ORIGINAL ARTICLE

ANTIHYPERAMMONEMIC EFFECT OF FISETIN ON HYPERAMMONEMIC RATS: A BIOCHEMICAL STUDY

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ABSTRACT

Ammonia is a key neurotoxin involved in the neurological complications of liver failure. Elevated ammonia leads to hyperammonemic condition which affects several important CNS functions. Fisetin is one of the naturally occurring flavonoids; it exhibits a wide variety of therapeutic benefits. In this present study, effect of fisetin on ammonium chloride (AC) induced hyperammonemic rats was aimed to establish the antihyperammonemic potential to modulate the alterations of hyperammonemia and/ or hepatic encephalopathy (HE). Amelioration of hyperammonemia and its complications of AC induced hyperammonemia by fisetin were measured by assessing the body mass, circulatory levels of ammonia, urea, lipid peroxidation products, antioxidant and brain glutamate and glutamine. Fisetin significantly (p<0.05; DMRT) normalized ammonia, urea, redox status, glutamine and glutamate in hyperammonemic rats this could be due to the antioxidant potential of fisetin.

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Key words: Hyperammonemia, fisetin, flavonoid, lipid peroxidation, antioxidant, glutamate

1.INTRODUCTION

Ammonia is the precursor molecule and essential substrate for biosynthesis of amino acids, proteins, and nucleic acids (Dimski, 1994). In mammals, at least 20 metabolic reactions generate ammonia (Cooper and Plum, 1987). Reduction in hepatocyte number or function in liver failure and inhibition or primary defect of urea cycle enzymes in inborn errors of metabolism (urea cycle defect) are the main causes of hyperammonemia (Brusilow and Horwich, 2001). Excessive ammonia accumulation due to liver failure and/or liver cirrhosis causes more deleterious effects on central nervous system (CNS) or hepatic encephalopathy (HE) (Widmer *et al.*, 2007). The mechanism of action of ammonia on CNS may include structural changes in blood brain barrier, changes in cerebral blood flow, effects on electrophysiological properties of CNS, interference with neurotransmitters, or interference with CNS biochemical pathways. Ammonia intoxication increases superoxide generation, indicating that oxidative DNA damage by nuclear superoxide radical in rats. The mechanism responsible for superoxide generation in liver and brain cells by NADH and NADPH cytochrome c reductase system (Kukielka and Cederbaum, 1992; Kosenko et al., 2004), and NMDA

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receptor-dependent increase of reactive oxygen/nitrogen species (ROS/RNS) lead to production and oxidation of mRNA and rRNA (Murthy *et al.*, 2001; Rao *et al.*, 2005). Thus, RNA oxidation by ammonia may affect local protein synthesis and gene expression and thereby provide another link between RNS production and ammonia toxicity (Gorg *et al.*, 2008).

The metabolic fate of blood-borne ammonia entering the brain is incorporated into amide group of glutamine in normal and chronic hyperammonemic rats. Glutamine synthesis by glutamine synthetase is the major route for removal of ammonia entering the brain from circulation (Brusilow *et al.*, 2010). Hyperammonemia increases brain glutamine, which leads to complex pathophysiological and neurotoxic responses. The route of glutamine mediated ammonia neurotoxicicity is by excess glutamine transport to mitochondria, wherein it is metabolized by phosphate-activated glutaminase (PAG) to glutamate and ammonia. The excess ammonia interferes with normal mitochondrial function and could lead to altered oxidative metabolism, lactate production, ATP depletion with the blockade in tricarboxylic acid cycle (TCA) enzymes, dysregulation of K^+ homeostasis, generating excessive reactive oxygen species/reactive nitrogen species (ROS/RNS), and induction of mitochondrial permeability transition (MPT) (Hawkins *et al.*, 1993; Brusilow *et al.*, 2010). A pathological

loss of selective permeability produces astrocyte dysfunction, including cell swelling and death.

The major drawbacks of many antihyperammonaemic agents/therapies are the reappearance of symptoms and serious adverse effects after the discontinuation and inadequacy of drugs (Srinivasan *et al*., 2001). Therefore, screening and development of drugs for antihyperammonemic activity are in progress. This can be achieved by focusing research on active principles (phytochemicals and/or flavonoids). The activities of flavonoids are related with their chemical structures mainly and the position and the degree of hydroxylation are important to exert their biological and pharmacological property (Armstrong and Doll, 1975; Hu *et al*., 1995). Fisetin (3, 3′, 4′, 7-tetrahydroxyflavone) is a dietary flavonoid (widely distributed in strawberries, apples, persimmons, grapes, onions and cucumbers) which displays a variety of pharmacological properties including antioxidant (Dimitric Markovic *et al*., 2011), anti-allergic (Goh *et al*., 2012), anti-inflammatory (Park *et al*., 2007), anti-cancer (Sung *et al*., 2005), neuroprotective (Zbarsky *et al*., 2005; Chiruta *et al*., 2012) and neurotrophic (Maher *et al*., 2006) characteristics.

