Research article

24-Hour rhythms in oxidative stress during hepatocarcinogenesis in rats: effect of melatonin or α-ketoglutarate

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To compare the effects of α-ketoglutarate (α-KG) and melatonin on 24-h rhythmicity of oxidative stress in N-nitrosodiethylamine (NDEA)-injected Wistar male rats, melatonin (5 mg/kg i.p.) or α-KG (2 g/kg through an intragastric tube) was given daily for 20 weeks. In blood collected at 6 time points during a 24-h period, serum activity of aspartate transaminase (AST) and alanine transaminase (ALT) and the levels of α-fetoprotein (α-FP) were measured as markers of liver function. To assess lipid peroxidation and the antioxidant status, plasma levels of thiobarbituric acid reactive substances (TBARS) and of reduced glutathione (GSH) were measured, together with the activity of erythrocyte superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione *S*-transferase (GST). NDEA augmented mesor and amplitude of rhythms in AST and ALT activity and plasma α-FP levels and mesor values of plasma TBARS, while decreasing mesor values of plasma GSH and erythrocyte SOD, CAT, GPx and GST. Acrophases were delayed by NDEA in all cases except for α-FP rhythm, which became phase-advanced. Co-administration of melatonin or α-KG partially counteracted the effects of NDEA. Melatonin decreased mesor of plasma TBARS and augmented mesor of SOD activity. The results indicate that melatonin and α-KG are effective in protecting from NDEA-induced perturbation of 24-h rhythms in oxidative stress. Melatonin augmented antioxidant defense in rats.

Keywords: Melatonin, α-ketoglutarate, N-nitrosodiethylamine, circadian rhythms, hepatocarcinogenesis, antioxidant status

INTRODUCTION

N-Nitroso compounds constitute one of the important groups of carcinogens frequently present in the human environment and food chain.[1,2] The presence of nitroso compounds and their precursors in the human environment together with the possibility of their endogenous formation in the human body have led to suggestions of their potential involvement in the pathogenesis of human cancer. N-Nitrosodiethylamine (NDEA) acts mainly by increasing oxidative stress and cellular injury due to involvement of free radicals.[1,2]

In a previous study, we reported that α-ketoglutarate (α-KG), an intermediate of the citric acid cycle, has a chemopreventive activity during NDEA-induced hepatocarcinogenesis.[3] α-KG decreased the levels of lipid peroxides and increased levels of antioxidants, like reduced glutathione (GSH) and the activities of glutathione

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peroxidase (GPx) and glutathione-*S*-transferase (GST), indicating that it positively modulates the oxidant–antioxidant imbalance during hepatocarcinogenesis. In addition, melatonin, another potent antioxidant, has also significant chemopreventive function in NDEA-induced hepatocarcinogenesis mainly by reducing lipid peroxidation and by increasing the antioxidant status in liver and blood.[4]

Disruption of circadian rhythms has been associated with cancer in experimental animals and humans.[5] One of the cellular processes that are regulated by circadian rhythm is cell proliferation, which often shows asynchrony between normal and malignant tissues. Several studies have shown that circadian system alterations are not only a risk factor for tumor incidence, but are also related to the progression of existing tumors.[6] Therefore, emerging data suggest that circadian regulation is an important prerequisite for the maintenance of host defenses against cancer.[5]

NDEA generally delayed acrophases of 24-h rhythms in circulating lipid peroxides and antioxidants, an effect partially counteracted by the co-administration of α-KG.[7] In view of the significant activity that melatonin has on the circadian system,[8,9] we considered it worthwhile to compare the effects of melatonin and α-KG on 24-h rhythmicity in antioxidant defenses in NDEA-injected rats.

Materials and Methods

Animals

Male Wistar albino rats (3 months old) weighing 150–170 g and bred in the Central Animal House, Rajah Muthiah Medical College, Annamalainagar, Tamil Nadu, India, were used. The animals were provided with standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum*. The animals (6 per group) were housed in plastic cages under controlled conditions of light (12 h light/12 h dark) with lights on from 06:00 to 18:00 h, humidity (50%) and ambient temperature (30 ± 2°C). 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). The guidelines are available at the web site <http://www.icmr.nic.in/bioethics/INSA_Guidelines.pdf>.

NDEA was purchased from Sigma Chemical Company (St Louis, MO, USA). Melatonin was purchased from Sisco Research Laboratories (Mumbai, India). α-KG was purchased from Hi Media Laboratories (Mumbai, India). All the other chemicals and solvents used in the study were of analytical grade and were obtained from the Sigma Chemical Company or Hi Media Laboratories.

Experimental design

To induce hepatic carcinogenesis, rats received a single necrogenic i.p. injection of NDEA at a dose of 200 mg/kg body weight in 1 ml saline followed by weekly s.c. injections of carbon tetrachloride for 6 weeks at individual doses of 3 ml/kg body weight.[10–12] Melatonin (5 mg/kg body weight) or its vehicle (0.5 ml of 5% ethanol in saline) was given i.p. daily 2 h before lights off for the whole experiment (20 weeks). α-KG was administered at a dose of 2 g/kg body weight by means of an intragastric tube.[13] The doses of melatonin and α-KG were selected on the basis of full prevention of macroscopically detectable hepatic tumors after NDEA treatment.[3,4] Appropriate controls administered with vehicle were included.

At the end of the 20-week period, blood samples were collected from the tail at 6 time points during a 24-h period and the rats were sacrificed, the liver being macroscopically inspected. Blood was collected in heparinized tubes and the plasma was separated by centrifugation at 1000 *g* for 15 min for further biochemical analysis. The packed cells were washed three times with physiological saline; 0.5 ml of the erythrocyte fraction was lyzed with 2.5 ml phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2500 *g* for 15 min at 2°C.

