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ORIGINAL ARTICLE

MODULATION OF CIRCADIAN RHYTHMS IN HEPATIC MARKER ENZYMES AND ANTIOXIDANTS IN NDEA INDUCED MICE

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ABSTRACT

Melatonin, the principle hormone of pineal gland plays an important role in several biological processes.Physiologic and pharmacologic concentrations of the pineal hormone melatonin have shown chemopreventive, oncostatic, and tumor inhibitory effects in a variety of *in vitro* and *in vivo* experimental models. In our study we aimed to examined the effect of melatonin in N-Nitrosodiethylamine (NDEA) induced mice. NDEA is an important carcinogen frequently present in human environment. Previous studies have also shown that melatonin is a component of the antioxidative defense system of organisms due to its free radical scavenging ability. Circadian rhythms of hepatic marker enzymes and antioxidant enzymes were altered carcinogen induced mice and were detectable. From these findings we suggest that the melatonin alter the circadian patterns of liver marker enzymes and metabolites which would play a key role in the inhibition of hepatocarcinogenesis.

Key words: N-nitrosodiethylamine, Circadian rhythms, Antioxidant enzymes, Melatonin

1.INTRODUCTION

The liver is the primary target site for hundreds of chemicals including pesticides, food additives, pharmaceuticals and industrial intermediates. Cirrhosis of liver is the most common type of chronic liver disease and is one of the most important health problems. The involvement of free radicals in the pathogenesis of liver injury has been investigated for many years in a few well-defined experimental systems (Poli, 1993). Many chemicals metabolized in the liver also induce liver damage (Park *et al*., 2005) and increase the risk of hepatocellular carcinoma (HCC). Liver cancer is third leading cause of cancer death worldwide (Thorgeirsson *et al*., 2002). N-nitrosodiethylamine (NDEA) has been used extensively in the past as a carcinogen and/or lesion initiator in animal model systems of carcinogenesis. It induces hepatic neoplastic and preneoplastic lesion in mice (Goldfarb *et al*., 1983). Administration of NDEA to animals has been shown to cause liver cancer and cancer at a low incidence in other organs. The involvement of ROS in NDEA-induced liver cancer has been extensively studied (Deal *et al*., 1989). HCC is induced by the chemical procarcinogen diethylnitrosamine (NDEA) in mice model and inflammation promotes hepatocarcinogenesis through production of cytokines that stimulate compensatory proliferation (Maeda *et al*., 2005; Naugler *et al*., 2007).

N-nitrosodiethylamine (NDEA), one of the most important chemical carcinogens is catalyzed by the cytochrome P-450, is involved in causing oxidative stress and high intracellular levels of reactive oxygen species (ROS) that can lead to damaged mitochondria and DNA modification resulting disruption of circadian clock coordination that contribute to the progression of HCC (Sundaresan and Subramanian 2003). NDEA is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis (Gey, 1993). NDEA undergoes metabolic activation by cytochrome P-450 enzymes to reactive electrophiles that are cytotoxic, mutagenic and carcinogenic (Archer,1989) It has been reported that NDEA is transported through blood and it causes hepatic injury (Bansal *et al.*, 1996).

Melatonin synthesis and secretion by the pineal gland surges during the night and is low during the day Melatonin (n-acetylmethoxytryptamine, MLT) is a principal hormone involved in regulation of circadian rhythms and produced mainly by the pineal gland and has antioxidant and prophylatic properties against oxidative stress in several experimental and clinical conditions (Reiter *et al*., 2003), and synthesized from tryptophan in a circadian rhythmic fashion, under the control of various enzymes that are inhibited by light and stimulated at night in the pineal gland (Jung , and Ahmad, 2006; Reiter, 1991). Earlier studies on the possible correlation and effects of melatonin and cancer can be traced back to the 1960s. The

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timing of melatonin administration seems to be an important factor in its chemopreventative properties. Melatonin, a hormone of the pineal gland has been known to be a chemopreventive, anticancer agent in in *vitro* studies and experimental animal models.Oxaliplatin(trans-/-diaminocyclohexane xalatoplatinum) is a water soluble platinum-compound with an oxalate as the hydrolysable ligand and a diaminocyclohexane (DACH) as a carrier ligand. The investigation of platinum (pt)-complex compounds as anti-tumor agents in the 1960s led to the sucessful development of cisplatin (Burchenal *et al*., 1979). Oxaliplatin is rapidly and non-enzymatically biotransformed to other molecular species.

