Disturbance of Circadian Rhythms Extensively Affects Blood Biochemical Parameters and Serum miRNA Profiles

Guo-Bin Huang
Sun Yat-sen University
Huan Ma
Sun Yat-sen University
Xi-Hui Gan
Sun Yat-Sen University Cancer Prevention and Treatment Center: Sun Yat-sen University Cancer Center
Si-Lin Li
Sun Yat-Sen University Cancer Prevention and Treatment Center: Sun Yat-sen University Cancer Center
Xiao-Hong Ma
Sun Yat-sen University
Si-Yu Chen
Sun Yat-Sen University Cancer Prevention and Treatment Center: Sun Yat-sen University Cancer Center
Hui-Ying Yang
Sun Yat-sen University
Liang Zhang
China Institute of Marine Technology and Economy
Zhi-Qiang Tian
Instrumentation Technology and Economy Institute
Xin Wang
Beijing University of Technology Institute of Recycling Economy
Zhen Liao
Instrumentation Technology and Economy Institute
Tuo-Yang Zhou
China Institute of Marine Technology & Economy
Yong-Cong Shao
Beijing Sport University
Ye-Bing Yang
Sixth Medical Center of PLA General Hospital
Hong-Mei Jiang
Sun Yat-sen University
Jin-Hu Guo (guojinhu@mail.sysu.edu.cn)

Sun Yat-Sen University

Research

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Abstract

Background: Circadian clock plays a critical role in synchronizing the inner molecular, metabolic and physiological processes to the environmental cues with a period of 24 h. Misalignment in the circadian rhythms leads to decreased adaptation and performance and increased risk of associated disorders. The non-24-h schedules and shift schedules are commonly used in maritime operations, both of which could result in disturbance of circadian rhythms.

Methods: In this study, we recruited volunteers and conducted two experiments: in one experiment 15 subjects followed an 8-h on and 4-h off schedule (non-24-h schedule), and in the second experiment 12 subjects followed a 3-d rotary schedule with consecutive shift in sleep time (phase-changing schedule). The serum/blood biochemical variables were measured and the serum miRNAs of the volunteers in the second experiment were subjected to transcriptomic miRNA sequencing.

Results: The results show both of the schedules caused comprehensive changes in the blood/serum biochemical variables. Notably, significant elevation in serum phosphate was observed in both experiments: 1.210 ± 0.141 in control and 1.330 ± 0.117 in recovery ($P = 0.014$) in the first experiment and 1.193 ± 0.152 in control and 1.343 ± 0.099 in recovery ($P = 0.007$) in the second experiment. In addition, a subset of serum miRNAs targeting genes involved in circadian rhythms, sleep homeostasis, phosphate metabolism and multiple critical physiological processes or pathways were identified in the second experiments.

Conclusions: This study reveals that non-24-h and shift schedules lead to changes in a large spectrum of blood/serum biochemical variables due to circadian misalignment. Schedules with frequent shift may cause remarkable changes in serum miRNAs which are involved in multiple physiological pathways. These findings would help understand the deleterious effects of shift schedules and develop optimized strategy to enhance welfare and performance of the shift workers.

Background

Circadian clocks are ubiquitous in most of the organisms on earth, which play critical roles in synchronizing the internal physiology with the external cycling environmental cues with a period of 24 h [1]. Environmental, genetic, physiological and pathological factors may cause disturbance in circadian rhythms [2]. In humans, circadian clock controls a broad variety of physiological, cognitive, emotional and behavioral features, and misalignment in circadian rhythms leads to extensive detrimental effects on health and performance [3].

The oscillation of many human physiological variables is governed by circadian clock [4, 5]. Not surprisingly, most blood biochemical contents are also under the circadian control, e.g., polymorphonuclears, lymphocytes, Ca^{2+}, Na^{+}, viscosity, sedimentation rate, etc. Overall, most of the variables of the quantity of blood cells and the concentration of electrolytes, hormones and metabolites display daily rhythmic profiles [6]. In the blood biochemical variables, the level of plasma phosphate

displays an overt diurnal rhythmicity with the nadir approximately at 11:00 AM, and the peak approximately at 12:30 AM [7].

In modern society, a substantial proportion of population (approximately 15–20%) live and work under schedules deleterious to their circadian rhythms [8]. Although numerous medical and ergonomic problems have been reported, rotatory shift schedules are still commonly employed in industrial society, including maritime tasks and the detrimental effects have not been sufficiently concerned. For instance, non-24-h and frequently rotating schedules are commonly used in maritime missions [9–12]. Circadian rhythms display certain adaptation to environmental cues with a period in a range deviated from 24 h, which is termed entrainment range. Within this range, the rhythms change in accordance to the cues; beyond this range, the rhythms show a superposed profile of the cues and endogenous period [10].

