Contents available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation

Correlation between inflammatory markers and impaired circadian clock gene expression in type 2 diabetes mellitus

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ARTICLE INFO

Article history:
Received 8 July 2019
Received in revised form
11 August 2019
Accepted 29 August 2019
Available online 30 August 2019

Keywords:
Type 2 diabetes
Clock gene
Inflammatory markers

ABSTRACT

Aim: Circadian rhythm controls a wide variety of physiological processes in the body. Disruption of the circadian clock in metabolic tissues may increase the risk of diabetes, obesity, and metabolic syndrome. The following study investigated whether the expression of clock genes of peripheral blood cells is impaired in type 2 diabetes (DT2) and whether inflammatory markers are associated with circadian clock gene expression in DT2 patients.

Materials and methods: Blood samples were obtained from 36 DT2 patients and 14 non-diabetic volunteers. Transcript levels of circadian clock genes were analyzed using real-time quantitative PCR; plasma inflammatory markers were measured by ELISA or clinical laboratory test.

Results: The CLOCK, BMAL1, PER1, CRY1 and CRY2 mRNA levels were decreased in the diabetic patients. In addition, HbA1c levels were negatively correlated with BMAL1, PER1 and CRY1 mRNA levels. The levels of IL-6, TNF-α and CRP were higher in diabetic subjects compared to control subjects. Impaired expression of circadian clock gene was interrelated with the elevated levels of plasma IL-6 and TNF. Moreover, a multiple linear regression showed that plasma IL-6 level was correlated with impaired expression of circadian clock gene.

Conclusions: Circadian clock genes are reduced in peripheral leucocytes of DT2 patients. Furthermore, impaired expression of circadian clock gene are interrelated with the elevated levels of plasma inflammatory markers.

1. Introduction

Circadian rhythms occur in almost all living organisms and control a wide variety of physiological processes, including sleep–wake cycles [1], feeding behavior [2], body temperature [3], cardiovascular [4] and endocrine system [5], as well as immune functions [6]. In mammals, circadian oscillators consist of a central pacemaker that is located in the suprachiasmatic nucleus (SCN) and peripheral clocks located in other brain regions and peripheral tissues, such as pancreas [7,8],

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https://doi.org/10.1016/j.diabres.2019.107831
0168-8227/

liver [9], skeletal muscle [10] and leucocytes [11]. The molecular clock is maintained by a transcription-translation feedback loop that oscillates with a near 24-h periodicity. At the core of this oscillator, two basic helix-loop-helix (bHLH) transcription factors, CLOCK and BMAL1 (also known as MOP3 or ARNTl) heterodimerize and subsequently bind to E-box promoter sites of their own repressors Period (Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2), which in turn induce rhythmic expression in the clock-controlled gene. PER and CRY proteins inhibit their own CLOCK: BMAL1-induced transcription after forming a complex and translocating to the nucleus [12].

Recent studies have identified the correlation between circadian clock function and metabolic diseases such as metabolic syndrome and DT2, which attracted much attention among scientists [13]. Intrinsic clocks have a critical role in the organization and information processing from genome to whole organisms. Disruption of the core clock genes could increase the risk of diabetes, obesity, and metabolic syndrome [14–16]. For example, shift work, as a risk factor, can cause obesity or disruption of glucose homeostasis [17,18]. In addition, studies on genome-wide association have revealed a causative role of Bmal1 variants in susceptibility to DT2 [19]. Nevertheless, although most of the studies have been performed to demonstrate that circadian clocks could regulate metabolic processes, only few studies have examined whether metabolic disorders influence the circadian system. Thus, it remains to be determined whether the expression of clock genes of peripheral blood cells is impaired in human patients with DT2.

