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Anti-hyperammonaemic effect of fisetin in rats: A chronotherapeutic study

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Abstract

Ammonia, a product of degradation of proteins and other nitrogen compounds is toxic at higher concentrations. Fisetin (a naturally occurring flavonoid) is known to exhibit a number of cytoprotective effects. In this study, chronotherapeutic effects of fisetin on ammonium chloride (AC) treated rats is aimed to establish the time point at which the maximum drug effect is achieved. Fisetin (50 mg/kg b.w oral) was administrated to rats at 06:00, 12:00, 18:00 and 00:00 h against AC (100 mg/kg.b.w. i.p) treated Wistar rats (180-200 g). Amelioration of pathophysiology of AC induced hyperammonaemic characteristics by fisetin at different time points were measured by assessing cellular architecture of liver and brain, lipid profile, lipid peroxidation products and antioxidants (superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione) in serum, plasma, liver and brain. The normalization of lipid profile and markers of reactive oxygen species (ROS) homeostasis indicated the protective effect of fisetin. Fisetin administration at 00:00 h showed significant effects on these indices than at other time points ($p<0.05$; DMRT). This might be due to chronopharmacokinetic and chronopharmacodynamic properties of fisetin along with its action on altered rhythms of lipid profile and markers of ROS homeostasis.

Keywords: Ammonium chloride, Antioxidants, Circadian, Chronotherapy, Fisetin, Hyperammonaemia

Introduction

Ammonia, the product of the metabolism of amino acids and other nitrogenous compounds is detoxified by conversion to urea in liver. Elevated ammonia in body leads to hepatic dysfunction and failure and brain encephalopathy by a variety of mechanisms [1]. Ammonia toxicity occurs partly via oxidative stress, which leads to lipid peroxidation and free radical generation.Extrahepatic tissues do not contain complete urea cycle; under hyperammonaemic conditions ammonia detoxification is performed by the astrocyte machinery, and astrocyte function and morphology are compromised [2].

The screening and development of drugs and successful therapeutic schemes for hyperammonaemia are still in progress and this can be achieved by focusing research (i) on active principles of plants and (ii) by the effective time-dependent administration of drug over the 24h period (chronotherapy). Flavonoids are polyphenolic substances which are abundant in several plant species; they exert wide range of pharmacological activities characterized by a diphenylpropane structure (C6-C3-C6). The two antioxidant structural features of flavonoids are the presence of a B-ring catechol group and the presence of a C2-C3 double bond in conjugation with an oxo group at C4; the first serves to donate hydrogen/electron to stabilize a radical species and the latter serves to bind metal ions such as iron and copper [3]. Fisetin (3, 3′, 4′, 7-tetrahydroxyflavone), a dietary plant flavonoid, displays a wide variety of pharmacological properties.

In mammals, the physiology and biochemistry vary greatly in a predictable manner throughout the 24 hour, regulated by endogenous biological clock. The biological clock provides time cues for a wide range of physiological processes and synchronizes metabolic reactions. The characteristics of circadian rhythms in mammals are altered by a variety of diseases and significant changes in response to time-dependent chronotherapy, is a common phenomenon [4]. Since all physiological functions oscillate rhythmically, the action, toxicity, and kinetics of a drug depend on its administration time, in relation to the enactment of relevant temporal variables. Chronotherapeutic approaches are useful for the treatment of cardiovascular diseases [5], colorectal cancer [6], asthma [7] and diabetes [8]. Circadian rhythms in gastrointestinal, liver, kidney and other bodily processes and functions are of great importance for effective therapeutics, in opting when to administer medications in relation to their pharmacokinetics [9]. There are no previous reports on chronopharmacological and chronotherapeutic effects of anti-hyperammonaemic drugs; the present study investigates the temporal influence of fisetin on cytoarchitecture of tissues (liver and brain), serum lipid profile, markers of reactive oxygen species (ROS) homeostasis in hyperammonaemic rats.

Materials and Methods

Experimental Animals

Adult male albino Wistar rats (180-200 g) were obtained from the Central Animal House, Department of Experimental Medicine,

Rajah Muthiah Medical College and Hospital, Annamalai University and maintained in an air-conditioned room (25±3ºC) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum*. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA, Approval No.: 737: 2.9.2010), Annamalai University.

Chemicals

Fisetin was purchased from Shanxi Jintai Biological (China). Ammonium chloride and other chemicals used in this study were of analytical grade and obtained from E. Merck and HIMEDIA, India.

