Modulation of biochemical circadian rhythms during long-term melatonin treatment in rats

Sankaran M, Subramanian P

ABSTRACT

Introduction: The influences of chronic administration of low and high doses of melatonin on the characteristics of circadian rhythms of glucose, reduced glutathione, total protein were studied, in order to investigate whether melatonin could modulate these rhythms differently.

Methods: Pharmacological doses of melatonin (0.5mg/kg and 1.0mg/kg body weight) were administered chronically for 45 days to Wistar rats, and 24-hour rhythms of glucose, reduced glutathione (GSH), total protein and melatonin (MLT) were studied under semi-natural (LD 12:12 hours) conditions.

Results: Exogenous melatonin administered caused delays in the acrophase of glucose, total protein and melatonin rhythms, whereas advances in the acrophases of reduced glutathione were observed. This indicated that the chronic administration of melatonin could act as the modulated internal *zeitgeber* and this could be the reason for altered acrophase (peak time of the variable) and other characteristics of rhythms in the melatonin-treated groups. Significant dose-dependent effects of melatonin were absent in the study.

Conclusion: The present study demonstrates that the exogenous administration of melatonin could influence the biochemical rhythms.

Keywords: circadian rhythm, glucose, melatonin, reduced glutathione, total protein

Singapore Med J 2006; 47(1):42-47

Department of Biochemistry
Faculty of Science
Annamalai University
Annamalainagar 608 002
Tamil Nadu
India

Sankaran M, MSc, MPhil
Research Scholar

Subramanian P, MSc, PhD
Reader

Correspondence to:
Dr Perumal Subramanian
Tel: (91) 4144 238 343 ext 210
Fax: (91) 4144 238 080
Email: psub@rediffmail.com

INTRODUCTION

The endogenous circadian rhythms govern most aspects of physiological and biochemical processes in mammals, including body temperature levels, endocrine functions and enzyme levels. The suprachiasmatic nucleus (SCN) constitutes the circadian pacemaker in mammals[1]. Melatonin secretion, regulated by SCN, is also thought to feedback upon these nuclei to modulate some circadian functions. Melatonin rhythm plays an important role in the time-keeping mechanism in the hypothalamo-hypophyseal-gonadal axis[2]. Further, melatonin rhythm plays a major role for internal signaling of photoperiodic changes, leading to the expression of seasonality of the physiological status[3]. The daily rhythm of melatonin secretion is regulated by the light-dark cycles: activated by darkness and inhibited by light. In most of the mammalian species studied to date, the nocturnal peak of melatonin secretion is positively correlated with the length of the dark period. Further, exogenously-administered melatonin was shown to influence a number of clock functions[4].

Melatonin (N-acetyl-5-methoxytryptamine) is a non-toxic naturally-occurring chemical mediator derived primarily from the pineal gland. It is a highly diffusable molecule and crosses the blood-brain barrier and penetrates into every type of cell organelle. The rhythmic secretion of melatonin acts as an internal synchroniser for the timing of daily events and is of promise for treatment of circadian disturbances[5]. Moreover, several lines of evidence, which strongly support the importance of melatonin in the regulation of circadian functions, include (1) the presence of high affinity melatonin receptors in the SCN, (2) physiological sensitivity of SCN to exogenous melatonin, and (3) entrainment of circadian rhythm by exogenous melatonin[6].

In mammals, the circadian patterns in the levels of a number of biochemical variables, hormones, oxidative and antioxidative status were reported[7,8]. However, the influences of long-term melatonin administration on temporal patterns of biochemical variables were not investigated so far. In the present study, the influences of chronic administration of low (0.5mg/kg body weight) and high (1.0mg/kg body weight) doses of melatonin on the characteristics (acrophase, amplitude and mesor) of circadian rhythms

of glucose, reduced glutathione, total protein studied, in order to investigate whether melatonin could modulate these rhythms differently.

METHODS

Adult male Wistar rats (180-200g) were obtained from Central Animal House, Faculty of Medicine, Annamalai University. The animals were treated and handled in accordance with the rules and instructions of Ethical Committee on Animal Care of Annamalai University and the Indian laws on animal care and use. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals[9]. The rats were housed in polypropylene cages (45x24x15cm) at room temperature (30±2ºC) under semi-natural light-dark (12:12 hours) conditions. In Annamalainagar, (11º24'N, 79º42'E) the light-dark (LD) cycle is almost 12:12 hours throughout the year. Animals were maintained in natural light-dark cycles (12:12 hours) in an experimental room simulating the natural conditions[10]. The animals received a diet of standard pellets (Hindustan Lever Ltd., Mumbai, India). Food and water were available *ad libitum* to the animals and replenished daily.

