OXIDATIVE STRESS IN ACUTE ANTI-PSYCHOTROPIC DRUG POISONING, AND THE EFFECT OF ANTIOXIDANTS ON CHOLINESTERASE LEVELS

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Abstract

Objective: To study the effect of alpha lipoic acid in reducing oxidative stress induced in acute amytriptyline poisoning cases.

Design and methods: We compared the effect of supplementation of treatment of poisoning cases with alpha lipoic acid alone or with vitamin C, with that of those receiving only routine standard treatment for poisoning and that of a healthy control group.

Result: A determination of the level of creatine kinase (CK) and cholinesterase (chE) (indirect indicators of oxidative stress) in both the supplemented and the non supplemented group was done. A decrease in the level of oxidative stress was observed among those supplemented with either alpha lipoic acid alone or along with vitamin C, with a slightly more decrease in oxidative stress in the later group.

Conclusion: The results provide evidence that the oxidative stress induced by acute amytriptyline poisoning is comparatively decreased by supplementation with antioxidants like alpha lipoic acid and Vitamin C, than those only on routine standard treatment.

Key words: **Alpha lipoic acid (ALA), Vitamin C, Acetyl cholinesterase (AchE), Creatine kinase (CK),**

INTRODUCTION

Acute poisoning is a common and urgent medical problem in all developed, and many developing, countries of the world. In Britain it accounts for 15-20% of all acute medical emergency admissions to hospital. The different types of acute poisoning are accidental (10%) and intentional (90%). In the intentional type only 10% are actual attempted suicide cases and 80% are self-poisoning cases. In older age groups the great majority are intentional. Acute poisoning is more common in females than in males in all age groups, the ratio of females to males being about 1.4: 1.0. The increase has been marked in patients of lower social class. Currently in all European Countries the main drugs causing death of self-poisoning patients admitted to hospital are analgesics, antidepressants and benzodiazepines.[1]

Stress-related diseases cost American Industry billions of dollars a year; several billion (psychotropic drugs and antidepressant) pills are prescribed in the world each year; and although it cannot be quantified, stress seems to be involved in much of our unhappiness, irritability and dissatisfaction.[2]

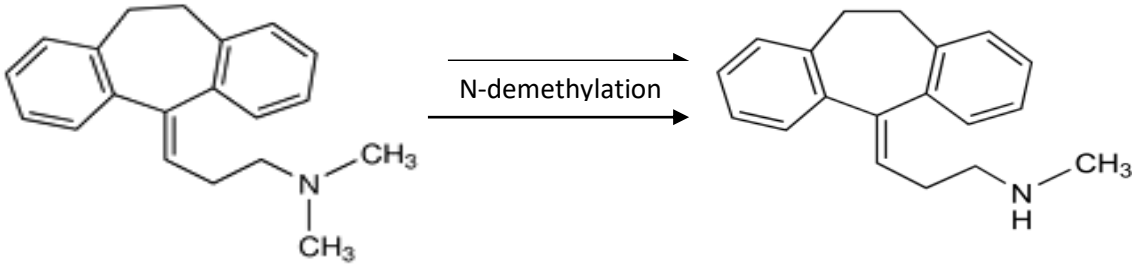
Attempted suicide is on a steady increase; in 1990 there were over 100,000 cases per year in Britain. Most suicide attempts are due to drug overdose, either prescribed or non-prescribed. Suicide attempts are commoner in women than in men and in young adults than in the elderly.[3] Drugs used to treat psychiatric disorders are known collectively as psychotropics. They are classified according to their main mode of action. The drugs used to modify or to correct pathological behavior, psychotic disorders, moods or thoughts are known as Psychotropic drugs,[4] which can be classified as follows:

I Antipsychotic drugs (Major Tranquilizers)
II Anti anxiety drugs
III Psychotogenic or Psychodelic drugs
IV Antidepressant drugs

The first antipsychotic drugs are further classified as phenothiazines, benzamides, etc. One of the most extensively used antipsychotic drugs in this group is Chlorpromazine. The second antianxiety drugs can be classified as benzodiazepines, nonbenzodiazepines, etc. Alprazolam and diazepam are widely used in this group. The third psychedelic drugs could be classified as (i) Those with indole ring e.g. lyseric acid diethylamide (LSD i.e. with indole ring) (ii) Without indole ring e.g. cannabis (marijuana) showing miscellaneous action (iii) Drugs showing anticholinergic action e.g. atropine. The fourth is antidepressant drugs. These drugs are classified as tricyclic, tetracyclic, monoamine oxidase inhibitors (MAOI) and lithium compounds. The tricyclic are divided into two groups:

i. Tertiary amines eg) Imipramine, Amitriptyline etc.
ii. Secondary amine eg) Desipramine, Nortreptyline and Protriptyline.