2.MATERIALS AND METHODS

Experimental Animals

Healthy adult male albino Wistar rats, body weight of 180-200 g reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the studies. The animals were housed in polypropylene cages and provided with food and water *ad libitum*. They were maintained in controlled environment under standard conditions of temperature and humidity with alternating light/dark (LD 12:12) cycle. All animals were fed with standard pellet diet (Hindustan Lever Ltd., Bangalore, India). The animals used in the study were approved by the ethical committee (Approval No.737; dated 02/09/2010), Annamalai University, India (Reg. No. 160/1999 /CPCSEA) and were in accordance with the guidelines of National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India.

Chemicals

Fisetin was purchased from Shanxi Jintai Biological (China). Ammonium chloride (NH4Cl), thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Company, St. Louis, USA. Butylated hydroxy toluene (BHT), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), phosphate buffered saline and ethylene diamine tetra acetic acid (EDTA) were purchased from S.D. Fine Chemicals Ltd., Mumbai, India. The rest of the chemicals and biochemicals utilized were obtained from local firms (India) and were of analytical grade.

Experimental induction of Hyperammonemia

Hyperammonemia was induced in Wistar rats by intraperitoneal injection of freshly prepared solution of ammonium chloride at a dose of 100 mg/kg body weight thrice in a week for 8 consecutive weeks (Essa and Subramanian, 2007; Subash and Subramanian, 2008).

Experimental Design

The animals were randomized and divided into five groups of six animals each as given below.

Group 1 : Normal rats received with 0.5% DMSO

Group 2 : Rats orally administered with fisetin (50 mg/kg b.w.)

Group 3 : Rats treated with NH_4Cl (100 mg/kg b.w, i.p injection)

Group 4 : Rats treated with NH_4Cl + Fisetin

At the end of experimental period (8th week), all animals were fasted overnight and sacrificed by cervical dislocation. Blood and brain tissue samples were collected for estimations of blood ammonia, plasma urea, brain glutamate and glutamine, nitric oxide and lipid peroxidation products.

Processing of blood and tissue samples

Plasma preparation

The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration, transferred into eppendorf tubes and stored at -20°C until analysis.

Tissue homogenate preparation

Brain tissue (250 mg) was sliced into pieces and homogenised in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for estimations.

Biochemical estimations

Blood ammonia was determined by enzymatic kinetic colorimetric assay of Wolheim (1984) and performed using automated Roche/Hitachi 912 kit. Plasma urea was determined by diacetylmonoxime method (Varley *et al*., 1998) was performed using automated Roche/Hitachi 912 kit. The amount of cellular glutamate and glutamine in brain was measured as previously reported (Lund 1986). Assay reagents (in kit) (Sigma, glutamine/glutamate determination kit) and prepared according to the manufacturer's instructions.

Estimation of lipid peroxidation, nitric oxide and antioxidant

The concentration of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides, conjugated dienes and reduced glutathione (GSH) in the plasma were estimated by the method of Fraga *et al*. (1988), Jiang *et al*. (1992), Klein (1979), Green *et al*. (1982) and Ellman (1959) respectively.

Statistical analysis

Statistical analysis was performed by One way Analysis of Variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Software Package for the Social Science (SPSS) software package version 12.00. Results were expressed as Mean ± S.D. for six rats in each group. *p* values <0.05 were considered significant.

3.RESULTS

The levels of blood ammonia and plasma urea in control and experimental rats are shown (Figure1 A&B). The brain

glutamate and glutamine levels are shown in control and experimental rats are shown (Figure 2 A&B). The levels of circulatory ammonia, brain glutamate and glutamine were

Table: Body weight changes in control and experimental rats.

Groups	Body weight changes (g)	
	Initial	Final
Normal	192±13.44	361±25.27*
AC treated	187±13.09	283±19.81***
AC+Fisetin treated	190±13.3	357±24.99**
Fisetin	185±12.95	335±23.45

significantly higher and plasma urea significantly decreased in AC-treated rats as compared with control rats. Hyperammonaemic rats treated with fisetin significantly normalized the levels of ammonia, urea, glutamate and glutamine, when compared with hyperammonaemic rats. The circulatory levels of lipid peroxidation products (TBARS, hydroperoxes and nitric oxide) and antioxidant (GSH) in control and experimental groups are given (Figure 3 A-D). The levels of lipid peroxidation markers were significantly increased and GSH was decreased in AC-treated rats, whereas these levels were significantly normalized in hyperammonaemic rats treated with fisetin. A significant decrease in final body weight observed in AC treated groups as compared to control rats (Table 1). Fisetin treated hyperammonemic rats showed nearly normalized body weights.

