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Diurnal rhythm of human semen quality: analysis of large-scale human sperm bank data and timing-controlled laboratory study

Kun Liu [1,2,†], Tianqing Meng[3,†], Qing Chen [1,†], Guizhong Hou[1,4], Xiaogang Wang [5], Siheng Hu[3], Xiuli Gu[3], Honggang Li [3], Yuyan Li [6], Chengliang Xiong [3,*], and Jia Cao [1,*]

[1]Key Lab of Medical Protection for Electromagnetic Radiation, Ministry of Education of China, Institute of Toxicology, College of Preventive Medicine, Third Military Medical University (Army Medical University), Chongqing, China [2]Center for Disease Control and Prevention, Southern Theater Command, Guangzhou, China [3]Hubei Province Human Sperm Bank, Center of Reproductive Medicine, Wuhan Tongji Reproductive Medicine Hospital, Wuhan, China [4]Institute for Vaccine Clinical Research, Hebei Province Center for Disease Control and Prevention, Hebei, China [5]Department of Chemical Defense Medicine, School of Military Preventive Medicine, Third Military Medical University (Army Medical University), Chongqing, China [6]Reproductive Medical Center, First Affiliated Hospital of Third Military Medical University (Army Medical University), Chongqing, China

*Correspondence address. Key Lab of Medical Protection for Electromagnetic Radiation, Ministry of Education of China, Institute of Toxicology, College of Preventive Medicine, Third Military Medical University (Army Medical University), Chongqing 400038, China.
E-mail: caojia1962@126.com (J.C.) https://orcid.org/0000-0001-7209-2993; Hubei Province Human Sperm Bank, Center of Reproductive Medicine, Wuhan Tongji Reproductive Medicine Hospital, Wuhan 430000, China.
E-mail: clxiong951@sina.com (C.X.) https://orcid.org/0000-0002-2321-1009

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STUDY QUESTION: Can we identify diurnal oscillations in human semen parameters as well as peak times of semen quality?

SUMMARY ANSWER: Human semen parameters show substantial diurnal oscillation, with most parameters reaching a peak between 1100 and 1500 h.

WHAT IS KNOWN ALREADY: A circadian clock appears to regulate different physiological functions in various organs, but it remains controversial whether diurnal rhythms occur in human semen parameters.

STUDY DESIGN, SIZE, DURATION: The medical record of a provincial human sperm bank (HSB) with 33 430 semen samples collected between 0800 and 1700 h from 1 March 2010 to 8 July 2015 was used to analyze variation in semen parameters among time points. A laboratory study was conducted to collect semen samples (n = 36) from six volunteers at six time points with identical time intervals (2 days plus 4 h) between 6 June and 8 July in 2019, in order to investigate the diurnal oscillation of semen parameters *in vivo*, with a strictly controlled abstinence period. Therefore, the sperm bank study with a large sample size and the *in vivo* study with a strictly controlled abstinence period in a 24-h time window could be compared to describe the diurnal rhythms in human semen parameters.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Samples were obtained from potential HSB donors and from participants in the laboratory study who were volunteers, recruited by flyers distributed in the community. Total sperm count, sperm concentration, semen volume, progressive motility and total motility were assessed using computer-aided sperm analysis. In addition, sperm chromatin integrity parameters (DNA fragmentation index and high DNA stainability) were assessed by the sperm chromatin structure assay, and sperm viability was measured with flow cytometry in the laboratory study.

MAIN RESULTS AND THE ROLE OF CHANCE: The 33 430 samples from the HSB showed a temporal variation in total sperm count, sperm concentration, semen volume, progressive motility and total motility (all $P < 0.001$) between 0800 and 1700 h. Consequently, the eligibility of semen samples for use in ART, based on bank standards, fluctuated with time point. Each hour earlier/later than 1100 h was associated with 1.14-fold risk of ineligibility. Similarly, the 36 samples taken during the 24-h time window showed diurnal oscillation. With the pre-collection abstinence period strictly controlled, most semen parameters reached the most favorable level between 1100 and 1500 h.

[†]These authors contributed equally to this publication.

LIMITATIONS, REASONS FOR CAUTION: Some of the possible confounding factors, such as energy intake, which might influence semen quality or diurnal rhythms, were not adjusted for in the analyses. In addition, the findings should be considered with caution because the study was conducted in a specific population, time and place, while the timing of oscillations could differ with changing conditions.

WIDER IMPLICATIONS OF THE FINDINGS: The findings could help us to estimate semen quality more precisely and to obtain higher quality sperm for use in ART and in natural conception.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the National Natural Science Foundation of China (81871208) and National Key R&D Program of China (2017YFC1002001). There are no conflicts of interest to declare.

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Key words: circadian clock / circadian rhythm / diurnal rhythm / ejaculation time point / semen quality / semen parameter / sperm chromatin integrity / human sperm bank / Homo sapiens / spermatozoa

Introduction

The circadian clock has been found to regulate different physiological functions in various organs (Bass and Takahashi, 2010; Harfmann *et al.*, 2015; Roenneberg and Merrow, 2016; Douma and Gumz, 2018). However, the male reproductive system might be an exception: it has been shown that the hypothalamus, pituitary and probably Leydig cells in the testis are regulated by circadian clocks (Sen and Hoffmann, 2020), but it remains controversial whether the spermatogenic cells as well as sperm possess strong circadian rhythms (Alvarez *et al.*, 2003; Morse *et al.*, 2003; Kennaway *et al.*, 2012; Gotlieb *et al.*, 2018; PeterLin *et al.*, 2019; Sciarra *et al.*, 2020). Our understanding of circadian clocks in male reproductive function lags behind the abundant work on other physiological functions, especially female reproductive function (Sen and Hoffmann, 2020). To date, it is still unclear whether there is a diurnal rhythm in human semen parameters.

Some studies have explored the temporal differences in semen parameters: for example, Cagnacci *et al.* (1999) recruited 54 males and compared their semen samples ejaculated in the morning (0700–0730 h) with those ejaculated in the afternoon (1700–1730 h); Xie *et al.* (2018) collected 12 245 semen samples from men of subfertile partnerships at different time points between morning and noon; and Biljan *et al.* (2005) investigated the semen collected from 570 males in the morning (0800–0900 h) and evening (1900–2000 h). Some of the studies showed differences in semen parameters such as total sperm count and sperm concentration, among different time points but these studies could not be compared because the time windows of semen collection were not identical. Valsa *et al.* (2016) firstly tried to analyze the differences in some semen parameters throughout 24 h and showed difference of semen parameters with time points. Unfortunately, some important technical details, such as recruitment criteria of subjects, control of the abstinence period and measurement methods of the semen parameters were not reported. Therefore it is unclear whether the observed diurnal variation is a true diurnal rhythm. Moreover, abstinence period, a vital determinant of semen parameters, is believed to highly correlate with the time point of semen collection, as it is plausible that ejaculation at later time points may indicate more hours or minutes for spermatogenesis. However, abstinence period was usually recorded in number of days, not hours, based on the memory of the volunteers in the previous studies (Cagnacci *et al.*, 1999; Biljan *et al.*, 2005; Xie *et al.*, 2018). It is not clear to what extent does the crude estimation of abstinence period will bias the analysis of diurnal rhythms in semen parameters.

To address this gap, we performed a laboratory study to investigate the diurnal rhythm of semen parameters *in vivo*, using semen samples which were sequentially collected with a strictly controlled identical abstinence period, and compared these results with 33 430 samples from a human sperm bank (HSB) collected within a specific time window (0800–1700 h) with a variable abstinence period (Fig. 1).

Materials and methods

Ethics approval and consent to participate

The procedures in this study were approved by the ethics committee of Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology, and the ethics committee of the First Affiliated Hospital of Third Military Medical University. Written informed consent was also obtained from each participant for use of their semen in research.

