Influences of aspartate on circadian patterns of lipid peroxidation products and antioxidants in Wistar rats

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ABSTRACT

Introduction: The aim of the present study was to demonstrate the effects of aspartate on the circadian patterns of lipid peroxidation products and antioxidants in aspartate-treated rats, in order to investigate the influences of aspartate and whether it could modulate these rhythms differently, since aspartate is an important excitatory neurotransmitter (present in retinohypothalamic tract and suprachiasmatic nuclei [SCN]) involved in the generation and regulation of circadian rhythmicity.

Methods: Aspartate (50 mg/kg body weight) was administered orally for 60 days to Wistar rats, and 24-hour rhythms of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase were studied under semi-natural (light/dark 12:12 hr) conditions.

Results: Exogenous aspartate administration caused acrophase advances in TBARS rhythms, and delays in GSH, SOD and catalase rhythms; altered MESOR and decreased amplitude values were also seen in all of these rhythms.

Conclusion: Our findings indicate that the orally-treated aspartate could reach the hypothalamus, and various brain centres possibly including SCN, and could modulate the circadian patterns of lipid peroxidation products and antioxidants.

Keywords: aspartate, circadian pattern, lipid peroxidation products, reduced glutathione, superoxide dismutase, thiobarbituric acid reactive substances

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INTRODUCTION

Circadian rhythms are widely found in prokaryotes (cyanobacteria), plants, animals and humans.[1] Circadian rhythms govern most aspects of physiology, behaviour, metabolism, endocrine, immune functions, biochemistry, and DNA synthesis.[2,3] In mammals, suprachiasmatic nuclei (SCN) present in the anterior hypothalamus of the brain[4] is referred to as the biological clock which regulates the circadian rhythms.[5] Endogenously-generated circadian (~ 24 hours) rhythms are adjusted (entrained) daily to light/dark cycles in the environment via information transmitted by retinohypothalamic tract (RHT) to SCN.[6] Aspartate is a putative excitatory neurotransmitter present in the retina, horizontal, amacrine and ganglion cells,[7] in RHT[8] and SCN.[9] Aspartate is involved in the transmission of light information from the retina to SCN via the RHT.[10] Derivatives of aspartate (such as N-acetyl aspartate, N-methyl-D-aspartate [NMDA], N-acetylaspartyl-glutamate) also act as a neurotransmitter in RHT.[6] D-aspartic acid is an endogenous amino acid occurring as a free acid in most of the vertebrates and invertebrates.[11] Aspartate modulates hormonal secretion, mainly leutinising hormone, growth hormone, prolactin and regulates the some circadian rhythms in mammals.[12,13]

Aspartic acid is one-half of the ubiquitous sweetener aspartame (Nutrasweet), which is used in diet desserts, low-calorie drinks, and chewing gum.[14] The presence of aspartic acid in aspartame causes an increased plasma concentration of aspartate and glutamate, which might in turn pose a risk of focal brain lesions.[15] Previous studies from our laboratory showed that aspartate could modulate circadian patterns of some biochemical rhythms in aspartame-treated rats.[16] Excess free aspartate in our diet causes serious chronic neurological disorders and a myriad of other acute symptoms, such as typical memory loss, confusion and mild intellectual deterioration.[14] The circadian phase-shifting effects of aspartate in various species are well-documented.[17] Amino acids and their derivatives are known as excitotoxins. They react with specialised neuron receptors such as glutamate receptors in the brain or spinal cord, and can cause injury or death to a variety of

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neurons.[18] Aspartate is a putative neurotransmitter and activates excitatory amino acid receptors, i.e., NMDA receptors.[19] Photic-like phase shifting (phase shifts induced by light pulses) has been reported in aspartate and glutamate administered rats due to its activation of NMDA receptors in SCN.[20] Aspartate administration to SCN causes circadian phase shifts in the free running rhythms of hamsters.[21]

Lipoid peroxidation products and antioxidants exhibit circadian rhythms.[22] The main objective of the present study is to investigate the influences of chronic administration of aspartate on the circadian rhythm characteristics (acrophase, midline estimating statistic of rhythm [MESOR] and amplitude) of lipid peroxidation products (TBARS) and antioxidants such as glutathione (GSH), superoxide dismutase (SOD) and catalase, and to investigate whether aspartate could affect these rhythms differently, as aspartate is an important excitatory neurotransmitter (present in RHT and SCN) involved in the generation and regulation of circadian rhythmicity.