Changes in sleep/wake status are accompanied by changes in gene expression. At the molecular level, circadian clock controls the expression and function of protein-coding genes and microRNAs (miRNAs) which are miRNAs are small, 20- to 22-nt non-coding RNA transcripts that base pair with mRNA to degrade it or inhibit its translation [13, 14]. Insufficient sleep elicits expression changes in serum miRNAs targeting circadian rhythm and genes associated with sleep homeostasis [15–17]. On the other hand, miRNAs play a conserved role in regulating sleep and circadian clock in human and other animals [18–20]. These evidences demonstrate that miRNAs are involved in the regulation of circadian clock, and vice versa, misalignment in circadian rhythms may affect the expression and subsequent the function of a subset of miRNAs.

In this work, we collected the blood/plasma samples from subjects who participated in two experiments following a non-24-h schedule and a schedule with consecutive shifts, respectively. The blood/serum biochemical variables of these samples were measured and the serum miRNAs from one experiment were sequenced. And the findings reveal extensive changes at the molecular and physiological levels due to circadian misalignment.

Methods

Participants and bioethics

Two experiments were carried out in this work, named experiment #1 and #2 for simplification. Experiment #1 was carried out from Dec 26, 2017 to Jan 04, 2018, and experiment #2 was carried out from Sep 2, 2017 to Oct 10, 2017. In total, 15 healthy men (age, 23.1 ± 0.8 year; height, 175.7 ± 4.8 cm; weight, 73.5 ± 7.2 kg; data are means ± SD) volunteered in experiment #1. No alcohol, smoking or caffeinated drinks and tea were allowed during this experiment. In experiment #2, the volunteers included 12 healthy men (age, 32.0 ± 4.4 year; height, 173.1 ± 4.6 cm; weight, 70.5 ± 9.8 kg; data are means ± SD). No alcohol and smoking were allowed, and caffeinated drinks and tea were allowed only during the experiment period but not control and recovery periods in this experiment.

Protocols and arrangements

The arrangement of experiment #1 contained four measurement time blocks: 1) control from 8:00 AM on the first day to 10:00 PM on the second day; 2) sleep deprivation (SD) period from 6:00 AM on the second day to 10:00 PM on the third day; 3) 8-h–on and 4-h–off schedule: from 6:00 AM on the fifth day to 2:00 AM on the eighth day. In control and recovery periods, the participants lived on a fixed schedule with a 24-h period (Fig. 1a).

In experiment #2, the participants were divided into three groups and worked on a 3-day shift rotation 3-day shift rotation. The arrangement of experiment #2 contained three periods: 1) control for 7 days; 2) 3-d rotatory shift for 30 days. The total sleep duration in every 3 days was 24 h though the time of sleep and waking up changed every day, which caused highly frequent shifts; 3) recovery for 7 days. In control and recovery periods, the participants lived on a fixed schedule with a period of 24-h (Fig. 1b).

The time of blood drawing after overnight fasting was between 7:00 AM and 8:00 AM for all of the subjects in control and recovery periods in both experiments. In experiment #1, blood drawing was performed on the second or third day in control and the first or second day in recovery. In experiment #2, blood drawing was performed on seventh day in control and the second day in recovery.

Biochemical analysis of blood/serum samples

The whole blood samples derived from experiment #1 and the serum samples from experiment #2 were subject to biochemical analysis. We mixed serum of seven volunteer, 150 μl serum of each person was used. AU5800 Series Chemistry Analyzers (Beckman Coulter, USA) was used for the analysis.

Transcriptomic analysis of serum exosomal miRNAs

For biochemical analysis, 100 μl of serum samples from each subject were pooled and the extracted miRNA was isolated with TreSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA), and ligated with adapters. After RT-PCR amplification, RNAs between 22 nt and 30 nt were excised and purified for library construction. The sequencing was performed on Illumina Hisseq2000/2500. The read of single end was 50 bp.

Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove the sequences of adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. The significance threshold was set to be 0.01 and 0.05. To predict the genes targeted by most abundant miRNAs, two computational target prediction algorithms (TargetScan 50 and Miranda 3.3a) were used to identify miRNA binding sites [21, 22]. Finally, the data predicted by both algorithms were combined and the overlaps were calculated. The KEGG Pathways of these most abundant miRNAs, miRNA targets were also annotated.

Statistics

In the analysis of blood/serum biochemical parameters, the results are presented as the means ± SD. Wilcoxon matched-pairs signed rank test (IBM SPSS Statistics 25) was used to analyze the values as indicated at certain time points. In the analysis of miRNA sequencing data, the miRNAs were normalized

using $\log_2$ relative expression, the miRNAs were further analyzed by using Fisher exact test (http://en.wikipedia.org/wiki/Fisher's_exact_test) and Chi-squared 2 × 2 test (http://en.wikipedia.org/wiki/Chi-squared_test) when the absolute value of relative expression fold change > 1. The significance of qRT-PCR results was detected by Student's t test. * represents the *P*-value of the statistical tests being less than a significance level of 0.05 ($P < 0.05$); ** $P < 0.01$, and # $P < 0.001$. Some of the samples failed to be detected which are not included in the analysis.