Participants

In the study of HSB samples, the medical record of HSB was derived from the Hubei HSB, which is the unique HSB in Hubei province, the ninth largest province/municipality with about 58 million people in China. Its running follows the standard requested by National Health and Family Planning Commission of China, as all other Chinese HSBs do (Chinese Ministry of Health, 2004a,b). Before the examination of semen, the sperm donation candidates were screened with the following criteria: Chinese nationality; 22–45 years old; height at least 165 cm; and educational attainment beyond high school. In the Hubei HSB, 33 430 semen samples were provided between 0800 and 1700 h from 1 March 2010 to 8 July 2015.

In the timing-controlled laboratory study, six male volunteers were recruited by flyers distributed in Chongqing, China from 6 June 2019 to 8 July 2019, which bordered Hubei and shared similar climatic characteristics and local residents' lifestyle with Hubei. The inclusion criteria were as follows: 18–49 years old; Chinese nationality; and local residents who were able to finish the study without interruption. The exclusion criteria were: history of incomplete orchiocatabasis, varicocele, mumps, testicular torsion, prostatitis or testicular hypoplasia syndrome diagnosed by urologist; history of inflammation, tuberculosis or injury of urogenital system diagnosed by urologist; history of anemia, hypertension, hyperlipidemia, cardiopathy, diabetes, epilepsy, thyroid disease, chronic nephritis, tuberculosis, venereal disease, hepatitis B, mental disease, high fever (in last 3 months), tumor or genetic disease;

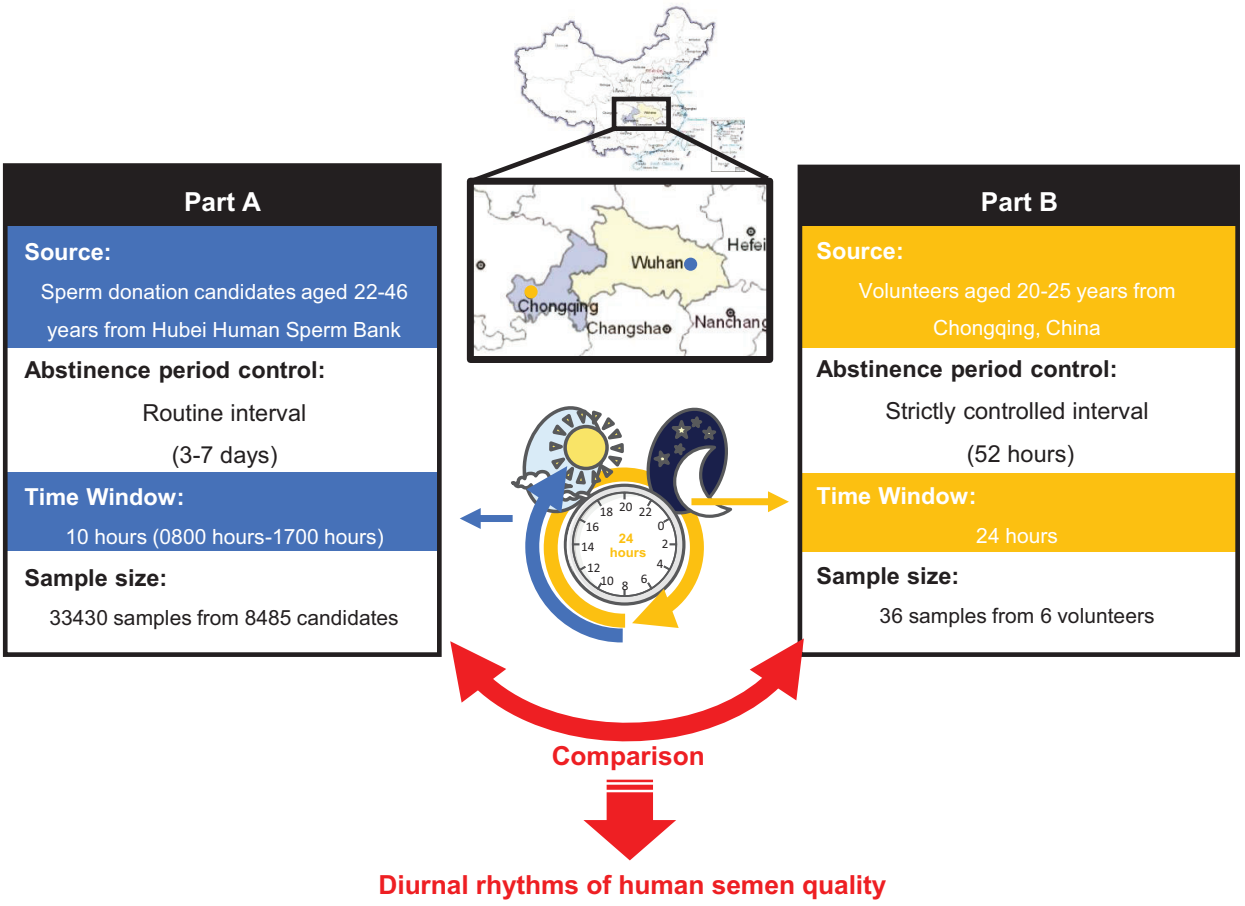

Figure 1. Study design for an investigation of diurnal rhythm in human semen quality. An analysis of samples from a human sperm bank (HSB) (**A**) and a laboratory study of community volunteers with a strictly controlled identical abstinence period (**B**) was conducted to investigate the temporal variation in semen parameters.

living or working in an environment with radioactive rays, high temperature (over 37°C), low temperature (lower than 0°C), noise or shaking of high intensity, high altitude, dust, pesticides, organic solvent, heavy metal, occupational exposure of off-gas from engines, intimate contact with livestock or pets. The sample size was based on a previous study (Valsa *et al.*, 2016). When recruited in the study, each subject finished a questionnaire containing demographic information (age, education level, family income) and lifestyle (consumption of tobacco, alcohol, tea, coffee and cola—a flavored carbonated drink), based on a questionnaire validated by us previously (Li *et al.*, 2009), through a face-to-face interview with the help of a well-trained interviewer. Body weight and height were also measured to calculate BMI as body weight (kg) divided by height squared (m^2).

Control and record of sampling time

In the study of Hubei HSB samples, candidates were informed to refrain from ejaculating (abstinence period) for a period of 3–7 days before providing semen for examination. The candidate age (in years) and abstinence period (in days) were recorded when semen samples were provided. Ejaculating time points were also recorded to an accuracy of 1 h for analysis.

In the timing-controlled laboratory study, each volunteer provided seven semen samples in the study center, with a strictly controlled abstinence period (52 h, i.e. 2 days plus 4 h) between the sequential samplings (Fig. 2). The first samples of each volunteer were withdrawn as the abstinence periods were not precisely controlled. The remaining six samplings were performed at different time points, at equal time intervals (0300, 0700, 1100 1500, 1900 and 2300 h). We did not collect all the samples in a single day with 4-h intervals (which is usually done in the diurnal analyses of other physiological functions) because it is not ethically acceptable and may put the volunteers in an unsafe situation. The effect of multiple sampling on semen parameters was not considered in the present study, because a previous study had shown that multiple sampling had no effect on the semen parameters as long as the abstinence periods were identical for each sampling (Mayorga-Torres *et al.*, 2015).

