

Circadian rhythms on hypothalamic–pituitary–adrenal axis hormones and cytokines of collagen induced arthritis in rats

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Abstract

This study is designed to identify whether circadian rhythms of the hormones of the hypothalamic–pituitary–adrenal (HPA) axis are associated with corresponding circadian fluctuations in cytokines in a rat model of collagen-induced arthritis (CIA). CIA is induced in Wistar rats by an intradermal injection of bovine type II collagen emulsified with complete adjuvant at the left foot. On day 33, in both the CIA and the control rats, circulating adrenocorticotropin hormone (ACTH) and corticosterone, tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and IL-1 β were evaluated at 6 h intervals from 00:00 to 24:00, and analyzed by statistics and cosinor-rhythmometry. The results showed that plasma corticosterone in CIA rats had a trough at 18:00 and reached a peak at 06:00 significantly. While peak values were presented in TNF- α at 24:00 and in IL-6 from 06:00 and 18:00 to 24:00. CIA rats exhibit abnormal circadian rhythms, with degrading amplitudes of corticosterone and IL-6, elevating amplitude of TNF- α , and marked phase shifts in corticosterone and IL-6. Our investigation suggests that the disorders of HPA axis in CIA rats may be related to the influence of inflammation mediators on hypothalamic centers. The circadian rhythms of hormones and cytokines in CIA rats may be reset due to the defective function of the HPA axis.

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Keywords: Circadian rhythms; Hypothalamic–pituitary–adrenal axis; Hormones; Cytokines; Collagen induced arthritis

1. Introduction

Collagen induced arthritis (CIA), which can be induced in rodents and primates by immunization with type II collagen (CII), has provided wide opportunities to study the nature of autoimmune reactions [1,2]. CIA is an ideal experimental model for human rheumatoid arthritis (RA), because it represents a true autoimmune reaction against a major joint component, class II major histocompatibility complex (MHC) genes association. It shows a chronic and progressive course and has other

compelling parallels with RA in pathology, immunology and genetics [3].

Neuroendocrine and immune responses to inflammatory stress represent important integrated physiologic circuits for the regulation of inflammation. In recent years, based on the work carried out in patients with RA and animal models of chronic inflammatory disease, the roles of cytokines and the hypothalamic–pituitary–adrenal (HPA) axis-immune system feedback loop have been exploited in several novel ways. Proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-6 released from inflammatory foci initiate a local inflammatory response and travel by way of the blood-stream to the central nervous system, where they trigger a variety of neuroendocrine counterregulatory mechanisms [4]. By evaluating diurnal cortisol secretion patterns in patients with RA, it has been found that there is an abnormal

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Abbreviations: ACTH, adrenocorticotropin hormone; CIA, collagen induced arthritis; HPA, hypothalamic–pituitary–adrenal; IL, interleukin; MHC, major histocompatibility complex; RA, rheumatoid arthritis; TNF, tumor necrosis factor

HPA axis response to immune/inflammatory stimuli, which may reside in the hypothalamus [5], or in a functional insufficiency of adrenal [6]. The defective response of the neuroendocrine system to inflammatory stimuli suggests a non-MHC genetic factor contributing to the pathogenesis of RA [7]. Some experimental models such as adjuvant-induced arthritis (AA) [8] and inbred Lewis (LEW/N) rats to group A streptococcal cell wall peptidoglycan polysaccharide (SCW) arthritis are also related to defective HPA axis responsiveness to inflammatory mediators [9].

Biological rhythms have been observed in a variety of physiological and pathological conditions in human rheumatoid arthritis. We investigated more than 1600 patients with rheumatic diseases. It is shown that the pain presents circadian rhythms [10], lunar month rhythms [11], annual rhythms [12], and the rhythm of its onset and relaxation on time [13]. Perhaps circadian variations in immunologic mediators, which have effects on the immune system, such as IL-6 [14], and hormones including adrenocorticotropin hormone (ACTH) and adrenal cortisol, correspond to the rhythms of symptoms and the synchronization mechanism of hypothalamus in human RA.

Recent studies show that disorder of rhythms may be responsible for the abnormalities of HPA axis function and immune system loop in RA. CII-induced arthritis in rats and mice is well known to have both arthritic signs and histological similarities to human RA [1,15]. However, up to now, the rhythm action in rat collagen-induced arthritis is not well understood. In this paper, circadian variations of HPA axis hormones such as ACTH, corticosterone, cytokines of IL-1beta, IL-6, and TNF-alpha in CIA rats were investigated. The impairment of the HPA axis and immune loop in CIA rats was studied.