METHODS

Adult male Wistar rats (160–180 g) were obtained from Central Animal House, Faculty of Medicine, Annamalai University. They were housed in polypropylene cages at room temperature (30 ± 2°C) under semi-natural conditions.[23,24] In Annamalainagar (11°24'N, 79°42'E), the light-dark cycle is almost 12:12 hours throughout the year. Animals were maintained in natural light-dark cycles (12:12 hr) in an experimental room simulating the natural conditions.[16] L-aspartic acid was obtained from Himedia Laboratories, Mumbai, India. The required amount of L-aspartic acid (50 mg/kg body weight) was dissolved by stirring it in distilled water and 1M NaOH solution was added dropwise to dissolve aspartic acid and obtain a final pH of 7.4, and then finally made up to the required volume with distilled water[25] (aspartic acid, a dicarboxylic amino acid is insoluble in its acidic form but soluble as sodium salts). All other biochemicals used in this study were of analytical grade.

The experimental animals were divided into two groups (n = 6 in each group): control (Group I) and aspartate-treated (Group II) rats. Aspartate 50 mg/kg[26] was orally administrated to group II rats every day (at irregular intervals) for 60 days. The experimental protocol was approved by the Committee for Research and Animal Ethics, Annamalai University (Vide No: 245/2005). The animals received a diet of standard pellets (Agro Corporation Private Limited, Bangalore, India). Food and water were available *ad libitum* and they were replenished daily. At the end of the experimental period, blood samples were collected from both groups at four-hour intervals continuously throughout a 24-hour period (00:00–24:00). Minimal amount of the blood was collected from the orbital sinus with great care using heparinised tubes.[27,28] Levels of TBARS[29] and GSH[30] were estimated in plasma; SOD[31] and catalase[32] activities were measured in haemolysate at the above-mentioned time intervals. Finally, both group animals were killed by decapitation and aspartate levels in the brain tissues[33] were measured.

The values of the variables (mean ± SD) were plotted against the time of blood collection. Measurements of acrophase (φ: measure of peak time of the variable studied), amplitude (A: corresponds to half the total rhythmic variability in a cycle), MESOR (M: rhythm-adjusted mean) and r values (correlation coefficient of the rhythm) were done by cosinor analysis using "cosinorwin" computer software programme.[23] From the r value, we calculate the p-value as follows:[34]

$$\left| \bar{t} \right| = \frac{r^2}{\sqrt{(1-r^2)}} \times \sqrt{n-2}$$

where n = number of samples taken. p-values were calculated from critical values of t distribution at $\alpha^{(1)}$ level at 0.05 significance.

RESULTS

Brain aspartate concentration was increased in group II (aspartate-treated) animals (5.25 ± 0.40 μmol/g tissue) compared to normal (group I) animals (1.30 ± 0.10 μmol/g tissue) (Table I). Control animals showed the maximum levels of TBARS at 19:16 hr; in aspartate-treated animals, the peak time was found at 18:46 hrs (~ 0.30 hr advance) (Figs. 1a & b). Increased MESOR and decreased amplitude values were shown in group II animals when compared with normal rats (Table II). GSH levels showed peak at 07:20 hrs in group I animals and aspartate treated rats at 08:28 hrs (~ 1.00 hr delay) (Figs. 2a & b). Acrophase of SOD was found at 12:24 hrs in normal rats, in aspartate-treated rats, the peak time was shown at 13:10 hrs (~ 0.45 hr delay) (Figs. 3a & b). Catalase levels showed peak at 07:25 hr in normal animals, in group II rats, it showed a maximum level at 09:52 hrs (~ 2.50 hr delay) (Figs. 4a & b). Decreased MESOR and amplitude were shown in GSH, SOD and catalase rhythms in group II animals compared to normal rats (Table II).