Monitoring of environment and physical parameters

In the study of Hubei HSB samples, monitoring data of daily average temperature and humidity within the study period were obtained from

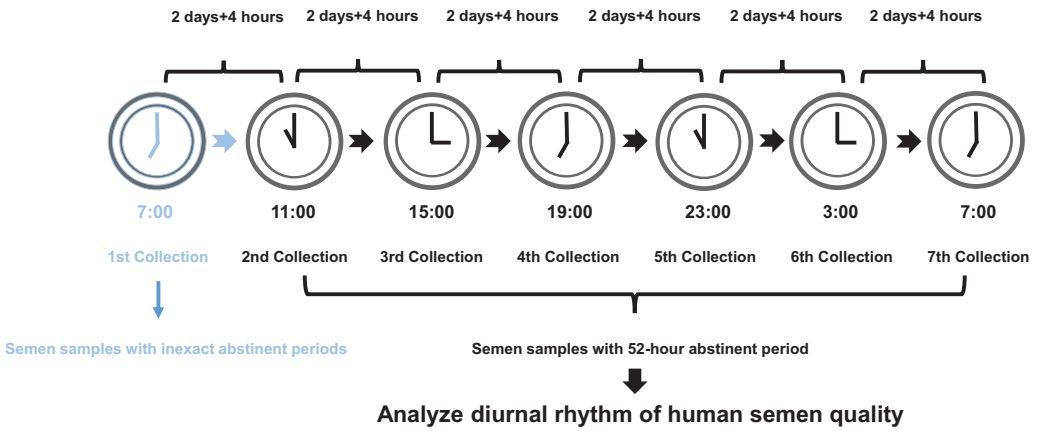

Figure 2. The pipeline for semen sample collection in the laboratory study. The first semen collection was at 0700 h, but the sample was not included in the analysis because of the unclear abstinence periods. Individuals were asked to remain abstinent between the following semen collections. Semen samples were then collected every 52 h at 1100, 1500, 1900, 2300, 0300 and 0700 h, which covered a circadian range of 24 h.

TuTiempo.net (http://en.tutiempo.net/climate/12-2010/ws-574940.html), as reported by the weather station: 574 940 (ZHHH).

In the timing-controlled laboratory study, diurnal signals for the circadian clock of volunteers were measured before each semen sample collection as follows: sleep duration of the volunteer was tracked with a sleep log; ear temperature was measured by infrared ear thermometer (Omron, China); heart rate and blood temperature were measured by electronic arm sphygmomanometer (Omron, China). Monitoring data of the daily highest\lowest temperature and weather were obtained from Tianqiwang (https://lishi.tianqi.com/chongqing/202006.html) reported by China Meteorological Administration.

Semen collection, timing and measurement

The semen was collected via masturbation in isolated rooms near the laboratories and was delivered to the laboratories immediately following ejaculation. The ejaculation time accurate to the minute was recorded and semen samples were kept in 37°C for liquefaction. Semen analysis was completed within 60 minutes after ejaculation.

In the study of Hubei HSB samples, semen parameters were measured based on the World Health Organization manual (World Health Organization, 1999) as described in detail elsewhere (Yang *et al.*, 2012; Rao *et al.*, 2015). 10 μl of well-mixed semen was put on a sperm-analyzing slide and transferred to the 37°C heating stage of automatic sperm analyzer WLJY-9000 (Weili Co Ltd, Beijing, China) for analysis of sperm concentration, progressive motility and total motility, following the instruction manual. The semen sample was examined immediately at a magnification of ×400. At least five areas or 200 sperm were scanned by the computer for each sample before an estimation of concentration or motility was given. Total sperm count was calculated as semen volume multiplied by sperm concentration. Semen volume was measured by weighing, assuming that the semen density was 1 g/ml. No substantial change in the materials or methods used happened during the study period. The internal quality control was implemented with a quality control semen sample made with fresh semen (at 15 million/ml and 50 million/ml). The certificated and well-trained staff measured five quality control samples at different concentrations, twice. Two-way ANOVA was carried out to estimate the system error and set up the target value. Thereafter, five quality-control samples at different concentrations were measured twice to the calculate coefficient of variation each month. A similar strategy was used for quality control of sperm motility, except that the tool was the standard video. The proficiency of the HSB was also surveyed by the key laboratory of male reproductive health of the National Health Commission.

In China, semen samples donated to HSB would be selected for use in ART. The semen samples considered to be suitable for use were required to have semen volume >2 ml, sperm concentration >60 million/ml and progressive motility >60% (Chinese Ministry of Health, 2004a).

In the timing-controlled laboratory study, the semen quality of volunteers was measured as below, and as recommended by the World Health Organization (World Health Organization, 2010). Sperm concentration and sperm motility parameters were measured by Computer-aided Sperm Analysis (Suiplus Software Co., Ltd, Beijing, China). 10 μl of well-mixed semen was put into a Disposable Sperm Counting Plate (Suiplus Software Co., Ltd, Beijing, China), and scanned by the computer-aided sperm analysis. At least six areas and 400 sperm were counted for estimation of sperm concentration. The samples with semen concentration over 50 million/ml were diluted to 2 to 50 million per milliliter with PBS, as suggested (Ashok *et al.*, 2016), and the fold dilution was recorded for the calculation of sperm concentration. Total sperm count was calculated as semen volume multiplied by sperm concentration. Semen volume was measured by weighing, assuming that the semen density was 1 g/ml. Each semen sample was measured by one certificated and well-trained technician.

Apart from the parameters measured in semen samples in HSB, sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were assessed by sperm chromatin structure assay, as previously described (Wang *et al.*, 2018). Sperm viability was assessed using a LIVE/DEAD Sperm Viability Kit (Thermo Fisher Scientific, USA).

Briefly, fresh sperm samples were diluted to 2 million/ml using Live Cell Imaging Solution (Thermo Fisher Scientific, USA) plus 10% BSA, and dyed with SYBR 14 and propidium iodide, following the manufacturer's instructions. Then sperm viability was assessed using a FACSCalibur system (FACSAria, BD Biosciences, CA, USA), and 10^4 sperm were counted in each sample.

Statistical analysis

In analysis of Hubei HSB samples, the Jonckheere–Terpstra test, Kruskal–Wallis test and chi-squared test was used in univariate analysis for the comparison of semen parameters collected at different time points. Linear regression or logistic regression was used in multivariate analysis with adjustment for abstinence period, age, month, year, temperature and humidity. As the semen parameters were of skewed distribution, they were analyzed on a logarithmic scale. Their regression coefficients were then back-transformed to obtain the percentage of change in each parameter per unit (h) of time. Missing data was left as it was because it accounts for a quite small proportion of the data (<0.1‰).

In the laboratory study, a mixed model or the Friedman test was used to analyze the diurnal variation of semen parameters, ear temperature, heart rate and blood pressure, with the intra-individual correlation of semen samples from the same volunteers under consideration. Semen parameters, ear temperature, heart rate and blood pressure were standardized by calculating the *z*-score before analysis. In addition, a cosinor analysis was performed to test whether their temporal distribution fitted a cosinusoidal curve, which is a classical oscillation pattern of diurnal rhythm. The cosinor analysis method provided a point and CI estimate of the cosine curve with the following parameters: acrophase (measure of timing of the peak value recurring in each cycle), double amplitude (2A, measure of extent of predictable change within a cycle) and midline estimating the statistic of the rhythm (MESOR, a rhythm-adjusted mean).

Statistical analyses were run with SPSS version 15.0 (SPSS Inc, Chicago, IL, USA), R version 3.6.2 and Cosinor (http://www.circadian.org/softwar.html). A value of $P < 0.05$ was considered statistically significant.

Results

Characteristics of the subjects

Demographic characteristics of HSB candidates and volunteers of laboratory study are listed in Tables I and II. In the HSB, 33 430 semen samples were provided between 0800 and 1700 h. The candidates had a median age of 26 (interquartile range (IQR) 23–30) years and a median abstinence period of 5 (IQR 4–7) days. In volunteers of the laboratory study, six healthy young males provided seven semen samples with a strictly controlled abstinence period (52 h) between the sequential samplings (Fig. 2). The volunteers had a median age of 24 years (IQR 21–24). Each of them had a high school degree or a bachelor's degree and a family income of 30 000–800 000 RMB/year. The volunteers were all had a low-level exposure to alcohol/cola/coffee/tea/cigarette consumption, and shift work. There was no drastic fluctuation in the climatic conditions within the study period (sunny, cloudy or with a moderate to light rain.): the highest temperature ranged from 23°C to 36°C, the lowest temperature ranged from 21°C to 25°C. A series of physical signals were recorded in the volunteers throughout the study period to allow us to understand the volunteers' comprehensive status of diurnal rhythm in the natural setting during the study. As expected, clear diurnal signals were observed in ear temperature, heart rate, blood pressure and sleep (Fig. 3). The sleep duration looks consistent for the individuals throughout the study period (Fig. 3).