2. Material and methods

2.1. Rats

Total of 86 outbred Wistar male rats, weight 154 ± 7 g, were purchased from research institute of experimental animals, Chinese Academy of Medical Science. Thirty rats, which were divided into two groups of 6 and 24, were used in Experiment (Expt) 1. Another 56 rats, which were divided into a normal group and a CIA group on average, were used in Expt 2.

2.2. Training of rats

Rats were housed in a temperature-, humidity- and light-controlled environment with free access to rodent chow and water. The light–dark cycle was 12 h:12 h with the light phase from 06:00 to 18:00. The rodent licence of

the laboratory (No. SYXK 11-00-0039) was issued by National Science and Technology Ministry of China.

2.3. Reagents

Soluble pure type II collagen was from Dr Rikard Holmdahl (Lund University, Sweden) and complete adjuvant was purchased from Sigma Corporation, USA. Enflurane was purchased from Yi-rui Ltd, Beijing, China. ACTH radioimmunoassay (RIA) test kit was purchased from DPC, USA, which contained buffer, zero standard, standard control, Anti-ACTH serum, ^{125}I -ACTH and separating medium. Corticosterone RIA test kit (containing buffer, zero standard, standard control, ^{125}I -corticosterone, Anti-corticosterone serum, and separating medium) was from Fu-rui Ltd, Beijing, and IL-6 (containing buffer, zero standard, IL-6 standard, ^{125}I -IL-6, Anti-IL-6 serum, and PR separating medium), IL-1beta (containing buffer, zero standard, IL-1beta standard, ^{125}I -IL-1beta, Anti-IL-1beta serum, and immunization separating medium) and TNF-alpha RIA test kit (containing buffer, TNF standard control, ^{125}I -TNF-alpha, Anti-TNF-alpha serum and PR separating medium) were from research institute of RIA, which affiliated the Chinese academy of military medicine science.

2.4. Induction of CIA

CIA was induced by bovine type II collagen 0.15 ml (1.5 mg) emulsified with equal complete adjuvant and 0.1 M acetic acid. Each rat was immunized by injection of mixture under fur skin at the left sole of the foot.

2.5. Anesthesia

Rats were under inhalation anesthesia by enflurane, which is always used for evaluating endocrine functions in human [16] and dog [17] in order to avoid the interference of narcotics in our study.

2.6. Blood sampling

In Expt 1, two blood sampling techniques were employed to evaluate the circadian rhythm of corticosterone in 30 rats. A chronic cannula was inserted in the right carotid of six rats using the method described previously [18] to allow sequential blood sampling at 06:00, 12:00, 18:00 and 24:00. Another 24 rats, divided into four groups on average, used an “interval-to-kill” method [19,20] and blood was taken from the abdominal aorta of rats under enflurane anesthesia every 6 h from 06:00 to 24:00, respectively. Plasma for the test of corticosterone was taken from 2 ml blood after 3000 revolutions per min (rpm) centrifugation for 12 min, which had resisted coagulation by 0.3 M (11%) edetic acid disodium, and kept at temperature of -20 °C.

Experiment 2: 33 days after the immunization, blood samples were collected every 6 h from 06:00 to 24:00 using an interval-to-kill method. Serum was taken by 2000 rpm for 15 min centrifugation from 6 ml blood for the test of IL-1beta, IL-6, and TNF-alpha. Plasma was taken from 2 ml blood according to the above-mentioned procedure for the test of corticosterone and ACTH. After rejection of the hemolytic samples, including three serum and four plasma samples from the control group, the remaining blood samples were kept at -20°C .

2.7. Measures

Serum TNF-alpha, IL-1beta, IL-6, plasma ACTH and corticosterone concentrations were assayed using mouse cytokine and hormone RIA test kits. The assay was performed in duplicate according to the manufacturer's recommended procedures. The results of the radioactivity count were recorded by an automatic Gamma counter (SN-695B), and expressed as mean \pm SD (picogram per milliliter) of individual rats.

2.8. Analysis

Using SPSS 10.0 software, ANOVA was used to determine significance in the data set. Student–Newman–Keuls test was employed for variables between both groups when equal variances assumed and Dunnett's *t* test for equal variances not assumed. The level of significance was set at $\alpha=0.05$. The time-series data was further analyzed by the methods of cosinor-rhythmometry [21] with Origin 6.0 software. Cosinor-rhythmometry, which can be used easily to detect shifts in the phase or timing of the circadian rhythm, is based on the mathematical model: $Y(t)=M+A\cos(2\pi t/T+\theta)$. The *M*, *A*, *T* and θ are the mean value, amplitude, period, and acrophase, respectively. These parameters represent the feature of biorhythm.