DISCUSSION

SCN contains a variety of neurotransmitters and neuropeptides, and many of them could influence the activities of the circadian pacemaker.[35] Aspartate and glutamate have excitatory effects on SCN neurons, both *in vivo* and *in vitro*.[19] The excitatory amino acid, aspartate, is involved in the neurotransmission and regulates
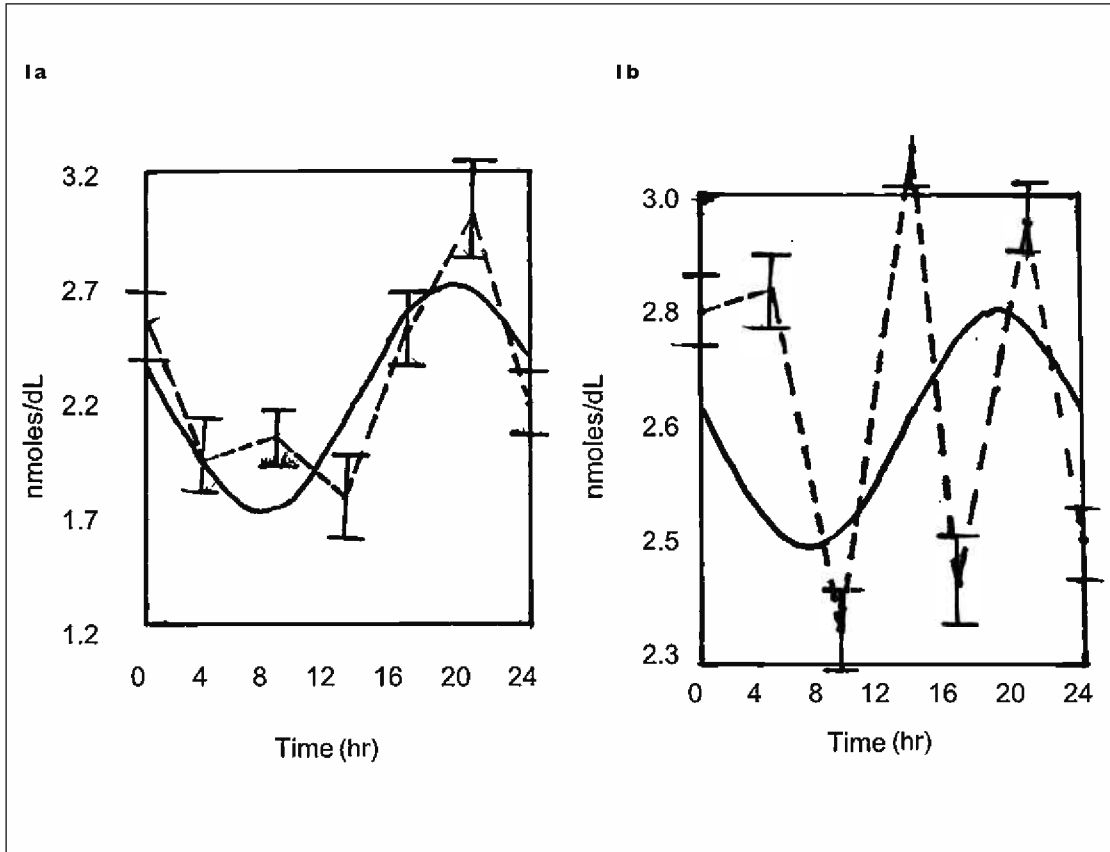
