Protective Effect of Diallyl disulphide on Hyperammonemic rats

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ABSTRACT
Hyperammonemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication. Garlic (Allium sativum. L) has been used as a spice, food and folklore medicine for over 4000 years, and it is the most widely used researched medicinal plant. Our aim is to investigate the effect of diallyl disulphide on blood ammonia, plasma urea, serum liver markers, lipid peroxidation and antioxidants status in tissues (liver and brain) of ammonium chloride induced hyperammonemic rats. Male albino Wistar rats (180–200 g) were used for the study. Hyperammonemia was induced by interaperitonial injection of ammonium chloride (AC) (100 mg/kg body weight); rats were treated with diallyl disulphide (DADS) (60mg/kg body weight) via oral administration. Administration of DADS in hyperammonemic rats reduced the levels of ammonia and urea. The antioxidant property of DADS was studied by assessing the activities of thiobarbituric acid reactive substances (TBARS), hydroperoxides (HP), nitric oxide (NO) and liver markers such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) and the levels of superoxide dismutase (SOD), reduced glutathione (GSH), in AC treated rats. Oxidative stress was effectively modulated by DADS administration. DADS significantly improved the status of antioxidants and decreased ammonia, urea, lipid peroxidation and liver markers enzymes as compared to AC treated group, as it has improved the maintenance of cellular integrity of liver and other tissues. However, the exact underlying mechanism has still to be investigated and isolation, investigating the efficacy of active constituents on hyperammonemia is desirable.

Keywords: Hyperammonemia, Diallyldisulphide, Antioxidant, Oxidative stress, Free radicals.

INTRODUCTION
Hyperammonemia is defined as an elevated ammonia concentration in blood, caused by an impairment of liver function resulting in inadequate ammonia detoxification.[1] It is a major contributing factor of neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication. Ammonia affects both excitatory and inhibitory synaptic transmission in the mammalian brain by a variety of mechanisms.[2] Ammonia toxicity results in free radical generation that leads to oxidative stress and tissue damage.[3] Previous reports stated that ammonium (chloride/acetate) salts induce ammonia toxicity partly via oxidative stress, which leads to lipid peroxidation and free radical generation. This could be the primary cause for central nervous system malfunction associated with hyperammonemia, such as, hepatic encephalopathies, Reye syndrome, irritability, somnolence, vomiting, seizures, and dearrangement of cerebral function, coma and death.[4] NO in physiological concentration has many beneficial roles. But, as it increases beyond this range, it will cause more damage to brain tissues.[5] Previous results showed that increased concentration of NO and nitric oxide synthase activity in brain region of animals subjected to ammonia toxicity.[6]

The greatest disadvantage of presently available potent conventional or synthetic antihyperammonemic agents/therapies lies in their toxicity and reappearance of symptoms after discontinuation. Furthermore, these drugs can cause serious adverse effects.[7] Hence, the screening and development of therapeutic agents from traditional medicinal plants for their antihyperammonemic activity is in progress worldwide.

Garlic is considered as a natural product that has an immense therapeutic potential in many pathologic conditions. The plant is rich in sulfur-containing amino acids.[8] Garlic and its products have many potential activities such as antibacterial,[9], anticarcinogenic,[10], hypolipidemic,[11], anti diabetic,[12], hepatoprotective,[13] and immunomodulatory effects.[14]

DADS is an organosulphur garlic compound, but it is not present in garlic cloves. It is present (60%) in garlic oil.[15] DADS is a product of allicin. Transformation of allicin produces DADS. Allicin is produced when an enzyme allicinase act on its substrate allin during garlic cutting or crushing.[16] DADS can be obtained from the less expensive commercial sources. Its antioxidant activities, accredited to the biologically active lipophilic sulfur bearing compounds like allicin, (DADS), (SAC) and (DAS). A survey of literature showed the lack of experimental evidence whatsoever to justify the antihyperammonemic role of DADS. This report is the first study to investigate the effect of DADS on blood ammonia, plasma urea, NO in plasma and tissues (liver and brain), liver marker enzymes, lipid peroxidation and antioxidant status in liver and brain tissues of hyperammonemic rats.

