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RESEARCH ARTICLE

Effects of *Pongamia pinnata* on Lipid Peroxidation Products and Antioxidants in Hyperammonemic Rats: with Reference to Circadian Variations

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ABSTRACT

Effects of *Pongamia pinnata,* an indigenous plant used in Ayurvedic Medicine in India on the temporal variations of circulatory lipid peroxidation products and antioxidants in ammonium chloride-(AC)-induced hyperammonemic rats has been studied. Experimental rats were divided into control, AC-treated, those treated with AC + ethanolic leaf extract of P. *pinnata* (PPEt), and PPEt-treated. The characteristics of 24 h rhythms (acrophase, amplitude and mesor) of lipid peroxidation products (thiobarbituric acid reactive substances – TBARS) and antioxidants (reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT)) were analyzed. Elevated lipid peroxidation (increased mesor of TBARS) associated with decreased activities of antioxidants (decreased mesor of GPx, GSH, SOD and CAT) were found in hyperammonemic rats. Differences were also found in amplitude and 'r' values between the hyperammonemic rats and other experimental groups. These alterations clearly indicate that temporal redox status could be modulated by PPEt during hyperammonemic conditions, which may also play a crucial role in disease development.

Keywords: *Hyperammonemia, Pongamia pinnata, Circadian, Lipid peroxidation, Antioxidants*

Pongamia pinnata (Linn) Pierre (Leguminosae), popularly known as 'Pongam' in Tamil and 'Karanja' in Hindi, is a medium sized glabrous tree, found throughout India and further distributed eastwards, mainly in the littoral regions of South Eastern Asia and Australia [1]. In the Indian Ayurvedic literature, various parts of this plant have been recommended as a remedy for various ailments. Different parts of the plant have been used in traditional medicines for bronchitis, whooping cough, and rheumatic joints and to quench dipsia in diabetes [2]. The leaves are digestive, laxative, anthelmintic and cure piles, wounds and other inflammations [2]. A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scrofulous enlargement [3]. Different extracts of leaves, roots and seeds (ethanol, petroleum ether, benzene extracts and others) of *Pongamia pinnata* have been reported to have anti-inflammatory activity and also used to treat infectious diseases such as leucoderma, leprosy, lumbago, muscular and articular rheumatism [4-6]. In addition, phytochemical examinations of this plant indicated the presence of furanoflavones, furanoflavonols, chromenoflavones, flavones, furanodiketones and flavonoid glucosides [7-10]. It is a well-documented fact that most medicinal plants are enriched with phenolic compounds and bioflavonoids that have excellent antioxidant property.

Hyperammonemia is a heterogenous group of disorders characterized by elevated levels of ammonia causing irritability, somnolence, vomiting, seizures, derangement of cerebral function, coma and death [11-13]. It is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication [11]. Ammonia toxicity results in free radical generation that leads to oxidative stress and tissue damage [14-16] and increased entry of ammonia to the brain is a primary cause of neurological disorders associated with hyperammonemia, such as hepatic encephalopathies, Reye syndrome, several other metabolic disorders, and some toxic encephalopathies [14,16]. Oxidative stress is evolving concept in ammonia neurotoxicity, and the potential involvement of oxidative as well as nitrosative stress in the deleterious effects of ammonia on the central nervous system has been recently reviewed [17]. Oxidative stress mediated lipid peroxidation is one of

Table 1. Changes in the temporal characteristics of TBARS in control and experimental rats

Groups	Acrophase (h)	Amplitude (nmoles/ml)	Mesor (nmoles/ml)	'r' value	'p' value
Normal	20:3	0.2	2.7	0.69^{dr}	<0.001
AC	16:7	0.3	4.5	0.21^{ns}	<0.5
AC+PPEt	18:59	0.5	3.0	0.49^{dr}	<0.05
PPEt	20:50	0.2	2.8	0.62^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.

Table 3. Changes in the temporal characteristics of SOD in control and experimental rats

Groups	Acrophase (h)	Amplitude (U^A/mg Hb)	Mesor (U^A/mg Hb)	'r' value	'p' value
Normal	12:00	0.4	2.9	0.79^{dr}	<0.001
AC	13:29	0.2	1.8	0.24^{ns}	<0.5
AC+PPEt	12:35	0.1	2.5	0.61^{dr}	<0.05
PPEt	11:33	0.4	2.8	0.86^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant
A- Amount of enzyme required to inhibit 50% of NBT reduction.

the key characteristic features of hyperammonemia [15,18]. Recent reports have demonstrated enhanced free radical production in cultured astrocytes exposed to pathophysiological concentrations of ammonia [13], and increased superoxide production and reduced activities of various antioxidant enzymes shown in the animal models of acute ammonia toxicity [14].