Results

Changes in plasma biochemical variables due to non-24-h schedule

The non-24-h schedules are commonly used in maritime tasks, including maritime oil & gas mining, commercial vessel, submarine and other fleets [9, 11]. A number of studies have demonstrated that 18-h or 12-h work schedules are deleterious to health and performance [8–11]. Although experiment #1 contained a period of 36-h SD, the sleep was compensated after SD which was supported by results from the psychomotor vigilance tests, therefore, the effects of SD could be neglected [11].

In experiment #1, a number of serum variables showed significant changes in recovery (after the non-24-h schedule) compared to control (before the non-24-h schedule). The increased serum variables include: phosphate (Pi) level, total bile acids level, triglyceride level, prealbumin level, aspartic aminotransferase level, apolipoprotein A-1 level, absolute neutrophil count, red blood cell distribution width, and white blood cell count. By contrast, the variables of sodium ion concentration, iron ion concentration, total protein level, albumin level, total bilirubin level, showed significant decrease after the experiment (Fig. 2; Supplemental table 1). Approximately 90% of Pi is present within the skeleton, 10% in the soft tissue and less than 1% in the serum [23]. In experiment #1, the average levels of serum Pi were 1.210 ± 0.141 in control and 1.330 ± 0.117 in recovery, respectively (Fig. 2b; supplemental table 1). The serum variables showed no significant change, including potassium ion concentration, chlorine ion concentration, glucose concentration, etc (supplemental Table 1).

Changes in whole blood biochemical variables due to frequent shift

Shift work and jet lag have been shown to affect circadian rhythms in many studies, and shift work is also called social jet lag [24]. Shift work is common in steel industries, petroleum industries, power plants, medicine and nursing, police forces and maritime [12, 245]. According to the 3rd international classification of sleep disorders (ICSD-3), the comprehensive inadaptations and disorders caused by shift schedules are defined as shift-work disorders [26].

A schedule with frequent shifts was used in experiment #2. The results revealed significant changes of many serum variables in recovery compared to those in control. The serum variables showed increased

levels included: glucose, Pi, cholesterol, creatinine, direct bilirubin, low density lipoprotein, gamma-glutamyl transferase, total protein, albumin, superoxide dismutase, alanine transaminase, alkaline phosphatase, glutamic oxalacetic transaminase, Apolipoprotein-B and total bilirubin. By contrast, the levels of homocysteine, urea, triglyceride and total bilirubin showed significant decrease after the experiment (Fig. 3; Supplemental table 2). The average levels of serum Pi were 1.193 ± 0.152 in control and 1.343 ± 0.099 in recovery, respectively (Fig. 3c; supplemental table 2). The serum variables showing no significant change included iron ion, potassium ion, etc, which are listed in supplemental Table 1. It is noteworthy that the significant elevation in Pi was observed in both experiments, suggesting that it may be a factor with high risk occurs in the disturbance of circadian rhythms (Fig. 2,3; supplemental Tables 1,2).

Transcriptomic analysis of serum exosome miRNAs

We conducted miRNA sequencing using the pooled serum samples derived from experiment #2, which identified 1008 and 1172 miRNAs in control and recovery periods, respectively. The control and recovery samples contain 852 miRNAs in common (Fig. 4a). In total, 249 miRNAs ($P < 0.01$) and 395 miRNAs ($P < 0.05$) showed differential expression before and after experiment, respectively (Fig. 4b,c; supplemental Table 3), and these differentially expressed miRNAs (DEMs) are implicated in comprehensive functional pathways, including multiple metabolic pathways, immunity, neural function (axon guidance) and cancer associated pathways (Fig. 4d).

We next investigated whether there were DEMs targeting the core circadian clock genes, as rotatory schedule causes misalignment in circadian rhythms, and DEMs targeting *PER1*, *PER2*, *CRY1*, *CRY2*, *BMAL1/ARNTL*, *BMAL2/ARNTL2*, *RORA*, *RORB*, *NPAS2* and *DEC1* genes were identified (Fig. 4e; supplemental Table 3). Quantitative reverse transcription polymerasec reaction (qRT-PCR) results confirmed the expression of four representative genes (Fig. 4f-i). Together, these data suggest that comprehensive changes occurred in circadian clock associated miRNAs due to the shift schedule.

Insufficient sleep leads to changes in the amplitude of several sets of genes associated with a variety of physiological processes [16]. In this study, the DEMs associated with sleep homeostasis were also observed, including miRNAs targeting the genes including *STAT3*, *KCNV2*, *IL6* and *CAMK2D* (supplemental Table 3).