It is believed that chronic inflammatory state is associated with DT2 [20]. High levels of proinflammatory cytokines may promote the development of insulin resistance via nuclear factor κB and Jun N-terminal kinase signaling [20,21]. Furthermore, some studies have suggested that IL-6 and TNF-α have a significant role in the pathogenesis of DT2 and insulin resistance [21]. In addition, epidemiological studies have found significantly elevated plasma inflammatory factors in diabetic patients [22]. Many immune parameters exhibit daily fluctuations in a circadian fashion, including plasma levels of cytokines and the number of immune cells. Moreover, the plasma inflammatory factors are not only regulated by the circadian rhythm, but may also affect circadian rhythms and alter the expression of clock gene [23]. Therefore, we speculated that proinflammatory cytokines are correlated with impaired circadian clock gene expression in subjects with DT2; and that the disruption of circadian clock genes may also indirectly increase glucose levels. Thus, in the current study, we analyzed the plasma from study subjects for inflammatory markers assay and peripheral leucocytes samples for their mRNA expression rhythms of clock genes comparison.

2. Materials and methods

2.1. Study participants

A total of 36 DT2 patients and 14 healthy volunteers (aged 30–60 years) who were admitted to Tianjin Metabolic Diseases Hospital, People's Republic of China between March 2017 and July 2017 were included in the current study. DT2 was diagnosed based on the Chinese Diabetes Association's diagnostic criteria for DT2. The exclusion criteria were the following: (1) an irregular schedule during the 2 weeks preceding the experiment (i.e., jet lag or shift work, irregular sleep or meal schedules, working during nights or staying up late); (2) initiation of any treatment with psychotropic medication within the previous 3 months; (3) pregnancy; (4) acute infection, acute complications and chronic inflammatory disease (rheumatoid arthritis or lupus), infectious disease. Additionally, subjects with malignancy or hematologic disorders were also excluded from the current study.

The study protocol was approved by the Ethics committee of Tianjin Metabolic Diseases Hospital and was in accordance with the Declaration of Helsinki. All individual participants provided written informed consent before enrollment.

2.2. Clinical investigations and laboratory determinations

All subjects were instructed to keep regular sleep and meal schedules for at least 2 weeks to ensure that the circadian pacemaker was stabilized relative to the sleep-wake schedule and diet cycle. Blood samples for leucocytes isolation and blood chemistry were obtained after an overnight fasting (>12 h) at 8:00 AM. Physical examinations and blood biochemical measurements were also determined on admission after overnight fasting. Plasma samples were separated from blood and stored at −80 °C; samples were consequently assayed for inflammatory markers IL-6 and TNF-α.

2.3. Isolation of leucocytes and purification of RNA

Venous blood samples were collected in the heparinized tube and immediately processed for cell isolation and purification using leucocytes separation kit. Briefly, leucocytes were separated by density gradient centrifugation at 800 g for 30 min, and then purified with lysing buffer to remove residual erythrocytes. Isolated cells were immediately frozen at −80 °C or processed for further use. Total RNA of purified leucocytes were extracted using Trizol reagent according to the manufacturer's protocol.

2.4. cDNA synthesis and real-time quantitative PCR

cDNA was synthesized with 1 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) after DNase treatment. Relative clock gene expressions were measured by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using the Light Cycler 480 system (Roche) and SYBR green fluorescent staining according to the manufacturer's recommendations. The oligonucleotide primers were designed and purchased from Thermo Fisher Scientific and the endogenous control gene (β-actin) was used for sample normalization. Real-time PCR data was analyzed using the comparative threshold cycle method. All results were run in triplicate. The primers used for PCR dosage were the following: BMAL1(forward 5′-GTAGGTGGCCCAAAGAGGAC -3′ reverse 5′-CTGGAGCCACAGC TAGAAGG-3′), CLOCK (forward 5′- CTGCCTAACGGGGCAAGTC -3′ reverse 5′- GCAAGCCGAGTCCGTGATT -3′), CRY1 (forward 5′- CATCTGCTGGTTGTCCACGA -3′ reverse 5′- CATCTGCTG