Biochemical assays

As marker enzymes for liver function in serum, the activities of aspartate transaminase (AST) and alanine transaminase (ALT) (expressed as IU/l plasma) were assessed.[14] Plasma α-fetoprotein (α-FP) concentration was measured using a Roche Elecsys 1010/2010 immunoassay analyzer. To assess lipid peroxidation and antioxidant status in liver and blood, the levels of thiobarbituric acid reactive substances (TBARS; expressed as nmol/100 g of tissue or nmol/ml of plasma)[15] and GSH levels (expressed as mg/100 mg of tissue or mg/dl of plasma)[16] were measured. In addition, the activities of superoxide dismutase (SOD),[17] catalase (CAT),[18] GPx,[19] and GST[20] were assessed in liver and erythrocyte fractions.

Enzyme activity was expressed as 'units' as follows: SOD (enzyme required for 50% inhibition of nitroblue tetrazolium reduction/min/mg protein or mg hemoglobin); CAT (μmoles of hydrogen peroxide utilized/min/mg protein or mg hemoglobin); GPx activity (μmoles of GSH utilized/min/mg protein or mg hemoglobin); and GST (μmoles of 1-chloro-2,4-dinitrobenzene–GSH conjugate formed/minute/mg protein or mg hemoglobin).

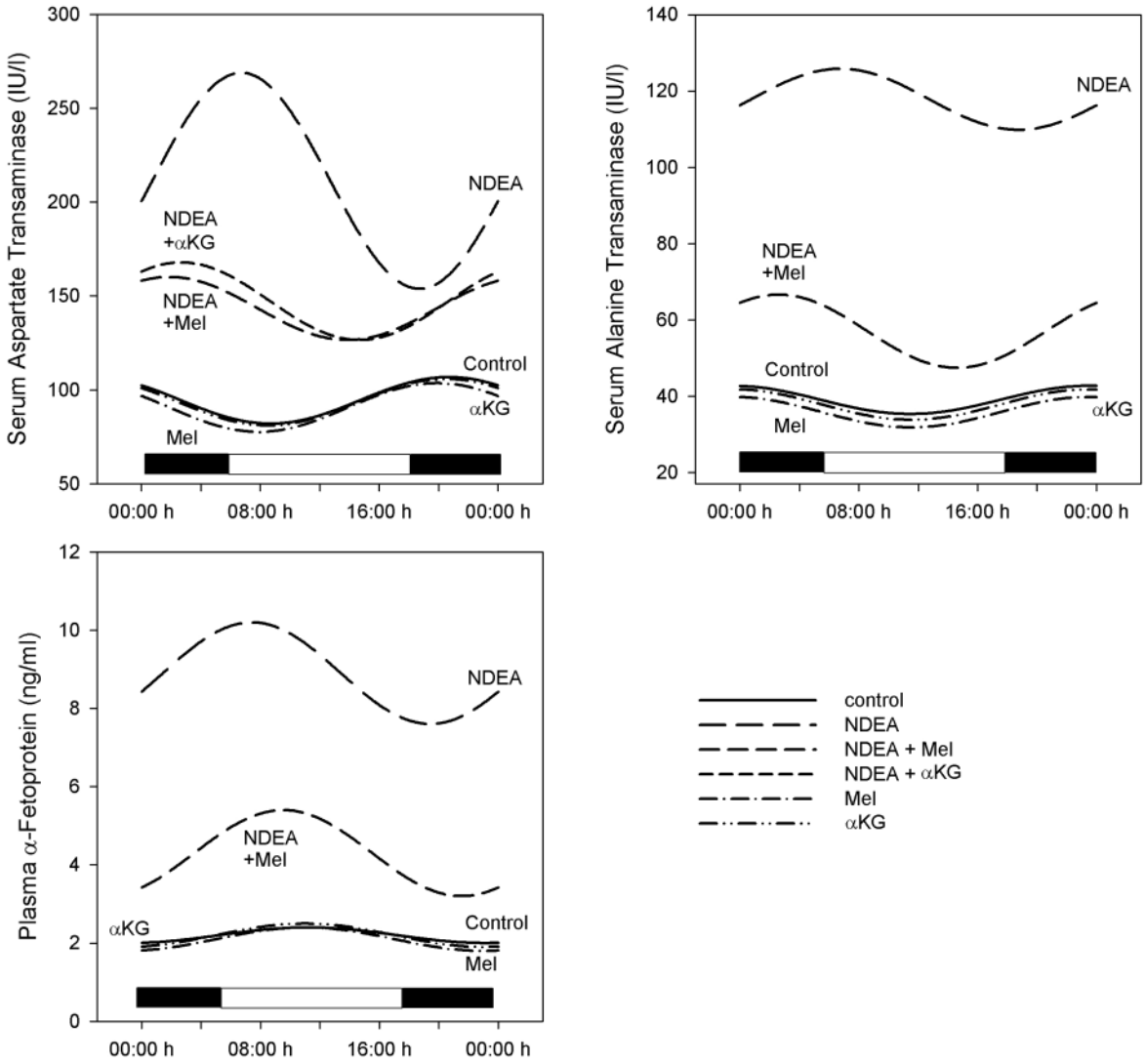

Fig. 1. 24-h Rhythmicity of plasma AST, ALT and α-FP in NDEA-treated rats. Effect of melatonin and α-KG. Cosine curves derived from the results given in Table 1 are shown.