3. Results

3.1. Rhythms from different blood sampling methods

In Expt 1, the rhythms obtained from different blood sampling techniques are compared. As shown in Fig. 1, although the levels of corticosterone fluctuated among six individuals, it did not reach significance between two blood sampling groups during one day (06:00, $P=0.702$; 12:00, $P=0.564$; 18:00, $P=0.549$; 24:00, $P=0.502$). Both show the significant circadian rhythm with the peak

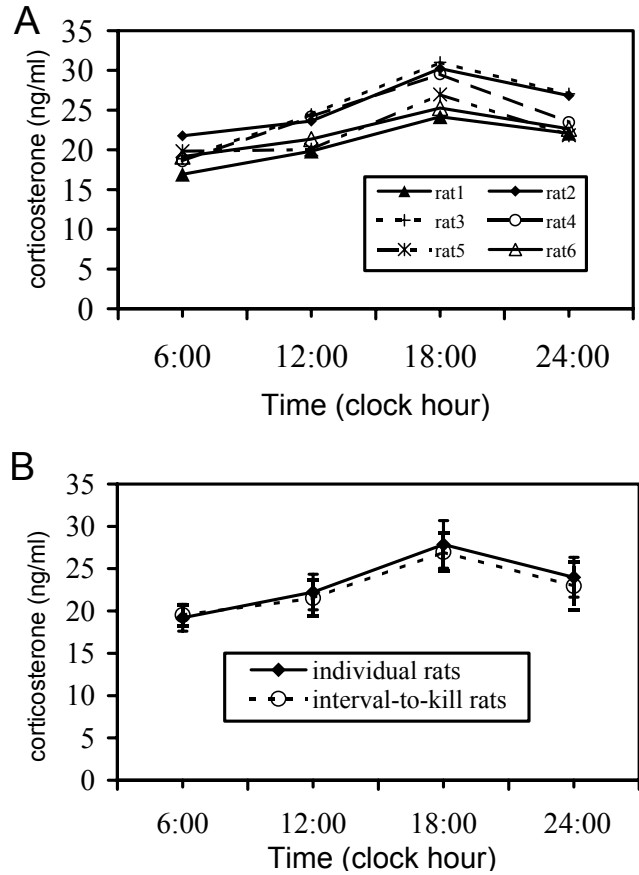


Fig. 1. Comparison with the levels of plasma corticosterone among six individual rats (A) and between two rhythmic blood sampling methods (B) during one day (06:00, $P=0.702$; 12:00, $P=0.564$; 18:00, $P=0.549$; 24:00, $P=0.502$) in Expt 1.

value at 18:00 and the valley value at 06:00 ($P<0.01$ in both groups, peak versus valley).

3.2. Arthritic signs

In Expt 2, all CIA rats immunized with CII developed arthritic signs such as chronic pain, swelling of ankle, rising temperature of the skin of the knee and ankle in both inflammatory and the corresponding inflammatory sides with the onset on days 7–8, and show a chronic and progressive disease course.

3.3. Diurnal variations of ACTH and corticosterone

As shown in Table 1, the control rats presented evident circadian rhythms for ACTH and corticosterone, whose peak values occurred at 18:00, the transition of light to dark phase; its nadir occurred at 06:00 ($P<0.01$). In the CIA rats, the level of ACTH at the four sampling times and the circadian variations were statistically similar to those of the controls. However, changes in plasma corticosterone were detected in the CIA group whose levels diminished at 18:00 ($P<0.01$),

Table 1

Diurnal changes in circulating adrenocorticotropin, corticosterone, interleukin 1beta, tumor necrosis factor-alpha and interleukin 6 during the study

	Normal rats	CIA rats
ACTH (ng/ml)		
06:00	72.8 ± 25.0	87.2 ± 26.5
12:00	102.9 ± 44.0	88.5 ± 30.2
18:00	136.7 ± 42.7 ^d	134.2 ± 44.3 ^c
24:00	93.0 ± 33.0	132.8 ± 46.5
Corticosterone (ng/ml)		
06:00	19.7 ± 2.8	25.4 ± 4.3 ^{a,c}
12:00	22.0 ± 4.7	20.7 ± 3.5
18:00	27.8 ± 4.6 ^d	21.3 ± 1.5 ^b
24:00	25.9 ± 4.6	22.7 ± 3.4
IL-1beta (ng/ml)		
06:00	0.16 ± 0.05	0.13 ± 0.07
12:00	0.13 ± 0.05	0.13 ± 0.06
18:00	0.14 ± 0.06	0.13 ± 0.04
24:00	0.2 ± 0.06	0.16 ± 0.07
TNF-alpha (ng/ml)		
06:00	1.34 ± 0.2	1.33 ± 0.2
12:00	1.42 ± 0.2	1.32 ± 0.3
18:00	1.41 ± 0.3	1.48 ± 0.3
24:00	1.34 ± 0.3	1.76 ± 0.3 ^{a,d}
IL-6 (ng/ml)		
06:00	11.9 ± 6.0	21.8 ± 5.9 ^a
12:00	18.7 ± 4.6	25.1 ± 5.9
18:00	12.6 ± 5.1	23.7 ± 7.2 ^a
24:00	13.9 ± 5.2	29.5 ± 5.8 ^{b,c}