Fig. I Temporal oscillations of TBARS at four-hour intervals in (a) control and (b) aspartate-treated rats. Dotted lines represent the raw data and solid lines represent the best-fitting cosinor curve ('cosinorwin' computer software programme). Note the significant advance (~ 0.30 hr) in the acrophase in group II (aspartate-treated) animals.
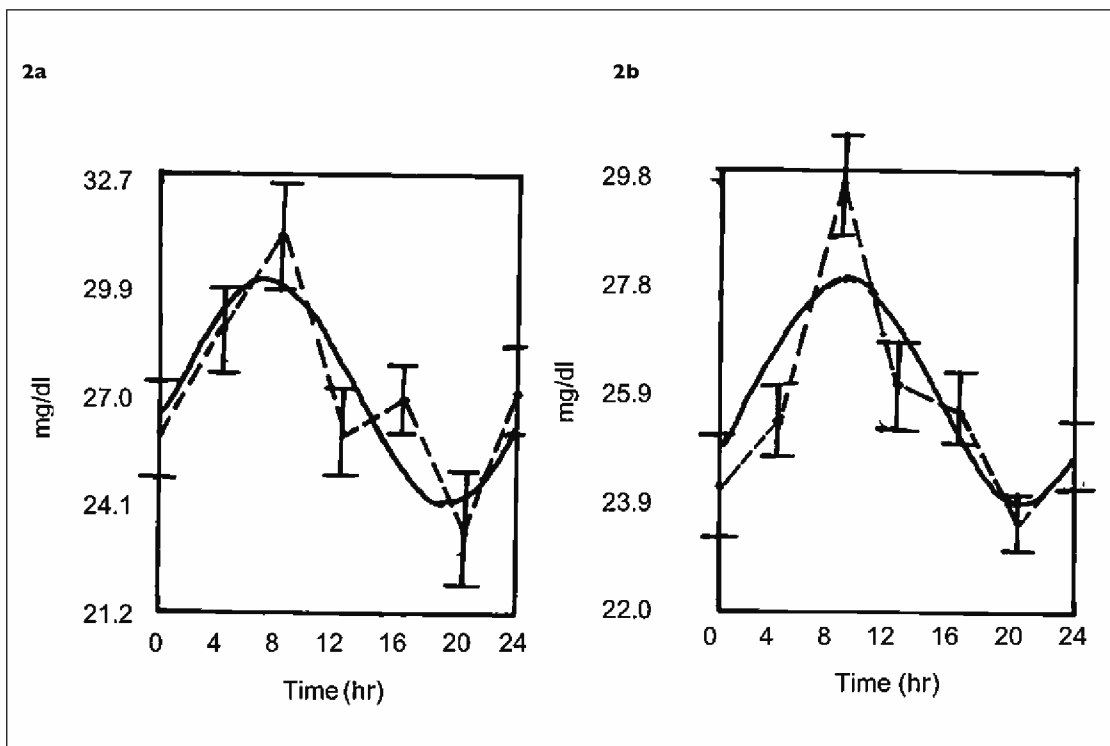