Figure 1. The (A) blood ammonia and (B) plasma urea level in control and experimental rats
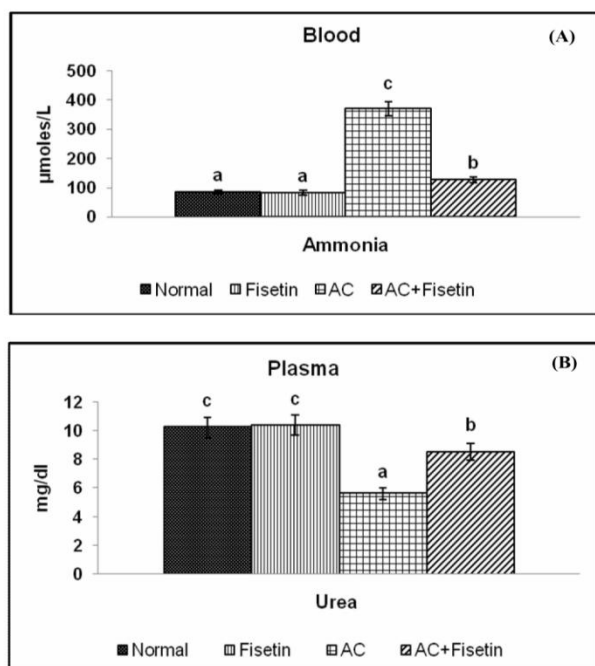
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

4.DISCUSSION

Liver contains all enzymes needed for urea cycle and thus eliminating ammonia. Liver failure and/or liver cirrhosis leads to partial or complete inactivity/ depletion of urea cycle enzymes and can predispose patients to life-threatening hyperammonemia and/ or hepatic encephalopathy (Enns, 2008). In the present study, ammonium chloride treated rats showed significant increase in the concentrations of circulatory ammonia and decrease in the level of urea. Increased levels of circulatory ammonia indicate a hyperammonemic condition in rats treated with ammonium chloride (Essa and Subramanian, 2007; Subash and Subramanian, 2012). Fisetin administered to hyperammonemic rats significantly decreased the levels of circulatory ammonia and increased the level of urea biosynthesis when compared with corresponding AC treated rats. The reduction in ammonia and increase in urea synthesis showed antihyperammonemic effects along with antioxidant and anti-inflammatory potencies of fisetin favouring the normalisation of urea cycle defect and lessens hyperammonemic complications. These observations clearly indicate that fisetin could exert potent antihyperammonemic effect by significant ammonia lowering effect in hyperammonemic rats (Jayakumar and Subramanian, 2013).

Hyperammonemia increased the lipid peroxidation and reduced activities of antioxidants via oxidative stress (Norenberg, 2003). A firm relationship between oxidative stress and hyperammonemia has been well established and evidenced with ammonium (acetate/chloride) induced hyperammonaemia partly via oxidative stress-mediated lipid peroxidation (Lena and Subramanian, 2004; Essa and Subramanian, 2007). Excess ammonia induces the enhanced production of thiobarbituric acid-positive substances in liver and brain leading to oxidative stress (Lena and Subramanian, 2004). The increased levels of TBARS, lipid hydroperoxides and conjugated dienes observed in plasma was associated with enhanced lipid peroxidation in AC treated rats that could be due to ammonia induced free radical generation, membrane damage, and further cell lysis.

GSH plays a major role in cellular metabolism as an antioxidant and free radical scavenger, and its depletion can result in cell degeneration due to oxidative stress (Lewerenz *et al*., 2010). Hyperammonemia mediated increase in extracellular glutamate leads to the inhibition of cystine (precursor for GSH) uptake due to the involvement of responsible mechanisms on cystine/glutamate antiporter and also competitive binding on the extracellular catalytic site of the enzyme (γ-glutamyl transpeptidase involves in the GSH biosynthesis) leading to a consequent decrease in the transport of cystine (Tan *et al*. 2001) and subsequent GSH depletion. This could be the proximate cause of toxicity induced by prolonged glutamate exposure causing an accumulation of oxidants and cell death (Pereira and Oliveira, 1997). The significant decrease in the level of GSH observed in hyperammonemic rats might be due to ammonia induced NMDA receptor mediated exicitotoxicity and metabolic oxidative insult. The significant increase in GSH level was observed in fisetin treated hyperammonemic rats indicates the capability of fisetin to maintain GSH level and can act itself as an antioxidant (Fotsis *et al*., 1998). The low molecular weight and highly hydrophobic nature of fisetin helps to readily pass through cell membranes and it