Transport of Pi across the renal proximal tubule is a crucial step in maintaining its homeostasis. In kidney, sodium-dependent Pi (Na/Pi) transport systems are localized in the brush-border membrane, and bulk of filtered Pi is re-absorbed in the proximal tubule. Sodium-dependent Pi (Na/Pi) transports mediate the rate-limiting step in the re-absorptive process. Three classes of Na/Pi cotransporters, NPTI, NPTII and NPTIII, have been identified in human. Of the NPT families, Pi homeostasis is maintained by NPT2A members (including *SLC34A1/NPT2A*, *SLC34A2/NPT2B* and *SLC34A3/NPT2C*) in kidney and intestine at the organism level and SLC20A1/PiT1 and SLC20A2/PiT2 at the cellular level [23, 7–29]. PHEX and FGF-23 are involved in regulation of Pi homeostasis and they have been identified in inherited and acquired

hypophosphatemia [28]. We searched potential miRNAs targeting the genes involved in the regulation of Pi regulation, including miRNAs targeting NPTII genes (*SLC34A1*, *SLC34A2* and *SLC34A3*) [28], Pi regulating gene with homologies to endopeptidases on the X chromosome (*PHEX*) and fibroblast growth factor-23 (*FGF23*) [Takeda et al 2004], and miRNAs targeting *SLC20A1* and *SLC20A2* were found.

Discussion

In this work, we conducted two experiments, and the schedules used in both of which are detrimental to circadian rhythms. However, the underlying mechanisms are different: experiment #1 provided a non-24 h cycling condition, while experiment #2 yielded highly frequent shifts. We demonstrate that these two schedules may cause extensive alterations of blood/serum variables, suggesting that circadian rhythmicity has been severely influenced.

In both experiments, the participants lived in the isolation condition, and most of the environmental cues including lighting, noise, ambient temperature and workload were controllable so that we could focus on the effects of circadian misalignment. Instead, in real condition like submarine, as the microenvironment differs between different locations or posts on board, the effects of circadian misalignment might be hard to be addressed [12].

The albumin level and total bilirubin level decreased in experiment #1 but increased in experiment #2, while the triglyceride level increased in experiment #1 but decreased in experiment #2 (Fig. 2,3; supplemental Tables 1,2), which may be owing to the individual difference or the different effects of between non-24-h schedule and rotatory schedule. Changes in metal elements (sodium, iron) and molecules associated with metabolism (glucose, cholesterol, urea and creatinine) were also found in one or both experiments although most of the changes are still within the normal ranges. By contrast, in experiment #1, the levels of total bile acids of five subjects were higher than the normal range (0.1–10.0 μmol/L) after the experiment (Fig. 2f; supplemental Table 1), suggesting that risk of dysfunctions in liver or hepatic duct should be taken into consideration [30].

Inorganic Pi is a vital component of bone mineralization, phospholipids in membranes, nucleotides that provide energy and serve as components of DNA and RNA, and phosphorylated intermediates in cellular signaling [31]. It is also required for skeletal development, mineral metabolism, and diverse cellular. The level of serum Pi is maintained within a narrow range - the normal plasma Pi concentration is 0.81–1.45 mmol/L, which is regulated by multiple factors, e.g., intestinal absorption, exchange with intracellular and bone storage pools, renal tubular reabsorption, and their interactions [28, 32]. Plasma Pi imbalance, including hypophosphatemia and hyperphosphatemia, are associated with disorders in many organs. Occurrence of hyperphosphatemia is reported in renal failure, hemolysis, tumor lysis syndrome, and rhabdomyolysis [33, 34].

LC20A1 and SLC20A2 are members of the mammalian type-III inorganic Pi transporters encoded by the SLC20 genes, which regulate cellular Pi concentration, Pi sensing, and transcription of downstream genes [23]. These two factors are the only members of this family in humans and they are conserved in many

kingdoms including fungi, bacteria and plants, animals [35, 36]. In mammals, SLC20A1 and SLC20A2 are highly expressed in brain although they are ubiquitously expressed [27, 37]. Mutations in SLC20A2 are associated with Fahr's Disease, characterized by the symmetric calcification in the basal ganglia and other brain regions, and this disease contains a wide spectrum of neuropsychiatric symptoms including headaches, psychiatric disorders, and movement disorders [27; 38]. In this study, some DEMs targeting SLC20A1 and SLC20A2 were identified, suggesting that frequently rotatory schedules might lead to Pi imbalance due to circadian misalignment.

miRNAs constitute a post-transcriptional layer in the regulation of circadian clock and sleep homeostasis [16, 39, 40]. In addition to Pi metabolism, we identified a substantial set of functional miRNAs which are implicated in multiple pathways or physiological processes, including circadian clock and sleep homeostasis, suggesting that these miRNAs may contribute to the misaligned circadian rhythms and affected sleep homeostasis. These DEMs may be causative or resultant of the disturbed circadian rhythms. In addition, DEMs associated with cancer, neural function and several metabolic pathways were also identified, which suggests that desynchronized circadian rhythms may increase the risks of many potential disorders through modulating the expression of corresponding miRNAs.