This biological clock generates signals of circadian rhythm, which are conducted to the supra-cervical sympathetic nucleus and the pineal body. Many physiological variables display rhythms with a period close to 24 h (Dunlap *et al.,* 2004). Circadian rhythms regulate many functions in the human body including sleep and wakefulness, body temperature, blood pressure, hormone production, digestive secretion and immune activity. Disruption of these rhythms can have profound influences on our health (Urs, 2002; Hastings, 2003; Fu, 2003). These rhythms are generated by endogenous circadian clocks. Dysregulation in the circadian pattern of lipid peroxidation and antioxidants has been reported in hepatocarcinogenesis in rats (Sundaresan and Subramanian 2003).

2. MATERIAL AND METHODS

Chemicals

NDEA, melatonin and oxaliplatin were purchased from Sigma chemical company, St. Louis, MO, USA. All the other chemicals and solvents used in the study were of analytical grade and were obtained from either Sigma Chemical Company or Hi Media Laboratories, Mumbai, India.

Animals

Adult male mice (*Mus booduga*) were captured from Madurai Kamarajar University campus. Locomotor activity was measured (Department of Animal Behavior and Physiology–MKU). Animal were kept in a temperature-controlled experimental cubicle maintained at 31±1^{o}C. Food comprised of millets, maize, grains and water available *ad libitum*. The animals (six per group) were housed in plastic cages under controlled conditions of light (12 hr light/12 hr dark), humidity (50%) and ambient temperature (30 ± 2C). The animals used in the present study were kept in accordance with the guidelines of National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and by the Animal Ethical Committee, Annamalai University (Reg. No. 160/1999/ CPCSEA).

Experimental induction of carcinogenesis

Hepatic carcinogenesis in mice was received i.p. injection of NDEA at a dose of 200 mg/kg body weight.

Experimental design

The animals were divided into 4 groups.

Group I:Control.

Group II:NDEA treated (20 mg/kg b.w. for 10 days)

Group III:NDEA+ melatonin dissolved in 0.1 ml of 10% ethanol insaline (0.5 mg/kg b.w) (thrice a week)

Group IV:NDEA+ oxaliplatin dissolved in water (2 mg/kg b.w.) (thrice a week) for 20 weeks.

Temporal biochemical determinations

After the experimental period, blood samples were collected from both groups at every four hour intervals (00:00, 04:00, 08:00, 12:00, 16:00, 20:00 and 24:00) throughout the 24-h period continuously. Minimal amount of blood was collected from the orbital sinus with great care using heparinized tubes (Essa and Subramanian, 2007). Lipid perioxidation in plasma was spectrophotometrically measured by estimating TBARS by the method of (Nichans and Samuelson 1986). The activities of superoxide dismutase (Kakkar *et al*., 1984), catalase (Sinha 1972), glutathione peroxidase (Rotruck *et al*., 1973), glutathione S-transferase, AST and ALT (Reitman and Frankel, 1957) were assayed at above-mentioned time intervals. The values of the variables (mean ± SD) were plotted versus the time of blood collection. Measurements of acrophase, amplitude, mesor and r-values were done by using the cosinorwin software program (Zar, 1984).

Acrophase, Amplitude and Mesor

The acrophase (ϕ) is the measure of peak time of the variable studied. The amplitude (A) corresponds to half of the total rhythmic variability in a cycle. The mesor (M) is the rhythm adjusted mean. It is equal to the arithmetic mean for equidistant data covering the 24 h period. The r -value referred as correlation coefficient of the rhythm. The *p* values were calculated from
r -values, by using the following formula.

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

Where n = number oɪ samples taken. p-values were calculated from critical values of t- distribution and ≤ 0.05 values were consider a significant. To draw the Cosine fitted curves of variables the following formula has been used.

$$Yti = M + A \cos (\omega t - \phi)$$

where,

Yti–Cosine function at the time point,

M–Mesor; A–Amplitude; ω–phase angle difference; t–Time; ϕ–Phase.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS software package 9.05. Results were expressed as mean ± SD from six animals in each group. P values < 0.05 were expressed as significant. The values of the biochemical variables (mean± SD) were plotted versus the time of blood collection. The characteristics of circadian rhythms (acrophase, amplitude, mesor) were analysed by cosinor analysis (using cosinorwin software program). Acrophase (time at which the level of variable is highest over a 24-h period) is expressed in hours. Amplitude (half the difference between maximum and minimum levels of variables) and mesor (mean value of the variable for equidistant data covering a 24-h period) were expressed in the same units of documented variables (Zar, 1984).