Amitriptyline: It is a 3-(10, 11 Dihydro - 5H - dibengo [a,d] cycloheptен- 5 ylidene) - N, N dimethylpropylamine,(C20H23N); with relative molecular mass of 277. Amitriptyline is very widely used tricylic antidepressant. It is metabolized by N-demethylation to nortriptyline, which is an antidepressant in its own right. Protriptyline is an analogue of amitriptyline.5 The tricylic antidepressant amitriptyline has been easily available and commonly abused for suicidal purposes in developing countries.



Amitriptyline exerts its antidepressant action by blocking the neuronal reuptake of nor adrenaline and serotonin. Amitriptyline has significant anticholinergic activity. It also has some sedative action. Endogenous depression responds to a greater extent than other types.

OXIDATIVE STRESS

The term oxidative stress refers to the situation of imbalance between production of free radicals and antioxidant defence[6] principally oxidative stress in human can result in diminished body antioxidant when the free radical production overwhelms the endogenous antioxidant levels, they cause considerable cell damage or death.

All major biomolecules like lipids, proteins, and nucleic acids react with free radicals, but lipids are probably the most susceptible.[7] The oxidative destruction of lipids (lipid peroxidation) is a destructive self-perpetuating chain reaction releasing malonyldialdehyde (MDA) as the end product.[8] Studies on acute amitriptyline poisoning cases indicate increased level of SOD, MDA and reduced antioxidant capacity of blood (FRAP assay) acute amitriptyline poisoning cases showed increased oxidative stress and reduced antioxidant status.

ALPHA LIPOIC ACID

Alpha lipoic acid initially classified as a vitamin discovered three decades earlier possessed potent antioxidant properties. It is more potent than the old guard antioxidants vitamin C and E; it even recycles these vitamins and enhances their effectiveness.

In addition to functioning as an antioxidant, this hard working nutrient assists B vitamins in producing energy from the proteins, carbohydrates and fats consumed through foods. As it dissolves in both water and fat this is called "UNIVERSAL ANTIOXIDANT". It can reach tissues composed of fat, such as the nervous system as well as those made mainly of water such as the heart.

A study was conducted with 600 mgs of alpha lipoic acid given daily to 9 Alzheimer's patients, on an average for 80 days. The treatment leads to stabilization of cognitive functions in the Alzheimer's study group. This was the first indication that alpha lipoic acid might be a successful neuroprotective therapy option for Alzheimer diseases and related dementias.[9] Alpha lipoic acid benefits anyone whose limbs tend to tingle or become numb or "fall asleep" due to nerve compression. In animal studies, alpha lipoic acid has been seen to increase the blood flow to the nerves and improved transmission of nerve impulses. Parkinson's disease (PD) is disorder of the central nervous system. Clinically the disease is characterized by disease in spontaneous movements, gait difficulties, postural instability, rigidity and tremor. PD may appear at any age but it is uncommon in people under 30 years of age. Oxidative stress appear to play an important role in neuronal degeneration associated with PD.[10] Depletion of glutathione (GSH) in the brain is the earliest indicator of oxidative stress presymptomatic PD.[11]

Studies both in vitro and vivo models have suggested that pretreatment with alpha lipoic acid increase cellular levels of GSH, probably by preventing its depletion thereby protecting mitochondria integrity. Results with previous studies suggest that alpha lipoic acid may be an effective neuro protective agent in age associated neuro degeneration utilizing the PC12 cell model system.