Temporal variation in semen parameters in the HSB samples

As shown in Fig. 4, statistically significant temporal variations were found in total sperm count, sperm concentration, semen volume, progressive motility and total motility (all $P < 0.001$). All these parameters showed an increase-to-decrease trend from 0800 to 1700 h. The peak of total sperm count (median: 160.0 million, IQR 108.0–234.0 million), and sperm concentration (median: 60.0 million/ml, IQR 40.0–66.0 million/ml) occurred at 1100 h; the peak of total motility (median: 65.0%, IQR 54.0–69.0%), progressive motility (median: 60.0%, IQR 48.0–63.0%) and semen volume (median: 3.2 ml, IRQ 2.4–4.0 ml), achieved at 1200 h. We also compared the proportion of semen samples that was eligible for use in ART, based on bank standards, collected at different time points, and a similar temporal variation was observed, with the peak at 1100 h (Fig. 4, $P < 0.001$). Then, we transformed the sampling time points into the length of time away from the peak time, to test whether there were any inverse U-shaped associations between ejaculation time points and semen parameters. As shown in Fig. 5, the timing length away from 1100 h was calculated for analysis of total sperm count, sperm concentration and eligibility proportion, and the timing length away from 1200 h was calculated for analysis of total motility, progressive motility and semen volume. Statistical significance of an inverse U-shaped trend was achieved in all these parameters ($P \leq 0.001$; Fig. 4). Multivariate analysis was performed with adjustment for abstinence period, age, month, year, temperature and humidity (Table III). The results indicated that each hour of the timing length away from the peak time was associated with a 2.97% (95% CI: 2.38, 3.55; $P < 0.001$) lower total sperm count, a 2.45% (95% CI: 2.05, 2.85; $P < 0.001$) lower sperm concentration, a 0.31% (95% CI: 0.08, 0.53; $P = 0.009$) lower total motility, a 0.43% (95% CI: 0.17, 0.69; $P = 0.001$) lower progressive motility and a 1.07% (95% CI: 0.58, 1.56; $P < 0.001$) lower semen volume. In addition, each hour of the timing length away from the peak time was also associated with a 1.14-fold (95% CI: 1.12, 1.16; $P < 0.001$) risk of the ineligibility of the semen samples.

Diurnal oscillation of semen samples in the abstinence-controlled laboratory study

As shown in Fig. 6, significant time-dependent oscillations were found in total sperm count ($P = 0.041$), sperm concentration ($P = 0.035$), progressive motility ($P = 0.006$) and total motility ($P = 0.009$). The cubic spline model showed that total sperm count, sperm concentration, total motility and progressive motility peaked at 1100–1500 h, which was similar to the results found from HSB samples (Fig. 6 and Supplementary Table SI). Setting the standardized semen parameters

Table I Basic information of participants in a study of diurnal rhythm in human semen quality.

Demographic characteristics	Candidates for the HSB (n = 33 430)		Volunteers of laboratory study (n = 6)	
	N	Distribution*	n	Distribution*
Age (year)	33 399	26 (23, 30)	6	24 (21, 24)
Abstinence period (day)	33 430	5 (4, 7)	6	2.17 (2.17, 2.17)
Temperature (°C)	33 160	18.9 (11.2, 24.6)		
Humidity (%)	33 160	71 (61, 81)		
Ejaculation time point	33 250	12 (11, 14)	6	13 (6, 20)
BMI (kg/m^2)			6	21.3 (20.0, 23.0)
Permanent shift work/Rotating shift work				
Never			6	100%
Ever			0	0%
Current			0	0%
Education level				
Junior school and below			0	0%
High school			2	33.3%
College and higher			4	66.7%
Family income (10 000 RMB/year)				
<3			0	0%
3–8			3	50.0%
8–15			2	33.3%
15–80			1	16.7%
>80			0	
Tobacco smoking				
Never			6	100%
Ever			0	0%
Current			0	0%
Alcohol drinking				
Never			3	50%
≤Once/week			3	50%
>Once/week			0	0%
Tea intake				
Never			6	100%
Ever			0	0%
Current			0	0%
Cola intake (bottles/week)				
Never			2	33.3%
<3/week			4	66.7%
≥3/week			0	0%
Coffee intake				
Never			5	83.3%
≤Once/week			1	16.7%
>Once/week			0	0%

*Data are presented as median (25th and 75th percentiles) or percentage. HSB, human sperm bank.

at 1100 h as the reference, total sperm count was lower at 2300 h ($\beta = -1.33$; 95% CI: −2.27, −0.39; $P = 0.010$) and sperm concentration was lower at 0700 h ($\beta = -0.98$; 95% CI: −1.92, −0.05; $P = 0.049$), 1900 h ($\beta = -1.11$; 95% CI: −2.04, −0.17; $P = 0.027$) and 2300 h ($\beta = -1.44$; 95% CI: −2.37, −0.50; $P = 0.005$). Setting the standardized semen parameters at 1500 h as the reference, sperm progressive motility was lower at 0700 h ($\beta = -1.83$; 95% CI: −2.71, −0.96; $P < 0.001$), 1100 h ($\beta = -0.98$; 95% CI: −1.85, −0.10;

Table II Demographic characteristics of the six male volunteers from Chongqing.

Demographic characteristics	No. 001	No. 002	No. 003	No. 004	No. 005	No. 006
Age (years)	21	20	24	24	24	25
BMI (kg/m^2)	19.8	22.9	22.3	20.0	20.2	23.3
Permanent shift work/rotating shift work	Never	Never	Never	Never	Never	Never
Education level	High school	High school	College	College	College	College
Family income (10 000 RMB/year)	8–15	15–80	3–8	8–15	3–8	3–8
Tobacco smoking	Never	Never	Never	Never	Never	Never
Alcohol drinking	Never	Never	≤once/week	≤once/week	Never	≤once/week
Tea intake	Never	Never	Never	Never	Never	Never
Cola intake (bottles/week)	Never	Never	<3	<3	<3	<3
Coffee intake	Never	≤once/week	Never	Never	Never	Never

$P = 0.037$) and 2300 h ($\beta = -1.18$; 95% CI: −2.06, −0.31; $P = 0.013$); and sperm total motility was lower at 0700 h ($\beta = -1.70$; 95% CI: −2.59, −0.82; $P < 0.001$) and 2300 h ($\beta = -0.93$; 95% CI: −1.82, −0.04; $P = 0.049$). Furthermore, when converted to a 24-h time point, an acrophase indicated a peak of semen quality at about 1100–1500 h. Cosine analysis showed that the variation in total sperm count fit a classic diurnal oscillation, with a cosinusoidal pattern that peaked at about 1100 h (with 56.4% amplitude and −182° acrophase, $P = 0.030$). Similarly, sperm concentration peaked at about 1100 h; total motility, progressive motility and viability peaked at about 1500 h. In addition, the acrophase indicated a nadir of sperm DFI and HDS at about 1100 h.

Discussion

The present study aimed to identify whether there is diurnal oscillation of human semen parameters. In the large clinical record of 33 430 semen samples collected in a partial time window in an HSB, most semen parameters were found to reach their most favorable level at the time point between 1100 and 1200 h. This temporal variation of semen parameters was replicated in a laboratory study. When collected with an abstinence period that was strictly controlled, the semen parameters still showed diurnal oscillation, and the peak time points (1100–1500 h) were quite close to those observed in the HSB data, Additionally, the laboratory study also found a nadir of sperm DFI and HDS (unfavorable indicators reflecting sperm DNA damage) at about 1100 h, indicating that the peak of semen quality was at about 1100–1500 h.