MATERIALS AND METHODS

ANIMALS
Adult male albino Wistar rats (180–200 g) and bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, India, were used. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 22°C ± 2°C and humidity of 45%–64%. Animals were fed with a standard pellet diet (Hindustan Lever Ltd, Mumbai, India) and water ad libitum. Studies were carried out in accordance with Indian National Law on Animal Care and Use, and ethical clearance was provided by Committee for the Purpose of Control and Supervision of
Experiments on Animals of Rajah Muthiah Medical College and Hospital (Vide. no: 588/2008/CPCSEA), Annamalai University, Annamalainagar, India.[6]

CHEMICALS
DADS was purchased from Lancaster, U.K. AC was purchased from Sisco Research Laboratories (Mumbai, India). All other chemicals used in the study were of analytical grade.

INDUCTION OF EXPERIMENTAL HYPERAMMONEMIA
Hyperammonemia was induced in Wistar rats by intraperitoneal injections of ammonium chloride at a dose of 100 mg/kg body weight thrice in a week for 12 consecutive weeks.[20]

EXPERIMENTAL DESIGN
In the experiment, a total of 32 rats were used. The rats were divided into four groups of eight rats each. Group I rats received physiological saline and considered as controls. Group II normal rats were administered with DADS (60 mg/kg body weight) using an intragastric tube[21], Group III rats were treated with AC (100 mg/kg body weight; i.p.)[20] and Group IV rats were treated with AC (100 mg/kg) and DADS (60 mg/kg) thrice in a week for 12 weeks.

At the end of 8th week, rats were fasted overnight and sacrificed by cervical dislocation. Blood was collected, and plasma and serum were separated by centrifugation. Liver and brain tissues were excised immediately and rinsed in ice-chilled normal saline, 500mg of the tissues were homogenized in 5.0 ml of 0.1 M Tris–HCl buffer (pH, 7.4). The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

BIOCHEMICAL ANALYSIS

DETERMINATION OF BLOOD AMMONIA AND PLASMA UREA
Blood ammonia was determined by enzymatic kinetic colorimetric assay Wolheim[22] by using an automated Roche/Hitachi 912 kit. Plasma urea was determined by diacetyl monoxime method[23] using automated Roche/Hitachi 912 kit. Urea reacts with diacetyl monoxime under strongly acidic conditions and gives a pink coloured complex and the colour developed was read at 540 nm.

ESTIMATION OF LIVER MARKERS ENZYMES
Activities of aspartate transaminase (AST) and alanine transaminase (ALT) were assayed by the method of Reit-man and Frankel.[24] AST catalyses the transfer of an amino group from L-aspartate to α-ketoglutarate to form oxaloacetate (unstable and gets converted into pyruvate) and L-glutamate. ALT catalyses the transfer of an amino group from L-alanine to α-ketoglutarate to form pyruvate and L-glutamate. The pyruvate liberated reacts with 2,4- dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone, which can be read at 540 nm. Alkaline phosphatase (ALP) was assayed by the method of Reit-man and Frankel.[25] ALP catalyses disodium phenyl phosphate into phenol and disodium hydrogenphosphate at pH 10. Phenol so formed reacts with 4-aminonitroantipyrine in alkaline medium in the presence of oxidizing agent, potassium ferricyanide, to form a red-coloured complex whose absorbance is proportional to the enzyme activity.

ESTIMATION OF LIPID PEROXIDATION
Plasma thiobarbituric acid reactive substances (TBARS) were estimated by the method of Yagi.[26] TBARS were quantitated by their reactivity with thiobarbituric acid (TBA) in acidic conditions to generate a pink coloured chromophore, which was read at 530 nm. TBARS in the liver and brain were estimated by the method Fraga.[27] In this method, malondialdehyde and other TBARS were measured by their reactivity with TBA in acidic conditions to generate a pink-coloured chromophore, which was read at 535 nm. Estimation of plasma, liver and brain tissues lipid hydroperoxides (HP) was done by the method of Jiang.[28] In this method, oxidation of ferrous ion (Fe[2+] ) under acidic conditions in the presence of xylene orange led to the formation of a chromophore, which was read at 560 nm.

ESTIMATION OF NITRIC OXIDE
NO were estimated by the private[29], to 0.5ml of plasma/tissue homogenate 0.1ml of sulphotetic acid was added vortexed well for 30 minutes. The samples were then centrifuged at 5000rpm for 15 minutes. The protein free supernatant, was used for the estimation of nitrite. To 200µl of the supernatant, 30 µl of 10% NaOH added followed by 300 µl of Tris-HCL buffer and mixed well, to this 530 µl of Gress reagent was added incubated in the dark for 10-15minutes and the absorbance was read at 540nm against a Gress reagent blank. Sodium nitrite solution was used as the standard.