The experimental animals were randomised and divided into three groups of six animals each: control (group I) treated with saline (ip) daily for 45 days, and melatonin (0.5 and 1.0mg/kg body weight, respectively for groups II and III) was injected intraperiotonially (ip) to Wistar rats every day between 17:00 – 18:30 hours (at irregular time points, to avoid injection as an additional time cue) for 45 days. Melatonin was obtained from Sisco Research Laboratories Private Ltd, Mumbai, India. The doses were selected based on previous investigations[11,12]. Melatonin was administered nearly at the end of light period/ onset of dark period since administration during day time was found to be ineffective and melatonin is prone to rapid degradation[4].

Blood samples were collected from animals (groups I, II and III) at four-hour intervals (00:00, 04:00, 08:00, 12:00, 16:00, 20:00 and 24:00 hours) throughout the 24-hour period continuously. Minimal amount of the blood (0.5ml) was collected from the orbital sinus with great care using heparinised tubes[10]. The biochemical variables chosen were studied separately in control and experimental rats. A low volume of blood was collected at each sampling time to minimise the effects of stress and disturbances that might influence the results[10]

Fig. 1 Effect of melatonin dose on glucose levels.

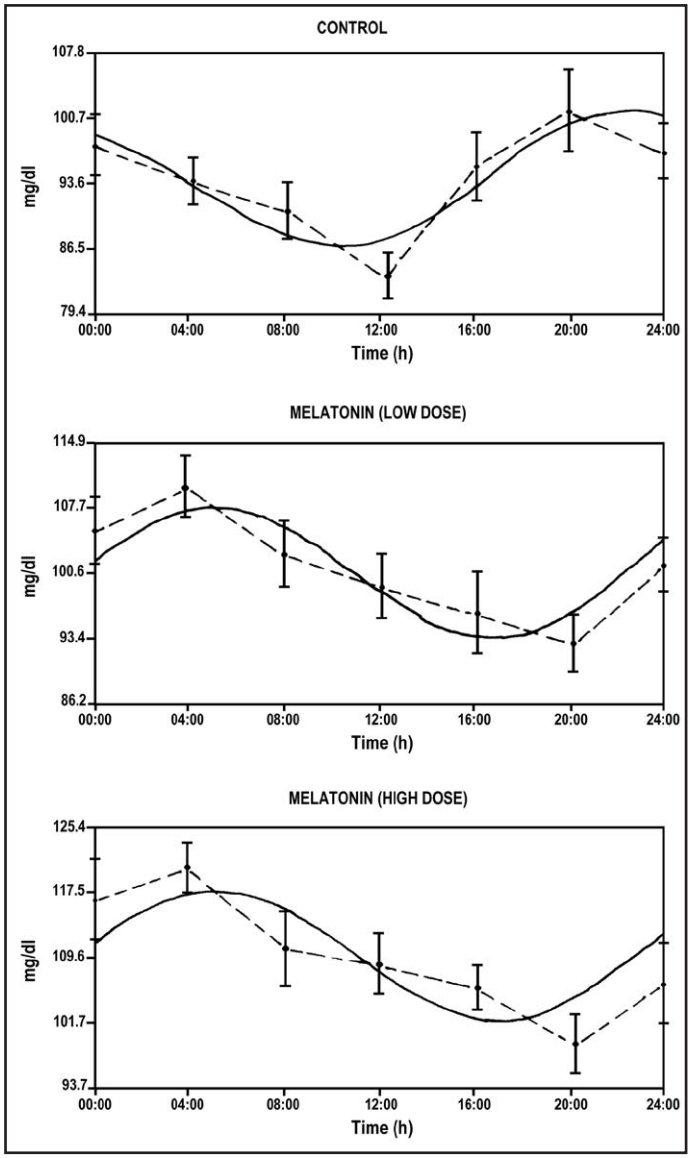


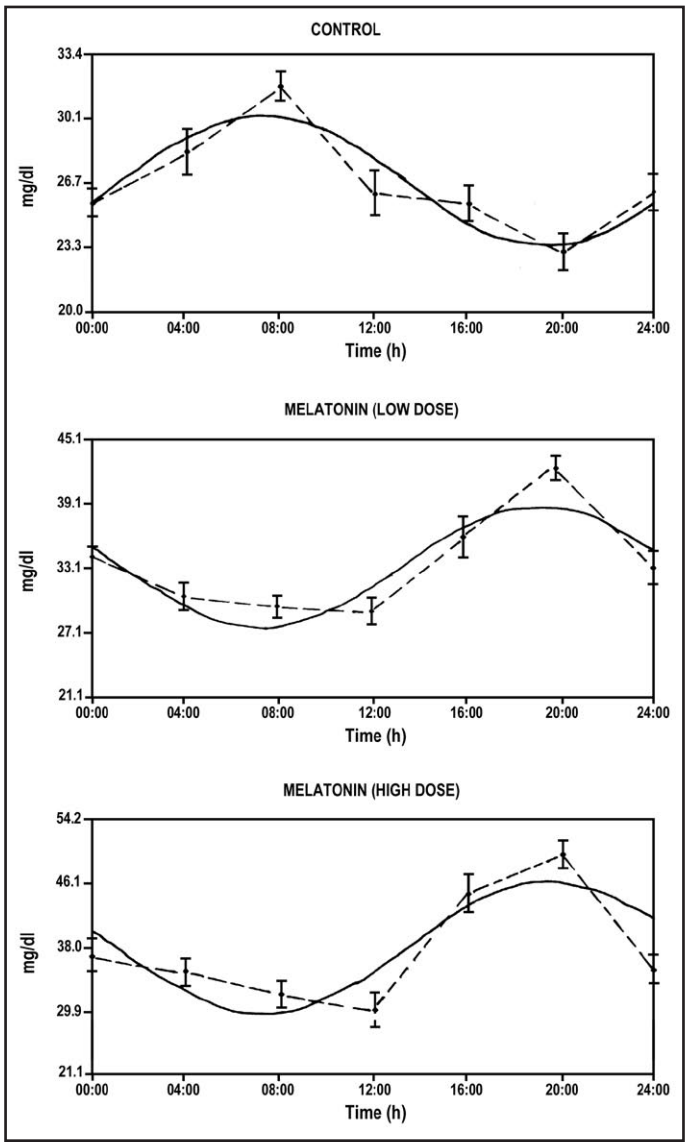
Table I. Average body weights of control and experiment animals.

