols in stroke patients have included vitamin therapy as a routine treatment for patients with high risk vascular disease[8].

The other causes of hyper homocysteinemia are methylene tetrahydrofolate reductase deficiency, folate, vitamin B12 and vitamin B2 deficiency which can also have therapeutic role in patients of hyperhomocysteinemia[9].The vascular pathology of hyperhomocystenemia was first described 35 years back by Mc Cully[10]. Since then the mechanisms by which homocysteine leads to vascular disease have been extensively studied. Homocysteine is shown to activate coagulation pathways therefore deranged levels of factor VIII, von willebrand's factor and antithrombin III has been reported in hyperhomocystenemia[11]. It also inhibits fibrinolysis[12, 13], inhibits endothelial nitric oxide synthase and causes endothelial dysfunction by modifying adhesive properties[10-13].

1.1.2 Hyper homocysteinemia as a risk factor of acute ischemic stroke

Significantly elevated total homocysteine levels are found in one third of patients with ischemic stroke or Transient Ischemic Attack (TIA)[14]. The levels are not related to hypertension, smoking, dyslipidemia blood

sugar or body mass index[15]. Neither age has any effect on plasma homocysteine levels[15].

1.1.3 Evidence of involvement of homocysteine in pathogenesis of ischemic brain damage

In one study, elevated plasma homocysteine was found to be associated with multiple infarctions and diffuse periventricular white matter lesions[16]. Homocysteine has been shown to cause microangiopathy in cerebral vessels[15,16].

1.1.4 Prognostic importance of plasma homocysteine levels in acute ischemic stroke

It is known that homocysteine is not an acute phase reactant; however its level during acute phase of ischemic stroke may have prognostic implications. This is because homocysteine can induce neuronal apoptosis and can increase the vulnerability of neurons to excitotoxicity by causing DNA damage (DNA strand breaks). It causes PARP (poly ADP ribose polymerase) and caspase activation, a decline in mitochondrial membrane potential and nuclear disintegration[17]. Homocysteine induces oxidative stress through its own oxidation. Oxidation of homocysteine generates hydrogen peroxide, superoxide and hydroxyl radial. Superoxide radical generated from homocysteine may inactivate nitric oxide and in process produce more potent oxidants like peroxynitrite[17]. The pro-oxidant effects of hyperhomocysteinemia may be accentuate by direct inhibitory effect of homocysteine on glutathione peroxidase[13,17]. Moreover, homocysteine also increases the levels of asymmetric dimethyl arginine [ADMA][18] an endogenous inhibitor of nitric oxide synthase, thus decreasing NO (Nitric Oxide) levels. Homocysteine has been implicated in generation of excitotoxic neurotransmitters like homocysteine acid, sulfinic acid leading to neuronal death[19]. Thus the oxidative stress imparted by homocysteine can lead to neurological deterioration of the patients which in turn should affect the functional outcome of the patients.

Previous studies regarding the correlation of homocysteine levels with the outcome of stroke patients have conflicting results. A study conducted by Mizrahi *et al* did not show any correlation between the levels of homocysteine and functional outcome of patients[20]. However a study by Kado *et al* has reported plasma homocysteine as a risk factor for functional decline in stroke patients[21].

1.2 Malondialdehyde in acute ischemic stroke

The reactive oxygen species damage the membrane lipids by forming lipid peroxides[22]. Since neurons are rich in polyunsaturated fatty acid they are more prone to free radical attack and formation of lipid peroxides like malondialdehyde. MDA is a volatile, low molecular weight (72.07), short chain 1,3 dicarbonyl compound which is a moderately weak acid (pKa 4.46). It is a product of peroxidation of arachidonic acid. MDA is a reactive molecule *in vivo* and forms stable derivatives with bio-molecules. It has an affinity for primary amino groups of proteins, nucleic acid bases, phospholipids and protein sulfhydryl group; and this property is responsible for its toxicity[23]. Formation of lipid peroxides in brain following ischemia has been documented in previous studies[24-26].

The present study was devised to estimate homocysteine and malondialdehyde in acute ischemic stroke comparing them with normal controls and correlating them with the stroke severity and clinical outcome.