Statistical analysis

The cosinor analysis was used to analyze general rhythmic parameters, *i.e.* acrophase (the maximum of the cosine function fit to the experimental data), mesor (the statistical estimate of the 24-h time series mean) and amplitude (half the difference between maximal and minimal values of the derived cosine curve). Statistical significance of the derived cosine curves was tested against the null hypothesis (*i.e.* amplitude = 0);[21] *P*-values lower than 0.05 were considered evidence for statistical significance.

Statistical analysis of group differences was made by a one-way ANOVA followed by a Student–Newman–Keuls test. *P*-values lower than 0.05 were considered evidence for statistical significance.

RESULTS

Macroscopically detectable tumors developed in the liver of all NDEA treated rats, an effect fully prevented by melatonin or α-KG administration (results not shown). Tables 1–3 and Figures 1–3 summarize the results obtained in the 24-h rhythm study.

Table 1 and Figure 1 depict the results obtained when 24-h changes of circulating AST, ALT and α-FP were measured. For the three markers, a similar profile was obtained, *i.e.* NDEA administration augmented significantly mesor and amplitude of 24-h rhythm. Acrophases for AST and ALT rhythms changed from the first to the second half of scotophase in NDEA-injected rats, while the rhythm of α-FP became phase-advanced by about 4 h. The administration of melatonin together with NDEA

Table 1. 24-h Rhythmicity of plasma AST, ALT and α-FP in NDEA-treated rats; effect of melatonin and α-KG

	Cosinor parameters	1. Control	2. NDEA	3. NDEA + Melatonin	4. NDEA + α-KG	5. Melatonin	6. α-KG
AST activity	Acrophase ϕ (h)	20:40	06:43	01:51	02:40	19:53	20:30
	Mesor (IU/l)	94.4 ± 8.3	211.3 ± 19.2[a]	143.3 ± 13.7[b]	147.2 ± 13.4	90.6 ± 8.1	93.4 ± 7.6
	Amplitude (IU/l)	12.4 ± 1.1	57.6 ± 4.7[c]	16.8 ± 1.5	20.7 ± 1.8	13.0 ± 1.5[d]	12.4 ± 1.1[d]
	r-value	–0.33[dr]	–0.69[dr]	0.59[dr]	0.54[dr]	–0.38[dr]	-0.33[dr]
	P-value	< 0.05	< 0.0001	< 0.0002	< 0.0007	< 0.03	< 0.05
ALT activity	Acrophase ϕ (h)	23:28	06:45	02:36	NS	23:31	23:31
	Mesor (IU/l)	39.0 ± 3.5	117.8 ± 11.0[c]	57.0 ± 5.8[e]	61.5 ± 5.4[e]	35.8 ± 3.2	37.8 ± 3.6
	Amplitude (IU/l)	3.7 ± 0.2	8.0 ± 0.6[b]	9.6 ± 0.9[b]	NS	4.0 ± 0.3	4.0 ± 0.4
	r-value	0.94[dr]	-0.77[dr]	0.33[dr]	0.28	0.92[dr]	0.92[dr]
	P-value	< 0.0001	< 0.0001	< 0.05	NS	< 0.0001	< 0.0001
α-FP	Acrophase ϕ (h)	11:11	07:25	09:32	NS	11:00	10:59
	Mesor (ng/ml)	2.2 ± 0.1	8.9 ± 0.7[c]	4.3 ± 0.3[e]	4.5 ± 0.4[e]	2.1 ± 0.1	2.2 ± 0.2
	Amplitude (ng/ml)	0.2 ± 0.01	1.3 ± 0.1[b]	1.1 ± 0.2[b]	NS	0.3 ± 0.02	0.3 ± 0.01
	r-value	–0.50[dr]	–0.45[dr]	0.33[dr]	0.23	0.54[dr]	–0.56[dr]
	P-value	< 0.01	< 0.006	< 0.05	NS	< 0.01	< 0.01

P-values denote fitness of data to the cosine function.
[dr]Detectable rhythmicity.
Superscripts show significant differences in a one-way ANOVA followed by a Student–Newman–Keuls test, as follows: [a]$P < 0.01$ versus groups 1, 5 and 6, $P < 0.05$ versus groups 3 and 4; [b]$P < 0.01$ versus groups 1, 5 and 6; [c]$P < 0.0001$ versus all groups; [d]$P < 0.05$ versus group 4; and [e]$P < 0.05$ versus groups 1, 5 and 6.

Table 2. 24-h Rhythmicity of plasma TBARS and erythrocyte SOD and CAT activity in NDEA-treated rats: effect of melatonin and α-KG

	Cosinor parameters	1. Control	2. NDEA	3. NDEA + Melatonin	4. NDEA + α-KG	5. Melatonin	6. α-KG
TBARS	Acrophase ϕ (h)	00:00	07:49	04:50	06:59	00:00	00:21
	Mesor (nmol/ml)	2.7 ± 0.1	4.7 ± 0.2[a]	2.9 ± 0.2	2.9 ± 0.2	2.1 ± 0.1[b]	2.4 ± 0.1
	Amplitude (nmol/ml)	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.03	0.4 ± 0.03[a]	0.2 ± 0.01	0.3 ± 0.02[c]
	r-value	0.96[dr]	-0.45[dr]	–0.43[dr]	+0.79[dr]	0.98[dr]	0.97[dr]
	P-value	< 0.0001	< 0.006	< 0.01	< 0.0001	< 0.0001	< 0.0001
SOD activity	Acrophase ϕ (h)	11:32	16:46	13:30	14:10	11:55	12:14
	Mesor (Units)	3.6 ± 0.2	1.5 ± 0.1[a]	2.5 ± 0.1[d]	2.2 ± 0.2[d]	4.2 ± 0.2	3.9 ± 0.1
	Amplitude (Units)	0.2 ± 0.01	0.3 ± 0.02	0.4 ± 0.03[e]	0.2 ± 0.01	0.3 ± 0.02	0.4 ± 0.03
	r-value	0.93[dr]	–0.71[dr]	0.87[dr]	0.49[dr]	0.94[dr]	0.94[dr]
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.01	< 0.0001	< 0.0001
CAT activity	Acrophase ϕ (h)	07:19	13:37	10:29	11:19	07:10	07:90
	Mesor (Units)	2.9 ± 0.2	1.6 ± 0.2[f]	2.2 ± 0.2	2.0 ± 0.2	3.3 ± 0.3	3.1 ± 0.2
	Amplitude (Units)	0.3 ± 0.02	0.2 ± 0.01	0.3 ± 0.03	0.3 ± 0.04	0.4 ± 0.1	0.3 ± 0.1
	r-value	–0.66[dr]	0.66[dr]	0.70[dr]	0.91[dr]	0.81[dr]	–0.75[dr]
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