Values are the mean ± SD level ($n=6$ or 7). ACTH, adrenocorticotropin; CIA, collagen induced arthritis; IL-1beta interleukin 1beta; IL-6, interleukin 6; TNF-alpha, tumor necrosis factor-alpha.

^d $P < 0.01$, compared with the valley value of the same group.

^c $P < 0.05$, compared with the valley value of the same group.

^b $P < 0.01$, compared with the normal rats of the same time.

^a $P < 0.05$, compared with the normal rats of the same time.

and increased at 06:00 ($P < 0.05$). Thus, the corticosterone of CIA showed a desynchronization compared with ACTH ($P < 0.05$) whose acrophase was inverted with a peak and valley at 06:00 and 18:00, respectively.

3.4. Diurnal variations of IL-1beta, TNF-alpha and IL-6

In our study, serum TNF-alpha and IL-6 in CIA rats showed a statistically significant circadian variation while these could not be detected in serum of the controls. As presented in Table 1, the serum concentrations of TNF-alpha increased significantly at 0:00 ($P < 0.05$), and IL-6 increased significantly at 06:00 ($P < 0.05$), 18:00 ($P < 0.05$) and 24:00 ($P < 0.01$) in CIA groups. Rhythm change was also found both in serum TNF-alpha and IL-6 of CIA rats. Serum TNF-alpha had a peak value at 0:00 and valley at 12:00 ($P < 0.01$). Peak value of IL-6 appeared at 24:00 and valley at 06:00 ($P < 0.05$). Compared with the controls, there were low concentrations, non-significant variations without

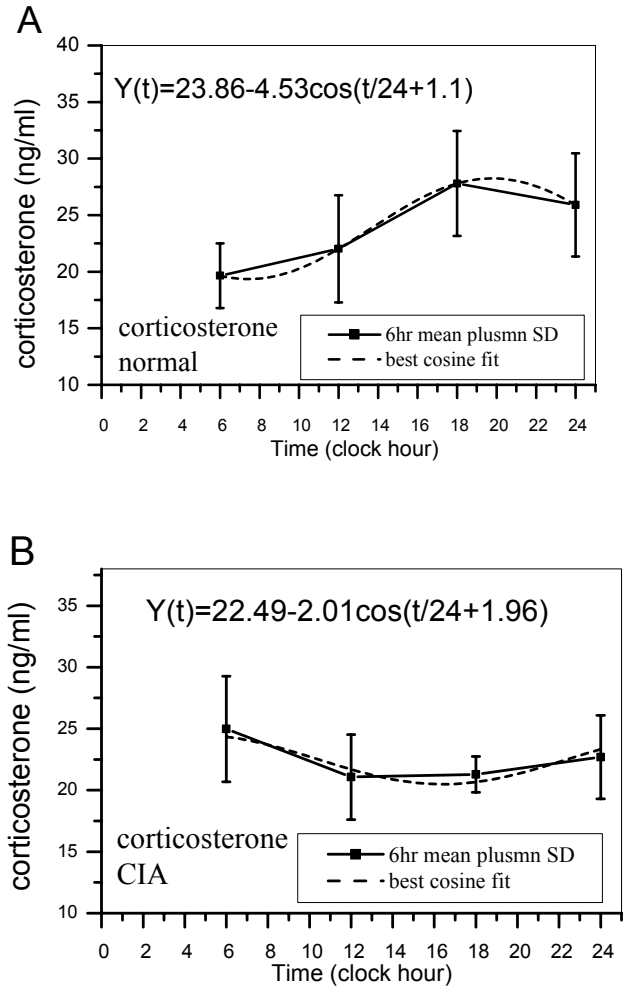


Fig. 2. Circadian rhythm of the plasma corticosterone in the normal rats (A) and the type II collagen induced arthritis (CIA) rats (B). Plasma corticosterone was collected at 6 h intervals from the light–dark cycle 12 h (h):12 h (light phase 06:00–18:00) when immunized at 33 days, and evaluated by radioimmunoassay (RIA) test. The curves with the mathematical model of $Y(t) = 23.86 - 4.53\cos(t/24 + 1.1)$ in the normal rats (A) and $Y(t) = 22.49 - 2.01\cos(t/24 + 1.96)$ in the CIA rats (B) are the best cosinor-fitting.

circadian rhythms of serum TNF-alpha and IL-6. The serum levels of IL-1beta did not show any significant differences within 24 h in both normal and CIA groups ($P > 0.05$).