Based on the above beneficial effects of alpha lipoic acid on nervous system it has been decided to supplement alpha lipoic acid with acute amitriptyline poisoning cases, which have increased oxidative stress and neuro toxicity, and to study the effect after supplementation of alpha lipoic acid.

CREATINE KINASE

Creatine Kinase (CK) is predominately found as a dimer of catalytic subunits, each with molecular weight of about 40 kDa; the two sub units are termed M (for muscle) and B (for Brain). The three resulting isoenzymes are CK_1 (BB), CK_2 (MB) and CK_3 (MM). Another structurally different form of CK, with molecular weight of 64 kDa is present in mitochondria, although it is seldom released to the circulation, it may also form oligomers. It is termed as macro CK_2 with molecular weights up to 250 kDa. CK is found in small amounts throughout the body, but is in high concentration only in muscle and brain, although CK, from brain virtually never crosses the blood-brain barrier to reach plasma.

In skeletal muscle, CK-MB comprises 0% to 1% of the total CK in type 1 fibers and 2% to 6% of CK in type 2 fibers. During regeneration of skeletal muscle, increased amounts of CK-MB are produced relative to CK-MM, similar to the pattern seen in total muscle.[12]

The most commonly used method for measuring CK-MB is mass immunoassay, either using two different antibodies or the "CONON" monoclonal antibody that specific for CK-MB isoenzyme.[15] There are slight but significant differences between different immunoassays.[16] CK by mass measurements is stable even at refrigerator temperatures, and shows only slight decrease when stored for many days at room temperature.[17]

Measurement of CK isoforms is usually accomplished using high-resolution electrophoresis. Because of the low activity of CK-MB isoforms, precision is generally poor for this purpose and careful control of assay conditions is necessary to achieve reproducible results; an automated, electrophoertic instrument is required for this purpose.[18] Immunoassays have also been used for quantifying the isoforms of CK-MB.[19]

PATIENTS AND METHODS

A total of 132 subjects were enrolled for the study and were divided into 5 groups. They were selected from IMCU and Toxicology Ward, Govt. General Hospital between Sept. 2005 and March 2008. Consent was obtained from the attendants of the patients. The study was approved by the ethical committee of Madras Medical College, Chennai-3.

Selection of subject: The patients were randomly selected. The Edinburgh scale was used to classify the depth or grade of coma of poisoned patients, graded as under:-

Grade 1	:	Patient drowsy but responding to verbal commands.
Grade 2	:	Patient unconscious but responding to minimal stimuli (for Example, shaking, shouting)
Grade 3	:	Patient unconscious and responding only to painful stimuli (For example, rubbing the sternum)
Grade 4	:	Patient unconscious with no response to any stimuli

The study was restricted only to the grade 1 patients from IMCU.

The groups were classified as follows:

- **Group I:** Consisted of 30 healthy volunteers (15 males and 15 females) mean age 32 years.
- **Group II:** Consisted of 30 patients (18 males and 12 females), mean age 34 years. These patients received only routine standard treatment (RST)
- **Group III:** Consisted of 21 patients (12 males and 9 females), mean age 32 years. These patients received routine standard treatment (RST) + Vitamin C supplementation.
- **Group IV:** Consisted of 27 patients (13 males and 14 females), mean age 31 years. These patients received routine standard treatment (RST) + alpha lipoic acid supplementation.
- **Group V:** Consisted of 24 patients (14 males and 10 females), mean age 34 years. These patients received routine standard treatment (RST) + Vitamin C and alpha lipoic acid supplementation.

Basal level of oxidative stress markers and enzymatic and non–enzymatic antioxidants were measured at the beginning of the treatment and followed up until the day of discharge from IMCU.

Exclusion criteria

Less than 18 years and more than 60 years are not included in this study. Patients those who have taken other drugs along with amitriptyline are not included in this study. Patients with TLC positive and spectra (uv-vis) negative are not included in this study.

Sample Collection

From each experimental subject 10 ml of venous blood was drawn from the antecubital vein. 5 ml of blood collected in a plain tube for enzyme analysis and 5 ml of blood collected in sterile heparin vacutainer tubes. The plasma was separated by centrifugation at 1500 x g for 10 minutes and this plasma was stored in a new clean storage vials and stored at - 80^{o}C and used for analysis of antioxidants and plasma cholinesterase. The cells were separated and washed with normal saline and RBCs were subjected to lyses and used for RBC cholinesterase estimation.