P-values denote fitness of data to the cosine function.
[dr]Detectable rhythmicity.
Superscripts show significant differences in a one-way ANOVA followed by a Student–Newman–Keuls test, as follows: [a]$P < 0.01$ versus all groups; [b]$P < 0.05$ versus group 1, $P < 0.01$ versus groups 3 and 4; [c]$P < 0.05$ versus all groups; [d]$P < 0.01$ versus groups 1, 5 and 6; [e]$P < 0.05$ versus groups 1, 2, 4 and 5; [f]$P < 0.05$ versus group 3, $P < 0.01$ versus groups 1, 4, 5 and 6.

partly restored mesor, amplitude and acrophase values of AST, ALT and α-FP 24-h rhythms. In the case of the simultaneous administration of NDEA and α-KG, cosinor analysis did not attain significance for rhythmicity of ALT or α-FP. Neither melatonin nor α-KG given alone changed cosinor parameters for AST, ALT and α-FP as compared to controls (Table 1 and Fig. 1).

As shown in Table 2 and Figure 2 (left upper panel), NDEA treatment augmented significantly plasma TBARS mesor and delayed the acrophase from the first to the second half of the scotophase. Co-administration of melatonin or α-KG partially counteracted the effects of NDEA. Melatonin administration to control rats decreased significantly mesor values of plasma TBARS. Erythrocyte SOD and CAT activities exhibited similar changes after treatment, *i.e*. NDEA decreased mesor values and phase delayed 24-h rhythms by about 3–5 h, both effects being partially counteracted by the simultaneous administration of melatonin or α-KG (Table 2 and Fig. 2, right upper and lower panels). Melatonin administration augmented significantly mesor of 24-h rhythm in SOD activity as compared to control.

Table 3 and Figure 3 summarize the results obtained when the 24-h rhythms of plasma GSH concentration and erythrocyte GPx and GST activities were measured. For all three parameters, the administration of NDEA decreased mesor values and phase delayed acrophases significantly, an effect that was partially counteracted by the concomitant administration of melatonin or α-KG.

Discussion

NDEA is a potent carcinogenic agent that induces liver cancer together with an increase in reactive oxygen species (ROS) generation. NDEA is metabolized pri-