Groups	Initial weight+ (g)	Final weight+ (g)	Weight gain+ (g)
Control	183 ± 15.50	230 ± 20.14	47 ± 4.11
Melatonin (0.5 mg/kg body weight)	184 ± 16.12	275 ± 21.96	90 ± 6.94*
Melatonin (1.0 mg/kg body weight)	180 ± 15.95	293 ± 24.41	103 ± 9.72*

*p: <0.001 (Students t – test).

+ Values are expressed as mean ± standard deviation (n=6).

Fig. 2 Effect of melatonin dose on reduced glutathione levels.



and plasma was separated immediately. Levels of glucose[13], reduced glutathione[14], total protein[15], and melatonin[16] were estimated at particular time points, immediately after the collection of blood.

Melatonin was assayed by double antibody radioimmunoassay using RIA kit (ALPCO American Laboratories, USA). Reverse-phase column extracted samples (plasma), standards and control were incubated with 10 μL of anti-melatonin antibody and ^{125}I melatonin. After 20 h incubation, 10 μL of solid-phase second antibody was added to the mixtures in order to precipitate the antibody bound fraction. After aspirating the unbound fraction, the antibody bound fraction of ^{125}I melatonin was counted using gamma scintillation counter.

The values of the variables (mean ± SD) were plotted versus the time of blood collection. Measurements of acrophase (ϕ-measure of peak time of the variable studied), amplitude (A-corresponds to half of the total rhythmic variability in a cycle), mesor (M-rhythm adjusted mean), r and p values were performed by using "cosinorwin" computer software program.