3. RESULTS

The biochemical variables chosen for the study in all groups showed marked fluctuations over the 24-h period. Variations in amplitude, r and p values were also noted (Table 1). The animals receiving NDEA + melatonin, activity of SOD was significantly augmented as compared to the control group. The circadian pattern of all parameters revealed a slight disturbed rhythmicity in all groups (Table 1). The temporal patterns of TBARS revealed detectable rhythmicity in control animals. No detectable rhythmicity was observed in NDEA +Melatonin and NDEA+Oxaliplatin when compared to control group. Elevated mesor value and advanced acrophase were found in NDEA induced group (Figure 1A). Amplitude value was found to be increased in NDEA induced animals whereas decreased in NDEA + Melatonin and NDEA+ Oxaliplatin when compared to control group. Delayed acrophase and decreased mesor values were found in melatonin or oxaliplatin treated group when compared to control group(Figure 1B). The temporal pattern of SOD showed disturbed rhythmicity was noticed in NDEA induced group. The advanced acrophase and decreased Mesor values were observed in melatonin or oxaliplatin treated group when compared to control group.

Circadian rhythm of CAT was found to be insignificant ($p<0.5$) in NDEA induced group. Acrophase was delayed in NDEA and advanced in melatonin or oxaliplatin treated group whereas, mesor value was decreased in NDEA and increased in melatonin or oxaliplatin treated group Figure 2 A). The rhythmicities of GPx and GST were found to be insignificant ($p<0.5$) in NDEA induced group. Acrophase values were delayed and mesor values decreased in melatonin or oxaliplatin treated group (Figure 2B) and Figure 3A). The characteristics of rhythms with r and p values indicating detectable rhythmicity or nonsignificant temporal variations over a 24-h period of control and experimental groups are given (Figures 3 B) and (C). Liver markers AST and ALT were found to be altering in NDEA induced animals ($p<0.5$). Acrophase of AST and ALT were delayed along with elevated mesor values in NDEA induced animals compared to control. Advanced acrophase and decreased mesor values of serum liver markers were found in NDEA + Melatonin and NDEA+ Oxaliplatin treated group when compared with NDEA treated animals.

Table 1. Characteristics of temporal patterns of hormonal variables in control and experimental animals