GTTGTCCACGA-3′), CRY2(forward5′-TAGGTCACTGGGCGGGC TAT -3′ reverse 5′- CGGAACCAGTGCACCGAAGA -3′), PER1(forward 5′- GGTTTGGAGGAGCCAGTGTAA-3′reverse5′-TGAGG GAGTGAGGTGGAAGAT -3′), PER2(forward 5′-TTCTTGGA CAGTCTCGCCTTC-3′ reverse 5′- CCGCGTATCCATTCATGCTG -3′), PER3(forward 5′- TACTTCCCCTCGGAGAGACG-3′ reverse5′-ACATGCTCACATCTGCCTGA-3′), NR1D1(forward 5′-ACCATGGAGGTGAATGCAGG-3′ reverse 5′-CAGAAGAGGAG GAGGACTGGA-3′).

2.5. *Plasma inflammatory marker assay*

Plasma levels of circulating IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA, ABclonal, Wuhan Aibotek Biotechnology. China) according to the manufacturer's instructions. CRP level was determined through immunological transmission turbidimetry in the Tianjin Metabolic Diseases Hospital Lab.

2.6. *Statistical analysis*

Continuous variables were presented as the mean ± standard deviation (SD) and were analyzed by student's *t*-test. Categorical variables were shown as frequency (percentage) and compared using chi-squared test. Prior to analyses, normality of data was assessed using the (Kolmogorov-Smirnov) KS test; and natural logarithm transformation of CRP was applied to improve normality. The value of clock genes expression was also analyzed after using natural logarithm transformation. Correlations between inflammatory markers (IL-6, TNF-α and CRP) and the expression of clock genes (CLOCK, BMAL1, PER1, PER2, PER3, CRY1, CRY2 and NR1D1) were evaluated using Pearson correlation analysis. Multiple linear regression analysis was used to assess the association between inflammatory markers with expression levels of clock genes. $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed with the software Statistical Package for a Social Science (version 20.0; SPSS Inc. Chicago, IL, USA).

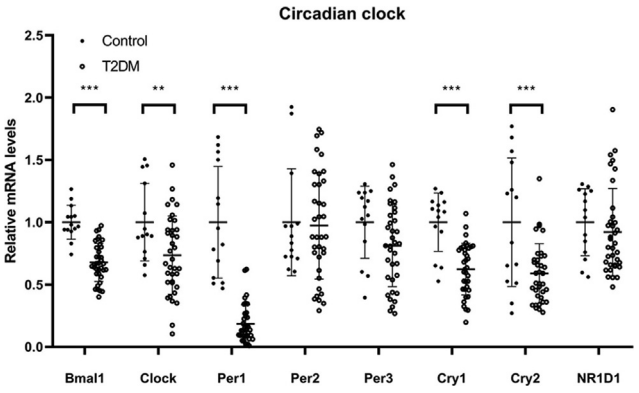

Fig. 1 – Clock genes expression in peripheral blood leucocytes. Q-PCR analysis of the clock genes expression (Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, NR1D1) in the human peripheral blood leucocytes of 36 T2DM patients and 14 normal subjects. The mean values of the control group were set at 1.0. Paired Student's t-test. Data are expressed as mean ± SD, $^{***}p < 0.001$, $^{**}p < 0.01$.

3. Results

The baseline clinical characteristics of all individuals included in the study are shown in Table 1. As expected, fasting blood glucose (FBG), 2-h postprandial blood glucose (P2BG) and HbA1c levels were significantly higher in patients with diabetes. In addition, diabetic group had significantly higher BMI, LDL, plasma levels of circulating IL-6, tumor necrosis factor α (TNF-α) and CRP compared with non-diabetic subjects; while there was no difference in age, SBP, DBP, AST, ALT, CR, TC and TG between groups (Table 1).

In addition, our data revealed that the mRNA levels of CLOCK, BMAL1, PER1, CRY1, CRY2 were significantly decreased in the diabetic patients; in contrast, no significant differences were found in the transcript levels of PER2, PER3 and NR1D1 (Fig. 1). As shown in Fig. 2, Pearson's correlation

Table 1 – The baseline clinical characteristics of all individuals.