It has long been noted that semen samples ejaculated by the same man have substantial intra-individual variation in semen parameters (Castilla *et al.*, 2006; Jarow *et al.*, 2013). However, the origin of the variation is not completely clear. The present results suggest that diurnal rhythm could be an important cause of the variation of semen parameters (e.g. total sperm count at 1100 h was 12.1 million more than total sperm count at 1700 h). Our results showed that total sperm count, sperm concentration, total motility and progressive motility reached a peak at 1100–1500 h. Furthermore, our results also showed that sperm DFI, an indicator of sperm DNA damage, reached its lowest level at about 1100 h. The oscillation pattern is in concordance with our previous analysis of 10 752 semen samples from a reproductive medical center and 630 semen samples from a community population (Ni *et al.*, 2019).

Our study makes up for some deficiencies in current knowledge. Previous studies on variation in semen parameters might not be comparable with each other owing to their narrow time windows. Our study illustrates the oscillation of semen parameters in a circadian range of 24 h. Our data support previous studies, which reported that semen parameters oscillated at different time points (Valsa *et al.*, 2016; Xie *et al.*, 2018; Shimomura *et al.*, 2020), and may help us to understand why Cagnacci *et al.* (1999) found higher semen parameters in the afternoon than in the morning, while Biljan *et al.* (2005) found no difference in semen parameters between morning and evening, suggesting that these semen parameters were dynamically changing in men, just as in other species (Giebultowicz *et al.*, 1989; Qin *et al.*, 2014). The peak time point in these studies was several hours different from that found in our study; this may be attributed to heterogeneity in different populations, for example, in some studies the participants were patients who might have different lifestyles (such as sleep–wake circle) and a different status of diurnal rhythms. Moreover, the difference in the abstinence period among studies was considered as one of the important biases in the assessment of semen parameters, which was not taken into consideration in previous studies. Abstinence periods for semen samples were always recorded in days. Therefore, the actual abstinence periods for semen samples collected at a later time point could be underestimated by several hours or minutes, if the recorded abstinence period (in days) and the time point of last ejaculation were identical. In our analysis of 36 semen samples, abstinence period (in hours) was strictly controlled and recorded accurately in order to be identical for each volunteer and for each time point of semen collection, which may help to rule out the influence of abstinence period as far as possible. This could also partly explain why the results from the laboratory study and HSB data were not identical, although the slight difference did not affect the overall conclusion that human semen parameters reached a better quality at 1100–1500 h.

In animal models, a circadian rhythm of semen parameters has been observed and has been partly attributed to regulation by a circadian clock in extra-testicular ducts (Giebultowicz *et al.*, 1989; Polanska

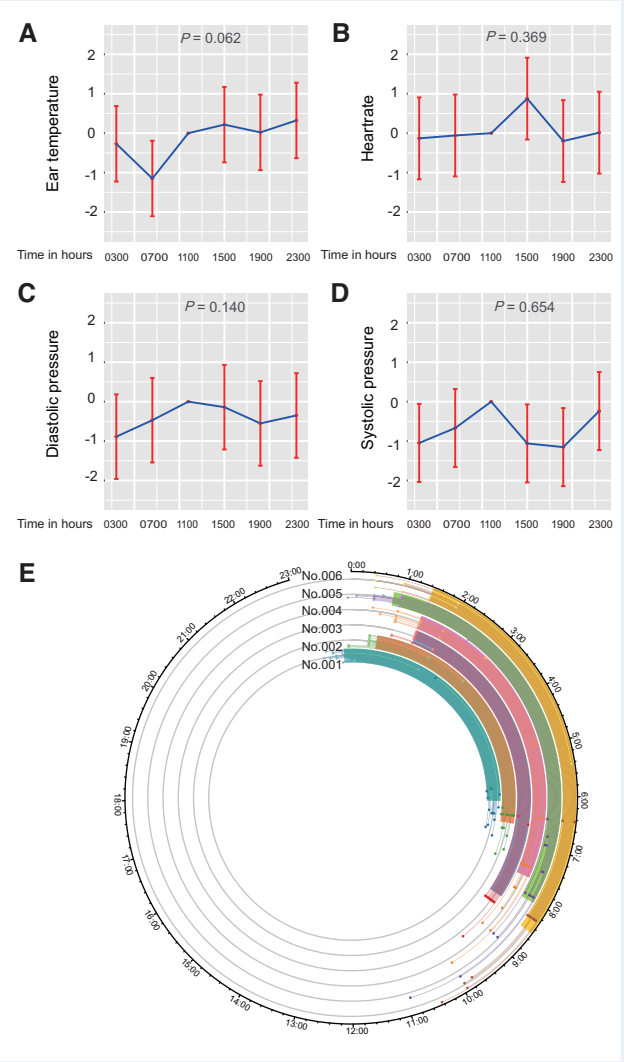

Figure 3. The diurnal signature of six volunteers. Time-dependent patterns of standardized ear temperature (**A**), heart rate (**B**), blood pressure (**C** and **D**) and the log of sleep (**E**) showed a clear diurnal signal in the six volunteers. Setting the semen parameters at 1100 h as the reference, a mixed model was used to analyze the diurnal variation of these signals. The bars of different colors in panel E represent the average sleep periods (from sleep to awakening) of each volunteer. Lines marked with starting points and terminal points represents sleep periods of each day. The Friedman test was used to analyze variation of the sleep/wake-up time point during the study.

et al., 2009; Kotwica et al., 2011; Kotwica-Rolinska et al., 2013; Qin et al., 2014; Zasiadczyk et al., 2015). Interruption of clock genes impairs spermatogenesis (Schoeller et al., 2016; Li et al., 2018; Lin et al., 2019) and the circadian rhythm of semen parameters in an animal model (Kotwica et al., 2009). In men, genetic polymorphisms of clock genes were found to be associated with semen quality and risk of infertility (Hodžić et al., 2013; Shen et al., 2015; Calhaz-Jorge et al., 2016), indicating a circadian clock in the human reproductive system. These clues support the hypothesis that there should be a diurnal rhythm in human semen parameters, which was observed in the present study. Interestingly, the diurnal rhythm of semen parameters seems to be in accordance with the rhythm of mating behavior of the species. For example, mating activity in the gypsy moth (*Lymantria dispar*) generally occurs in darkness, when the sperm are released from testis to vas deferens and seminal vesicles, and both the diurnal rhythm of mating behavior and sperm release was controlled by a circadian clock (Giebultowicz, Riemann, Raina and Ridgway, 1989; Silvegren et al., 2005). This may maximize the success rate of conception. Interestingly, while better semen quality was found at daytime in our study, human intercourse most often takes place at night (Palmer et al., 1982; Refinetti, 2005). This discrepancy might be because, for most people today, the selection of intercourse timing is restricted by strong external forces such as work and family schedules (Refinetti, 2005). Nevertheless, the short evolutionary time frame of modern work/family practices may not be sufficient to impact the long-established clock-controlled oscillation pattern of semen parameters. This may explain why better semen quality was found at daytime in our study.

Our findings may have important clinical significance. Only a minority (15–30%) of the volunteers visiting HSBs could meet the strict screening standards (Akinrinola et al., 2003; Paul et al., 2006; Jie et al., 2011; Guan et al., 2014). Most of the ineligible candidates were rejected owing to unfavorable semen quality, especially inadequate sperm count and sperm motility (Paul et al., 2006; Jie et al., 2011; Guan et al., 2014; Zhang et al., 2019). This high rejection rate leads to a substantial resource wastage and a shortage of semen for assisted reproduction, while the demand continues to grow (Guan et al., 2015). Obtaining semen with higher semen quality is a serious challenge for the sperm banks. Currently, the most common method used to increase sperm in semen is prolonging the abstinence period, however this may lead to a decrease of sperm motility (Menegassi et al., 2015), mainly because of aging of the accumulated sperm. The present study suggests that ejaculating at 'optimal' time points could be a novel way to obtain more sperm without the decrease in progressive motility; this may also be helpful to boost natural conception rates.