The concepts of chronobiology have been studied in various diseases in an attempt to improve the therapeutic index of drugs [19]. Circadian rhythms of a number of biological variables influence drug efficacy of disease treatments. Furthermore, a lack of synchronization, or an alteration of circadian clock function, make rhythm peaks and troughs unpredictable and may require specific measures for chronotherapy to improve therapeutic index. Hence, assessment of the relevance of a normal circadian system for a favourable outcome of chronotherapy is desirable [20]. Failure to recognize the biochemical temporal organization may introduce unnecessary heterogencity and hinder the full understanding of biological processes during diseased conditions. It is in this context, in the current study, that the variations in the temporal characteristics of lipid peroxidation products: thiobarbituric acid reactive substances – TBARS and antioxidants such as reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in control, hyperammonemic rats, and PPEt treated rats were analyzed.

MATERIALS AND METHODS

Plant Material and Extraction

The mature green leaves of *Pongamia pinnata* were collected from Chidambaram, Cuddalore District, Tamil Nadu, India. The plant was identified and authenticated at the Herbarium of Botany Directorate in Annamalai University. A voucher specimen (No.3670) was deposited in the Botany Department of Annamalai University. The shade-dried and powdered leaves of *Pongamia pinnata* were subjected to extraction with 70% ethanol under reflux for 8 h and concentrated to a semi-solid mass under reduced pressure (Rotavapor apparatus, Buchi Labortechnik AG, Switzerland). The yield was about 24% (w/w) of the starting crude material. In the preliminary phytochemical screening, the ethanolic extract of PPEt gave positive tests for sterols, tannins, flavones and glycosides [21]. The residual extract was dissolved in sterile water and used in the investigation.

Animals

Male albino Wistar rats weighing 180-200 were used for the study. They were housed in polycarbonate cages under standard conditions (22 ± 2˚C, humidity of 45-64%, 12 h light/dark cycles). They were given standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum.* All animal experiments were approved by the ethical committee (Vide. No. 273/2004), Annamalai University, India and were in accordance with the guidelines of the National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India. Ammonium chloride was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used in the study were of analytical grade.

Experimental Design

Hyperammonemia was induced in Wistar rats by daily intraperitoneal injections of ammonium chloride (AC) at a dose of 100mg/kg body weight for eight consecutive weeks [22]. Rats were divided into four groups, eight animals each. Group 1: Control rats. Group 2: Rats intraperitoneally treated with AC (100 mg/kg body weight) [22]. Group 3: Rats treated with AC (100 mg/kg; intraperitoneally) + PPEt (300 mg/kg; orally)

Table 2. Changes in the temporal characteristics of GSH in control and experimental rats

Groups	Acrophase (h)	Amplitude (mg/dl)	Mesor (mg/dl)	'r' value	'p' value
Normal	6:1	2.8	26.7	0.66^{dr}	<0.001
AC	9:2	1.4	15.9	0.19^{ns}	<0.5
AC+PPEt	7:38	3.5	22.5	0.49^{dr}	<0.05
PPEt	5:25	3.1	25.6	0.62^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.

Table 4. Changes in the temporal characteristics of GPx in control and experimental rats

Groups	Acrophase (h)	Amplitude (U^A/mg Hb)	Mesor (U^A/mg Hb)	'r' value	'p' value
Normal	8:00	1.4	23.4	0.69^{dr}	<0.001
AC	11:1	0.6	12.5	0.21^{ns}	<0.5
AC+PPEt	9:33	1.2	20.8	0.58^{dr}	<0.05
PPEt	7:39	1.5	24.6	0.64^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.
A- micromoles of GSH utilized/g Hb.

Table 5. Changes in the temporal characteristics of CAT in control and experimental rats.

Groups	Acrophase (h)	Amplitude (U^A/mg Hb)	Mesor (U^A/mg Hb)	'r' value	'p' value
Normal	10:10	1.1	2.1	0.70^{dr}	<0.001
AC	13:59	0.7	1.6	0.28^{ns}	<0.5
AC+PPEt	11:2	1.0	1.9	0.74^{dr}	<0.05
PPEt	8:50	1.2	2.0	0.78^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant
A- µmoles of H_2O_2 consumed/min/mg/Hb

[6,22]. Group 4: Rats orally administered with PPEt (300 mg/kg).

Biochemical rhythms

Biochemical parameters such as plasma TBARS [23] and hemolysate GSH [24], GPx [25], SOD [26] and CAT [27] were performed after the eight weeks of experimental study. Blood samples were collected after every 4 h from each group of experimental and control rats (00:00, 04:00, 08:00, 12:00, 16:00, 20:00 and 24:00 h) throughout the 24 h period continuously. Minimal amount of blood was collected from orbital sinus with great care using heparinized tubes. The values (mean ± SD) obtained from each group were plotted versus the time of blood collection. The characteristics of circadian rhythms (acrophase, amplitude and mesor) were analysed by cosinor analysis. Acrophase was expressed in h and mesor and amplitude were expressed in the same units of documented variables.