Variables	Subjects with type 2 diabetes	Non-diabetic subjects	T values or χ^2 values	P values
Male (%)	12(33.3%)	5(35.71%)	0.025	0.873
Age (years)	49.47 ± 7.88	45.93 ± 7.16	1.463	0.150
BMI (kg/m^2)	23.30 ± 3.04	22.31 ± 1.44	1.162	0.251
SBP (mmHg)	129.58 ± 12.67	127.14 ± 6.71	0.682	0.499
DBP (mmHg)	79.03 ± 9.47	76.07 ± 6.56	1.069	0.290
FBG (mmol/L)	10.65 ± 3.58	4.96 ± 0.40	9.384	0.000
P2BG (mmol/L)	15.55 ± 6.60	5.92 ± 0.59	8.662	0.000
HbA1C (%)	8.86 ± 2.19	5.09 ± 0.41	9.878	0.000
TC (mmol/L)	4.82 ± 0.86	4.66 ± 0.50	0.793	0.432
TG (mmol/L)	1.35 ± 0.33	1.33 ± 0.26	0.215	0.831
LDL (mmol/L)	3.08 ± 0.65	2.65 ± 0.36	2.384	0.021
AST (U/l)	18.46 ± 7.04	18.63 ± 3.30	0.118	0.907
ALT (U/l)	19.96 ± 9.10	20.00 ± 3.51	0.025	0.980
CRP (mg/L)	2.58 ± 3.19	1.34 ± 0.69	2.188	0.034
IL-6 (ng/L)	4.77 ± 2.24	1.34 ± 0.73	8.164	0.000
TNF-α()	4.05 ± 1.87	1.89 ± 0.74	5.840	0.000