3.5. Cosinor-rhythmometry analysis

Cosinor-rhythmometry provides a powerful and accurate means for ascertaining (with statistical confidence) how much a circadian rhythm may deviate from 24 h within a short sample. Meanwhile, adding a 12-h or 18-h harmonic cosine substantially enhances fit to most circadian variables, and this has become a standard and accepted procedure [22]. Fig. 2 shows the circadian rhythm and the best cosinor-fitting of the plasma corticosterone in the control and CIA rats. The fitting

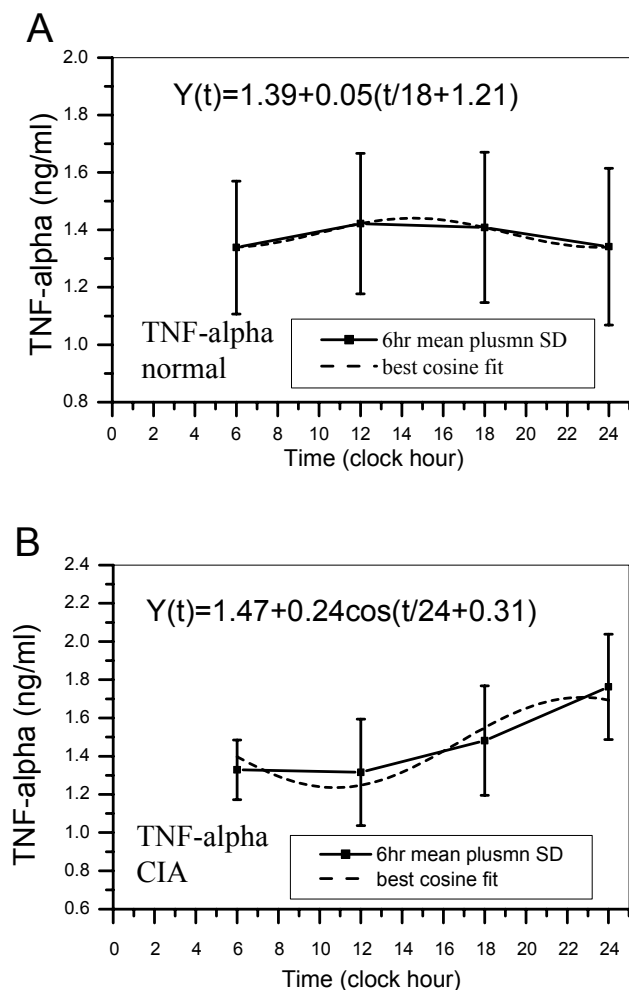


Fig. 3. Circadian rhythm of the serum tumor necrosis factor-alpha (TNF-alpha) in the normal rats (A) and the type II collagen induced arthritis (CIA) rats (B). Serum TNF-alpha was collected at 6 h intervals from the light-dark cycle 12 h (h):12 h (light phase 06:00–18:00) when immunized at 33 days, and evaluated by radioimmunoassay (RIA) test. Values are the mean+SD. The curves with the mathematical model of $Y(t) = 1.39 + 0.05 \cos(t/18 + 1.21)$ in the normal rats (A) and $Y(t) = 1.47 + 0.24 \cos(t/24 + 0.31)$ in the CIA rats (B) are the best cosinor-fitting.

oscillator in both groups has a periodicity of 24 h. The mean activity level of plasma corticosterone in CIA rats ($M = 22.49$ ng/ml) is similar to the control ($M = 23.86$ ng/ml). While the amplitude of corticosterone in CIA rats ($A = 2.01$ ng/ml) decreases obviously compared with the control rats ($A = 4.53$ ng/ml). Moreover, the acrophase of the CIA rats is inverted.

Fig. 3 presents the circadian rhythm and the best cosinor-fitting of the serum TNF-alpha in the normal and CIA rats. For the normal group, the serum TNF-alpha is at a lower level with gentle oscillation. The periodicity of 18 h has the best fitting result. The fitted mean activity level, amplitude and acrophase are 1.39 ng/ml, 0.05 ng/ml and 3:48 clock, respectively. The serum TNF-alpha of the CIA rats exhibits the

periodicity of 24 h according to cosinor fitting. The mean activity level, amplitude and acrophase are 1.47 ng/ml, 0.24 ng/ml and 0:58 clock, respectively (Table 2).