Experimental Design

Hyperammonemia was induced in Wistar rats by intraperitoneal injections of a freshly prepared solution of ammonium chloride (AC) at a dose of 100 mg/kg body weight thrice a week for 8 consecutive weeks [10, 11]. Fisetin was dissolved in 0.5% DMSO and administered orally (1ml) thrice in a week in tandem with AC.

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

Group I : Control rats orally administered with 0.5% DMSO
Group II : Rats orally administered with fisetin (50 mg/kg b.wt)
Group III : Rats treated with AC (100 mg/kg b.w, i.p injections)
Group IV : Rats treated with AC + Fisetin to be administered at 06:00 h
Group V : Rats treated with AC+ Fisetin to be administered at 12:00 h
Group VI : Rats treated with AC+ Fisetin to be administered at 18:00 h
Group VII : Rats treated with AC+ Fisetin to be administered at 00:00 h

At the end of the experimental period (8th week), all animals were sacrificed by cervical dislocation. The liver and brain tissues were excised at 09:00, 15:00, 21:00 and 03:00 h from groups IV to VII respectively, (at 07:00 h from groups I to III after overnight fasting), washed with chilled isotonic saline, stored at -80 C and used for further analyses. Serum and plasma were separated by centrifugation. Tissues (liver and brain) were homogenized in 5ml of cold 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 56 g for 10 minutes at 4 C in a cooling centrifuge. The supernatant was collected in separate test tubes and used for analysis of various parameters.

Histopathological examination

The liver and brain tissues obtained from control, fisetin, AC and AC+fisetin treated (at 00:00 h) rats were fixed for 48 hour in 10% (v/v) formalin-saline and dehydrated by passing successfully in different mixtures of ethyl alcohol and water, then cleaned in xylene and embedded in paraffin. Sections of tissues were prepared by using a rotary microtome and stained with hematoxylin and eosin dye, examined with a high-power microscope with a CCD cold camera.

Biochemical Estimations

Estimation of cholesterol, phospholipids, free fatty acids and triglycerides

The levels of total cholesterol and triglycerides (TG) were estimated by the methods of Zlatkis *et al.* [12] and Foster and Dunn [13]. The levels of free fatty acids (FFA) and phospholipids in serum and tissue (liver and brain) were estimated according to the methods of Falholt *et al.* [14] and Zilversmit and Davis [15] respectively.

Estimation of markers or ROS homeostasis

Thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides in the plasma and tissues (liver and brain) were estimated by the method of Fraga *et al.* [16] and Jiang *et al.* [17] respectively. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were assayed by the standard procedures of Kakkar *et al.* [18], Sinha [19] and Rotruck *et al.* [20] respectively; plasma and tissue reduced glutathione (GSH) was estimated by the method of Ellman [21].

Statistical Analysis

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

Results

Histopathological changes of the liver and brain in control and AC-induced hyperammonaemic rats were shown in Figure 1 (A-H). AC-treated rats showed a gross distortion of the normal architecture, prominence, widening of sinusoids and influx of inflammatorycells when compared to control rats (Figure 1 B&F). Treatment of fisetin effectively attenuated these pathological abnormalities toward near-normal morphology (Figure 1 C&G). Control rats treated with fisetin showed normal architecture of liver and brain devoid of any pathological changes (Figure 1 D&H).

Total cholesterol, free fatty acids, triglycerides and phospholipids were significantly reduced in AC-induced rats ascompared with control rats (Figure 2 A-I). The hyperammonaemic rats treated with fisetin at 00:00 h showed significant ($p<0.05$) normalization of lipid levels, than at other time points (06:00, 12:00 and 18:00 h).

The chronotherapeutic effect of fisetin on circulation and tissue (liver and brain) levels of TBARS, reduced glutathione and antioxidants were shown (Figure 3 A-F and Figure 4 A-G).