2. MATERIALS AND METHODS

The study was conducted in the Department of Biochemistry, Maulana Azad Medical College, New Delhi and Department of Neurology, GB Pant Hospital, New Delhi. Prospectively consecutive acute ischemic stroke patients were included in the study. At least one and a half times number of age and sex

matched normal controls were taken. Informed consent was taken from all the cases and controls. A detailed history and evaluation of risk factors were done. Baseline NIHSS score was calculated in all patients. A standard battery of investigations (complete haemogram, Renal Function Tests, Liver Function Tests, random blood sugar, lipid profile, urine routine/microscopy, X ray chest, ECG and carotid doppler) were done in all the patients.

Blood samples for estimation of homocysteine and malondialdehyde were collected as early as possible after admission and confirmation of stroke (within 72 hours of onset of stroke). A second sample was taken on 7th day following the first sampling and NIHSS (National Institute of health stroke scale) score was calculated on both occasions. Three measures of outcome were assessed:

1. Recurrence of stroke or death: Clinically all patients were under a close follow up for a period of six months and any recurrence of stroke or other vascular events and death were recorded.
2. Modified Rankin Scale (MRS) [27]: The disability was assessed using MRS on 7th day (during second sampling) and after six months.
3. Barthel Index (BI) [28]: Functional independence was assessed using BI on 7th day and after six months.

2.1 Inclusion Criteria

All patients of either sex aged more than 12 years presenting within 72 hours with focal neurological deficit and clinical signs consistent with WHO definition of stroke[29] and all patients proven to have acute ischemic stroke by CT head or MRI of brain were included in the study.

2.2 Exclusion Criteria

Patients presenting with CT/MRI proven hemorrhagic stroke; patients presenting with transient ischemic attack only (deficits resolving completely within 24 hours); patients having fever at the onset or history of fever in the recent past(one week prior to stroke); patients with a history of rheumatologic diseases, auto immune diseases, or any kind of acute or chronic infection; patients on immunosuppressive therapy like corticosteroids, or regular analgesic uptake and patients with severe impairment of hepatic and renal functions were completely excluded from the study.

2.3 Selection of Controls

Healthy individuals of either sex, aged more than 12 years, with no history of any major surgery or acute or chronic infection in the recent past, no history of immunosuppressive therapy, analgesic abuse or other drug abuse; no history of cardiovascular or cerebro-vascular events in the past and with no history of rheumatologic or autoimmune diseases were selected.

The investigations for diagnosis of ischemic stroke included DWI MRI (Diffusion Weighted Imaging Magnetic Resonance Imaging), intracranial angiography, carotid doppler and trans-thoracic echocardiography.

2.4 Collection of Samples: Five ml. of venous blood was collected in EDTA vials and the plasma was separated by centrifuging at 3000 rpm for 10 minutes. Hemolyzed samples were discarded.

MDA was estimated using the method of Ashakawa and Matshushita[30]. MDA, a product of fatty acid peroxidation reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm. Butylated Hydroxy Toluene, an antioxidant, is added to prevent MDA formation during the assay which can result in relatively elevated TBA reactivity. Ferric chloride provides ferric ions which act as catalyst to improve the sensitivity of the method. Plasma homocysteine was estimated using competitive ELISA. Reference range: 5-15 micromols/litre.

3. RESULTS

MDA: The mean MDA on the 1st day in the case group was 5.01±4 (nmol/ml) and ranged from 0.4-19.2(nmol/ml), while the mean MDA in the control was 2.52±1.66 (nmol/ml). The difference was found to be statistically significant (p <0.0001). The mean MDA on 7th day was 3.26±2.38 (nmol/ml). The difference of the mean MDA at 7th day between cases and controls was not statistically significant (Table 1)

Homocysteine: The mean homocysteine on the 1st day in the case group was 26.58±17.7 µmol/L and ranged from 4-76µmol/L. The mean homocysteine in control group was 14.06±12µmol/L and ranged from 2-56 µmol/L. The difference was statistically significant. The mean on 7th day was 25.94±16.71 µmol/L and the difference with controls was also significant (Table 1).