Fig. 3 displays the circadian variation of the serum IL-6 in both groups. The periodicities were set as 12 h and 18 h for CIA and normal rats, respectively, to obtain the best fitting results. Serum IL-6 in CIA rats shows a significant circadian rhythm with the mean activity level of 25.02 ng/ml, the amplitude of 1.51 ng/ml, and the acrophase (θ) of 0:04 clock. This circadian rhythm reaches its peak at 0:00; the subsequent peak occurs 12 h later. In the control rats, the levels of serum IL-6 decreased significantly. The fitting parameters are $M = 14.73$ ng/ml, $A = 3.96$ ng/ml and $\theta = 6:43$ clock.

4. Discussion

Type II collagen induced arthritis, a model which mimics human RA, is well known to provide useful information on the characterization of immunopathogenic mechanisms and the nature of autoreactivity. However, few previous works were engaged in the study of the circadian rhythms in the HPA axis and the immune system loop in a Wistar CIA rat. In this study, two blood sampling methods are adopted to investigate the circadian rhythms in rats. One is individual sampling by using a chronic carotid cannula [18]. Another is an “interval-to-kill” method [19,20] that has been used in both blood and tissue sampling. Our results in Expt 1 showed the negligible rhythmic individual variability among the normal rats and between both methods, while the levels of corticosterone fluctuate slightly. This agrees with the result in Ref. [23], and validates that the circadian pacemaker has stability and precision within a given species of plants and animals [24]. To avoid the continuing stress by the implanted cannula and the repeated changes in blood volume for the detection of many indexes, we adopted the “interval-to-kill” technique in Expt 2 to evaluate the average rhythm pattern of the CIA rats.

As shown in Table 1, the circadian variation of plasma ACTH and corticosterone of the control rats had the same acrophase at 18:00 and nadir at 06:00. This result is identical to the marked circadian rhythm of hypothalamic corticotropin releasing factor activity, which peaked around 16:00, the minimal level was at around 08:00 followed by almost parallel changes of plasma corticosterone in the rats [25]. On day 33, after immunization with CII, the secretion of plasma ACTH in CIA rats was similar to that of the controls, however, the level of corticosterone significantly decreased at 18:00 and increased at 06:00. The cosinor-rhythmometry analysis in Fig. 2 shows small amplitude for corticosterone, inverted acrophase, and the desynchronization with ACTH in CIA rats. The dysfunction of HPA axis

Table 2
Circadian rhythms of circulating adrenocorticotropin, corticosterone, interleukin-6 and tumor necrosis factor-alpha during the study

Rats	Index	Mean (ng/ml)	Amplitude (ng/ml)	Period (h)	Acrophase (clock)
Normal	ACTH	105.6	34.4	24	3:16
	Corticosterone	23.9	4.53	24	3:27
	IL-6	14.7	3.96	18	6:43
	TNF-alpha	1.39	0.05	18	3:48
CIA	ACTH	109.7	34.5	24	2:06
	Corticosterone	22.5	2.01	24	6:10
	IL-6	25.0	1.51	12	0:04
	TNF-alpha	1.47	0.24	24	0:58

Values are the parameters of a circadian rhythm. Analysed by the cosinor-rhythmometry which based on the mathematical model as $Y(t)=M+A\cos(2\pi t/T+\theta)$. The M , A , T and θ are the mean value, amplitude, period and acrophase. ACTH, adrenocorticotropin; CIA, collagen induced arthritis; IL-6, interleukin 6; TNF-alpha, tumor necrosis factor-alpha.

was also observed in RA patients with high activity whose erythrocyte sedimentation rate was higher than 80 mm/h. Inappropriately low secretion of cortisol in relation to inflammation is a typical feature of inflammatory disease in patients with RA [26]. Neeck et al. reported that the maximal and minimal levels of cortisol shifted to an earlier time of the day whereas the circadian rhythm of cortisol was lost or reduced in RA patients [27]. Dallman et al. found that mild chronic stressors characteristically increased circadian trough corticosteroid concentration in rats and in humans and the elevation in trough concentration was often accompanied by a reduction of peak concentrations [28]. The effect of sex hormones on the pathogenesis of RA are often contradictory and have yet to crystallize into a coherent hypothesis with testable predictions [7], so we used male Wistar rats for the induction of CIA.