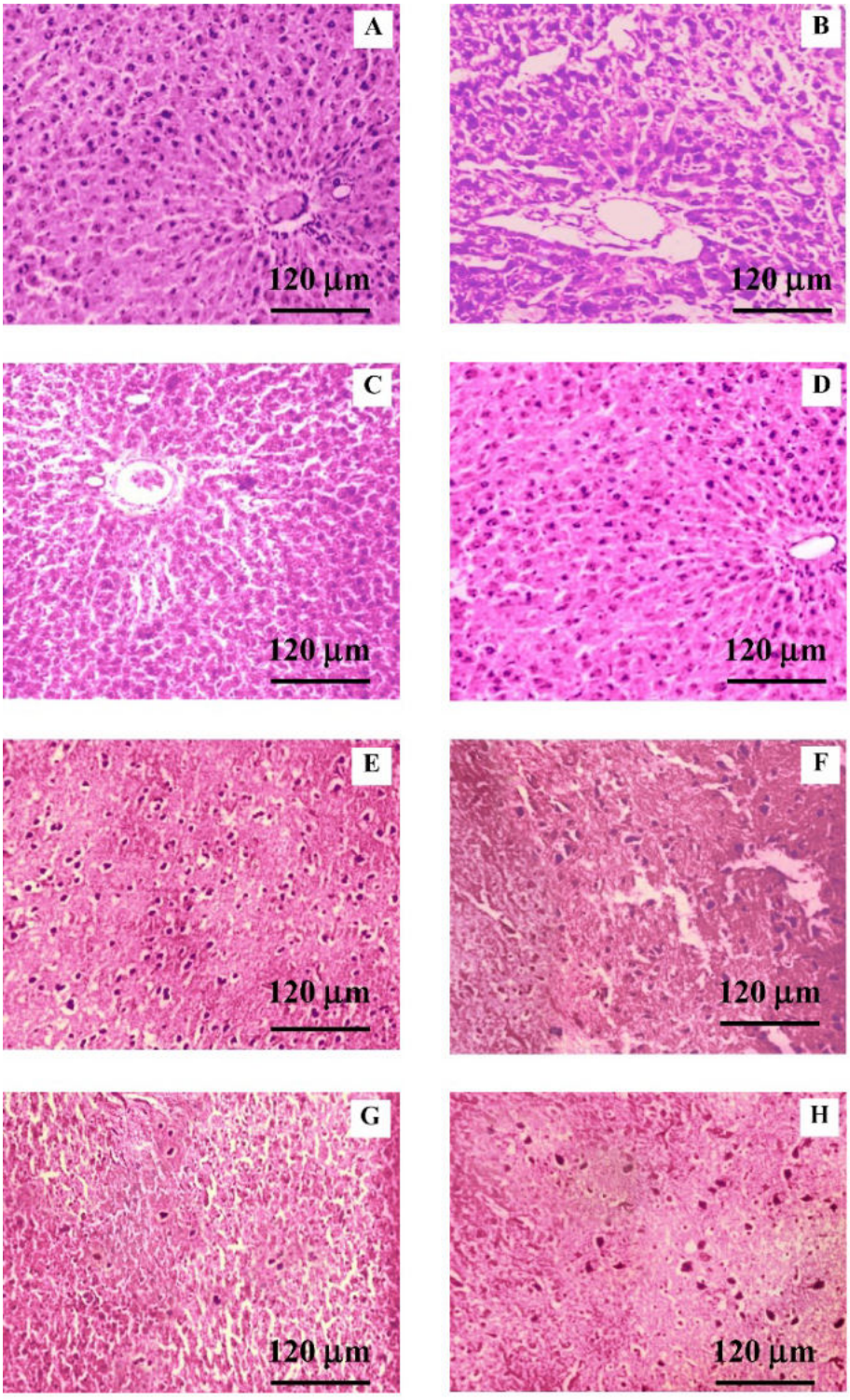
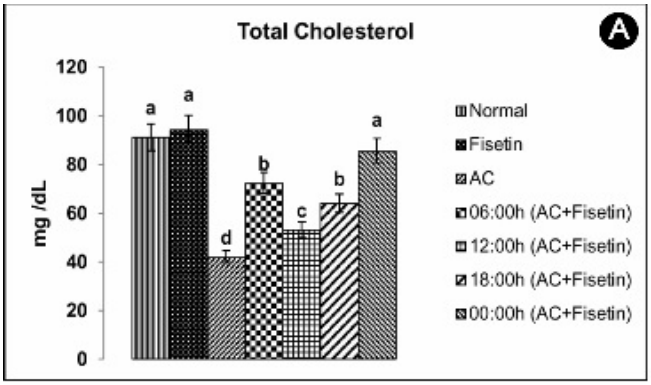
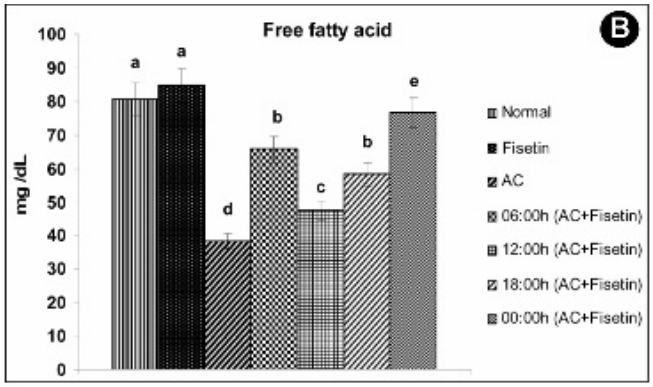
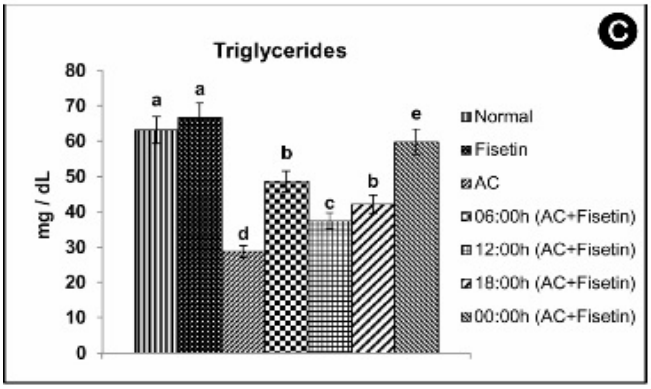
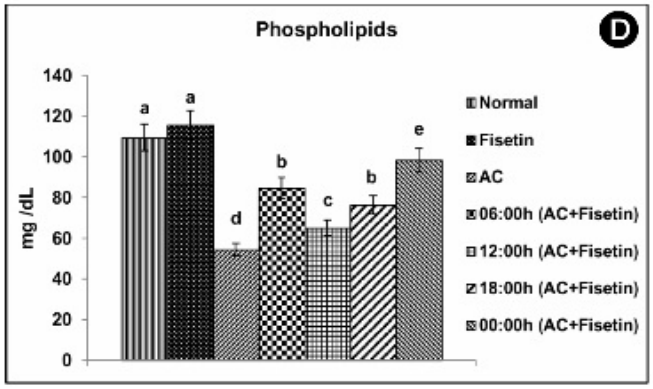