Figure 2. The brain (A) glutamate and (B) glutamine level in control and experimental rats
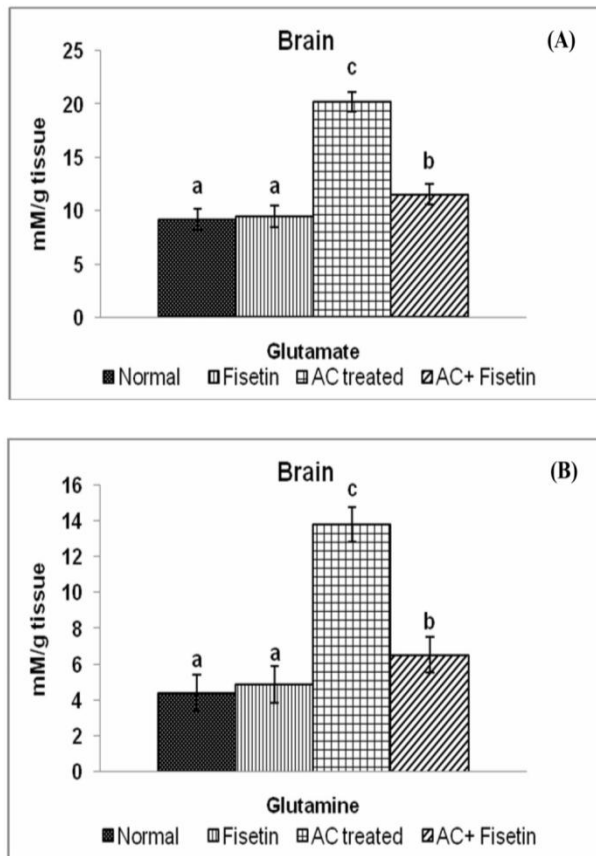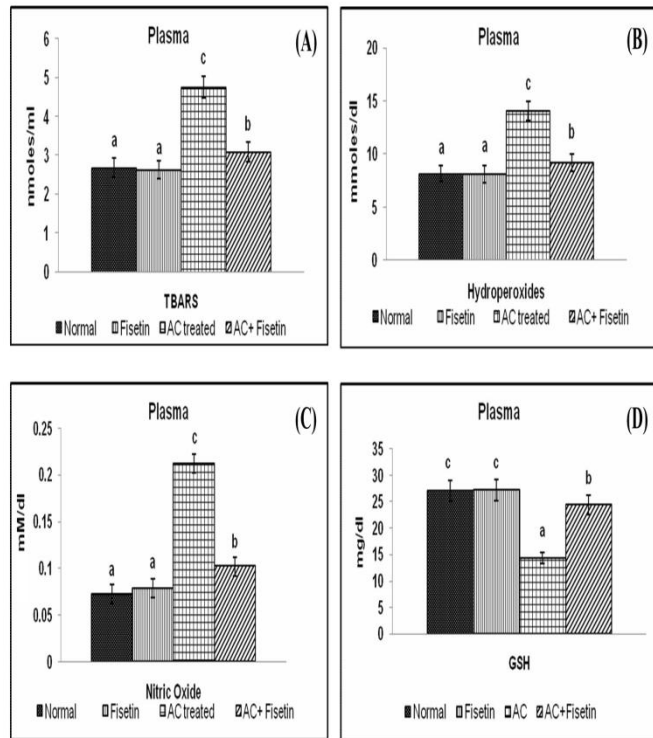

Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

accumulates intracellularly protecting most vulnerable liver and brain cells from oxidative stress (Chiruta *et al*., 2012; Maher *et al*., 2007). Therefore the possible underlying mechanisms by which fisetin modulates hyperammonemic conditions could be attributed to its ammonia lowering effect by radical scavenging properties, maintaining antioxidant reserve and cellular integrity.