RESULTS

The body weights of melatonin (0.5mg and 1.0mg/kg body weight) treated rats were increased significantly when compared to control rats (Table I). Control animals showed the maximum levels of glucose at 22:18 hours; in melatonin-treated animals, the peak time were at 04:52 hours and 04:59 hours, respectively (~ six hours advance) (Fig. 1). Amplitude and mesor values were increased in melatonin-treated animals (Table II). The levels of

Table II. Characteristics (acrophase, amplitude and mesor) of circadian patterns of biochemical variables in control and melatonin-treated rats.

Biochemical variables	Characteristics of rhythms	Control animals	Melatonin-treated animals	
			0.5 mg/kg*	1.0 mg/kg*
1. Glucose (mg/dL)	Acrophase ϕ (h)	22:18	04:52	04:59
	Amplitude	7.0	7.1	7.9
	Mesor	93.6 ± 7.11	100.6 ± 8.12	109.6 ± 8.21
	r-value	0.63[dr] (p<0.01)	0.69[dr] (p<0.005)	0.66[dr] (p<0.005)
2. Reduced glutathione (mg/dL)	Acrophase ϕ (h)	07:15	19:20	19:21
	Amplitude	3.3	5.9	8.1
	Mesor	26.7 ± 2.43	33.1 ± 2.81	38.0 ± 2.88
	r-value	0.67[dr] (p<0.005)	0.61[dr] (p<0.01)	0.6[dr] (p<0.01)
3. Total protein (mg/dL)	Acrophase ϕ (h)	07:27	13:39	13:45
	Amplitude	0.8	0.5	0.5
	Mesor	5.2 ± 0.38	5.6 ± 0.41	5.9 ± 0.45
	r-value	0.61[dr] (p<0.01)	0.65[dr] (p<0.005)	0.62[dr] (p<0.01)
4. Melatonin (pg/ml)	Acrophase ϕ (h)	22:17	23.55	23:59
	Amplitude	5.5	6.4	6.8
	Mesor	31.4 ± 2.71	51.0 ± 4.24	60.6 ± 5.33
	r-value	0.63[dr] (p<0.010)	0.98[dr] (p<0.001)	0.98[dr] (p<0.001)

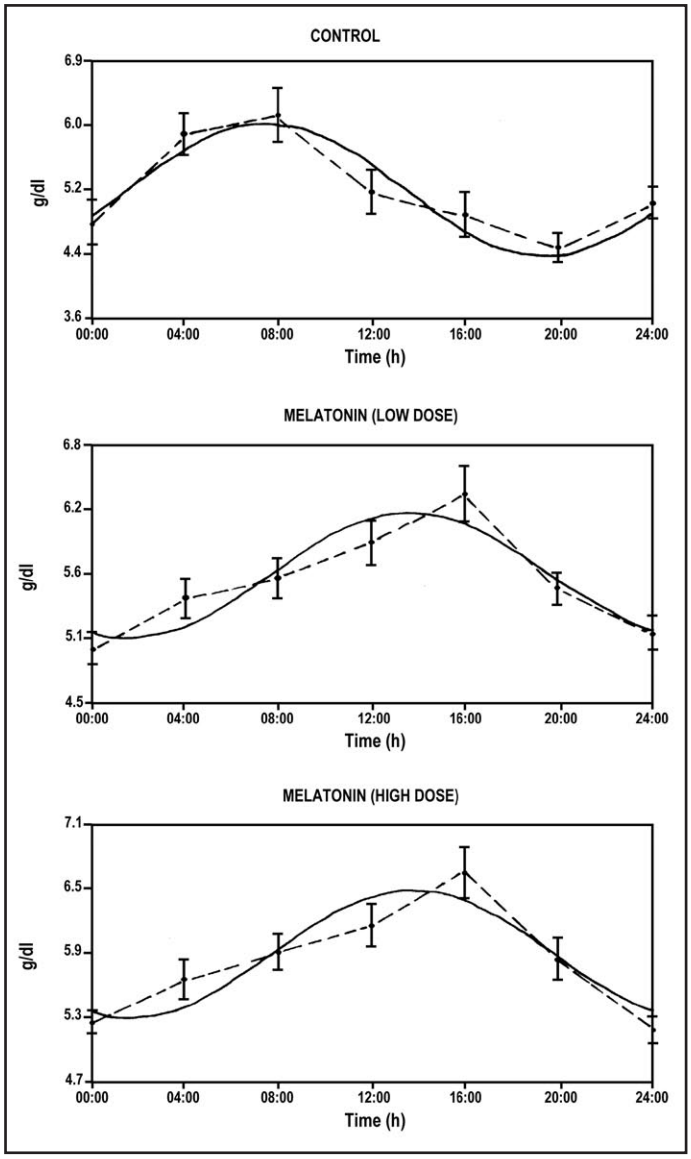
[dr] detectable rhythmicity.