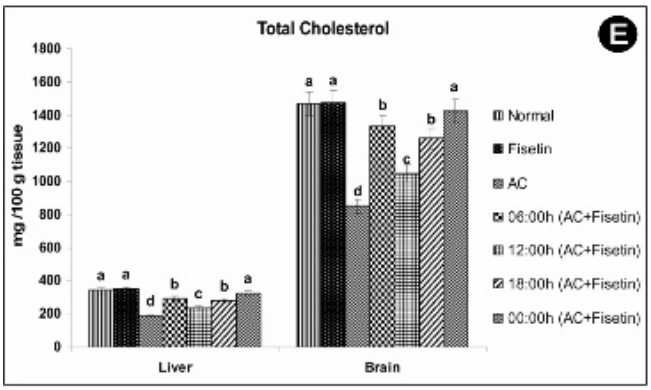
Figure 1.Histopathological changes of liver and brain (stained with hematoxylin and eosin). A) Section of control liver showing (i) normal architecture with hepatocytes radiating from the central vein and sinusoids and (ii) no pathological changes, B) AC-treated rat liver showing the gross distortion of the normal architecture with inflammatory cells, prominence and widening of sinusoids, C) AC+fisetin-treated liver (00:00 h) showing the amelioration of distorted architecture, D) Fisetin treated liver with normal architecture, E) Control rat brain with typical appearance of the cerebral cortex with no pathological chanhes, F) AC-treated brain showing disorganization of the cerebral cortex, G) AC+fisetin-treated (00:00 h) brain showing amelioration of disorganized architecture and H) Fisetin treated rat brain with normal architecture. Scale bar: 5 µm.