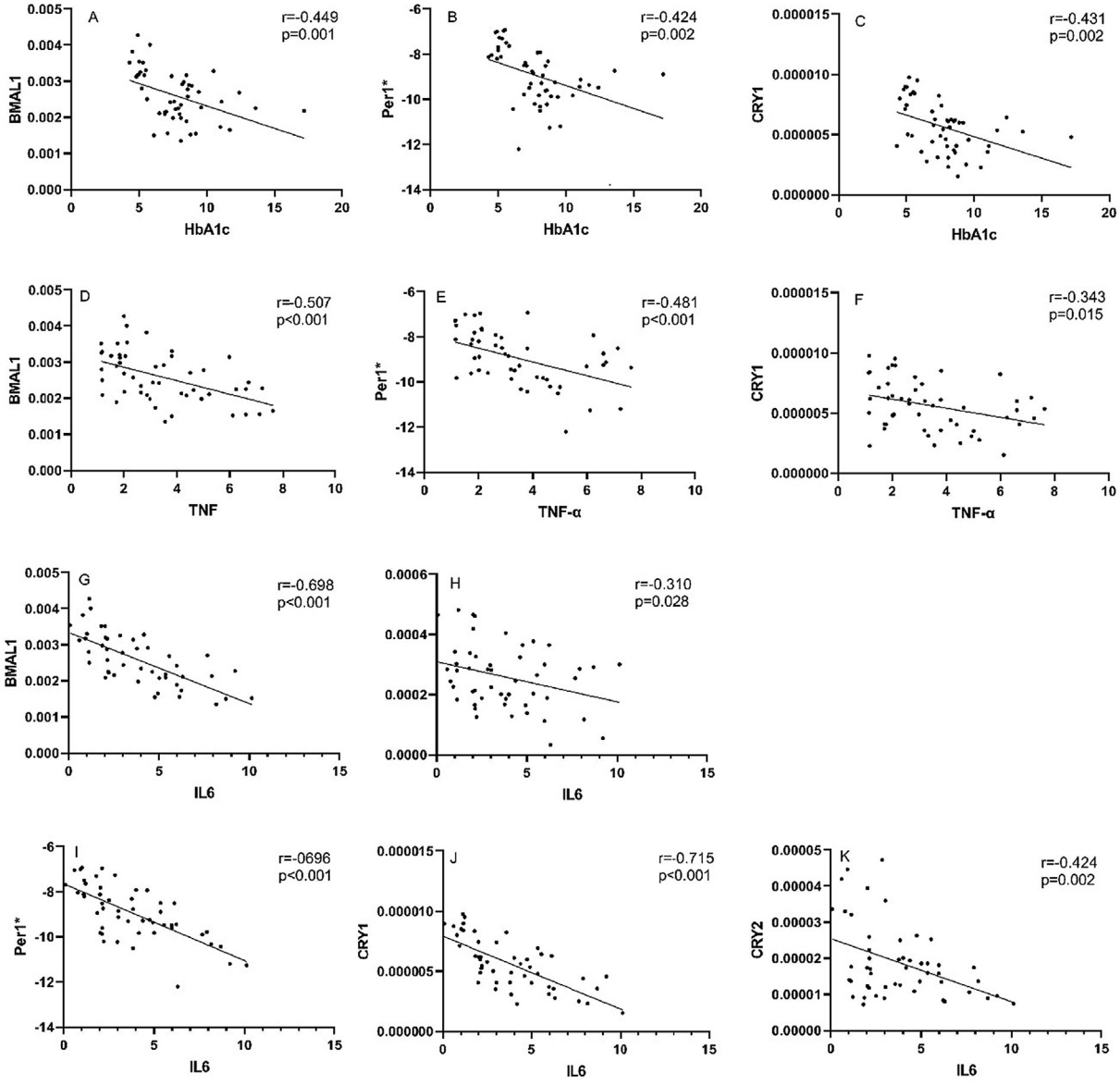

Fig. 2 – Correlation coefficients between Clock genes expression or blood glucose levels and inflammatory markers. *Variables were subjected to natural Log transformation (A–C) The correlation between clock genes and HbA1c. (D–F) The correlation between clock genes and TNF-α. (G–K) The correlation between clock genes and IL-6. r: correlation coefficient. p-value of Pearson's product-moment correlation coefficient.

analysis revealed a significant negative correlation between BMAL1, PER1, CRY1 and HbA1c mRNA levels (r = −0.449, P = 0.001; r = −0.424, P = 0.002; r = −0.431, P = 0.002). Moreover, the levels of IL-6 were inversely correlated with BMAL1, CLOCK, PER1, CRY1 and CRY2 mRNA levels (r = −0.698, P = 0.000; r = −0.310, P = 0.028; r = −0.696, P = 0.000; r = −0.715, P = 0.000; r = −0.424, P = 0.002). The TNF-αlevels were negatively correlated to the mRNA levels of BMAL1, CRY1, PER1 (r = −0.507, P = 0.000; r = −0.481, P = 0.001; r = −0.343, P = 0.015). However, the levels of CRP was not significantly associated with the expression of clock genes. Furthermore, multiple linear regression models were established to examine cross-sectional relationships among blood glucose, the mRNA levels of clock genes and individual inflammatory markers. Regression analyses model 1 was used to assess the association between blood glucose and clock genes levels. Individual clock genes were used as dependent variable, and the blood glucose was used as independent variable, adjusted for the expected confounder factors, including gender, age, BMI, SBP, and TG, TC, LDL. Results of multiple linear regressions showed that FBG levels were negatively related to the mRNA levels of BMAL1 (β = −0.561, P = 0.000). P2BG levels were negatively correlated to the mRNA levels of CRY1 (β = -0.479, P = 0.000), and were inversely correlated with the mRNA levels of PER1 (β = − 0.466, P = 0.001) (Table 2).

In addition, multiple linear regression analyses model 2 was established to test the interaction between the mRNA levels of clock genes and individual inflammatory markers. Clock gene levels were used as dependent variable and inflammatory markers were individually used as independent

Table 2 – Multiple linear regression model 1 analysis between blood glucose and clock gene expression.