50ml of gastric aspirate was collected from all patients who are directly admitted to IMCU and poison centre GGH, Chennai-3. This gastric aspirate was taken for TLC (thin layer chromatography) identification.

Methods

Plasma Cholinesterase estimation: The cholinesterase levels were determined in plasma and RBC lysate. Estimation was done by colorimetric method using acetyl choline (SD fine chemicals) as substrate (Venkataraman et al. 1993)[20]. Both true and pseudo cholinesterase would hydrolyse the substrate and produce choline and acetic acid. The change in colour of the indicator bromothymol blue (SD fine chemicals) caused by the liberated acetic acid from cholinesterase was read by spectrophotometer at 620 nm.

Bromothymol blue 0.5ml solution was diluted with 3.8 ml of distilled water and 0.2 ml of 15% acetyl choline chloride was added. To it 100µl of plasma was added and the change in colour was read at 620 nm at 37 degree c after 30 minutes. A standard graph was plotted using acetic acid 0.15 N in concentration of 10, 20, 50,100 and 200 micro moles.

In RBC cholinesterase estimation RBCs were extracted by first adding distilled water 3 ml followed by precipitation of hemoglobin with acetone 2 ml and centrifugation at 3000 rpm. The supernatant was used for estimation of RBC cholinesterase.

Total Antioxidant Status[21]

Total antioxidant status of sample was measured by commercial kit, supplied by Randox.

Principle: $ABTS^R$ (2-2' – Azino-di-[ethylbenzthiazoline sulphonate] incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^R$. This has a relatively stable blue green colour, which was measured at 600 nm. Antioxidants in the added sample cause suppression of this colour production to a degree which was proportional to their concentrations.

20 µl of plasma was added to 1 ml of chromogen and incubated at RT for 1 minute. The initial Absorbance (A_1) measured at 600 nm. 200 µl of substrate added to it and incubated at RT for 3 minutes. The final absorbance A_2 was measured at 600 nm.

A Blank and a standard were run simultaneously, the initial absorbance A_1 and final absorbance A_2 was measured at 600 nm for both blank and standard.

A_2-A_1 =ΔA of the sample

A_2-A_1= ΔA of the blank

A_2-A_1= ΔA of the standard

$$\text{Factor} = \frac{\text{Concentration of the Standard}}{\Delta A \text{ blank} - \Delta A \text{ standard}}$$

Total Anti oxidant status in m.mol/I = Factor X (ΔA Blank-ΔA sample)

Drug extraction from stomach wash contents

The stomach wash contents obtained from the patient who have been suspected to have consumed drug was taken for Thin Layer Chromatography analysis, 10 ml of the collected stomach wash contents was taken in the vortex tube of 11 cm length and 2.5 cm diameter and to it was added equal volume of 10 ml of chloroform: propane-2-ol (9:1) mixture which was allowed to stand for 5 minutes. The solution was thoroughly mixed by the vortex mixer (cyclomixer) for about 5 to 10 minutes.

The mixed solution was poured into a separating funnel where it was allowed to stand for 30-45 minutes for the aqueous and organic layer to separate. The drug compound suspected get extracted into the chloroform layer, which remained at the bottom of the separating funnel. The separated organic layer containing chloroform and drug was removed from the separating funnel by slowly opening the knob where by the lower layer was allowed to run into a funnel with filter paper 2 gm of sodium sulphite present on the filter paper removes the water molecules and polar substances from the layer and the rest of the solution poured into a beaker, where it is evaporated to near dryness in a water-bath at 60°c. Few drops of chloroform were added in the breaker and the extract was ready for application.

TLC application

About 5 µl of the extracted sample was spotted to the TLC plate at a position 2.0 to 2.5 cm from the bottom edge of the plate with a help of a specialized capillary tube. The circular spot about 2 to 6 mm in diameter was spotted on a line parallel to the standard drug substance. The two spots of standard and test sample were plotted at a distance of more than 1.5 cm distance with much to prevent any smearing.