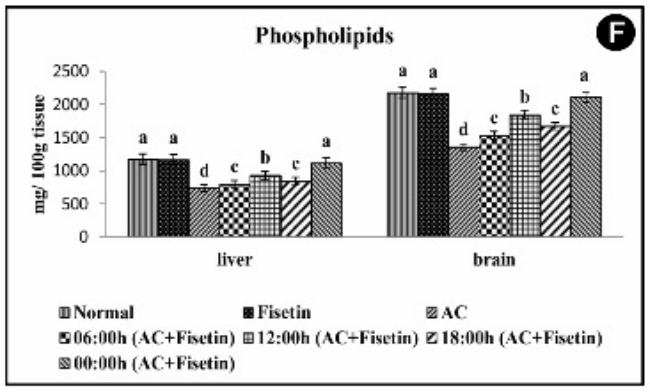

A
Total Cholesterol
mg /dL
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


B
Free fatty acid
mg /dL
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


C
Triglycerides
mg / dL
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


D
Phospholipids
mg /dL
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


E
Total Cholesterol
mg /100 g tissue
Liver
Brain
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


F
Phospholipids
mg/ 100g tissue
liver
brain
Normal
Fisetin
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06:00h (AC+Fisetin)
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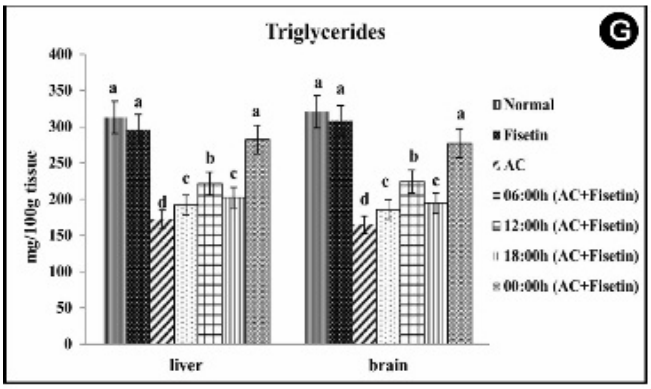
G
Triglycerides
mg/100g tissue
liver
brain
Normal
Fisetin
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06:00h (AC+Fisetin)
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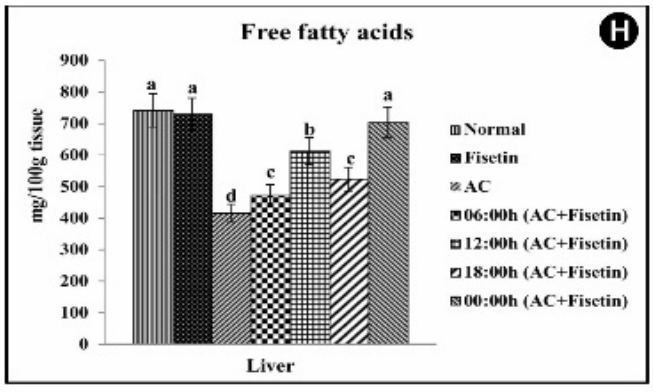
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)


H
Free fatty acids
mg/100g tissue
Liver
Normal
Fisetin
AC
06:00h (AC+Fisetin)
12:00h (AC+Fisetin)
18:00h (AC+Fisetin)
00:00h (AC+Fisetin)

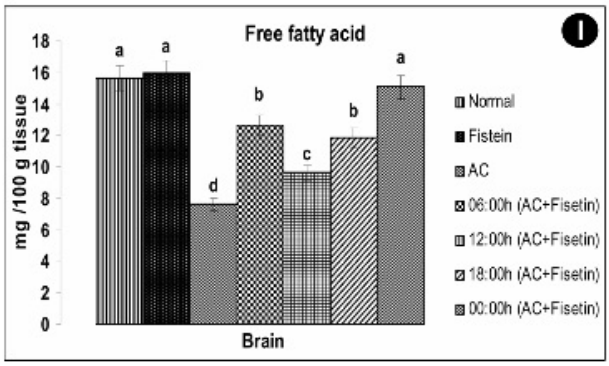


Figure 2.Chronotherapeutic effect of fisetin on serum cholesterol (A), free fatty acids (B), triglycerides (C), phospholipids (D)and tissue(liver and brain) cholesterol (E), phospholipids (F), triglycerides (G) and free fatty acids (H and I) in normal and experimental rats (Mean ± SD).Values not sharing a common superscript letter differ significantly ($p<0.05$).