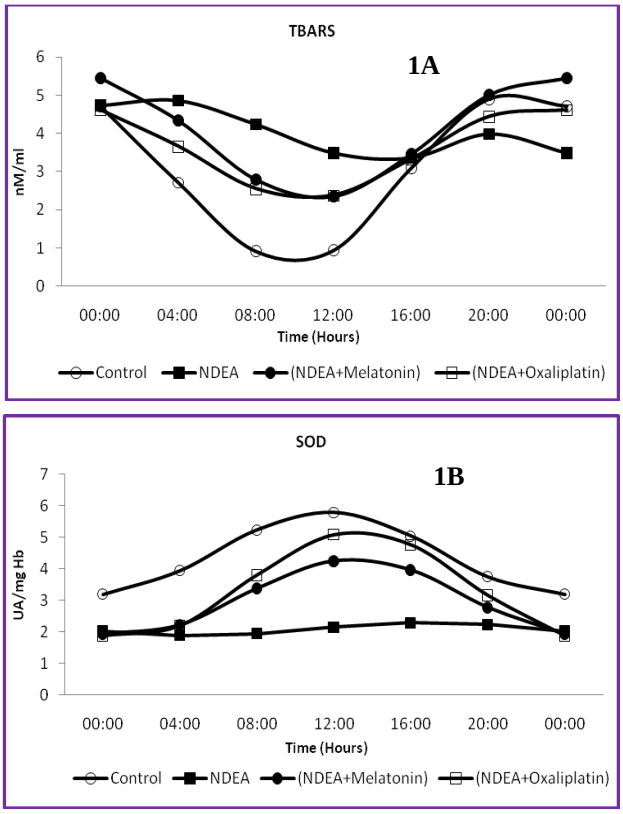
	Characteristics of circadian rhythms	CONTROL	NDEA	NDEA+ MELATONIN	NDEA+ OXALIPLATIN
TBARS (nmoles/dl)	Acrophase ϕ (hr)	21:38	2:39	23:02	22:31
	Amplitude (A)	2.2	0.8	1.6	1.2
	Mesor	2.9	4.1	3.9	3.5
	r-value	0.33^{dr}	0.06^{ns}	$0.0.42^{dr}$	0.27^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.5)	(P<0.05)
SOD (U^A/mg Hb)	Acrophase ϕ (hr)	11:40	16:45	13:5	13:19
	Amplitude (A)	1.3	0.2	1.2	1.7
	Mesor	4.5	2.1	3.1	3.5
	r-value	0.33^{dr}	0.16^{ns}	0.47^{dr}	0.61^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.5)	(P<0.05)
CAT (U^B/mg Hb)	Acrophase ϕ (hr)	07:08	12:00	08:48	08:01
	Amplitude (A)	2.7	0.8	1.5	1.5
	Mesor	4.2	2.7	3.7	3.5
	r-value	0.72^{dr}	0.15^{ns}	0.62^{dr}	0.81^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.5)	(P<0.001)
GPx (U^C/mg Hb)	Acrophase ϕ (hr)	04:43	17:07	06:46	06:16
	Amplitude (A)	5.1	3.7	6.50	5.5
	Mesor	31.3	15.0	24.6	27.6
	r-value	0.69^{dr}	0.64^{ns}	0.20^{dr}	0.59^{dr}
	p-value	(P<0.001)	(P<0.001)	(P<0.5)	(P<0.001)
GST (U^D/mg Hb)	Acrophase ϕ (hr)	13:59	19:33	15:49	15:21
	Amplitude (A)	1.9	1.2	2.2	1.5
	Mesor	5.5	2.3	4.1	4.5
	r-value	0.66^{dr}	0.68^{ns}	0.19^{dr}	0.61^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.5)	(P<0.001)
AST (IU/L)	Acrophase ϕ (hr)	20:00	03:5	01:27	02:41
	Amplitude (IU/L)	05	32	18	19.8
	Mesor (IU/L)	94.5	211.7	114.6	105.5
	r-value	0.27^{dr}	0.22^{ns}	0.46^{dr}	0.06^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.05)	(P<0.05)
ALT (IU/L)	Acrophase ϕ (hr)	23:48	02:5	01:9	01:23
	Amplitude (IU/L)	5.0	1.8	4.2	4.4
	Mesor (IU/L)	46.4	85.1	50.1	50.2
	r-value	0.29^{dr}	0.06^{ns}	0.28^{dr}	0.27^{dr}
	p-value	(P<0.001)	(P<0.5)	(P<0.5)	(P<0.005)

^{dr} – detectable rhythmicity, ^{ns} – no significant rhythmicityU^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.

U_c - µg of glutathione consumed/minute.

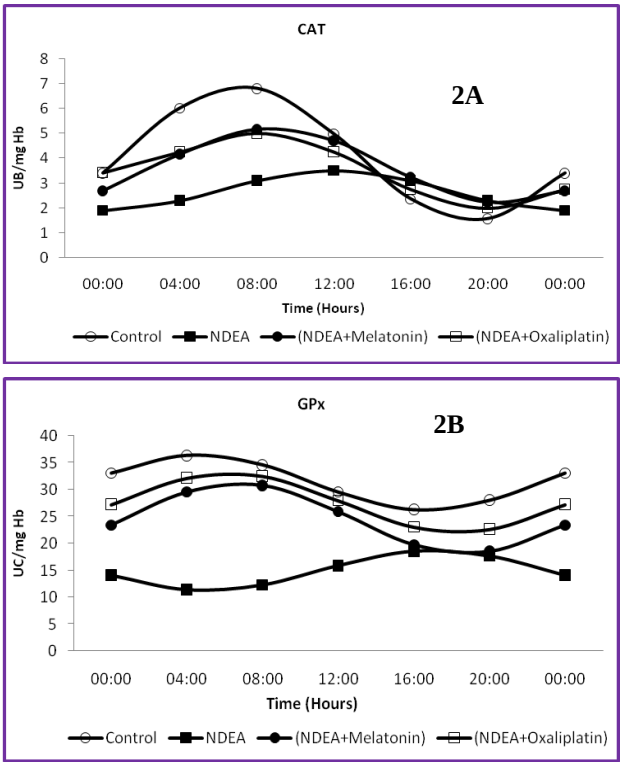
4.DISCUSSION

Figure.1 Temporal oscillations of (A) TBARS and (B) SOD in control and experimental animals



U^A - one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 minute. Cosine- fitted curves derived from the results given in Table 1 are shown.

