EXPERIMENTAL STUDY

The Impact of Estrogen Supplementation to Autonomic and Sleep Modulations in Free-Moving Spontaneously Hypertensive Rats

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Summary
Sleep and estrogen levels have an impact on neural regulation and are associated with cardiovascular (CV) events. We investigated the effects of estrogen on heart rate variability (HRV) and circadian cycle in spontaneously hypertensive rats (SHRs). Polysomnographic recording was performed in seven male and seven female SHRs during sleep. The electroencephalogram (EEG) and electromyogram (EMG) were evaluated to define active waking (AW), quiet sleep (QS), and paradoxical sleep (PS) stages. Cardiac activities were measured by RR interval of the electrocardiogram (ECG), mean arterial pressure (MAP), and power spectrum of HRV.

In ECG, estrogen prolonged the RR interval in total sleep when compared with that at baseline in male SHRs (203.74 ± 6.61 versus 181.30 ± 8.06 ms, P < 0.001) and in female SHRs (169.21 ± 6.43 versus 160.76 ± 10.66 ms, P < 0.05). In HRV, the estrogen increased the high frequency (HF) in total sleep when compared with that at baseline in male SHRs (1.03 ± 0.28 versus 0.60 ± 0.43 ln (ms²), P < 0.001) and in female SHRs (0.71 ± 0.26 versus 0.42 ± 0.19 ln (ms²), P < 0.05).

In male SHRs, estrogen increased the frequency of QS (26.50 ± 4.85 versus 20.79 ± 5.07, P < 0.01) and PS (25.64 ± 5.18 versus 20.14 ± 4.75, P < 0.05) stages when compared with baseline. In female SHRs, estrogen increased the percentage of delta waves in total sleep (79.87% ± 3.10% versus 76.71% ± 2.74%, P < 0.05) when compared with that at baseline.

In HRV, estrogen leads to neuromodulation by increased parasympathetic tone in all SHRs, suggesting a lower risk to CV events. In sleep analyses, estrogen in male SHRs caused poor sleep quality. In contrast, estrogen in female SHRs demonstrated improved quality of sleep and decreased risk of hypertension.

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Key words: Estrogen replacement therapy, Hypertension, Sleep disorders, Circadian rhythm, Autonomic nervous system, Sleep stages

While humans spend one-third of their life span on sleep, studies of an association between hypertension (HTN) and sleep have emerged. Sleep analysis includes tools of electroencephalography (EEG) to determine the stages of the sleep cycle, such as active awaking (AW), quiet sleep (QS), and paradoxical sleep (PS). Animal studies of SHRs have demonstrated higher vasomotor activity during sleep causing amplified risk of sleep-related cardiovascular (CV) events.1-2 It was suggested that the autonomic imbalance during sleep may reflect the actual body condition due to less external influence as it is in the conscious state.3

In previous studies, estrogen has cardioprotective and vasoprotective effects in both men and women.4,5 Hormone imbalance may lead to HTN, especially in postmenopausal women and older men.6,7 The Framingham Study has also confirmed that premenopausal women tend to have lower blood pressure (BP) in comparison to men with the same age.8-10 It was also demonstrated that men with imbalance in estrogen have a higher risk of mortality from congestive heart failure and cerebrovascular events.11

Over the years, the effect of estrogen therapy has gained great interest owing to the extended life expectancy and favorable outcomes of estrogen supplementation in postmenopausal HTN. The mechanism of hormone supplement is not fully understood, but evidence of changes

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in the estrogen-androgen ratio, renin-angiotensin-aldosterone system (RAAS) activation, and sympathetic hyperactivity have concluded that estrogen plays an important role.12-14) Hence, we aimed to evaluate the autonomic modulation after estrogen therapy in the changes of sleeping cycles in ambulatory SHR model.

Methods
Animal preparation and hypertensive rat model: In this study, the experimental protocol was approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital (IACUC number: 2017-173), and the investigation was conducted in accordance with the US National Institute of Health’s Guide for the Care and Use of Laboratory Animals. Seven adult male SHRs and seven adult female SHRs which are 10-13 weeks old were selected as our experimental model. In female SHRs, the bilateral ovariectomy was followed by the head circuit implantation under the same anesthetic procedure to minimize excessive operation. Bilateral ovariectomy was achieved through a middle ventral abdominal approach. The rats were obtained from the Animal Center of National Yang-Ming University of Taiwan under the guidelines established by the Position of the American Heart Association on Research Animal Use. The rats were placed in a soundproof room with consistent temperature (22°C ± 2°C) and humidity (40%-70%) with a 12:12 hour (08:30 am to 08:30 pm) of circadian rhythm.

Head circuit installation, BP monitoring, and signal recording: The animals were anesthetized with Pentobarbital (50 mg/kg, IP) and placed under a standard stereotaxic apparatus with exposure of the dorsal skull (Figure 1).16) Two stainless screws were driven through the bilateral parietal skull with the EEG electrodes overlaying the parietal cortex region (2.0 mm posterior and 2.0 mm lateral to the bregma) without penetrating the dura mater, and a reference electrode was implanted 2 mm caudal to the lambda (Figure 2). The ECG was recorded through microwires implanted subcutaneously over the dorsal thoracic and lumbar levels. A connector to document all information was anchored onto the head. A telemetry transmitter (TA 11PA-C40, Data Sciences, St. Paul, MN) was implanted in the abdominal aorta to measure arterial pressure. After the procedure, chlorotetracycline, an antibiotic agent, was administered followed by a 2-week recovery.