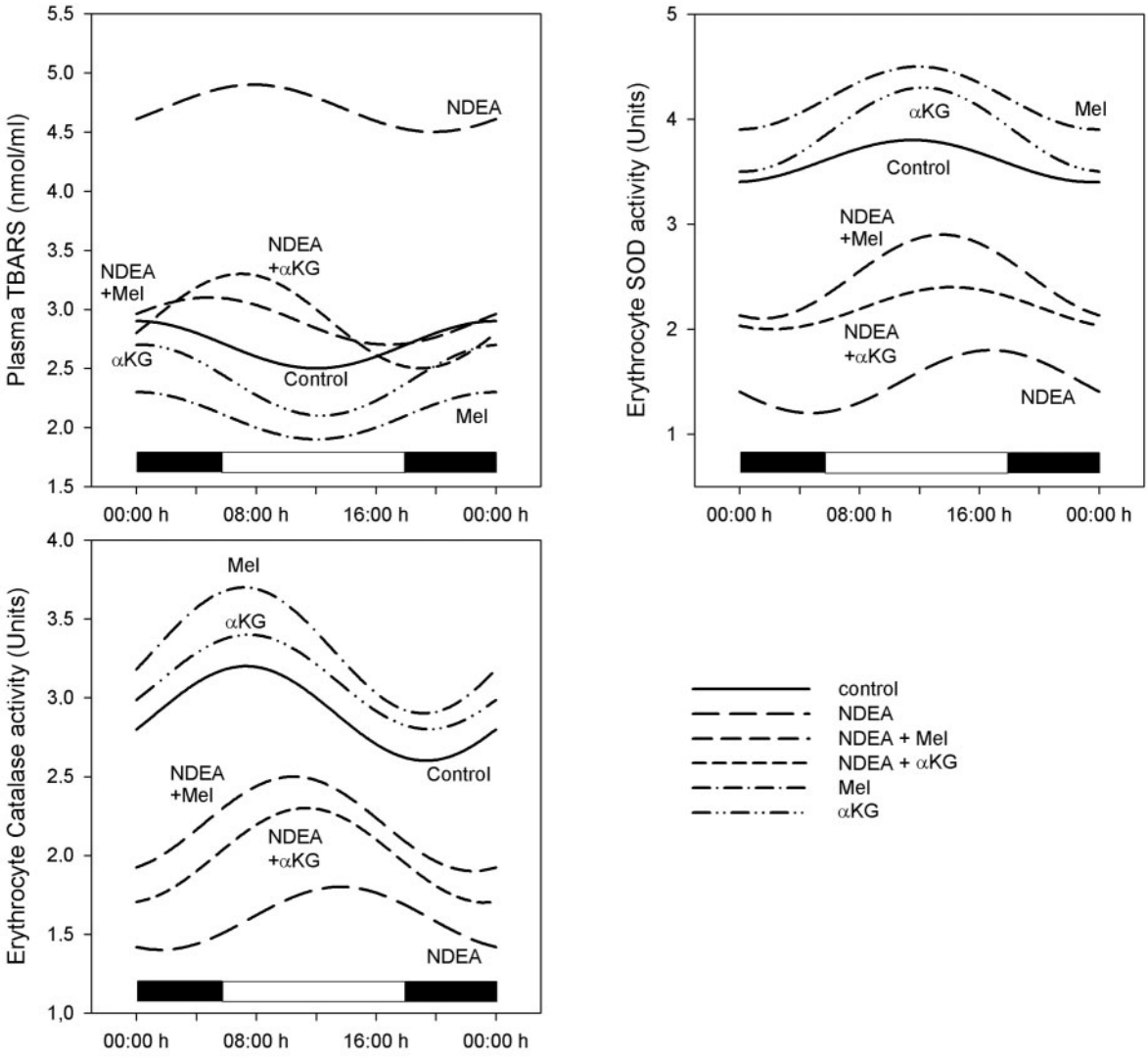

Fig. 2. 24-h Rhythmicity of plasma TBARS and erythrocyte SOD and CAT activity in NDEA-treated rats. Effect of melatonin and α-KG Cosine curves derived from the results given in Table 2 are shown.

Table 3. 24-h Rhythmicity of plasma GSH and erythrocyte GPx and GST activity in NDEA-treated rats: effect of melatonin and α-KG

	Cosinor parameters	1. Control	2. NDEA	3. NDEA + Melatonin	4. NDEA + α-KG	5. Melatonin	6. α-KG
GSH	Acrophase ϕ (h)	07:48	19:17	11:31	13:54	07:18	07:46
	Mesor (mg/dl)	37.9 ± 3.0	15.4 ± 1.1[a]	24.7 ± 2.2[b]	23.9 ± 2.2[b]	40.9 ± 3.5	40.8 ± 3.1
	Amplitude (mg/dl)	5.1 ± 0.4	3.0 ± 0.3[c]	4.5 ± 0.4	2.4 ± 0.2[d]	5.5 ± 0.4	5.8 ± 0.5
	r-value	–0.48[dr]	–0.71[dr]	0.96[dr]	0.56[dr]	–0.71[dr]	–0.50[dr]
	P-value	< 0.004	< 0.0001	< 0.0001	< 0.0005	< 0.0001	< 0.002
GPx	Acrophase ϕ (h)	04:28	17:50	10:22	11:16	04:33	04:28
	Mesor (Units)	30.3 ± 1.8	14.8 ± 1.1[e]	20.8 ± 1.6[c]	18.5 ± 1.1[c]	35.1 ± 2.0	31.0 ± 1.9
	Amplitude (Units)	4.3 ± 0.3	2.9 ± 0.3[f]	3.5 ± 0.2	3.0 ± 0.3	4.6 ± 0.4	4.3 ± 0.4
	r-value	–0.6[dr]	–0.84[dr]	0.69[dr]	0.92[dr]	–0.64[dr]	–0.61[dr]
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
GST	Acrophase ϕ (h)	13:46	19:34	16:57	17:32	13.47	13:53
	Mesor (Units)	5.8 ± 0.4	2.0 ± 0.1[g]	4.5 ± 0.3[h]	4.2 ± 0.3[h]	5.9 ± 0.4	5.8 ± 0.5
	Amplitude (Units)	1.6 ± 0.1	0.8 ± 0.06[g]	1.6 ± 0.1	2.4 ± 0.2[i]	1.5 ± 0.1	1.5 ± 0.2
	r-value	0.62[dr]	–0.59[dr]	–0.71[dr]	–0.94[dr]	0.63[dr]	0.59[dr]
	P-value	< 0.0001	< 0.0003	< 0.0001	< 0.0001	< 0.0001	< 0.0003

P-values denote fitness of data to the cosine function.
[dr]Detectable rhythmicity.
Superscripts show significant differences in a one-way ANOVA followed by a Student–Newman–Keuls test, as follows: [a]$P < 0.001$ versus groups 1, 5 and 6, $P < 0.05$ versus groups 3 and 4; [b]$P < 0.001$ versus groups 5 and 6; [c]$P < 0.01$ versus groups 1, 5 and 6; [d]$P < 0.001$ versus groups 1, 3, 5 and 6, $P < 0.05$ versus group 2; [e]$P < 0.001$ versus groups 1, 5 and 6, $P < 0.05$ versus group 3; [f]$p < 0.05$ versus groups 1 and 6, $P < 0.01$ versus group 5; [g]$P < 0.01$ versus all groups; [h]$P < 0.05$ versus groups 1, 5 and 6; and [i]$P < 0.05$ versus all groups.

marily in the liver by cytochrome P450 enzyme activity to ethyl-acetoxyethyl-nitrosamine.[1,2] This intermediate can be metabolized by the phase II enzymes to a non-toxic compound or it can produce ethyl-diazonium ion, which directly ethylates cellular macromolecules.[10] Metabolic activation of NDEA by cytochrome P450 enzymes is responsible for its cytotoxic, mutagenic and carcinogenic effects.[22] Instrumental to them is the increased in ROS production caused by NDEA.