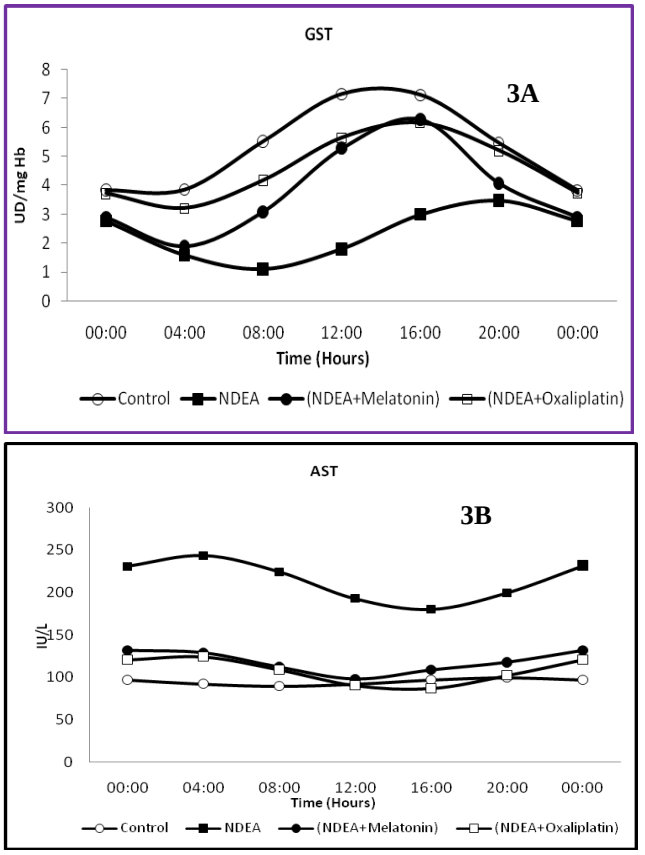
Figure.2 Twenty-four hour patterns of (A) CAT and (B) GPx in control and experimental animals



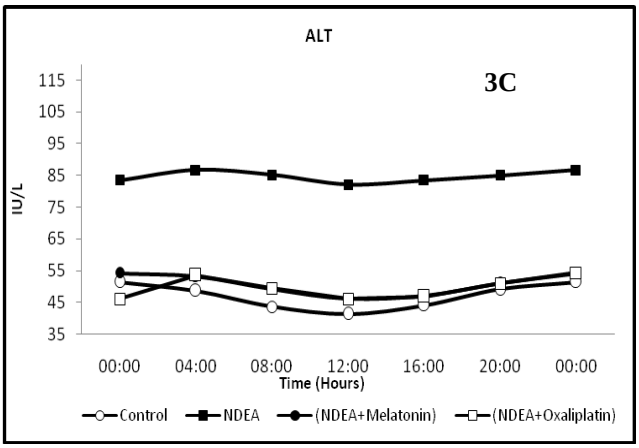
U^B - µ mole of hydrogen peroxide consumed/minute; Uc - µ mole of CDNB-GSH conjugated formed/min/mg Hb;Cosine- fitted curves derived from the results given in Table 1 are shown.

The liver is an important site of protein synthesis and it has highest rate of synthesis of tissue. Major protein mass of the organism is severely affected by cancer.

Figure.3 Rhythms of (A) GST, (B) AST, (C) ALT in control and experimental animals



U_D - µ mole of CDNB-GSH conjugated formed/min/mg protein.
Cosine- fitted curves derived from the results given in Table 1 are shown.



Cosine- fitted curves derived from the results given in Table 1 are shown.