Table 1: Mean Levels of MDA and homocysteine in cases and controls

Details	Cases (n=45)	Control (n=80)	P(2 tailed)
MDA-1(nmol/ml)			
Range	0.4-19.2	0.6-7.4	
Mean±SD	5.01±4.0	2.52±1.66	**<0.0001**
MDA-7(nmol/ml)			
Range	1 - 10.1		
Mean±SD	3.26± 2.38		0.086
Hcy-1(µmol/L)			
Range	4 - 76	2 - 56	
mean±SD	26.58±17.7	14.06±12	**<0.0001**
Hcy-7(µmol/L)			
Range	6 - 75		
mean±SD	25.94±16.71		**<0.0001**

Mann-Whitney U test was applied, p<0.05 is significant.

Table 2: Correlation of biochemical parameters with the severity of stroke as determined by NIHSS score

a) Correlation of day-1 Parameters with NIHSS on the day of admission

Parameters	Correlation coefficient	P(2-tailed)
MDA	0.442	**0.002**
Hcy	0.022	0.883

b) Correlation of biochemical parameters on 7th day with NIHSS score at 7th day

Parameters	Correlation coefficient	P(2-tailed)
MDA	0.335	**0.024**
Hcy	0.122	0.442

Spearman correlation coefficient was used.

Plasma MDA and homocysteine levels on day 1 and day 7 were correlated with the stroke severity assessed by NIHSS score and it was found that MDA levels correlated with stroke severity on corresponding days.

To find out the correlation of biochemical parameters with the recurrence of stroke /TIA or death within 6 months, two groups were created in this study population. In group-1, cases with no recurrence of TIA or death were included and group-2 consisted of cases with recurrence of stroke/TIA/ death within 6 months (Table 3a & 3b)

Table 3 a: Difference in biochemical parameters between group 1 and group 2

Groups	Male	Female
Group-1(n=32)	19(59%)	13(41%)
Group-2(n=13)	7(53.8%)	6(46.2%)

Table 3 b: Difference in biochemical parameters between group 1 and group 2

Parameters (Mean± SD)	Group-1	Group-2	P(2 tailed)
MDA-1: (nmol/ml)	4.603±4.086	6.038±3.743	0.280
MDA-7: (nmol/ml)	2.866±2.035	4.254±2.954	0.076
Hcy-1: (µmol/L)	27.07±19.37	25.4±14.73	0.779
Hcy-7: (µmol/L)	26.26±17.68	25.29±14.73	0.843

There was no significant difference in biochemical parameters between group 1, cases without recurrence of stroke/TIA/ death within 6 months and group 2, cases with recurrence of cerebrovascular events/death within 6 months of follow-up.

Table 4: Correlation of biochemical parameters with Modified Rankin Scale on 7th day post stroke

Parameters	Correlation coefficient	P(2 tailed)
MDA-1	0.370	**0.012**
MDA-7	0.342	**0.022**
Hcy-1	-0.029	0.850
Hcy-7	0.029	0.852

Table 5: Correlation of biochemical parameters with Barthel Index at 7 day's post stroke

Parameters	Correlation coefficient	P(2 tailed)
MDA-1	-0.385	**0.009**
MDA-7	-0.320	**0.032**
Hcy-1	-0.043	0.779
Hcy-7	-0.108	0.480

On correlating the biochemical parameters with the outcome of cases it was found that plasma

malondialdehyde (MDA) on day 1 and around 7th day correlated with the outcome measured by Modified Rankin scale and Barthel Index. Homocysteine levels did not have any correlation with the outcome of the patients (Table 4 & 5).

Table 6: Correlation of biochemical parameters with Modified Rankin Scale at 6 months

Parameters	Correlation coefficient	P(2 tailed)
MDA-1	0.369	**0.012**
MDA-7	0.322	**0.030**
Hcy-1	0.0734	0.632
Hcy-7	0.109	0.476

Table 7: Correlation of biochemical parameters with barthel index at 6 months

Parameters	Correlation coefficient	P(2 tailed)
MDA-1	-0.284	0.58
MDA-7	-0.258	0.86
Hcy-1	-0.109	0.473
Hcy-7	-0.156	0.306

On correlating the parameters with long term outcome of stroke patients (at 6 months), it was found that plasma MDA levels on day 1 and day 7 correlates with outcome on Modified Rankin Scale at 6 months. However, the values do not correlate with functional independence of the cases assessed by Barthel Index. Homocysteine levels did not show any correlation with outcome of patients (Table 6 & 7).