Calculation of Rf (Relative front or ratio front)

The distance travelled by solvent mixer is marked and this distance is first measured in cm next the distance travelled by the solute is marked as a rounded spot. The distance from the centre of the spot to the point of its spotting, which was measured in cm is the distance travelled by the solute. Rf value for the drug was calculated using the following formula.

$$Rf = \frac{\text{Distance travel by solute in cms}}{\text{Distance travel by solvent in cms}}$$

METHODS FOR TOTAL AND ISOENZYMES ANALYSIS

Twenty cases were selected randomly among all the groups and divided into two groups as group A and group B for CK study. The blood samples were collected on admission and analyzed for total and isoenzymes of CK.

Total CK analysis

Total CK was analyzed by semi auto analyzer Micro lab 200 by UV kinetic IFCC method.

Principle: CK catalyzes the conversion of Creatine Phosphate and ADP to Creatine and ATP. The ATP produced and glucose is converted to ADP and glucose-6-phosphate by hexokinase (HK) in the second reaction. In the final reaction, glucose-6-phosphate dehydrogenase (G-6-PD) oxidizes the glucose-6-phosphate produced in the second reaction and reduces NADP. The NADPH produced in the final reaction is proportional to the creatine produced in the initial reaction. The rate of increase in NADPH absorption at 340 nm is thus directly proportional to the CK activity.

$$\text{Creatine Phosphate + ADP} \xrightarrow{CK} \text{Creatine + ATP}$$

$$\text{ATP + D-Glucose} \xrightarrow{HEXOKINASE} \text{Glucose-6-Phosphate+ADP}$$

$$\text{Glucose-6-Phosphate+NADP} \xrightarrow{G\text{-}6\text{-}PDH} \text{NADPH + 6-Phosphoglyconolactone}$$

Reagents

Reagent 1 (Enzymes)

N-Acetyl-L-Cysteine 20 mmol/L, ADP – 2mmol/L, AMP-5mmol/L, NADP-2mmol/L, D-Glucose-20mmol/L, Diadenosine Pentaphosphate-10µmol/L, EDTA-2 mmol/L, Hexokinase-≥3500 U/L, G-6-PDH-≥2000 U/L, Creatine Phosphate-30mmol/L.

Reagent 1A (Buffer)

Imidazole buffer, pH 7.10 – 100 mmol/L, Magnesium Acetate-10 mmol/l.

Procedure: 3ml of Reagent 1A into Reagent 1 bottle and mix by gentle swirling till completely dissolved. This is working Reagent. 1000µl of working Reagent and 20µl of serum sample are added and the analysis is carried out in semi auto analyzer by kinetic mode at 340nm. Initial absorbance A_0 after 10 minutes and repeat the absorbance change per minute (ΔA/min).

Calculation

Total CK activity in µ/l at 37°C = ΔA/min x 6666

CK Isoenzyme analysis

Analysis of Isoenzyme of CPK was carried out by HYDRASYS system SEBIA, PN 1210.

The HYDRASYS SEBIA system is a semi automated multi-parameter, Electrophoresis system. Commercial kits hydragel 7 Iso-CK are available for the analysis. The test was carried out as per the instruction provided in the kit by the manufacturer.

Principle: Creatine kinase isoenzymes consist of two subunits: M ("muscle") and B ("brain"), assembled in dimers. The three resulting combinations constitute the three isoenzymes. MM is principally located in cardiac and skeletal muscles, MB in cardiac muscle and BB in cerebral tissues. Each subunit has a specific electric charge which confers characteristics mobility to the individual CK isoenzymes. On HYDRAGEL 7 ISO CK and HYDRAGEL ISO CK 15/30 gels, the BB fraction is the most anodic, the MM fraction is the most cathodic and the MB is intermediary.

All CK isoenzymes catalyze the same reaction that is utilized in their visualization. In the HYDRAGEL, 7 ISO-CK kits, the serum samples were electrophoresed and the separated CK isoenzymes were visualized using a specific chromogenic substrate according to the following reactions:

Creatine Phosphate + ADP $\xleftrightarrow{CK}$ Creatine + ATP

ATP + D-Glucose $\xleftrightarrow{HEXOKINASE}$ Glucose-6-Phosphate+ADP

Glucose-6-Phosphate+NADP $\xleftrightarrow{G\text{-}6\text{-}PDH}$ NADPH + 6-Phosphoglyconolactone

NADPH + PMS $\longleftrightarrow$ NADP+ PMSH

PMSH+TNBT $\longleftrightarrow$ PMS+ TNBT reduced precipitate of formazan

The amount of resulting formazan precipitate is proportional to the CK enzymatic activity.