* per body weight.

GSH showed a peak at 07:15 hours (group I) and they were maximum at 19:20 hours and 19:21 hours, respectively, in melatonin-treated rats (groups II and III) (~ 11 hours advance) (Fig. 2). The amplitude and mesor values were increased in both groups II and III rats (Table II).

Peak time of total protein levels was at 07:27 hours in control animals, in contrast to that in groups II and III at 13:39 and 13:45 hours, respectively (~ 6 hours delay) (Fig. 3). Further mesor values were increased, whereas acrophase values were decreased in melatonin-treated animals (Table II). Control animals showed acrophase of melatonin at 22:17 hours and acrophase values were found at 23:55 and 23:59 hours, respectively, in melatonin-treated animals in groups II and III (~ 1.5 hours delay) (Fig. 4). Amplitude and mesor values were significantly increased in melatonin-treated rats (Table II).

Fig. 3 Effect of melatonin dose on total protein levels.



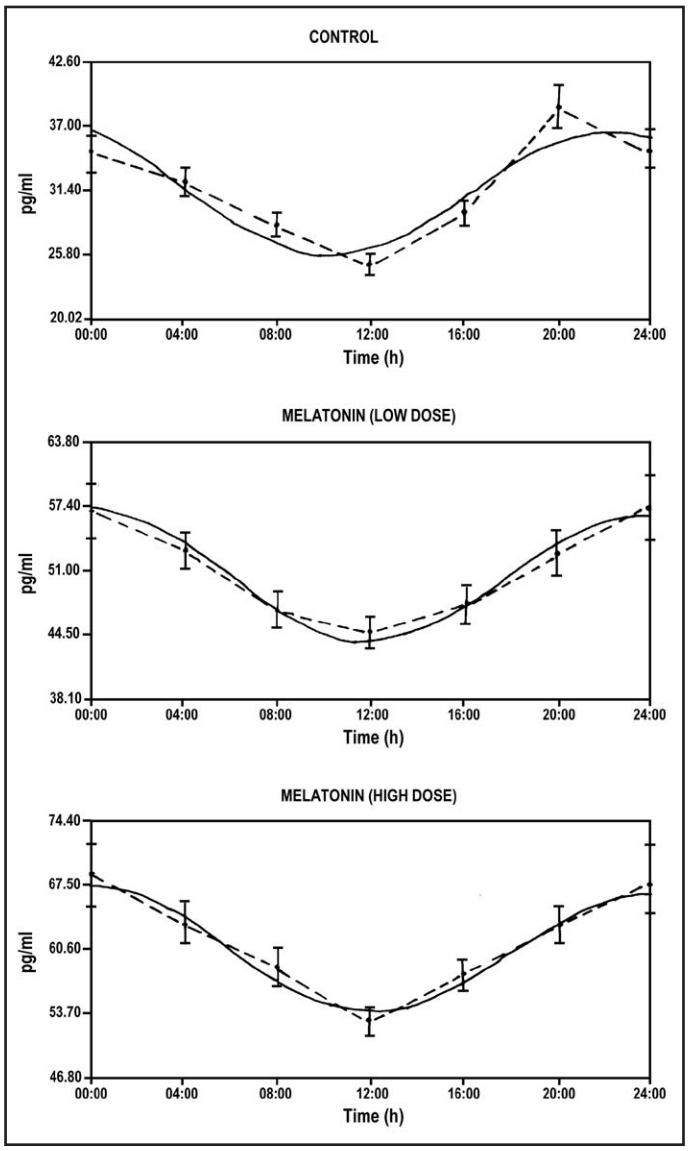
DISCUSSION

Body weights of melatonin-treated rats were higher compared to control rats. Many studies have revealed that exogenous melatonin could increase the energy consumption as well as the deposition of fat. Moreover, melatonin significantly influenced the absolute and relative weights of white fat, periovarial fat and organ (liver, heart and brain) muscles[17]. Thus, it could influence food consumption. The possibilities of reduced metabolism, reduced activity and soporific effect of melatonin could cause an increase in body weight in animals. Further, melatonin was found to cause increases in the levels of growth hormone[18] which could be the reason for increased body weights in melatonin-treated rats.