Dependent variable	Independent variables	Unstandardized coefficients (β)	Standardized coefficients (β)	t values	P values
BMAL1	FBG	−9.858E-05	−0.561	−4.649	0.000
PER1*	P2BG	−0.079	−0.466	−3.607	0.001
CRY1	P2BG	−1.408E-07	−0.479	−3.742	0.000

*Variables were subjected to natural Log transformation.

Table 3 – Multiple linear regression model 2 analyses between inflammatory markers and clock gene expression.

Dependent variable	Independent variables	Unstandardized coefficients (β)	Standardized coefficients (β)	t values	P values
BMAL1	IL-6	0.000	−0.700	−6.726	0.000
CLOCK	IL-6	−1.341E-05	−0.313	−2.258	0.029
PER1*	IL-6	−0.341	−0.696	−6.654	0.000
CRY1	IL-6	−6.026E-07	−0.715	−7.019	0.000
CRY2	IL-6	−1.768E-6	−0.431	−3.275	0.002

*Variables were subjected to natural Log transformation.

variable, even after adjusting for the above mentioned risk factors. The results revealed that plasma IL6 was a negative correlated with BMAL1, CLOCK, PER1, CRY1, CRY2 expression (β =--0.700, P = 0.000; β = −0.313, P = 0.029; β = −0.696,P = 0.000; β = −0.715,P = 0.000, β = −0.431, P = 0.002). The obtained results demonstrated that mRNA expression of a subset of clock genes was diminished in patients with DT2, especially in those with poorly controlled blood glucose. In addition, the mRNA expression of a subset of clock genes also showed a tendency to incrementally increase with increasing plasma IL6 and TNF. Impaired expression of circadian clock gene was interrelated to the elevated levels of plasma IL-6 and TNF. Impaired expression of circadian clock gene was interrelated to the elevated levels of plasma IL-6 and TNF (Table 3).

4. Discussion

Recent studies have demonstrated bilateral interaction between circadian clock function and the development of metabolic diseases such as DT2 [13]. Accumulating evidence has indicated that dysfunction of the circadian clock contributes to the development of DT2 and metabolic disorder [13]. Yet, few studies have assessed the expression of clock genes on peripheral blood cells in human patients with these metabolic diseases. In the present study, we obtained peripheral leucocytes from patients with or without DT2 mellitus and compared their mRNA expression rhythms of clock genes. Our results showed that the mRNA expression of clock genes was reduced in peripheral leucocytes of patients with DT2, particularly in those with poorly controlled blood glucose. Moreover, after adjusting for multiple confounding factors, the levels of FBG, P2BG and HbA1c were all negatively correlated to the mRNA levels of BMAL1, CRY1, PER1, respectively.

Many studies have shown that core clock genes are closely related to the glucose homeostasis. Ando et al have indicated that clock gene rhythmic expression is disturbed in the liver and adipose tissues of obese and diabetic ob/ob mice [24]. In human islets, the mRNA levels of PER2, PER3, and CRY2 are significantly lower from donors with DT2 mellitus [25]. A positive correlation was also found between insulin content and the PER2, PER3, CRY2 mRNA levels, whereas negative correlation was observed between glycated hemoglobin levels and the expression of PER3 and CRY2 [25]. Furthermore, high glucose concentration has been shown to negatively affect the mRNA level of PER3 [25]. The above results suggest that clock function is impaired in patients with these metabolic diseases.

The exact mechanisms underlying disturbance of peripheral clock gene expression in human with DT2 mellitus still remains unclear. Inflammation is currently considered as one of the causes of circadian rhythms disruption. Chronic low-grade inflamation, also known as 'metaflamation' is the main characteristic of DT2 that induces persistent proinflammatory program. The chronic condition has the same cellular and molecular mechanisms as inflammation and can promote the development of both diabetes and its complications [26]. In our study, a significantly higher expression of proinflammatory cytokines IL-6, TNF-α and CRP were found in diabetic patients. Furthermore, the levels of IL-6 were inversely correlated with BMAL1, CLOCK, PER1, CRY1 and CRY2; and TNF-α levels were negatively correlated with BMAL1, CRY1, PER1 mRNA levels. Multiple linear regression analysis revealed that the plasma IL6 levels were a negative factor for the levels of BMAL1, CLOCK, PER1, CRY1, CRY2 after adjusting for multiple variables. Based on the above results, we speculate that impaired expression of circadian clock gene is interrelated to the elevated levels of plasma IL-6 and TNF.