ESTIMATION OF ANTIOXIDANTS
Superoxide dismutase (SOD) activity in liver and brain tissues was assayed by the method of Kakkar.[30] Superoxide radicals react with nitroblue tetrazolium in the presence of reduced nicotinamide adenine dinucleotide and produce formazon blue. SOD removes the superoxide radicals and inhibits the formation of formazon blue. The intensity of the colour is inversely proportional to the activity of the enzyme and read at 560 nm. Estimation of reduced glutathione (GSH) in liver and brain tissues was done by the method of Ellman.[31] This method is based on the development of yellow colour, when dithionito-benzoic acid is added to compounds containing sulphydryl groups. The colour developed was read at 412 nm.

STATISTICAL ANALYSIS
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using SPSS software package 9.05. Result were expressed as mean ± SD from 8 rats in each group. P values <0.05 were considered as significant.

RESULTS
Table 1 shows the levels of TBARS and HP in plasma and tissues (brain and liver) of normal and experimental rats. TBARS and HP levels were found to be significantly increased in plasma and tissues of AC- induced rats when compared to normal rats. The levels found to be significantly reduced in hyperammonemic rats treated with DADS when compared with AC induced rats.

Table 1. Effect of Diallyl disulphide on changes in the plasma (HP &TBARS) of normal and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma</th>
<th>Liver</th>
<th>Brain</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TBARS (nM/mI)</td>
<td>HP (x 10^-9 M/dl)</td>
<td>TBARS (mM/100 g wet tissue)</td>
</tr>
<tr>
<td>Normal</td>
<td>4.85±0.20</td>
<td>0.18±0.75</td>
<td>1.55±0.18</td>
</tr>
<tr>
<td>Normal + DADS</td>
<td>4.60±0.14</td>
<td>0.71±0.69</td>
<td>2.20±0.22</td>
</tr>
<tr>
<td>(60 mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC (100 mg/kg)</td>
<td>5.95±0.27</td>
<td>1.15±0.93</td>
<td>1.01±0.51</td>
</tr>
<tr>
<td>DADS (60 mg/kg)</td>
<td>4.90±0.22</td>
<td>1.07±0.89</td>
<td>0.95±0.04</td>
</tr>
<tr>
<td>AC (100 mg/kg)</td>
<td></td>
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<tr>
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ANOVA followed by Duncan’s multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at $P \leq 0.05$
Tables 2 display the levels of enzymatic antioxidants SOD and nonenzymatic antioxidant GSH in tissues (brain & liver). Both enzymatic and non-enzymatic antioxidants levels were found to be significantly decreased in rats induced with AC. The levels were reverted back to near normal levels in hyperammonemic rats treated with DADS. There was no significant changes observed in DADS treated when compared with AC- induced rats.

NO levels were found to be significantly increased in plasma and tissues (brain & liver) of AC induced rats when compared to normal rats. These levels were found to be significantly reduced in hyperammonemic rats treated with DADS when compared with AC-treated rats were shown in the figure 1 (A&B).

ANOVA followed by Duncan’s multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at \( P \leq 0.05 \)

Table 2. Effect of Diallyl disulphide on the activities of SOD and GSH in the liver and brain of normal and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD (U/mg protein)</td>
<td>GSH (mg/100 g wet tissue)</td>
</tr>
<tr>
<td>Normal</td>
<td>7.25 ± 0.43 (^a)</td>
<td>56.88 ± 3.97 (^a)</td>
</tr>
<tr>
<td>Normal + DADS (60 mg/kg)</td>
<td>9.91 ± 1.41 (^a)</td>
<td>58.66 ± 2.16 (^a)</td>
</tr>
<tr>
<td>AC (100 mg/kg)</td>
<td>5.01 ± 0.38 (^b)</td>
<td>28.44 ± 1.03 (^b)</td>
</tr>
<tr>
<td>DADS (60 mg/kg) + AC (100 mg/kg)</td>
<td>9.60 ± 1.18 (^c)</td>
<td>44.43 ± 3.10 (^c)</td>
</tr>
</tbody>
</table>

Fig 1. Levels of Nitric oxide in (A) Plasma and (B) Tissues (liver and brain) in normal and experimental rats. Analysis of variance (ANOVA) followed by least significant difference (LSD), a, a- not significant (group II is compared with group I), b \( \leq 0.05 \) (group III is compared with group I), c \( \leq 0.01 \) (group IV is compared with group III).

Fig 2. Change in the levels of (A) Blood ammonia and (B) Urea in normal and experimental rats. Analysis of variance (ANOVA) followed by least significant difference (LSD), a, a- not significant (group II is compared with group I), b \( \leq 0.05 \) (group III is compared with group I), c \( \leq 0.01 \) (group IV is compared with group III).