In the pathogenesis of organ-specific autoimmune diseases, hormones regulate immunologic reaction principally by adjusting the dynamic balance of T-helper lymphocytes (Th) 1 and 2. The characteristic cytokines of T-helper cells include TNF-alpha, IL-6 and IL-1beta. Their circulating levels are well correlated with the activity of rheumatic arthritis [29]. So it is necessary to examine the level and rhythm of several types of cytokines. Our investigation, as shown in Table 1, demonstrates that the circulating levels of IL-6 in CIA rats increased significantly at 06:00, 18:00 and 24:00 compared with those of the controls. The level of serum IL-6 in the RA patients was highly correlated to the amount of ESR and C reactive protein [30], the contents of immunoglobulin, and leukocyte in synovial fluid and serum [31]. A significant increase of plasma IL-6 levels was found in RA patients during the early morning hours [32]. Takagi et al. suggested that IL-6, produced after CII immunization, might play an essential role in immunity to CII in mice with CIA [33].

There is an interaction between IL-6 and hormones. Circulating IL-6 is a potential activator of the human HPA axis. On the other hand, synthetic glucocorticoids, such as dexamethasone, can significantly reduce the rate

of IL-6 gene transcription [34]. A pronounced circadian variation of plasma IL-6 levels and a positive temporal correlation between plasma levels of IL-6 and ACTH/cortisol were presented in early untreated RA patients, with IL-6 preceding ACTH and cortisol by 1 and 2 h, respectively [32]. In our study, the significant decrease of plasma corticosterone and the increase of serum IL-6 occurred simultaneously at 18:00 in CIA groups. This behavior is similar to that of RA patients since there are about 180° difference in the circadian acrophases of plasma corticosteroids between humans and rats [35]. The levels of plasma corticosterone and serum IL-6 increase significantly at 06:00 clock in CIA rats, suggesting that the HPA axis of CIA apparently is insufficient to inhibit ongoing inflammation, although endogenous IL-6 may stimulate the secretion of corticosterone.

TNF-alpha is a cytokine at the top of the inflammatory cascade [36]. Williams investigated the effects of anti-TNF-alpha, anti-IL-1, and combined anti-TNF-alpha/anti-CD4 therapy in CIA. When treatment was initiated after onset of arthritis, anti-TNF-alpha, anti-IL-1beta, and anti-IL-1R were all found to be effective in reducing the severity of arthritis [37]. In our study, the level of serum TNF-alpha also increased significantly at 24:00 showing the progression of arthritis in CIA rats 33 days after immunization. The levels of serum IL-1beta within 24 h in CIA rats were similar to those in normal rats (Table 1). This may be due to the decreased activation of the whole immune system compared with local IL-1beta immunoreactions [38], or the existence of IL-1 inhibitors in the synovia [39]. Studies on humans also indicate that glucocorticoids suppressing cytokine production has a hierarchy of sensitivity, with TNF-alpha having the greatest sensitivity, IL-1beta having intermediate sensitivity, and IL-6 being resistant [40].

It is known that the production of cytokines exhibits diurnal rhythm. Peak productions of the pro-inflammatory cytokines interferon (IFN)-gamma, TNF-alpha, IL-1 and IL-12 occur during the night and early morning at the time when plasma cortisol is the lowest [41]. Statistically, circadian variation in levels of IL-6