Hyperammonemia could be an important factor for abnormal expression of inducible nitric oxide (iNOS) in astroglial cells. Astrocytic induction of iNOS and further increase of nitric oxide (NO) may contribute to neuronal damage in chronic neurodegenerative disorders through generation of RNS (Wright *et al*., 2007). Nitric oxide plays a vital role as signalling molecule under normal physiological conditions but abnormal synthesis under hyperammonemia mediated excitotoxicity and calcium dependent activation of iNOS leading to more deleterious effects on functional proteins. Increased NO level and its reactive products (RNS) formation reduced the glutamine synthetase (GS) activity there by resulting reduction in the glutamine synthesis and subsequent accumulation of ammonia (Ayers *et al*., 1996). The results of the present study showed a significant increase in NO level in

AC-induced rats suggesting a hyperammonemia-mediated alteration in Glutamate-NO-cGMP pathway with neuroinflammatory responses. After treatment with fisetin in hyperammonemic rats showed a significant decrease in the levels of NO.

Figure 3. The (A) TBARS, (B) lipid hydroperoxide, (C) nitric oxide and (D) GSH level in plasma of control and experimental rats

Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

Increased level of extracellular glutamate is a consistent feature of hepatic encephalopathy (HE) associated with liver failure and other hyperammonemic pathologies. Reduction of glutamate uptake has been described in ammonia-exposed cultured astrocytes, synaptosomes, and in animal models of hyperammonemia (Chan *et al*., 2003). Glutamate transporters are vital components of glutamatergic system that act to regulate the balance of intracellular/extracellular glutamate levels in both astrocytes and neurons. In order to perform its various functions in nerve tissue, glutamate must be compartmentalized into distinct pools (Fonnum *et al*., 1984; Chan *et al*., 2003). The loss of astrocytic and neuronal glutamate uptake as a result of hyperammonemia impairs removal of glutamate from extracellular milieu and may be ultimately implicated in the pathogenesis of brain edema, a major complication of liver failure and congenital hyperammonemia in humans and experimental animal models (Swain *et al*., 1992). Glutamine is capable of forming free radicals in astrocytes. Glutamine has been implicated in brain astrocytic swelling by creating an osmotic load. Increased glutamine entering into mitochondria also leads to elevation of ammonia and a transient increase in glutamate by phosphate

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activated glutaminase and subsequent blockade in the oxidative respiratory enzymes leads to depletion of ATP synthesis. Reduced glutamine synthesis might cause an increase in extracellular glutamate in hyperammonemic rats. The increase in cerebral ammonia levels inhibits the high-affinity uptake of glutamate by inhibiting the glutamate receptors and transporter proteins and mRNA synthesis (Chan *et al*., 2000). Hence the alterations in the glutamine and glutamate levels observed in the present study indicates homeostatic imbalance due to severe alterations and/ or defect in the receptors of neurotransmitters under hyperammonemia. Fisetin treatment with hyperammonemic rats lead to a significantly increased expression of glutamine synthetase and reduced ammonia by converting into glutamine and might lead to attenuation in glutamine and glutamate homeostasis in brain.

Changes in lipid composition could have far reaching implications on the functionality of brain, making this an important focus in hyperammonemia and/ or HE (Miu *et al*., 2004; Kieseier *et al*., 2005). Changes in lipid composition of brain have been implicated in HE along with changes in CSF fatty acid composition (Minana *et al*., 1989; Osada *et al*., 1990). Oxidative stress has been reported in mitochondria and cortex during HE (Reddy *et al*., 2004). The high lipid content in myelin is particularly susceptible to oxidative changes like lipid peroxidation. These studies though provide initial evidence in the direction of lipid changes being involved in pathogenesis of HE (Swapna *et al*., 2006). The pyruvate dehydrogenase may also be inhibited by ammonia and this will further limit the availability of substrates of TCA cycle, and prevent detoxification of ammonia by conversion of glucose to glutamine. Ammonia is a potent inhibitor of α-KGDH, the rate limiting enzyme in TCA cycle causing of α-KG accumulation stimulating the formation of glutamate in both astrocytes and neurons (Lai and Cooper, 1986), These earlier observations in changes of lipids and proteins under hyperammonemia and/ or HE and its consequences are consistent with the alterations in lipids and protein responsible for the link between body mass changes and hyperammonemia. The body mass showed significant decrease in AC treated rats. Treatment with fisetin in hyperammonemic rats showed normalized body mass as compared to hyperammonemic rats. This amelioration in body mass regulation by fisetin treated rats might be due to the maintenance of lipids and proteins by its hepatoprotective and neuromodulatory effects throughout oxidative insult s mediated by hyperammonemia.

5.CONCLUSSION

These findings showed that fisetin might be a representative agent that could effectively ameliorate hyperammonemia and its complications. The therapeutic effects of fisetin on hyperammonemia might be due to the modulatory potency on ammonia detoxifying enzymes, inflammatory markers and redox homeostasis. Thus the beneficial effect of fisetin on ammonia detoxification offers exciting opportunity to further investigations and clinical trials.

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