The foregoing results indicate that macroscopically detectable hepatic tumors after NDEA were prevented if α-KG or melatonin were simultaneously injected. NDEA administration augmented significantly mesor and amplitude of 24-h rhythms of AST and ALT activity and plasma α-FP levels (all indexes of liver damage). Acrophase of AST and ALT activity rhythm was delayed by NDEA treatment while that of α-FP became phase-advanced. The administration of α-KG or melatonin partially counteracted the effects of NDEA on 24-h liver damage parameters.

ROS generation is a major factor involved in all steps of carcinogenesis, including initiation, promotion, and progression (for references see elsewhere[23–26]). Lipid peroxidation by-products have a crucial role in the early phases of tumor growth if they are excessively generated. The ability of lipid peroxidation in generating mutagenesis and DNA adducts formation suggest their possible role in carcinogenesis.[27] The foregoing results indicate that NDEA augmented significantly mesor values of 24-h rhythm in plasma lipid peroxidation (TBARS), and decreased mesor values of 24-h rhythms in plasma GSH concentration and erythrocyte SOD, CAT, GPx and GST activities. Acrophases were phase-delayed by NDEA treatment in all cases. Again, the co-administration of melatonin or α-KG partially counteracted the effects of NDEA. In addition, melatonin, but not α-KG, decreased significantly plasma lipid peroxidation and augmented significantly mesor of SOD activity as compared to controls.

Melatonin has long been recognized as the chief pineal secretory product and later it has emerged as a widely distributed compound, locally synthesized in several organs and tissues to serve as an autacoids.[28] In pharmacological amounts, melatonin effectively reduces oxidative stress through several mechanisms (for references see elsewhere[28,29]). Melatonin scavenges hydrochlorous acid, detoxifies highly toxic hydroxyl radical and peroxyl radical *in vitro* and scavenges peroxynitrite. Melatonin has also been reported to increase the synthesis of GSH and of several antioxidant enzymes. As a result of oxidation, melatonin gives rise to a cascade of antioxidant compounds like cyclic 3-hydroxymelatonin,

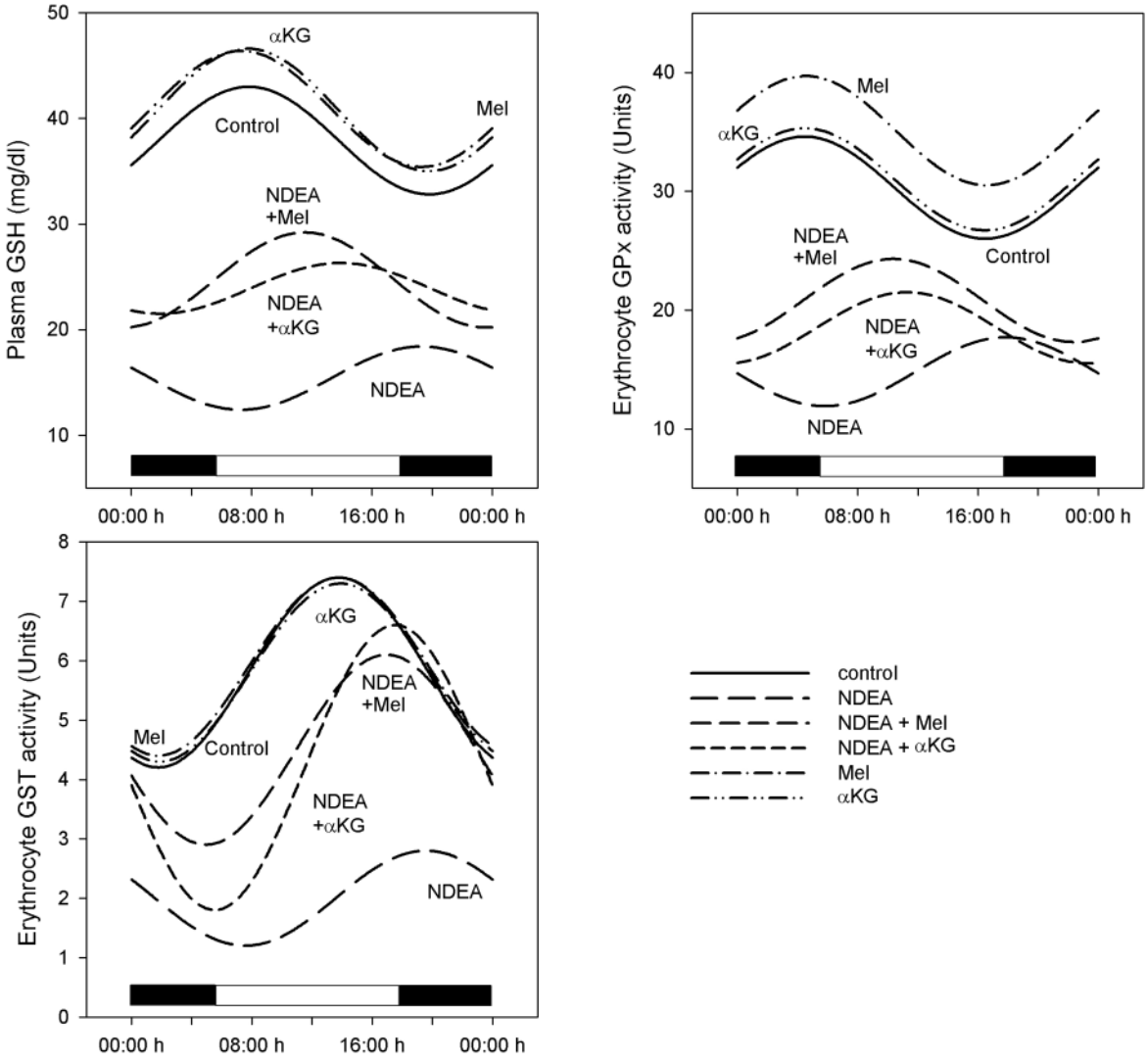

Fig. 3. 24-h Rhythmicity of plasma GSH and erythrocyte GPx and GST activity in NDEA-treated rats. Effect of melatonin and α-KG. Cosine curves derived from the results given in Table 3 are shown.