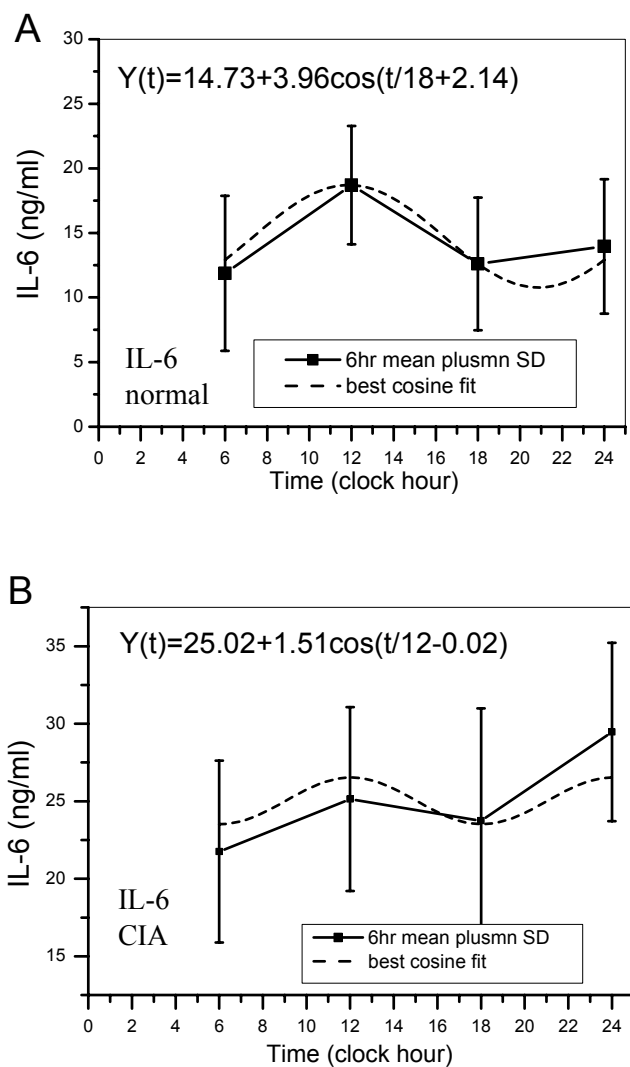


Fig. 4. Circadian rhythm of the serum interleukin 6 (IL-6) in the normal rats (A) and the type II collagen induced arthritis (CIA) rats (B). Serum IL-6 was collected at 6 h intervals from the light–dark cycle 12 h (h):12 h (light phase 06:00–18:00) when immunized at 33 days, and evaluated by radioimmunoassay (RIA) test. Values are the mean±SD. The curves with the mathematical model of $Y(t)=14.73+3.96\cos(t/18+2.14)$ in the normal rats (A) and $Y(t)=25.02+1.51\cos(t/12-0.02)$ in the CIA rats (B) are the best cosinor-fitting.

was found in RA patients with peak values appearing in the morning and low values of the afternoon and evening. While the levels of serum IL-6 decreased significantly after treatment with prednisolone, the circadian rhythm was constant [14]. A similar phenomenon in CIA rats observed in our study is the lower amplitude of plasma corticosterone, and the abnormal circadian variations of TNF-alpha and IL-6. Cosinor rhythmometry demonstrates that there are significant differences in circadian rhythm of circulating corticosterone, IL-6 and TNF-alpha between the CIA rats and controls. It can be seen from Table 2 and illustrated in Figs. 2–4 that the amplitude of corticosterone and is lower TNF-

alpha in CIA rats is higher than that of the controls. The period of IL-6 becomes shorter followed by a shift of acrophase in CIA rats.

The activation of the HPA axis in mammals has been proposed as a biological clock [42]. The inflammatory cytokines TNF-alpha, IL-1beta and IL-6 are potential activators of the HPA axis. These cytokines stimulate the ACTH production from the pituitary gland and corticosterone from the adrenal glands. They also stimulate the HPA axis by inducing the secretion of corticotropin releasing hormone (CRH) [43]. For instance, intravenous injections of TNF-alpha stimulate plasma ACTH and corticosterone secretion in a dose dependent fashion. CRH is a major mediator of the HPA axis which responds to TNF-alpha. The primary site of action of TNF-alpha appears to be the hypothalamic CRH-secreting neuron [44]. IL-6 is possibly involved in the interaction between the neuroendocrine and immune system because IL-6 stimulates not only the secretion of ACTH through the CRH, but also adrenal cells directly [45]. Subsequently, our data allow us to predict an impairment of HPA axis in CIA rats.

Our investigation shows an earlier and higher IL-6 and TNF-alpha peak in the CIA group. As expected, the cytokine stimulus (24 h) is responsible for the earlier peak, for both ACTH and corticosterone in CIA groups, when compared with controls. Therefore, the proinflammatory stimulus of cytokines both of the level of ACTH production (hypothalamus) and corticosterone production (adrenal gland) is obtained and here nicely observed. Of course, the IL-6 level in the CIA group is high and that means a strong stimulation of the HPA and adrenals, but at the same time adrenal insufficiency, as Cutolo describes a similar condition in humans [46]. It suggests that the disorders of HPA axis in CIA rats may be related to the influence of inflammation mediators on hypothalamic centers.

Similar to the human RA patients [5], we also found a normal ACTH secretion and abnormal corticosterone secretion in CIA rats, which imply a hypothalamic and adrenal defect and normal pituitary function. Concomitantly, the abnormal circadian rhythms of corticosterone, IL-6 and TNF-alpha could affect the progress of joint inflammation in CIA rats. These further demonstrate the impairment of HPA axis-immune system feedback loop of CIA Wistar rats, and CIA can mimic the defective function of HPA axis in human RA. In addition, animal studies and some early genetic studies in RA patients indicated that insufficient HPA axis response to inflammatory stimuli might increase susceptibility to, or severity of, rheumatic diseases [47], which showed evidence for the possibility of altered HPA function preexisting the human RA disease onset. And the dysfunction of the HPA in RA could be related to previous factors such as chronic stress or even the altered photoperiod influencing both cortisol and

melatonin [48] secretion. Thus, the impairment of the synchronization role of hypothalamic formations is assumed of importance in the genesis of neuroendocrine dysfunction in CIA rats, and a clear delineation of the role of the neuroendocrine-immune system in disease pathophysiology of CIA, and the possible interference on circadian rhythm exerted by other neuro-hormones, such as melatonin, are still required.

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