In the figure 2 (A&B) concentrations of circulatory ammonia, urea was increased significantly in AC treated rats. AC and DADS-treated rats showed significantly low levels of circulatory ammonia, urea, when compared with the corresponding AC treated rats. Rats treated with DADS alone showed no significant differences in levels of ammonia, urea, when compared with control rats.

Fig 3. Change in the activities of ALT, ASP and AST in normal and experimental rats. Analysis of variance (ANOVA) followed by least significant difference (LSD), a, a- not significant (group II is compared with group I), b \( \leq 0.05 \) (group III is compared with group I), c \( \leq 0.01 \) (group IV is compared with group III).
Concentrations of circulating liver marker enzyme AST, ALT, ALP levels were found to be significantly increased in serum were shown in the figure 3. AC induced rats, when compared to normal rats. The levels were found to be significantly reduced in hyperammonemic rats treated with DADS when compared with AC alone induced rats.

DISCUSSION
In liver, ammonia is removed either in the form of urea in periporal hepatocytes and/or as glutamine in perivenous hepatocytes. The increased levels of blood ammonia and plasma urea indicate hyperammonaemic condition in rats treated with AC, which may be due to liver damage caused by ammonia intoxication. Elevated levels of ammonia in blood and brain result in dearrangement of cerebral function. Reports have shown that excess ammonia induces nitric oxide synthase, which leads to the enhanced production of NO, which in turn leads to oxidative stress and liver damage. Decreased levels of blood ammonia and plasma urea in DADS and AC treated rats may be due to the antioxidant potential of DADS. Previous reports suggested that DADS has been known to have free radical scavenging effect and it could be a potential therapeutic or modulating agent for oxidative damage induced diseases. It is a well documented fact that most medicinal plants are enriched with phenolic compounds and bioflavonoids that have excellent antioxidant property.

In our study, the elevated levels of circulating liver markers and lipid peroxidation and NO products in AC-treated rats might be due to the liver damage caused by ammonia-induced free radical generation. Reports have shown that excess ammonia intoxication leads to excessive activation of NMDA receptors leading to neuronal degeneration and death. The mechanisms by which excessive activation of NMDA receptors lead to neuronal degeneration and death are caused by increased Ca$^{2+}$ concentration in the postsynaptic neuron. Ca$^{2+}$ binds to calmodulin and activates nitric oxide synthase, increasing the formation of NO that contributes to the neurotoxic process. Activation of NMDA receptors also leads to increased production of superoxide radical, which has been also proposed under in vivo conditions. Superoxide and NO have the ability to generate hydroxyl radicals. This leads to oxidative stress, which causes tissue damage. Decreased levels of circulating liver markers and lipid peroxidation products and NO in DADS administered rats may be due to its free radical scavenging property. Many studies have shown that oxidative stress and free radical production mediated lipid peroxidation could be involved in the mechanism of ammonia toxicity.

In this study, AC-induced rats exhibited decreased activities of SOD and catalase in liver and brain. SOD plays an important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by catalase to molecular oxygen and water. The decrease in the activities of these antioxidant enzymes might be due to damage of these tissues (liver and brain). DADS administration significantly normalized the activities of SOD and catalase in liver and brain of a AC-induced rats. The metal-GSH conjugation process is desirable in that it results in the excretion of the toxic metal into the bile. However, it depletes the GSH from the cell and thus decreases the antioxidant potential. Administration of DADS increased the levels of GSH. The enhancement of GSH may be due to the presence of DADS, which is a glutamine precursor from which GSH is formed.

The free radical scavenging effect of DADS has been reported in previous studies. DADS could enhance the levels of SOD, CAT, GSH in hepatocarcinogenesis. In fact, it has been shown that DADS have antioxidant properties both in vivo and in vitro. DADS changed the rat hepatic glutathione related antioxidant enzyme activities by increasing the GSH. It has been suggested that the number of sulfur atoms and allyl groups may play a determining factor on the biological activities of garlic organosulfur compounds. The biochemical findings obtained from this study indicates that DADS, exerts protection to AC-induced hyperammonemic rats against oxidative stress. This could be due to prevention or inhibition of lipid peroxidative system by its antioxidant and hepatoprotective effect and by maintaining the cellular integrity of hepatocytes and other tissue. In summary, DADS has been shown to possess antihyperammonemic effect in AC-induced hyperammonemia in rats by means of ammonia detoxification and antioxidant properties.

REFERENCES