4. DISCUSSION

In the present study, the MDA levels were found to be higher in cases of acute ischemic stroke than controls. Previous studies have also reported a higher MDA levels in stroke patients, compared to controls[31, 32]. In the study by Beg *et al*, the initial mean MDA level in cases was 4.8±2.1 nmol/ml, which is comparable to the values obtained in the present study (5.01±4.0 nmol/ml)[31]. The timing of the 1st sample estimation is important. In their study Sharpe *et al*, did not get an initial rise in MDA levels when the samples were taken within 6 hours of onset of stroke; however the levels were higher in the samples taken 48 hours following the event. The 1st sample in our study was taken within 72 hours of onset of stroke (mean time of sampling was 33 hours); therefore the initial levels of MDA were higher in our cases and compares well with the findings of Sharpe *et al*[26].

The levels of MDA in second sample (done 7 days after the 1st estimation) were not different from controls. This is possibly explained by the fact that after 7-10 days of stroke the production of free radicals decreases and the antioxidant defense mechanisms successfully scavenge the free radicals. Similar finding were reported by Demirkaya *et al*, who estimated MDA as well as antioxidant levels (glutathione peroxidase, ascorbate, superoxide dismutase) in the RBCs of acute ischemic stroke patients on the day of admission and after 7 days. They reported a rise in the levels of MDA on the day of admission accompanied by a fall in the levels of antioxidant enzymes, while after 7 days the MDA levels were normal and the levels of antioxidant enzymes were raised[33]. The MDA levels on the 7th day in our study were higher in males compared to females. This is possibly because MDA levels are influenced by smoking[33] and about 22% of males in our study were smokers.

In our study, 60% of cases had hyperhomocysteinemia (defined as values more than 15µmol/L). Most of the previous studies have reported 22-40% cases of hyperhomocysteinemia in their cohort of acute ischemic stroke patients. This difference is possibly because hyperhomocysteinemia is more prevalent in Indian population due to greater prevalence of cobalamin and folate deficiencies and greater incidence of polymorphisms in MTHFR(Methylene Tetra Hydro Folate Reductase) alleles[34]. In our study, homocysteine levels were higher in cases, both on the day of admission and after 7 days compared to controls. The mean homocysteine levels in the study group were 26.58µmol/L. However, in most of the previous studies, mean homocysteine in cases ranged from 10-15µmol/L. The higher values obtained in the present study may be due to greater prevalence of

hyperhomocysteinemia in India as mentioned previously[34]. Ideal timing of measurement of homocysteine following a cardiovascular/ cerebrovascular event has been debated. A study done on acute myocardial infarction documents that homocysteine levels show a false rise during acute phase in response to oxidative stress[35], while in another study by Lindgren *et al*, it was found that homocysteine levels fall during acute phase and after 7 days an increased level was observed[14].

Correlation Studies with Plasma MDA: In the present study, MDA levels on the day of admission and after 7 days correlated strongly with the NIHSS scores on the corresponding days. Demirkaya *et al* also reported significant correlations between MDA levels and stroke severity on NIHS scale[32]. MDA is a biomarker of lipid peroxidation resulting from oxidative stress. As already mentioned there is enormous generation of free radicals during ischemia in the penumbral tissue which has a minimum supply of oxygen[36]. The degree of free radical induced damage depends on the antioxidant defense mechanism[36]. In severe stroke due to greater amount of tissue damage the available antioxidants cannot cope up with the sudden increase in free radicals. The antioxidant enzymes are inducible enzymes hence their transcription and synthesis takes time[36.] Therefore, in the initial phase, a failure of defense mechanisms leads to increase in lipid peroxidation which reflects the amount of tissue damage and hence the severity of stroke [31,37]. In our study, MDA levels were not increased in patients who had recurrence of stroke or who expired within 6 months compared to those who survived and did not have any further vascular events. Similar findings were described by Sharpe *et al* who found no difference in MDA levels in patients who expired compared to those who survived, however their follow-up period was limited to the duration of stay in the hospital[26]. Our study therefore has extended the data increasing the time frame of assessment of outcome up to 6 months. However in a study by Lorento *et al* it was reported that MDA levels in patients with malignant middle cerebral artery infarction were associated with mortality within 30 days[38]. Their study included all the patients in comatosed condition, but in our study only 6 patients were unconscious during presentation.