Conclusions

In this work, our findings would add important evidence supporting that sleep deprivation, non-24-h schedules and shift schedules may lead to remarkable changes in the blood biochemical parameters, suggesting that the health and performance are prone to be influenced. Some of the findings may elicit important questions, for instance, what are the mechanisms underlying the increased blood Pi caused by circadian misalignment?

Circulating microRNA profile has been proposed as a potential biomarker for obstructive sleep apnea diagnosis [41]. We found that a subset of plasma miRNAs are involved in circadian rhythms, sleep homeostasis and Pi metabolism, which may better the investigation of the underlying molecular mechanisms of circadian misalignment and serve as biomarkers in further study.

Abbreviations

RT-PCR
reverse transcriptase-polymerase chain reaction; miRNA:microRNA; SD:sleep deprivation; Phosphate:Pi; PHEX:phosphate regulating gene with homologies to endopeptidases on the X chromosome; FGF-23:fibroblast growth factor-23; Pi:phosphate; DEM:differentially expressed miRNA.

Declarations

Authors' contributions

YS, YY, XW, LZ, ZT and JG designed and organized the experiments. GH, HM, SL and TZ performed experiments. XM, XG, SC, LZ, HJ, HY and JG helped with data collection and statistical analysis. JG wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The research methods and procedures of experiment #1 and #2 were in accordance with the guidelines and approved by the Ethics Committee of the Beihang University. All participants provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]State Key Laboratory of Biocontrol, Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China. [2]China Institute of Marine Technology & Economy, Beijing 100081, China. [3]Sixth Medical Center, General Hospital of Chinese People's Liberation Army, Beijing 100048, China. [4]School of Psychology, Beijing Sport University, Beijing 100084, China. *Present address: School of Biological Science and Medial Engineering, Beihang University, Beijing 100191, China.

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Figures

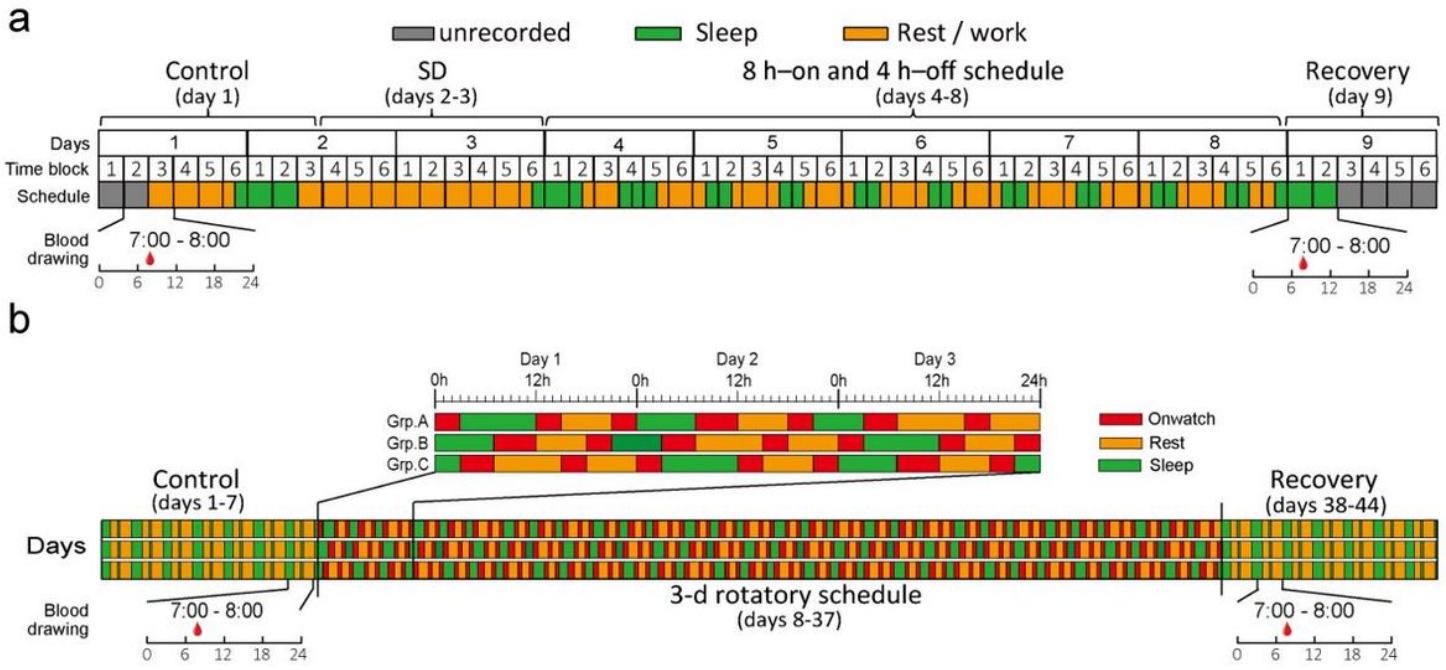

Figure 1

Experimental setup and arrangement. a Diagram of experiment #1. The time points of blood drawing in control are indicated. Every day is divided into six equal time blocks (1-6) and the duration of each is 6 h. Legends are labeled on the top. b Diagram of experiment #2. The time points of blood drawing are

denoted. A 3-d rotatory schedule is shown (days 1-3) in a zoomed view as an example. Legends are labeled on the top.