The biochemical variables chosen in this study showed striking fluctuations over the 24-hour period. The results also indicated that long-term, systemic administration of melatonin could alter the characteristics of biochemical rhythms. Recent studies have demonstrated a direct phase-advancing effect of melatonin and indicated that the phase-shifting effects of melatonin[2]. These findings suggest that melatonin could modulate the functions of the biological clock, either directly or indirectly via neural inputs. This hypothesis is supported by neuroanatomical findings that melatonin receptors are found in SCN and hypothalamo-hypophyseal axis, the exogenous melatonin could influence biochemical rhythmicity (as an modulated internal *zeitgeber*) by acting on these areas of the brain[2]. The melatonin rhythm is hypothesised as an important hormonal signal driven by the clock. Modulation of this rhythmicity due to exogenous melatonin administration could very well influence the blood glucose, reduced glutathione and total protein rhythms.

Russell et al (1983) showed that some circadian rhythms were synchronised by meal timing. It has also been reported that the timing of food availability and intake exerted powerful effects on the temporal characteristics of biochemical rhythmic phenomena[19]. In the present study, although food was available *ad libitum*, 80% food intake occurred during the night time only. Further, adult Wistar rats normally commence eating soon after 18:00 hours. Eating would be most pronounced during the hours of the dark phase[19]. The peak times of enzymes involved in glycolysis were found to lie at the dark phase. Moreover, melatonin acts

Fig. 4 Effect of melatonin dose on melatonin levels.



directly on target cells of hepatocytes and pancreatic β-cells and could affect glucose metabolism[20]. In the present study, the acrophase of glucose at 22:18 hours can be attributed to food intake, digestion and accumulation of glucose in blood. Many recent studies have shown that melatonin inhibits insulin secretion since pinealactomy decreases blood glucose levels[20]. Increase in the amplitude and mesor values of glucose in melatonin-treated groups may be due to changes in growth hormone levels induced by melatonin[18].

A circadian pattern of GSH in plasma has been reported[21]. GSH showed maximal activity at 07:15 hours in control animals. Increased mesor and amplitude values in melatonin-treated rats could be due to its capability in increasing glutathione levels, by stimulating the rate limiting enzyme γ-glutamyl cysteins synthase[22]. Moreover, melatonin at both physiological and pharmacological levels stimulates glutathione peroxidase (GPx) and glutathione reductase (GRd), in the brain of rats[23]. Both GPx and GRd are involved in the redox cycling of glutathione.

Diurnal rhythms of total protein were reported in humans and mice[24]. In the present study, acrophase of the protein occurs at 07:27 hours. The positive and negative balance between synthesis and degradation of proteins may be responsible for this rhythmic phenomenon. Further, the signal transduction cascade involved in the melatonin-mediated phase shifts of circadian rhythms in the SCN and stimulates protein kinase C (PKC) activity at dusk and dawn[25], which may lead to elevated mesor values in melatonin-treated animals.

Significant dose-dependent effects of melatonin were absent in the doses administered (0.5 and 1.0mg/kg body weight) although dose dependent effects of melatonin were observed (0.5 and 2mg/kg body weight) in liver glycogen content in rats[21]. As used in previous studies on the actions of exogenous melatonin, we used pharmacological doses of the substance[23]. The quantities of melatonin given in our experiments have been reported to cause blood concentrations that far exceed the highest levels achieved from endogenous sources[23]. It still remains to be proven how modulated endogenous rhythm of melatonin could influence biochemical rhythms investigated in the present study.

In conclusion, biochemical variables chosen in this study showed significant temporal variations over the 24-hour period. The circadian variations of biochemical variables during chronic melatonin treatment could be due to: (1) the presence of high affinity melatonin receptors in the SCN; (2) physiological sensitivity of SCN to exogenous melatonin; (3) simulation of the conditions of altered photoperiod; (4) effect on the internal *zeitgeber*; and (5) effect on regulation of sleep mechanism and several metabolic activities.

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