In the present study, the levels of MDA levels on the day of admission as well as after 7 days predicted the disability of the patients on 7th day and after 6 months. But the levels did not predict functional outcome (Barthel Index) of stroke patients after 6 months. This difference may possibly reflect the inherent differences between the two scales. Modified Rankin scale uses gross assessment of abilities (and hence disabilities) whereas Barthel Index uses assessment of functional components in everyday activities. Cherubini *et al* found a significant correlation between the initial MDA levels in stroke patients during the acute phase and functional outcome after 1 week[39]. Tsai *et al* reported a significant correlation between MDA levels in acute ischemic stroke cases and outcome at 3 months based on modified Rankin scale[40]. They did not assess Barthel Index in their study and the follow-up was only for 3 months, whereas in our study the follow up was extended upto 6 months. To the best of our knowledge none of the previous studies correlated MDA levels with disability and functional independence after 6 months.

Correlation Studies with Homocysteine:

Homocysteine is a well known risk factor for cerebrovascular events and various epidemiological studies have shown it to be an independent risk factor for stroke. In the first part of the study, we had shown that levels of homocysteine was higher on both day 1 and day 7 compared to controls. We hypothesized that

as homocysteine produces oxidative stress, it may also have a prognostic bearing in ischemic stroke.

But we did not find any significant correlation between the levels of homocysteine and severity of stroke, nor did it correlate with the outcome in terms of recurrence of stroke/TIA or death, disability and functional independence. The studies on homocysteine and outcome after ischemic stroke have conflicting results. Boysen *et al* conducted a case controlled study with 1039 cases and found that homocysteine level was an independent explanatory variable for recurrent stroke after 15 months[41]. Similar findings were reported by Del Ser *et al* who determined Hcy levels after 3 months following stroke and found that Hcy levels were higher in cases that had recurrence of stroke expired within 15 months[42]. In the present study, follow up was done only upto 6 months. In another study by Kado *et al*, it was found that the cases with high Hcy levels during the acute phase of stroke had greater functional decline[21]. However Mizrahi *et al* reported that elevated serum Hcy levels do not serve as a predicting factor for functional outcome at discharge and rehabilitation gains after 6 months[20]. Recently one study conducted by Xu-Qing Wu *et al* has reported a significant correlation between homocysteine levels and NIHSS score as well as outcome using modified Rankin scale and Barthel Index in atherothrombotic stroke[43]. Their study included only atherothrombotic subtype of stroke which comprised only 19 cases in our study. Therefore, from the above discussion it is clear that no definite opinion can be formed regarding the predictive value of homocysteine in stroke patients. Further studies with larger number of patients are required to resolve this issue.

Limitations of the Present Study:

1. The sample size was small, compared to other studies.

2. The timing of our first sample was within 72 hours, which was slightly longer than other studies, but we chose the time on logic grounds. However, on sub-analysis of this time window, we did not find much difference in the value of the biochemical parameters.

5. CONCLUSION

MDA and homocysteine levels rise in the acute phase following ischemic stroke. The levels of MDA on the day of admission and after 7 days correlate positively with the severity of stroke as measured by NIHSS score. MDA on the day of admission and around 7 days correlate with the short term prognosis (around one week) of the patients (measured by MRS and BI). MDA levels on day 1 and day 7 correlates with outcome on Modified Rankin Scale at 6 months. However the values do not correlate with functional independence of the cases assessed by Barthel Index. Homocysteine levels do not have any correlation with the outcome of patients.

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