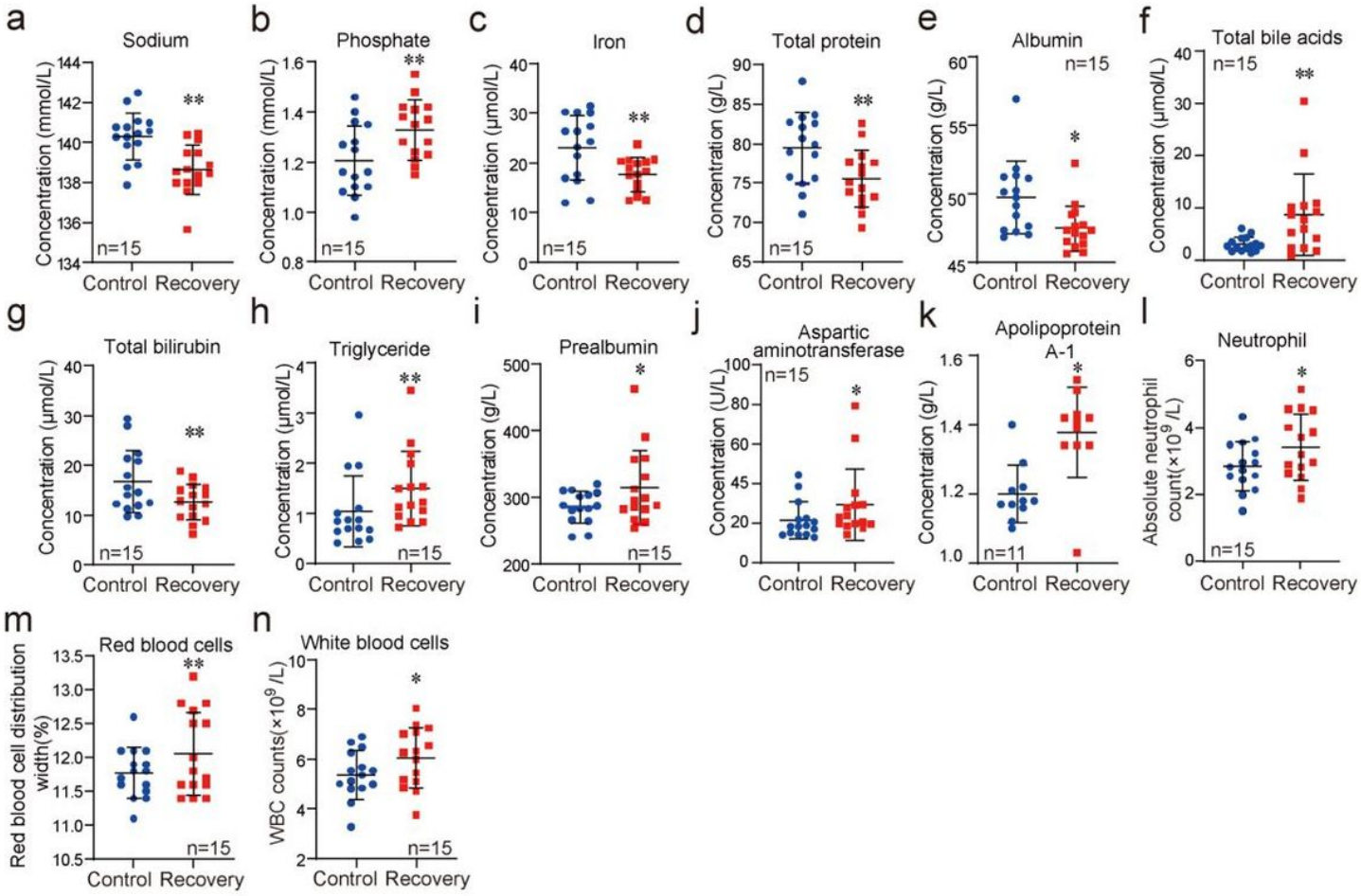

Figure 2

Results of blood biochemical analysis. Scatter plots with bars of blood parameters from experiment #1 in control and recovery. Panels a to s are the results of sodium (a), phosphate (b), iron (c), total protein (d), albumin (e), total serum bile acids (f), total bilirubin (g), triglyceride (h), prealbumin (i), aspartic aminotransferase (j), apolipoprotein A-1 (k), neutrophil (l), distribution width of red blood cells (m), white blood cell counts (n). Data are mean ± SD. * $p < 0.05$, ** $p < 0.01$.