N[1]-acetyl-*N*[2]-formyl-5-methoxykynuramine and, with a highest potency, *N*[1]-acetyl-5-methoxykynuramine. Therefore, melatonin is considered to be a broad spectrum antioxidant that is more powerful than GSH in neutralizing ROS and that can protect cell membranes more effectively than other antioxidants.[28] It must be noted, however, that under certain circumstances, a pro-oxidant effect of melatonin has been reported. For example, pro-oxidant actions of melatonin take place in the human liver cell line HepG230 while melatonin promoted fas-induced cell death in human leukemic Jurkat cells via a pro-oxidant effect.[31] Moreover, the pro-oxidant activity of the melatonin metabolite 6-hydroxymelatonin, involving a unique non-o-quinone type of redox cycle, has also been documented.[32]

Several studies support the efficacy of melatonin to prevent the development of liver cancer. For example, melatonin treatment decreased preneoplastic liver lesions in F344 rats administered with NDEA[33] and concurrent NDEA administration and constant light exposure, resulting in inhibition of melatonin synthesis, accelerated malignant transformation and growth and shortened survival of rats.[34] Melatonin administration had a significant chemopreventive function in NDEA-induced hepatocarcinogenesis in rats.[4]

In addition to its antioxidant properties, melatonin is the prototype of the chronobiotics, *i.e.* substances capable of shifting the phase of the circadian time system; therefore, it can act by re-entraining circadian rhythmicity.[8,9] This activity can be relevant if circadian periodicity is taken as an index of host and tumor cell proliferation and for response to cancer treatments.[5] In patients with metastatic colorectal cancer, severe alterations of the rest–activity circadian rhythm predicted for

a 5-fold increase in the risk of death as compared to a normal rest-activity pattern.[35] Similarly, an abnormal cortisol rhythm in patients with metastatic breast cancer predicted for a doubling of the risk of death as compared to those with a normal cortisol pattern.[36] Being a potent phase shifter in humans, melatonin administration must strictly be performed at the correct time in the phase–response curve to avoid circadian disruption.[37]

It is possible that the circadian system negatively controls malignant processes through circadian physiology and/or molecular clocks.[38] Disruption of clock gene function may increase cancer risk.[39] In mice, clock genes stabilize the genome and help maintain important repair mechanisms such as the apoptosis of damaged cells.[6] Per-2 gene deprived mice lack a circadian rhythm[40] and have been shown to develop cancer rapidly and spontaneously. Therefore, one mechanism through which melatonin may prevent hepatic cancer development is through maintenance of circadian rhythmicity. In addition, other mechanisms by which melatonin can exert its oncostatic actions could involve its antioxidant action,[28,29] enhancement of immune mechanisms[41] or uptake inhibition of key factors for tumor growth and tumor growth signaling molecules (*e.g*. linoleic acid).[42]

Conclusions

Collectively, the foregoing data indicate that melatonin or α-KG modulate 24-h rhythmicity of circulating lipid peroxidation and antioxidants. We previously demonstrated (in liver and brain of rats treated for 45 days with 0.5 or 1.0 mg/kg of melatonin) a significant decrease in lipid peroxidation and in the levels of cholesterol, phospholipids, triglycerides and free fatty acids in both tissues.[43] Concomitantly, treatment with melatonin augmented the activity of the brain and liver SOD, CAT and GPx as well as increased GSH levels, resembling the decrease in lipid peroxidation the augmented SOD activity reported herein in melatonin-injected rats.