Fig. 2 Diurnal rhythms of GSH at four-hour intervals in (a) control and (b) aspartate-treated rats. Note the approximate one hour delay in the acrophase in aspartate-treated rats.
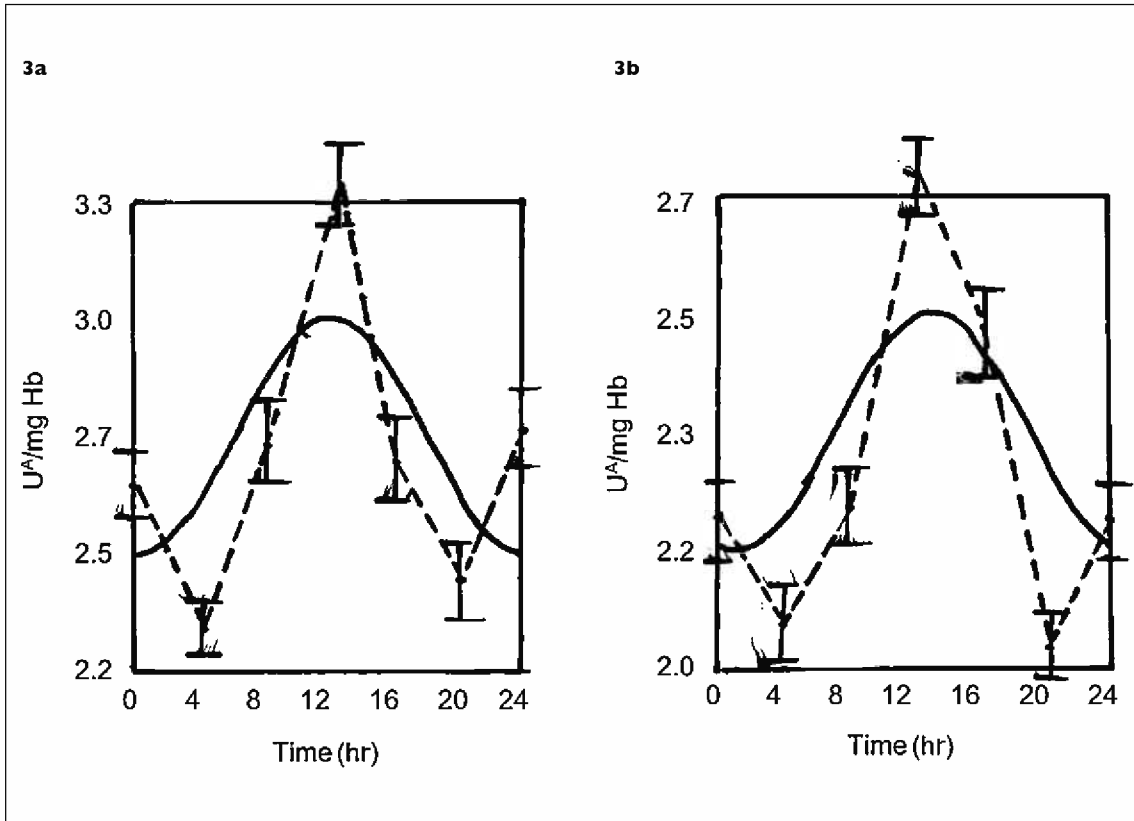
Fig. 3 24-hour rhythms of SOD at four-hour intervals in (a) control and (b) aspartate-treated rats. Acrophase of the SOD in aspartate-treated animals are delayed by ~ 0.45 hr.
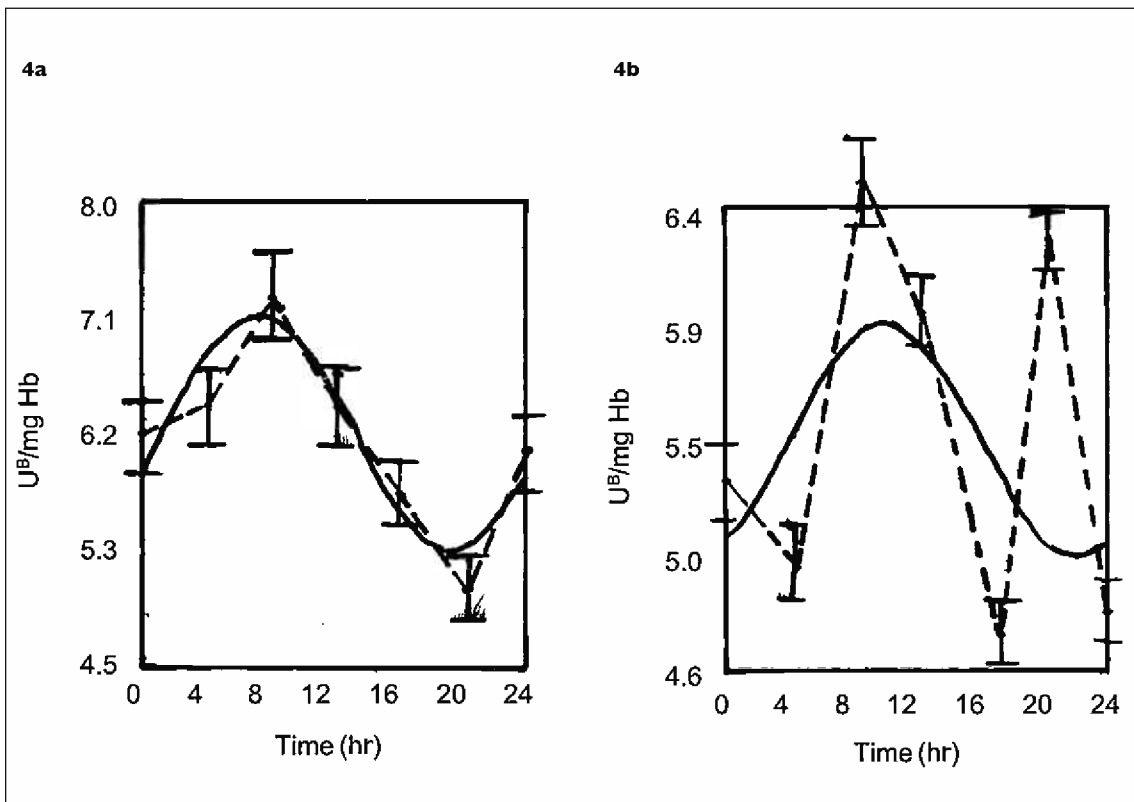