Inflammation has a significant role in regulating Clock function. Numerous studies, including both preclinical and clinical trials have indicated that inflammatory responses can lead to altered expression of clock gene in various tissues and cells, including peritoneal macrophages, suprachiasmatic nuclei and peripheral blood leukocytes [27–29]. Lipopolysaccharide (LPS), a potent pro-inflammatory molecule, causes transient suppression of rPer1 and rPer2 mRNA levels in rats. It is possible that LPS alters the expression levels of clock genes via an indirect effect through inflamma-

tory cytokines such as IL- 6, TNF-α [30]. Clinical and laboratory investigations have shown that acute systemic inflammatory response is triggered by intravenous administration of an appropriate dose of endotoxin to healthy subject. The results showed that endotoxin suppresses the expression of Clock, Cry1-2, Per3, Rora and Rev-erb gene in human peripheral blood leukocytes, neutrophils, and monocytes. The expression of Per1 and Per2 reach the peak simultaneously with the levels of plasma IL- 6 and tumor necrosis factor [28]. In addition, NF-κB, a master regulator of immune and inflammatory responses, is crucial for circadian homeostasis, and clock function is particularly vulnerable to direct and indirect triggers of NF-κB activation. The inflammation signaling TF NF-κB represses transcription of CLOCK/BMAL1 target genes by directly binding to the promoters of the core clock repressors Period, Cryptochrome, and Rev-erb genes. The above results revealed that impaired clock function is closely related to the inflammation and NF-κB in response to inflammatory stimuli leads to marked inhibition of clock repressors [23].

The current study has few limitations. Frist, we were unable to assay the circadian oscillation patterns of clock genes expression during the day. Expression of the circadian clock was mainly observed at the usual time of activity and light exposure. Blood samples for leucocytes isolation and clock gene expression analysis were obtained at 8 AM, which was considered the most profitable time point for exploring the correlation between mRNA levels of clock genes and blood biochemical and inflammation markers. Another limitation was that the experiments were restricted by the sample size and lack of replications, thus the obtained results might lack robustness and may not be generalizable to all ages. Accordingly, the presented results should be further verified by future studies with a larger sample size.

In conclusion, our study defined a severe dampening effect of peripheral clock gene expression in peripheral leucocytes of patients with DT2. This is the first study to evaluate the relationship between expression of clock genes in peripheral leucocytes and inflammatory factor in patients with type 2 diabetes during chronic inflammatory status. Our results revealed that inflammatory factor IL-6, TNF-α, but not CRP, were correlated with clock gene expression. Therefore, the presented data may contribute to better understanding of the pathophysiology of DT2 in humans with impairment of the circadian clock.

Funding

The Natural Science Foundation of China (Grant No. 81600628/H0713), the Natural Science Foundation of Tianjin (Grant no. 16JCYBJC25700), Tianjin Education Commission Science Foundation (Grant no. 2018KJ071) and Scientific Research Funding of Tianjin Medical University Chu Hsien-I Memorial Hospital (Account number 2012DX05) supported this study. We would like to thank all the nurses for helping with blood collection.

Declaration of competing interest

The authors declared that there is no conflict of interest.

Author contribution

Rongguo Yu and Linlin Tian carried out the studies, participated in collecting data, and drafted the manuscript. Yi Ding and Yali Gao performed the statistical analysis and participated in its design. Daiqing Li and Yunzhao Tang participated in acquisition, analysis, or interpretation of data and draft the manuscript. All authors read and approved the final manuscript.

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