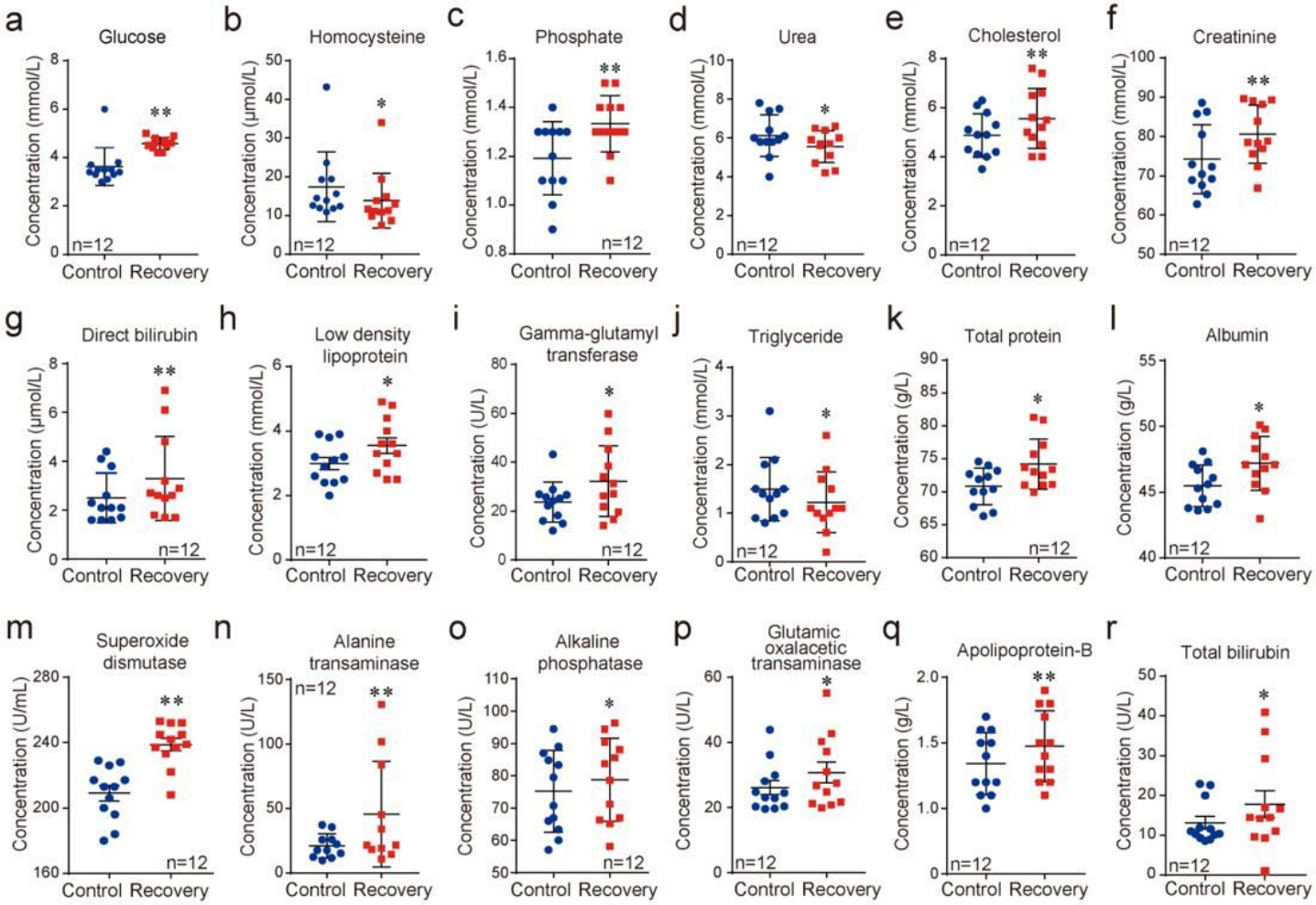

Figure 3

Results of plasma biochemical analysis. Scatter plots with bars of blood parameters from experiment #2 in control and recovery. Panels a to s are the results of glucose (a), homocysteine (b), phosphate (c), urea (d), cholesterol (e), creatinine (f), direct bilirubin (g), low density lipoprotein (h), gamma-glutamyl transferase (i), triglyceride (j), total protein (k), albumin (l), superoxide dismutase (m), alanine transaminase (n), alkaline phosphatase (o), glutamic oxalacetic transaminase (p), apolipoprotein-B (q), total bilirubin (r). Data are mean ± SD. * $P < 0.05$, ** $P < 0.01$.