Fig. 4 Circadian oscillations of catalase at four-hour intervals in (a) control and (b) aspartate-treated rats. Note the significant delay (~ 2.50 hr) in the acrophase of aspartate-treated rats.

the circadian rhythm via photic transmission to the biological clock (SCN).[36] In our study, in group II rats, aspartate concentration in the brain was found to be increased as compared with normal rats; this could be due to orally-treated aspartate, which could cross the blood brain barrier and reach the brain.[21] The biochemical variables (TBARS, GSH, SOD and catalase) chosen in this study exhibited marked fluctuations over a 24-hour period, i.e. maximum and minimum levels were observed within the 24-hour period. Lipid peroxidation is a unique mode of oxidative injury which is triggered and promoted by different radical and non-radical members of the reactive oxygen species (ROS) family or by the catalytic decomposition of preformed lipid hydroperoxides in tissues by several agents.[37] The temporal patterns of TBARS depend on the nature of the diurnal rhythms of antioxidant enzymes (such as SOD and catalase).[27,38] In our study, the peak time of TBARS was found to be at 19:16 hrs in normal rats, corroborating previous reports.[39]

Table I. Aspartate levels in the brain after treatment.

Group (n = 6)	Aspartate level (μmol/g tissue) Mean ± standard deviation
Normal (group I)	1.30 ± 0.10
Aspartate-treated (group II)	5.25 ± 0.40

p-value: < 0.05 (Student's t-test)

Antioxidant enzymes, such as SOD, catalase and glutathione peroxidase, are important for cellular protection due to their ability to detoxify free radicals, such as ROS.[40] In normal rats, the antioxidants, such as GSH and catalase, show maximal activity at 07:20 hrs and 07:25 hrs (around 08:00 hrs in both cases); this is similar to the previous report,[39] and maximal levels were found around 08:30 hrs and 10:00 hrs in aspartate-treated rats. Peak activity of SOD was at 13:10 hrs in group II (~ 0.45 hr delay) animals. In our study, increased MESOR and decreased amplitude of TBARS rhythm, and decreased MESOR, amplitude and r values of GSH, SOD and catalase, were found in aspartate-treated rats when compared with normal rats. Increased MESOR of lipid peroxidation products (TBARS) might be due to the administration of aspartate, which could cause excessive stimulation of NMDA receptors[41] and excessive Ca^{2+} concentration inside neurons. It could also be caused by the activation of nuclear enzymes (endonucleases) that lead to apoptosis, also known as cell suicide, which results in the production of nitric oxide (NO) by the body.[42] This NO could react with peroxynitrate; and both of them are potent causes of cellular damage.[43] The NO free radicals and its related species may damage a variety of cell macromolecules, including the electron transport system, thus disrupting mitochondrial function.[44,45] This mitochondrial inhibition would potentiate ONOO–formation, leading to the depletion of antioxidant defences (mainly GSH)[46] and

Table II. Characteristics of temporal patterns of biochemical variables in rats.

Biochemical variables	Characteristics of rhythm	Normal	Aspartate-treated
Thiobarbituric acid reactive substances	Acrophase φ (hr)	19:16	18:46
(TBARS)	Amplitude (A)	0.5	0.1
	MESOR M (nmol/ml)	2.2	2.6
	r-value	0.89[dr] (p < 0.01)	0.13[ns] (p < 0.20)
Reduced glutathione (GSH)	Acrophase φ (hr)	07:20	08:28
	Amplitude (A)	2.8	1.9
	MESOR M (mg/dL)	27	25.9
	r-value	0.70[dr] (p < 0.05)	0.10[ns] (p < 0.20)
Superoxide dismutase (SOD)	Acrophase φ (hr)	12:24	13:10
	Amplitude (A)	0.2	0.1
	MESOR M (UA/mg Hb)	2.7	2.3
	r-value	0.68[dr] (p < 0.05)	0.45[ns] (p < 0.50)
Catalase	Acrophase φ (hr)	07:25	09:52
	Amplitude (A)	0.8	0.4
	MESOR M (UB/mg Hb)	6.2	5.5
	r-value	0.69[dr] (p < 0.05)	0.21[ns] (p < 0.50)

dr: detectable rhythmicity; ns: no significant rhythmicity

Enzyme activities are expressed as:

SOD: one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT (nitroblue tetrazolium) reduction in one minute; catalase: μmoles of hydrogen peroxide consumed/min/mg protein.

initiation of lipid peroxidation;[47] this finally leads to insufficiency of the protective antioxidant systems.[46]

As enzymatic and non-enzymatic antioxidants are involved in scavenging the products of lipid peroxidants, decreased MESOR values of antioxidants might be caused.[48] Altered rhythms (amplitude, acrophase and r values) in group II rats may be due to aspartate and could convey the photic information to SCN and entrain the circadian pacemaker. From the present data, we hypothesise that increased aspartate levels in the brain, possibly including SCN, could alter the characteristics of biochemical rhythms in aspartate-treated rats.

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