Sample for Analysis

Fresh serum samples are taken for analysis. Sample can be stored at 2 to 8°C up to one week. 500 µl serum sample with 5 µl of activation solution are mixed and incubated for 10 mts at room temperature.

Procedure: The steps include processing of Hydragel agrose gels in the following sequences.

Sample application, Electrophoresis migration, Incubation with substrate, Stopping the enzymatic reaction, Blotting and final drying of the gel, Gel Scanning. By identifying the pattern we can evaluate the fraction of CPK isoenzyme which is elevated.

NOTES:

NAD : Nicotinamide Adenine Dinucleotide

PMS : Phenazine Moethosulfate

NBT : Nitro Blue Tetrazolium.

STATISTICAL ANALYSIS

Statistical evaluation was carried out using SPSS (Version 14.0) Data obtained from the study groups were compared by the parametric student's t-test; correlation analysis between variables were made by Pearson test; P value <0.001 was considered statistically significant. All the results were expressed as means with their standard deviation (mean ± SD). Statistical analysis was also performed by using standard deviation and ANOVA.

The effect of vitamin C, alpha lipoic acid and both combined were analyzed for each groups and expressed as percentage of benefit with and without supplementation of vitamin C, alpha lipoic acid and both combined. Multiple comparison of each group with the normal was carried out using Bonferoni-t-test.

RESULTS

Identification of amitriptyline overdoses in gastric aspirate in all cases admitted directly to intensive medical care unit (IMCU) and toxicology ward, Government General Hospital between September 2005 and March 2008 are carried out by thin

layer chromatography (TLC). The samples along with controls are run simultaneously and based on their rf values in the TLC chromatogram, the amitriptyline overdoses are detected and confirmed in all the groups.

CONCLUSION

Antioxidants levels were increased during treatment after supplementation with vitamin C and alpha lipoic acid. We suggest that antioxidant status of acute amitriptyline poisoning cases should be considered for more effective recovery and that diets low in antioxidants may render slow in recovery.

This study provides quantitative recommendations for the intake of vitamin C and alpha lipoic acid in fast recovery and in increasing the antioxidant status of glutathione.

This study shows that by oral supplementation with Vitamin C and alpha lipoic acid to acute Amitriptyline poisoning cases considerable oxidative stress was reduced, and it also enhances the total antioxidant levels of these patients. This will help the acute Amitriptyline poisoning patient to recover faster, and stay in intensive care will be reduced, also supplementation of vitamin C and alpha lipoic acid reduces the long term side effects such as Amitriptyline induced delayed Neuropathy (AIDN).

The effect of supplementation of vitamin C and alpha lipoic acid was maximum in Group V(Routine standard treatment+ vitamin C+ alpha lipoic acid) acute Amitriptyline poisoning cases, on these group received the maximum dosage of vitamin C and alpha lipoic acid (5-13 days). It is clear that the supplementation should be for longer period to have the maximum beneficial effect (reduced oxidative stress and increased total antioxidant status). In our opinion oral supplementation will be more effective for chronic Amitriptyline poison cases for a longer duration and for acute Amitriptyline poisoning cases I.V. form of vitamin C and alpha lipoic acid will be more effective.

The recovery and regeneration of plasma cholinesterase was rapid when compared to RBC cholinesterase in all groups of acute Amitriptyline poisoning cases.

On the above discussion it could be noticed incidentally that the elevated CPK in Group A (on admission randomly selected 10 cases for CPK study) and Group B(on admission randomly selected 10 cases for CPK study) which may be due to pulmonary infarction not due to cardiac or skeletal muscle involvement.

The average age of acute Amitriptyline poisoning cases were 33 for male and 32 for female, and this study shows that the Amitriptyline poisoning is seen more in male [55.3%] than female [44.7%].

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