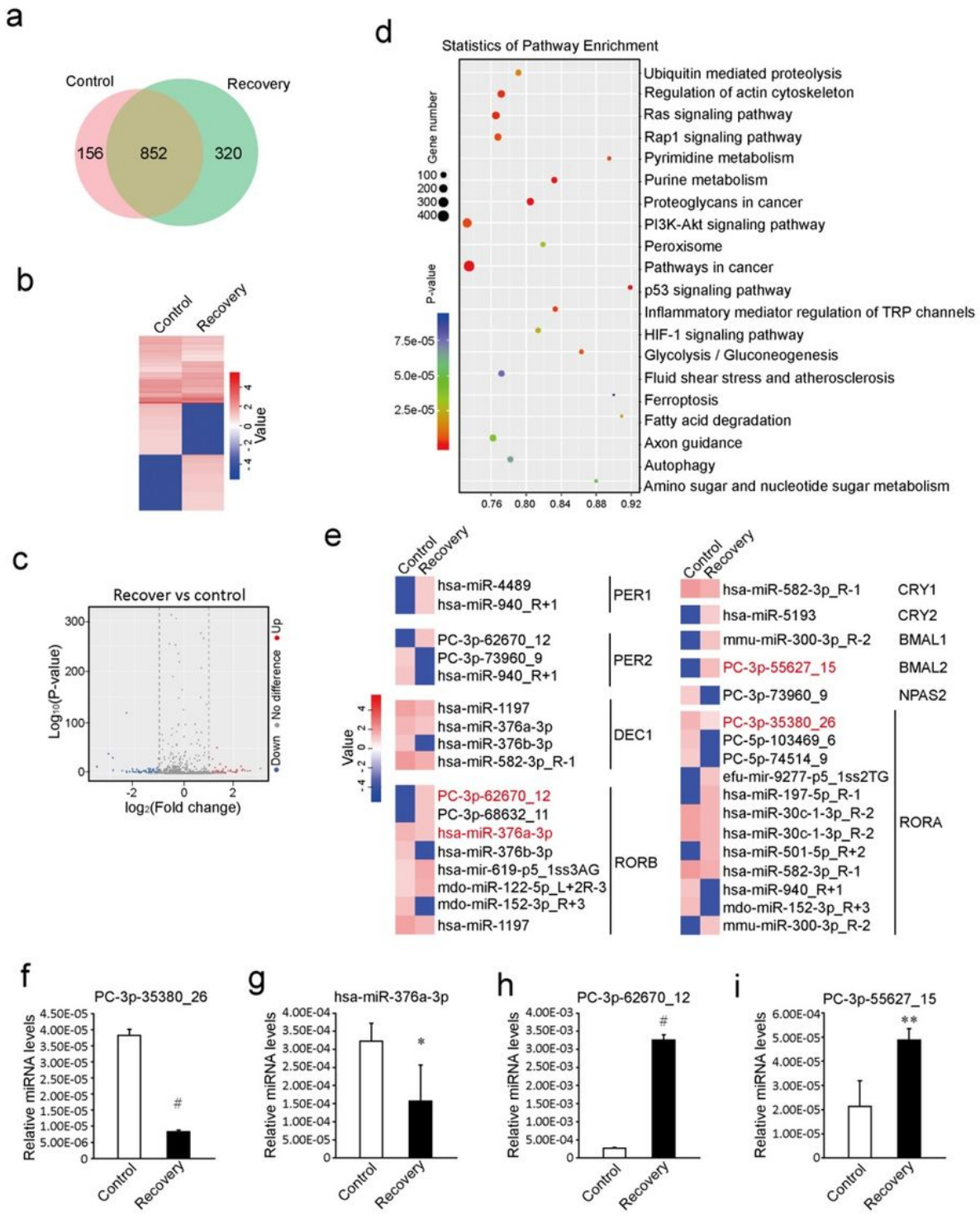

Figure 4

Transcriptomic analysis of plasma exosomal miRNAs. a Venn diagram of expressed miRNAs in pooled sera in control and recovery periods. b Heat map of all miRNAs showing changed levels in control (Ctrl) and recovery (Reco). P < 0.01. c Volcano plot of differentially expressed serum miRNAs constructed using P-values and fold change values. The abscissa represents the log2 transformation value of the differential expression fold change and the dashed vertical lines correspond to 2.0-fold up and down,

respectively. d KEGG enrichment scatter plot of differentially expressed miRNAs. e Heat map of examples of differentially expressed serum miRNAs targeting core circadian clock genes between control (Ctrl) and recovery (Reco). Only those DEMs with P values < 0.05 are shown. Four miRNAs in red were subjected to qRT-PCR validation. f-l q-RT-PCR validation of four indicated miRNAs. Data are mean ± SE. n = 4. * P < 0.05, ** P < 0.01, # P < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaltable1.pdf
- Supplementaltable2.pdf
- Supplementaltable3.pdf