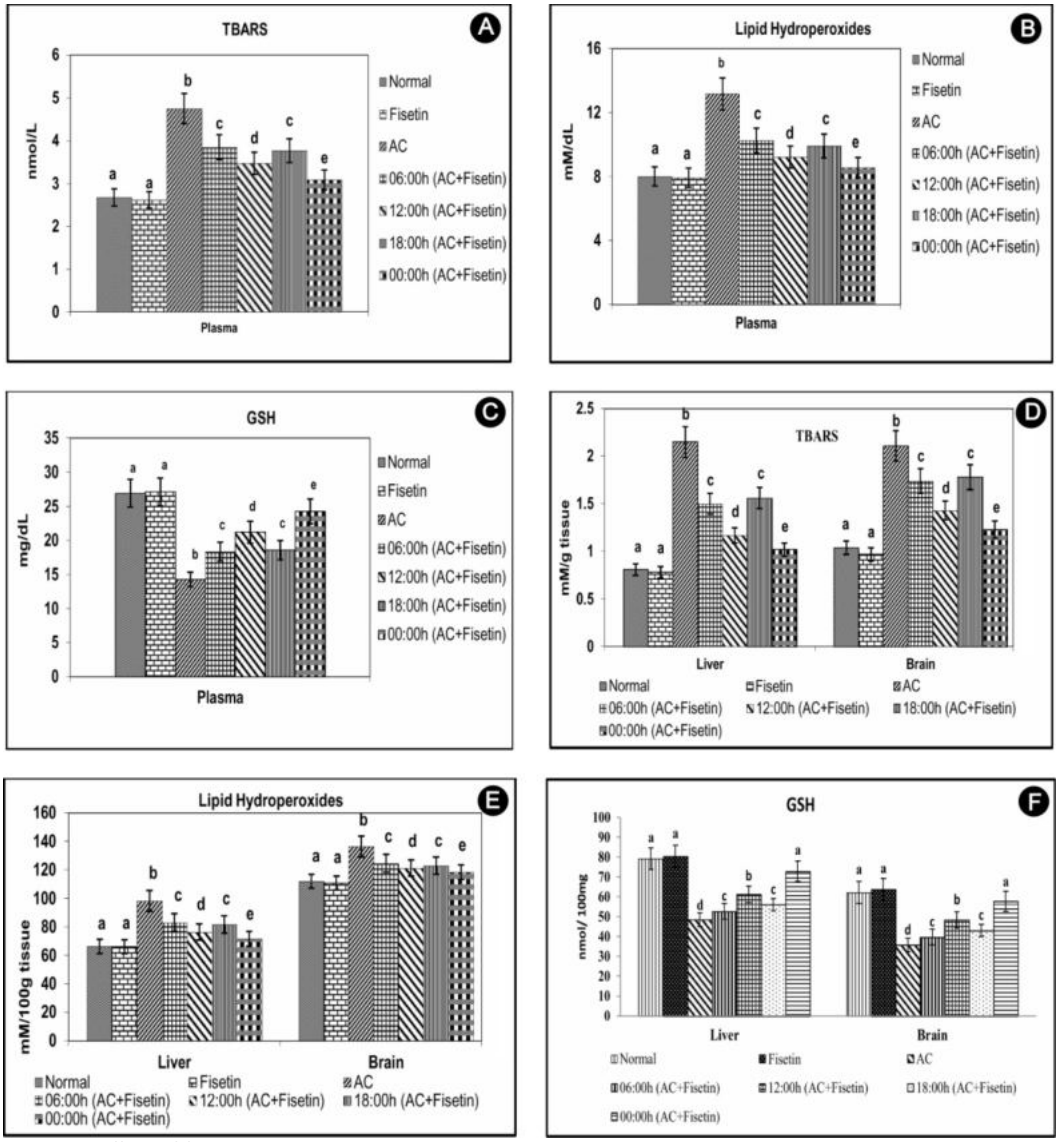


Figure 3.Chronotherapeutic effect of fisetin on plasma TBARS (A), lipid hydroperoxides (B) and GSH(C) and tissue (liver and brain) TBARS (D), lipid hydroperoxides (E) and GSH (F) in normal and experimental rats (Mean ± SD).Values not sharing a common superscript letter differ significantly ($p<0.05$).