RESULTS

The biochemical variables chosen for this study in all the groups showed marked fluctuations over 24 h period. The characteristics of rhythms, r and p values indicating detectable rhythmicity or non-significant temporal variations over a 24 h period of all the groups are mentioned in Tables 1-5. The circadian patterns of TBARS revealed detectable rhythmicity in control and group 3 and 4 (Table 1). Detectable rhythmicity was found to be insignificant ($p<0.5$) in group 2 rats. Elevated mesor values and advanced acrophase was found in AC treated rats. Delayed acrophase and decreased mesor values are found in groups 3 and 4 when compared with controls. Amplitude values in groups 2 and 3 showed increases and decreases in (group 4) when compared to group 1.

The temporal patterns of GSH showed a detectable rhythm in control and groups 3 and 4 (Table 2). Detectable rhythmicity was disturbed in AC treated rats. Acrophase was delayed in group 2 and advanced in groups 3 and 4 when compared to control rats. Mesor values were decreased in group 2 and increased in group 3 and 4 (Table 2). The detectable circadian rhythm of SOD was found to be insignificant ($p<0.5$) in AC treated rats (Table 3). Acrophase was delayed in group 2 and advanced in group 3 and 4. Mesor values were decreased in group 2 and increased in groups 3 and 4 (Table 3). The detectable rhythmicities of GPx and CAT were found to be insignificant ($p<0.5$) in AC treated rats (Table 4 and 5). Acrophase values were delayed in group 2 and advanced in group 3 rats. Mesor values were decreased in group 2 and increased in groups 3 and 4.

DISCUSSION

The biochemical variables chosen in the study exhibit marked fluctuations over the 24 h period and the results of the present study indicated that control and experimental group (AC and PPEt treated) rats differ in the temporal characteristics. Alterations in period, amplitude, mesor and acrophase were detected in DNA synthesis of spleen, liver and bone marrow of diseased mice [28]. Our results also revealed that the rhythms in animals are not synchronized / exhibited a phasing with that of normal rats. This lack of synchronization reflected as an alteration of circadian clock function in hyperammonemic rats and may require specific measures for chronotherapy to improve therapeutic index.

Oxidative stress and related lipid peroxidation; associated membrane damages are the key features of AC induced toxicity [14,18]. Enhanced lipid peroxidation indicated as TBARS in AC treated rats might result in increased mesor values. Circadian or exogenous daily variations in metabolism including those related to locomotor and brain activities, should result in corresponding temporal patterns of oxidant formation [29]. Oxidative and antioxidative indices show circadian variations [30]. Desynchronised rhythms of oxidative and antioxidative indices reported in diseased conditions such as glomerulonephritis [31] and diabetes [32]. The temporal patterns of TBARS depend on the nature of 24 h rhythms of (I) lipid peroxidation levels and (II) SOD and CAT activities in blood [33,34]. The acrophase alterations observed in group 2 to 4 indicate that exogenous perturbations could influence the temporal organization of TBARS levels by influencing one or more factors mentioned above. Decrease in mesor value found in group 3 indicate that PPEt could decrease the deleterious effects of AC by reducing the formation of TBARS during hyperammonemic conditions [22,35-36] and also reported that PPEt to inhibit lipid peroxidation and scavenge reactive oxygen species [35-40].

The increased mesor values of antioxidants in PPEt administered to hyperammonemic rats showed that PPEt could elevate the levels enzymatic and non-enzymatic antioxidants (35-40). The antioxidant such as GSH and CAT also exhibit circadian rhythms and showed peaks at 08:00 h in experimental animals [30,33-34] and the circadian rhythms of SOD and CAT were also previously reported in liver and blood [41]. Circadian fluctuations in plasma [34] and tissue GSH concentrations including the liver, brain [42], heart, stomach [30], kidney [29], gut [43] etc., were reported. Circadian changes in glutathione concentration are related to rhythmic sensitivities towards radical inducers such as, toluene and dioxane [44], carbon tetrachloride [45], cisplatin,

choloroform and acetominophen etc., were also reported.

The diminished GSH levels and decreased activities of SOD, CAT and GPx in hyperammonemic rats reflected in decreased mesor values could be due to over utilization of these antioxidants to scavenge the products of lipid peroxidation. The present study also revealed that non-significant rhythm characteristics in hyperammonemic rats could influence the temporal patterns of lipid peroxidation and other antioxidant levels in rats. Alterations in the circadian variation in the mitotic index and oxidant and antioxidant status of various liver diseases in rats were reported [34,46]. Increased mesor values of GSH, SOD, CAT and GPx observed in group 3 indicate that PPEt may have the ability to control the formation of free radicals by elevating the levels of antioxidants during hyperammonemic and other conditions [35-40]

Knowledge of the circadian rhythms in normal and in pathological conditions can be used to improve the understanding of pathophysiological process and therapeutic approach to illness. Adapting chemotherapy delivery to circadian rhythms has indeed achieved meaningful clinical improvements in chemotherapy tolerability and efficacy. The detectable circadian rhythms of lipid peroxidation products and antioxidants and their alterations during AC/PPEt treatments, in the present study, needs further investigation for the diagnosis and for improve the therapeutic efficacy of hyperammonemia.

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