N-nitrosodiethylamine (NDEA) is a major environmental carcinogen increases the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Bartsch *et al.*, 1989). It is a DNA alkylating agent leading to the formation of mutagenic DNA adducts. The genotoxic drug diethylnitrosamine (NDEA) is used since the 60s to induce HCCs in rodents (Rajewsky *et al*., 1966), and widely used chemical to induce liver cancer in mice. NDEA is bioactivated by cytochrome P-450 can generate reactive oxygen species (ROS) (Qi *et al.*, 2008), which damage DNA, proteins and lipids and lead to hepatocyte death. A remarkable body of evidence indicates that melatonin exerts antioxidant protection in

different experimental systems both *in vitro* and *in vivo* (Reiter *et al.*, 2007; Tan *et al*., 2007). Several studies have been investigated the relationship between NDEA treatment and the multistage steps of the hepatocarcinogenesis process in rats (Tsuda *et al*., 2003;Williams *et al.*, 1993). In contrast, similar studies in mouse have been limited (Vesselinovitch *et al*., 1984). Circadian rhythms are rhythmic oscillations in various biological processes that are regulated by an endogenous clock (Fu and Lee 2003). It has been reported that aging alters certain rhythmic cellular functions which have biological outcomes such as changes in sleeping patterns, alterations in melatonin synthesis and release, and disruptions in the levels of a variety of circulating hormones (Pandi-Perumal *et al.*, 2002). AST and ALT are reliable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred. (Whittby *et al*., 1984) Serum AST and ALT are the most sensitive markers employed in the diagnosis of hepatic damage because they are cytoplasmic in location and hence released into the circulation after cellular damage. Analysis of these enzymes reflects mechanisms of cellular damage, subsequent release of proteins, their extracellular turnover and mechanisms of neoplastic processes (Jahan *et al*., 2011). Production of free radicals during NDEA metabolism, thus damaging the hepatocellular membrane and these cytoplasmic enzymes are released into the systemic circulation. Treatment with melatonin may reduced these enzymes might be due to scavenge the free radicals, thus preventing the hepatocellular damage caused by NDEA.

The antioxidant enzymes, SOD, CAT, GPx, and GST have been shown to be sensitive indicators of increased oxidative stress and increased activities of these antioxidant enzymes are known to serve as protective responses to eliminate ROS (Reiter *et al.*, 2007). The evidence for a direct effect is seen when melatonin acts as a power-free radical scavenger in isolated cell-free-systems (Tan *et al*.,2002) there are, however, reports that melatonin can act as a prooxidant in such systems (Buyukavci *et al*., 2006). Melatonin increases activities of antioxidative enzymes and reduces oxidative damage (Rodriguez *et al.*, 2004; Samantaray *et al*., 2008; De Filipis *et al*., 2008). Melatonin (5 mg/day) in hypertension patients increased SOD,CAT, and glutathione reductase activities and reduced malonylodialdehyde (MDA) levels (Ke dziora *et al.*, 2008). Melatonin inhibits oxidation reactions catalyzed by metal ions and scavenges reactive oxygen species, thereby reducing lipid peroxidation. Likewise, melatonin metabolites are all potent antioxidants and also inhibit lipid peroxidation (Tan *et al.*, 2007).

It was also shown melatonin could inhibit mutagenesis and clastogenic effect of a number of chemical mutagens (Anisimov *et al*., 2006). Melatonin reduces the free radical-induced alteration of microsomal membrane fluidity during lipid peroxidation. Melatonin activity includes up-regulation of antioxidant enzymes (glutathione peroxidase, glutathione reductase, γ-glutamylcysteine synthase, glucose 6-phosphate dehydrogenase, superoxide dismutase and catalase) and down-regulation of prooxidant enzymes (NO synthases, lipoxygenases) (Reiter *et al*., 2007; Tan *et al.*, 2007). Furthermore, It has been reported to increase the activities and expression of mRNA encoding GPx and SOD in the rat cerebral cortex. We previously found that long-term melatonin administration to mice increased GPx activity in brain and liver homogenates (Okatani *et al.*, 2002). Circadian rhythms are an adaptation to the solar changes of light and dark and it is the suprachiasmatic nucleus of the anterior hypothalamus which is responsible for this function (Jan *et al*.,1998). From these findings we concluded that the melatonin may suppress the formation of NDEA induced hepatotoxicity in mice by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants. When the animals were treated with melatonin, it improved the activities of antioxidant enzymes and also caused recovery in GSH and reduction in LPO. The results shows that melatonin act as a potent antioxidant agent and capable of ameliorating NDEA-induced oxidative stress and perturbation of mitochondrial antioxidants. Melatonin treatment can effectively stimulated cell proliferation and also act as a potent protector of mitochondrial functions. Furthermore, it can efficiently reduce intracellular ROS levels of hepatocytes, thus preventing oxidative stress-induced cellular damage.

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