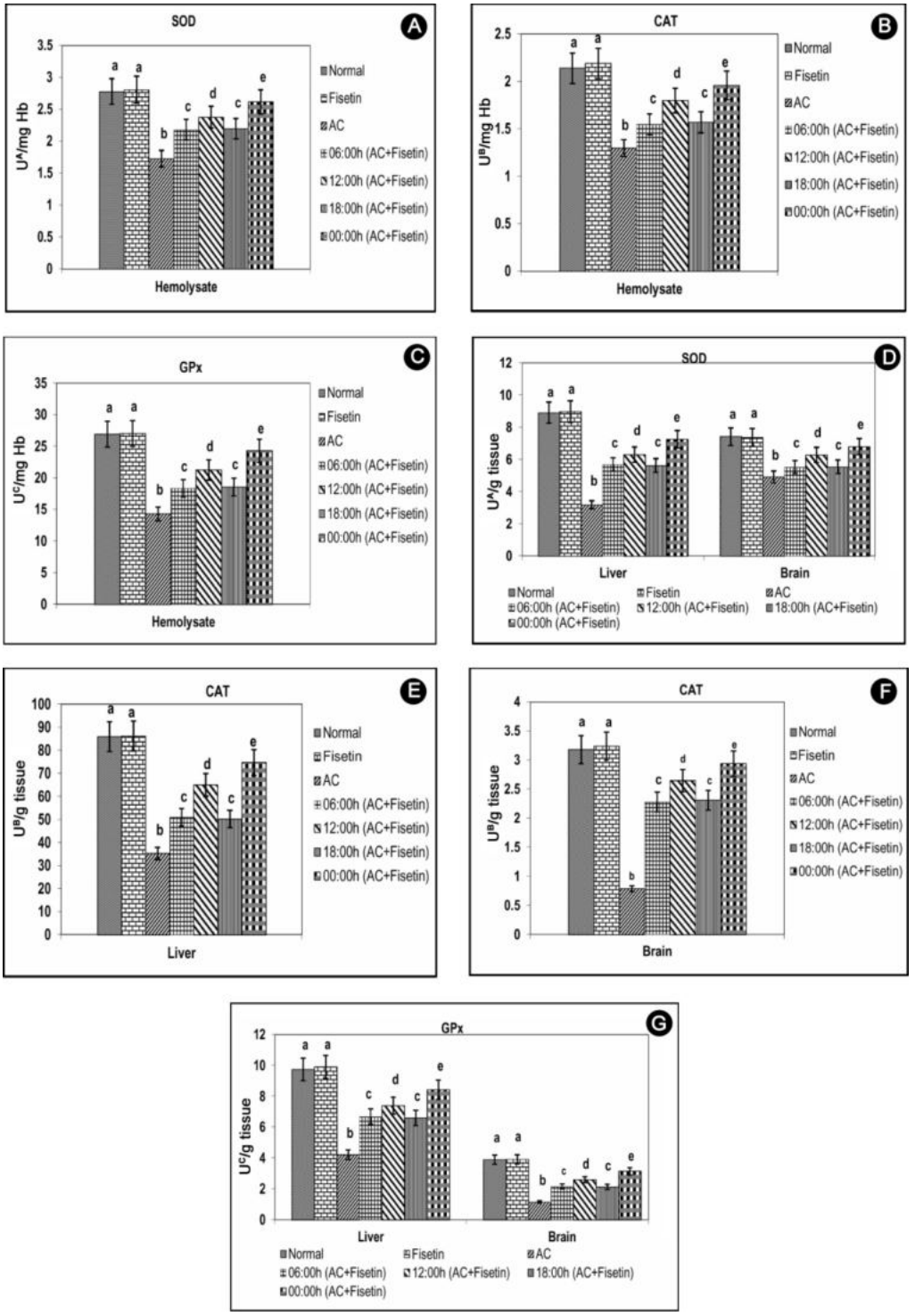


Figure 4.Chronotherapeutic effect of fisetin on antioxidants, SOD (A), CAT (B) and GPx (C) in haemolysate and in tissue (liver and brain),SOD (D), CAT (E and F) and GPx (G)in normal and experimental rats (Mean ± SD).Values not sharing a common superscript letter differ significantly ($p<0.05$).

U^A - one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 minute

U^B - µ mole of hydrogen peroxide consumed/minute

U^C - µg of glutathione consumed/minute

The levels of total TBARS and lipid hydroperoxides were significantly increased and SOD, CAT, GPx and GSH were significantly decreased in AC-induced rats (compared to control rats). Hyperammonaemic rats treated with fisetin at 00:00 h showed significant ($p<0.05$) normalization of redox status than at other time points.

Discussion

Several fold accumulation of ammonia in circulation occurs if there is any defect in liver (hepatic failure/ cirrhosis), leading to hyperammonaemia with symptoms of impaired memory, shortened attention span, sleep-wake inversions, brain edema, intracranial hypertension, seizures, ataxia and coma [22]. Hyperammonemia increased lipid peroxidation and reduced activities of antioxidant via the oxidative stress. 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 [23, 22]. The increased levels of TBARS and lipid hydroperoxides observed in plasma and tissues (liver and brain) observed in the present study could be due to ammonia induced free radical generation, membrane damage, and cell lysis; amelioration of lipid peroxidation is observed in fisetin treated hyperammonaemic rats due to enhanced antioxidant activity. Maximum level of lipid peroxidation occurs in dark with acrophase lying at 00:00 h [24]. Administration of fisetin at this point could attain maximal effect of detoxification of lipid peroxidation products and other variables and protection of cells (Figure 3 A-F and Figure 4 A-G).