There are several main limitations in the present study. First, some potential confounders were not available in the analysis of HSB samples. In China, running of the HSB is directed by law and staff were only allowed to collect information according to the recruiting/excluding criteria. For example, possible confounders, such as energy intake, which might influence the semen quality or diurnal rhythms, were not adjusted for. The candidates were asked whether they have severe cardiac disease and those with disease would be immediately excluded, with no examination of semen quality. The conditions were similar for information on heavy smoking, alcohol addiction and medication. Hence, the characteristics of the study subjects were relatively homogeneous, and generalizability of the findings may be restricted. Second, candidates for the HSB were screened by height, education and age, and were more likely to have low-to-moderate economic status (as the HSBs would provide them compensation). Third, the volunteers in the abstinence-controlled study were all young, and routinely stayed awake, to about 0100 h, during the study (Fig. 3). Their sleep pattern suggested a late chronotype, like most young men, but it might not be most representative of the general population, especially for

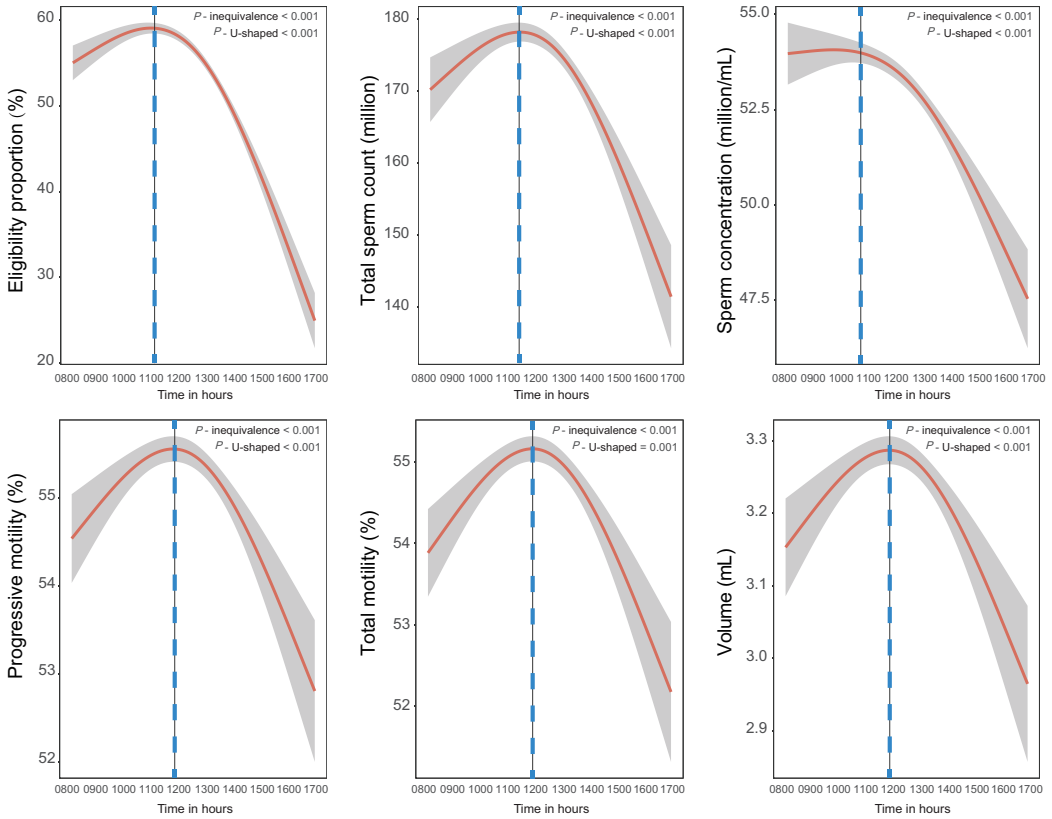

Figure 4. Temporal variation of the semen parameters in samples from potential human sperm bank donors (n = 33 430). The figures are based on a cubic spline model. The red lines represent the change of semen quality from 0800 to 1700 h, and the shadowed area represents the 95% CIs. Tests for inequivalence among different time points were performed using Kruskal–Wallis test or chi-squared test. Tests for U-shaped association between semen parameters and the timing length away from the peak time were performed using the Jonckheere–Terpstra test or chi-squared test.

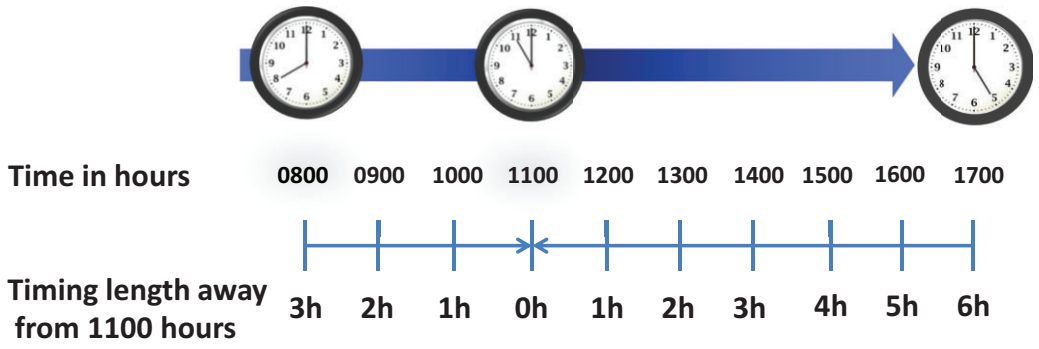

Figure 5. Calculation of the timing length away from 1100 h. To test whether there is a turning point at 1100 h for the association between ejaculation time point and the semen parameters (i.e. a positive association before 1100 h and a negative association after 1100 h), timing length away from 1100 h was calculated. For example, 0800 h is 3 h away from 1100 h, and 1700 h is 6 h away.

those of more advanced age. This circadian characteristic may have an effect on oscillation pattern of their semen parameters. Fourth, it is interesting that the peak time of some semen parameters over 24 h found in this study differed from a previous study (Valsa *et al.*, 2016).

Table III Multivariate analysis of the association between timing length away from the peak time and semen parameters, based on samples from candidates for the human sperm bank.

	N	Percentage change (95% CI)	P-value
Total sperm count[a]	32 561	−2.97 (−3.55, −2.38)	<0.001
Sperm concentration[a]	32 576	−2.45 (−2.85, −2.05)	<0.001
Progressive motility[b]	32 522	−0.43 (−0.69, −0.17)	0.001
Total motility[b]	32 534	−0.31 (−0.53, −0.08)	0.009
Volume[b]	32 660	−1.07 (−1.56, −0.58)	<0.001

Analyzed with linear regression (total sperm count, sperm concentration, progressive motility, total motility and volume) adjusted for abstinence period, age, month, year, temperature and humidity.
[a]The association of the timing length away from 1100 h and semen parameters were analyzed.
[b]The association of the timing length away from 1200 h and semen parameters were analyzed.

Since some technical details of semen analysis (e.g. abstinence period, sampling method) and the subjects' overall status of diurnal rhythm, such as sleep–wake cycle or other physical signs of a circadian rhythm, were not reported in that study, it is difficult to identify the reasons for the difference between the two studies. Further studies are needed to reveal the potential factors affecting the peak time of the diurnal oscillation in semen parameters. Fifth, the 24-h oscillation curve of sperm count in our study illustrated the relative difference, not the accumulating change of semen parameters among different time points, because the abstinence periods were identical. For example, a man may obtain more sperm at time points later than 1500 h because the sperm could still be released from the testis at a slower speed. Sixth, the study was observational, so reverse causation cannot be ruled out although it appears unlikely that the semen parameters would affect the time when a man provided semen.