References

1. IARC. N-nitrosodiethanolamine. *IARC Monogr Eval Carcinog Risks Hum* 2000; **77**: 403–438.
2. Loeppky RN. The mechanism of bioactivation of N-nitrosodiethanolamine. *Drug Metab Rev* 1999; **31**: 175–193.
3. Dakshayani KB, Subramanian P, Manivasagam T, Mohamed EM. Metabolic normalization of alpha-ketoglutarate against N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. *Fundam Clin Pharmacol* 2006; **20**: 477–480.
4. Subramanian P, Mirunalini S, Dakshayani K, Pandi-Perumal SR, Trakht I, Cardinali DP. Prevention by melatonin of hepatocarcinogenesis in rats injected with N-nitrosodiethylamine. *J Pineal Res* 2007; **43**: 305–312.
5. Levi F, Schibler U. Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol* 2007; **47**: 593–628.
6. Fu L, Lee CC. The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer* 2003; **3**: 350–361.
7. Dakshayani KB, Subramanian P. Alpha-ketoglutarate modulates the circadian patterns of lipid peroxidation and antioxidant status during N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. *J Med Food* 2006; **9**: 90–97.
8. Arendt J, Skene DJ. Melatonin as a chronobiotic. *Sleep Med Rev* 2005; **9**: 25–39.
9. Cardinali DP, Furio AM, Reyes MP, Brusco LI. The use of chronobiotics in the resynchronization of the sleep-wake cycle. *Cancer Causes Control* 2006; **17**: 601–609.
10. Muzio G, Marengo B, Salvo R, Semeraro A, Canuto RA, Tessitore L. Liver cancer is induced by a subnecrogenic dose of DENA when associated with fasting/refeeding: role of glutathione-transferase and lipid peroxidation. *Free Radic Biol Med* 1999; **26**: 1314–1320.
11. Sundaresan S, Subramanian P. *S*-Allylcysteine inhibits circulatory lipid peroxidation and promotes antioxidants in N-nitrosodiethylamine-induced carcinogenesis. *Pol J Pharmacol* 2003; **55**: 37–42.
12. Dakshayani KB, Subramanian P, Manivasagam T, Essa MM, Manoharan S. Melatonin modulates the oxidant-antioxidant imbalance during N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *J Pharm Pharm Sci* 2005; **8**: 316–321.
13. Velvizhi S, Dakshayani KB, Subramanian P. Protective influences of alpha-ketoglutarate on lipid peroxidation and antioxidant status in ammonium acetate treated rats. *Indian J Exp Biol* 2002; **40**: 1183–1186.
14. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; **28**: 56–63.
15. Niehaus Jr WG, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem* 1968; **6**: 126–130.
16. Ellman GL. Tissue sulphydryl groups. *Arch Biochem Biophys* 1959; **82**: 70–79.
17. Kakkar P, Das B, Viswanathan A. A modified spectrophotometric assay of superoxide dismutase. *Ind J Biochem Biophys* 1984; **21**: 130–132.
18. Sinha KA. Colorimetric assay of catalase. *Anal Biochem* 1972; **47**: 3889–3894.
19. Rotruck JT, Pope AL, Gauther HE, Swanson AB, Hafeman DG, Hoekstna WG. Selenium: biochemical roles as component of glutathione peroxidase. *Science* 1973; **179**: 588–590.
20. Habig WH, Pabst MJ, Jakoby WB. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130–7139.
21. Nelson W, Tong YL, Lee JK, Halberg F. Methods for cosinor-rhythmometry. *Chronobiologia* 1979; **6**: 305–323.
22. Archer MC. Mechanisms of action of N-nitroso compounds. *Cancer Surv* 1989; **8**: 241–250.
23. Ozben T. Oxidative stress and apoptosis: impact on cancer therapy. *J Pharm Sci* 2007; **96**: 2181–2196.
24. Flora SJ. Role of free radicals and antioxidants in health and disease. *Cell Mol Biol (Noisy-le-grand)* 2007; **53**: 1–2.
25. Hirst DG, Robson T. Nitrosative stress in cancer therapy. *Front Biosci* 2007; **12**: 3406–3418.
26. Serafini M, Villano D, Spera G, Pellegrini N. Redox molecules and cancer prevention: the importance of understanding the role of the antioxidant network. *Nutr Cancer* 2006; **56**: 232–240.
27. Otteneder MB, Knutson CG, Daniels JS *et al*. *In vivo* oxidative metabolism of a major peroxidation-derived DNA adduct, M1dG. *Proc Natl Acad Sci USA* 2006; **103**: 6665–6669.

28. Reiter RJ, Tan DX, Terron MP, Flores LJ, Czarnocki Z. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. *Acta Biochim Pol* 2007; **54**: 1–9.
29. Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J Pineal Res* 2007; **42**: 28–42.
30. Osseni RA, Rat P, Bogdan A, Warnet JM, Touitou Y. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. *Life Sci* 2000; **68**: 387–399.
31. Wolfler A, Caluba HC, Abuja PM, Dohr G, Schauenstein K, Liebmann PM. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. *FEBS Lett* 2001; **502**: 127–131.
32. Sakano K, Oikawa S, Hiraku Y, Kawanishi S. Oxidative DNA damage induced by a melatonin metabolite, 6-hydroxymelatonin, via a unique non-o-quinone type of redox cycle. *Biochem Pharmacol* 2004; **68**: 1869–1878.
33. Imaida K, Hagiwara A, Yoshino H *et al*. Inhibitory effects of low doses of melatonin on induction of preneoplastic liver lesions in a medium-term liver bioassay in F344 rats: relation to the influence of electromagnetic near field exposure. *Cancer Lett* 2000; **155**: 105–114.
34. van den HS, Depres-Brummer P, Barbason H, Claustrat B, Reynes M, Levi F. The tumor promoting effect of constant light exposure on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Life Sci* 1999; **64**: 2523–2534.
35. Levi F. Circadian chronotherapy for human cancers. *Lancet Oncol* 2001; **2**: 307–315.
36. Sephton SE, Sapolsky RM, Kraemer HC, Spiegel D. Diurnal cortisol rhythm as a predictor of breast cancer survival. *J Natl Cancer Inst* 2000; **92**: 994–1000.
37. Wirz-Justice A, Krauchi K, Cajochen C, Danilenko KV, Renz C, Weber JM. Evening melatonin and bright light administration induce additive phase shifts in dim light melatonin onset. *J Pineal Res* 2004; **36**: 192–194.
38. Moser M, Schaumberger K, Schernhammer E, Stevens RG. Cancer and rhythm. *Cancer Causes Control* 2006; **17**: 483–487.
39. Filipski E, Li XM, Levi F. Disruption of circadian coordination and malignant growth. *Cancer Causes Control* 2006; **17**: 509–514.
40. Lee CC. Tumor suppression by the mammalian Period genes. *Cancer Causes Control* 2006; **17**: 525–530.
41. Anisimov VN, Popovich IG, Zabezhinski MA, Anisimov SV, Vesnushkin GM, Vinogradova IA. Melatonin as antioxidant, geroprotector and anticarcinogen. *Biochim Biophys Acta* 2006; **1757**: 573–589.
42. Blask DE, Dauchy RT, Sauer LA. Putting cancer to sleep at night: the neuroendocrine/circadian melatonin signal. *Endocrine* 2005; **27**: 179–188.
43. Subramanian P, Mirunalini S, Pandi-Perumal SR, Trakht I, Cardinali DP. Melatonin treatment improves the antioxidant status and decreases lipid content in brain and liver of rats. *Eur J Pharmacol* 2007; **571**: 116–119.