The antioxidant nature of the polyphenolic compounds may sustain the body's defences against hyperammonaemia mediated free radical reactions. The chemical structure, position and degree of hydroxylation are the important factors to exert the biological and pharmacological properties of flavonoids. The low molecular weight and highly hydrophobic nature of fisetin helps it to readily pass through cell membranes and to accumulate intracellularly, which protect most vulnerable liver and brain cells from oxidative stress.The significant decrease observed in the activity of SOD, catalase and GPx in hyperammonaemic rats (Figure 4 A-G) might be due to NMDA receptor mediated exicitotoxicity and metabolic oxidative insult. Most of the enzymes within the detoxification pathway have been shown to display circadian fluctuation throughout the day [25] and circadian variations of redox pathway enzymes has been evidenced in rats, (including SOD, catalase and GPx) [24, 26, 27]. The significant increase in GSH level in fisetin treated hyperammonaemic rats (Figure 3 C&F) indicates the capability of fisetin to maintain GSH level by blocking glutamate toxicity and promoting cystine (GSH precursor and excellent source for thiol group) uptake into brain by its free radical scavenging and cytoprotective effects. In many cases, circadian rhythms in activity and gene expression of antioxidant enzymes are mutually coinciding and could help to overcome the oxidative imbalance [26, 27]. Enhanced effect of fisetin at 00:00 h might be due to its temporal bioavailability and on its action on rhythmic pattern of markers of ROS homeostasis. Earlier reports demonstrated well-established links between hyperammonaemia, circadian pattern of cellular detoxifying enzymes, immune outputs and of signaling cascades and could add supporting evidences for the time dependent effect of fisetin [28].

In hyperammonaemic rats lipid content is reduced to 50% [29], suggesting an alteration in lipid metabolism and subsequent decrease in blood carnitine and increased long-chain acylcarnitine. Hyperammonaemia could probably impair the mitochondrial glucose oxidation in brain leading to progressive impairment of energy metabolism with several fold increase in lactate. This brain energy deficit in hyperammonemia might be due to inhibition of the TCA cycle at the level of -ketoglutarate dehydrogenase [30]. The significantly decreased levels of serum and tissue lipids (cholesterol, triglycerides, free fatty acids and phospholipids) observed might indicate a greater degree of utilization of lipids probably to compensate the energy requirement for ammonia detoxification. The rhythmic patterns of lipoprotein transporters of serum cholesterol, free fatty acids, and triglycerides in rats showed acrophase in dark period [31]. Further HMG CoA reductase and lipoprotein lipase activity are increased at the midpoint of the dark period, causing a drop of serum lipids [32]. The maximum effects of fisetin at 00:00 h (Figure 2 A-I) could be supportive in maintaining a homeostatic level of lipids.

Circadian clock influences the pharmacokinetics, bioavailability, efficacy and adverse effects of drugs through its rhythmicities on a wide variety of biological processes [9]. Circadian variations have been reported in the plasma levels of IgA, IgG, IgM, and IgE in different populations of T total, T helper, and T killer lymphocytes in the inflammatory reaction [33, 34]. Disruption of circadian coordination could be manifested by hormone imbalance, psychological span and in contrast, resetting of circadian rhythms has led to well-being and increased longevity. Previously it has been reported that hyperammonaemia would be a main contributor to adrenal function insufficiency and impairment that leads to circadian changes in cellular physiology and metabolism [35]. These modified physiologic, metabolic and inflammatory circadian responses could play a significant role in the chronotherapeutic effects of fisetin; however, comprehensive studies on the dysregulated/modified circadian responses are desirable to understand the time dependent effects of drugs.

Conclusion

The possible mechanism by which fisetin caused its ammonia lowering effect could be by its free radical scavenging properties, and by maintaining the cellular integrity of liver and brain cells.The significant effect of fisetin attained at 00:00 h as compared with other time points could be due to (i) appropriate circadian changes in free radical/biochemical/physiological/immune functions and (ii) temporal variations in its pharmacokinetic and pharmacodynamic attributes.

Acknowledgement

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