In summary, based on an analysis of a large number of HSB samples and a laboratory study with a strictly controlled abstinence period, we have shown that human semen parameters exhibit substantial diurnal oscillation during 24 h. Most semen parameters reached the most favorable level between 1100 and 1500 h. The findings should be considered with caution because the study was conducted in a specific population, time and place, while the timing of oscillations could differ with changing conditions. Considering the importance of semen parameters in successful pregnancy and the shortage of

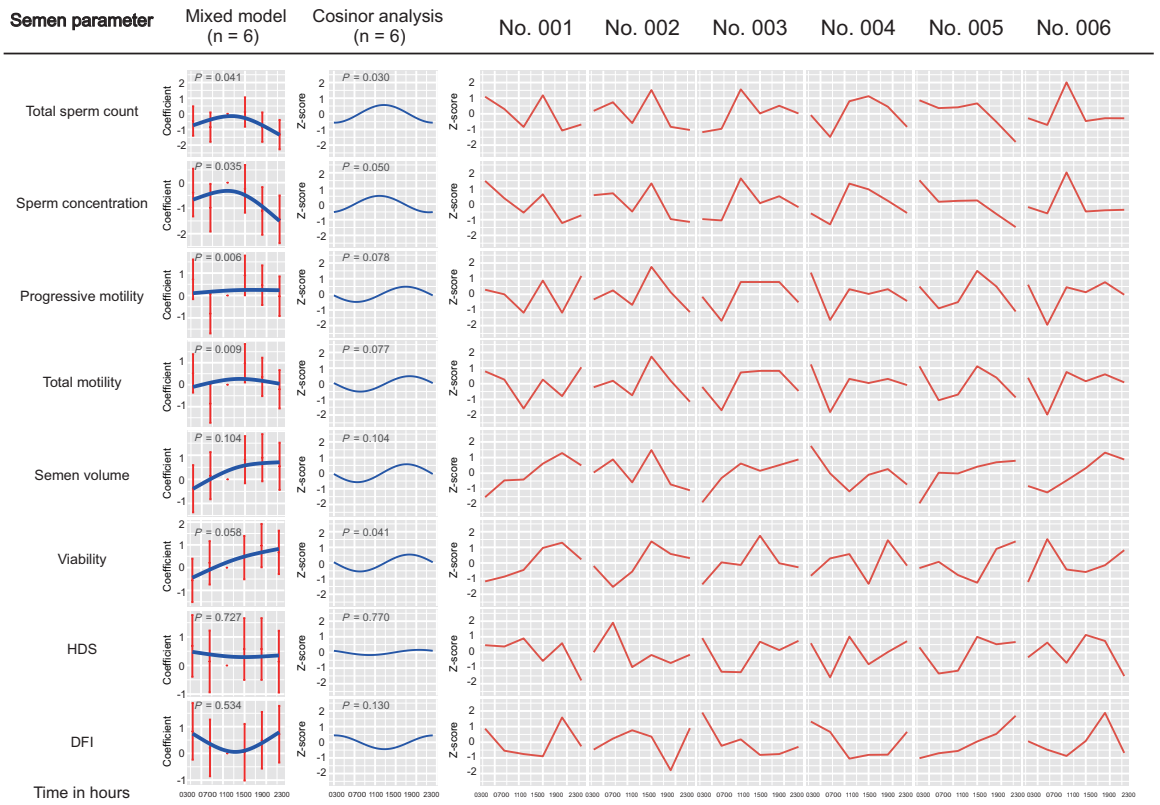

Figure 6. Diurnal rhythm of semen parameters in the six volunteers. The *X*-axis represents ejaculation time points. The first column (left) shows time-of-day dependent differences in total sperm count, semen volume, sperm concentration, total motility, HDS, DFI and sperm viability (n = 6), and the *Y*-axes show regression coefficients, using a mixed model. The middle column shows the fitness of the semen parameters with a cosinusoidal curve and the estimated oscillation phase, including the peak time point. The six columns on the right show the variation in standardized semen parameters of each volunteer. HDS, high DNA stainability; DFI, DNA fragmentation index.

eligible semen for assisted reproduction, our findings dmerit validation in future studies.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

J.C., C.X. and Q.C. formulated the overarching research goals and aims. K.L., T.M., Q.C., G.H., S.H. X.G., H.L. and Y.L. developed the methodology and conducted the research. Each author joined in the writing of the article.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Ashok A, Sajal G, Rakesh S. Andrological evaluation of male infertility. In: A Ashok, G Sajal, S Rakesh (eds). *Semen Analysis Using Sperm Class Analyzer (SCA v5) for Computer Assisted Semen Analysis (CASA)*. Switzerland: Springer International Publishing, 2016, 59–67.

Akinrinola OA, Melie NA, Ajayi RA. Poor acceptance rate for semen donors to a private cryobank in Nigeria. *Afr J Reprod Health* 2003; **7**:12–16.

Alvarez JD, Chen D, Storer E, Sehgal A. Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. *Biol Reprod* 2003;**69**:81–91.

Bass J, Takahashi JS. Circadian integration of metabolism and energetics. *Science* 2010;**330**:1349–1354.

Biljan MM, Tkalec DD, Lachgar H. Absence of diurnal variation in semen parameters in normospermic men. *Fertil Steril* 2005;**83**: 477–479.

Cagnacci A, Maxia N, Volpe A. Diurnal variation of semen quality in human males. *Hum Reprod* 1999;**14**:106–109.

Calhaz-Jorge C, de Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T, Scaravelli G, Wyns C, Goossens V. Assisted reproductive technology in Europe, 2012: results generated from European registers by ESHRE. *Hum Reprod* 2016;**31**: 1638–1652.

Castilla JA, Alvarez C, Aguilar J, González-Varea C, Gonzalvo MC, Martínez L. Influence of analytical and biological variation on the clinical interpretation of seminal parameters. *Hum Reprod* 2006;**21**: 847–851.

Chinese Ministry of Health. Basic standards and technical norms of human sperm banks. *Chin J Reprod Health* 2004a;**15**:68–71.

Chinese Ministry of Health. Ethical principles of assisted human reproductive technology and human sperm banks. *Chin J Reprod Health* 2004b;**15**:72–74.

Douma LG, Gumz ML. Circadian clock-mediated regulation of blood pressure. *Free Radic Biol Med* 2018;**119**:108–114.

Giebultowicz JM, Riemann JG, Raina AK, Ridgway RL. Circadian system controlling release of sperm in the insect testes. *Science* 1989; **245**:1098–1100.

Gotlieb N, Moeller J, Kriegsfeld LJ. Circadian control of neuroendocrine function: implications for health and disease. *Curr Opin Physiol* 2018;**5**:133–140.

Guan HT, Wan Z, Zhang L, Meng TQ, Xiong CL, Li CL. Analysis of the screening results for 3,564 student sperm donors in Hubei Province, China. *J Reprod Med* 2015;**60**:409–414.

Guan HT, Wang Z, Meng TQ, Xia W, Xiong CL, Li CL. Screening results and causes of uncompleted donation process in 1145 sperm donors. *Zhonghua Nan Ke Xue* 2014;**20**:124–128.

Harfmann BD, Schroder EA, Esser KA. Circadian rhythms, the molecular clock, and skeletal muscle. *J Biol Rhythms* 2015;**30**:84–94.

Hodžić A, Ristanović M, Zorn B, Tulić C, Maver A, Novaković I, Peterlin B. Genetic variation in circadian rhythm genes CLOCK and ARNTL as risk factor for male infertility. *PLoS One* 2013;**8**:e59220.

Jarow JP, Fang X, Hammad TA. Variability of semen parameters with time in placebo treated men. *J Urol* 2013;**189**:1825–1829.

Jie LI, Zhu WB, Fan LQ, Zhang K. Analysis of the screening results from 7738 donors of semen. *Chin J Mod Med* 2011;**21**:2269–2276.

Kennaway DJ, Boden MJ, Varcoe TJ. Circadian rhythms and fertility. *Mol Cell Endocrinol* 2012;**349**:56–61.

Kotwica J, Bebas P, Gvakharia BO, Giebultowicz JM. RNA interference of the period gene affects the rhythm of sperm release in moths. *J Biol Rhythms* 2009;**24**:25–34.

Kotwica J, Joachimiak E, Polanska MA, Majewska MM, Giebultowicz JM, Bebas P. Diurnal rhythm in expression and release of yolk protein in the testis of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Insect Biochem Mol Biol* 2011;**41**:264–272.

Kotwica-Rolinska J, Gvakharia BO, Kedzierska U, Giebultowicz JM, Bebas P. Effects of period RNAi on V-ATPase expression and rhythmic pH changes in the vas deferens of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Insect Biochem Mol Biol* 2013;**43**: 522–532.

Li Y, Lin H, Ma M, Li L, Cai M, Zhou N, Han X, Bao H, Huang L, Zhu C *et al*. Semen quality of 1346 healthy men, results from the Chongqing area of southwest China. Hum Reprod 2009;**24**: 459–469.

Li C, Xiao S, Hao J, Liao X, Li G. *Cry1* deficiency leads to testicular dysfunction and altered expression of genes involved in cell communication, chromatin reorganization, spermatogenesis, and immune response in mouse testis. *Mol Reprod Dev* 2018;**85**:325–335.

Lin Z, Chen L, Chen X, Zhong Y, Yang Y, Xia W, Liu C, Zhu W, Wang H, Yan B *et al*. Biological adaptations in the Arctic cervid, the reindeer (*Rangifer tarandus*). *Science* 2019;**364**:eaav6312.

Mayorga-Torres BJ, Camargo M, Agarwal A, du Plessis SS, Cadavid ÁP, Cardona Maya WD. Influence of ejaculation frequency on seminal parameters. *Reprod Biol Endocrinol* 2015;**13**:47.

Menegassi SR, Barcellos JO, Dias EA, Koetz C Jr, Pereira GR, Peripolli V, McManus C, Canozzi ME, Lopes FG. Scrotal infrared digital thermography as a predictor of seasonal effects on sperm traits in Braford bulls. *Int J Biometeorol* 2015;**59**:357–364.

Morse D, Cermakian N, Brancorsini S, Parvinen M, Sassone-Corsi P. No circadian rhythms in testis: period1 expression is clock independent and developmentally regulated in the mouse. *Mol Endocrinol* 2003;**17**:141–151.

Ni W, Liu K, Hou G, Pan C, Wu S, Zheng J, Cao J, Chen Q, Huang X. Diurnal variation in sperm DNA fragmentation: analysis of 11,382 semen samples from two populations and in vivo animal experiments. *Chronobiol Int* 2019;**36**:1455–1463.

Palmer JD, Udry JR, Morris NM. Diurnal and weekly, but no lunar rhythms in humans copulation. *Hum Biol* 1982;**54**:111–121.

Paul S, Harbottle S, Stewart JA. Recruitment of sperm donors: the Newcastle-upon-Tyne experience 1994-2003. *Hum Reprod* 2006; **21**:150–158.

Peterlin A, Kunej T, Peterlin B. The role of circadian rhythm in male reproduction. *Curr Opin Endocrinol Diabetes Obes* 2019;**26**: 313–316.

Polanska MA, Maksimiuk-Ramirez E, Ciuk MA, Kotwica J, Bebas P. Clock-controlled rhythm of ecdysteroid levels in the haemolymph and testes, and its relation to sperm release in the Egyptian cotton leafworm, *Spodoptera littoralis*. *J Insect Physiol* 2009;**55**: 426–434.

Qin F, Zhang J, Cao H, Guo W, Chen L, Shen O, Sun J, Yi C, Li J, Wang J *et al*. Circadian alterations of reproductive functional markers in male rats exposed to 1800 MHz radiofrequency field. *Chronobiol Int* 2014;**31**:123–133.

Rao M, Meng TQ, Hu SH, Guan HT, Wei QY, Xia W, Zhu CH, Xiong CL. Evaluation of semen quality in 1808 university students, from Wuhan, Central China. *Asian J Androl* 2015;**17**:111–116.

Refinetti R. Time for sex: nycthemeral distribution of human sexual behavior. *J Circadian Rhythms* 2005;**3**:4.

Roenneberg T, Merrow M. The circadian clock and human health. *Curr Biol* 2016;**26**:R432–R443.

Schoeller EL, Clark DD, Dey S, Cao NV, Semaan SJ, Chao LW, Kauffman AS, Stowers L, Mellon PL. Bmal1 is required for normal reproductive behaviors in male mice. *Endocrinology* 2016;**157**: 4914–4929.

Sciarra F, Franceschini E, Campolo F, Gianfrilli D, Pallotti F, Paoli D, Isidori AM, Venneri MA. Disruption of circadian rhythms: a crucial factor in the etiology of infertility. *Int J Mol Sci* 2020;**21**:3943.

Shen O, Ding X, Nie J, Xia Y, Wang X, Tong J, Zhang J. Variants of the CLOCK gene affect the risk of idiopathic male infertility in the Han-Chinese population. *Chronobiol Int* 2015;**32**:959–965.

Sen A, Hoffmann HM. Role of core circadian clock genes in hormone release and target tissue sensitivity in the reproductive axis. *Mol Cell Endocrinol* 2020;**501**:110655.

Shimomura Y, Shin T, Osaka A, Inoue Y, Iwahata T, Kobori Y, Ide H, Soh S, Okada H. Comparison between semen parameters in specimens collected early in the morning and in the evening. *Syst Biol Reprod Med* 2020;**66**:147–150.

Silvegren G, Löfstedt C, Qi Rosén W. Circadian mating activity and effect of pheromone pre-exposure on pheromone response rhythms in the moth *Spodoptera littoralis*. *J Insect Physiol* 2005;**51**: 277–286.

Valsa J, Skandhan KP, Sumangala B, Amith S, Skandhan Avni KP. Effect of different timings of the day on semen and calcium and magnesium in it. *Urologia* 2016;**83**:207–210.

Wang X, Chen Q, Zou P, Liu T, Mo M, Yang H, Zhou N, Sun L, Chen H, Ling X *et al*. Sleep duration is associated with sperm chromatin integrity among young men in Chongqing, China. *J Sleep Res* 2018;**27**:e12615.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions*, 4th edn. Cambridge: Cambridge University Press, 1999.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva: World Health Organization, 2010.

Xie M, Utzinger KS, Blickenstorfer K, Leeners B. Diurnal and seasonal changes in semen quality of men in subfertile partnerships. *Chronobiol Int* 2018;**35**:1375–1384.

Yang Y, Jiang H, Zhang H. Correlation of age, sperm DNA fragmentation with semen parameters in infertile males. *Chin J Hum Sex* 2012;**21**:17–19.

Zasiadczyk L, Fraser L, Kordan W, Wasilewska K. Individual and seasonal variations in the quality of fractionated boar ejaculates. *Theriogenology* 2015;**83**:1287–1303.

Zhang HT, Zhang Z, Cao J, Tang WH, Zhang HL, Hong K, Lin HC, Wu H, Chen Q, Jiang H. Ambient ozone pollution is associated with decreased semen quality: longitudinal analysis of 8945 semen samples from 2015 to 2018 and during pollution-control period in Beijing, China. *Asian J Androl* 2019;**21**:501–507.