Estrogen silastic capsule production and implantation: Silastic capsule containing 60 μg of 17β-estradiol benzoate in 60 μL of sesame oil was selected for our method for hormone administration. We aimed to assess the influence of estrogen during the sleep cycle; therefore, a slow-release formulation may avoid undesirable pulsatile concentrations of estrogen and daily animal manipulation.17) Silastic capsule implantation was performed in all male and female SHRs in the same procedure as in the animal preparation. The silastic capsules were artificially made through a 30-mm-long laboratory tubing (inner/outer diameter: 1.6/3.2 mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) containing 60 μg of 17β-estradiol benzoate in 60 μL sesame oil. The silastic capsules were sealed with wooden applicators and immersed overnight in sesame oil containing the same concentration as that inside the capsules.18,19) One silastic capsule was implanted subcutaneously in each rat over the posterior
The implantation was followed by a 1-week recovery. The slow release of 17β-estradiol benzoate from the silastic capsules maintained the physiological range of serum estrogen for 1 month.

**Experimental procedure:** In this study, we aimed to achieve the maximum authenticity of the natural sleeping cycle and behavior; therefore, all SHRs were not physically restricted during sleep.

In all SHRs, after a 1-week recovery from the head circuit implantation, a 24-hour signal recording was performed before and after the implantation of estrogen pump for comparison. The head circuit in each rat contained wireless sensors for EMG recordings to confirm the sleeping status by recognition of minimal physical motion. The EMG and EEG recordings were both investigated to facilitate our final confirmation of the sleeping-awake status. The signals during the waking period were excluded due to the interference of increased physical activity and white noise. One hour after confirmation of the sleeping status, 6 consecutive hours of signals were collected and analyzed at baseline and after estrogen pump implantation.

**ECG and BP measurement:** ECG signals were amplified to 500-folds with different sections for filtered at 0.72 to 103 Hz. The mean arterial pressure (MAP) measurements were obtained through the abdominal aorta where the signals were transmitted to a receiver (CATALOG# 272-6001, Data Sciences, St. Paul, MN, USA). The pulse signals were filtered at 10 to 100 Hz and a voltage converter (KY1C, K&Y Lab, Taiwan) reconstructed the pulse signals into continuous waveforms and then integrated the arterial pulse contour. These signals were synchronized by a 12-bit analog-digital converter connected to an IBM PC compatible computer at sample rates of 500 Hz. Finally, all signals were transmitted at a frequency of 2.4 GHz to a data recorder (KY1C, K&Y Lab, Taipei, Taiwan, size: 25 x 21 x 13 mm, weight: 8.6 g) for documentation.

**Cardiovascular variability analysis:** The protocol for heart rate variability (HRV) analysis has been described in previous studies. The R-R (RR) interval was estimated continuously from the digitized ECG signals. The stationary of RR interval was resampled and interpolated at 64 Hz to provide continuity in the time domain and were then truncated into 16-second time segments with a 50% overlap. These sequences were analyzed by fast Fourier transform after the application of a Hamming window. The low-frequency power of the BP (BLF, 0.06-0.6 Hz) spectrogram and the high-frequency power (HF, 0.6-2.4 Hz) and normalized low-frequency power (LF, 0.06-0.6 Hz) of the RR interval spectrogram were quantified. The BLF, LF, and HF indicate sympathetic vasomotor activity, cardiac sympathetic modulation, and cardiac vagal activity, respectively.

**Sleep cycle analysis:** We defined the consciousness status via electroencephalogram (EEG) recordings. Continuous power spectral analysis was applied to the EEG signals using a Hamming window of 16 seconds (50% overlap), from which the mean power frequency of the EEG was quantified. The consciousness status was categorized into stages of active waking (AW), QS, and PS. The QS stage is widely known as the slow-wave sleep, whereas the PS stage is known as the rapid eye movement (REM) sleep. Frequent transitions between sleep stages indicate arousal in sleep which is documented by the number of each stage during sleep.

In these sleep stages, we quantified the delta wave (1-4 Hz), theta wave (4-8 Hz), alpha wave (8-13 Hz), and beta wave (13-32 Hz) of EEG spectrum based on fast Fourier transform. Figure 2 presents an example of semi-automatic computing analysis, which demonstrates the ECG waveforms, RR interval (ms), delta wave (1-4 Hz), theta wave (4-8 Hz), alpha wave (8-13 Hz), and beta wave (13-32 Hz) of EEG spectrum and the HRV parameters.

The quality of sleep was evaluated by several parameters, such as the comparison of the accumulation time of each sleep stage, the mean duration of each sleep stage, the frequency of transition between stages, and the percentage of sleep waves within each stage. The transition between the sleep stages was considered a normal phenomenon. However, increased transition between...
stages may indicate increased arousal during sleep. High-quality sleep was demonstrated by increased accumulation time and increased mean duration of the QS stage, which indicated a deep and effective sleep. During sleep, the increase of delta waves and the suppression of beta waves also indicated a better sleep quality. In addition, the decreased frequency of switching between sleep stages suggested less arousal during sleep. The theta waves were correlated with active behavior during awake or REM sleep, whereas the alpha waves were associated with the REM sleep and arousal state. Sleep disorders or underlying diseases may also demonstrate increased alpha waves during sleep.

Statistical analysis: The statistical results are expressed as mean ± SD with $P < 0.05$ indicating statistical significance. Parameters of LF/HF, LF, and HF were logarithmically corrected to the skewness of the distribution, and the two sets of data were assessed by least significant difference test using the IBM SPSS Statistics version 22 for comparison.

Results

Analyses of ECG and BP: In all SHRs, the estrogen supplementation demonstrated a significant prolongation of RR intervals at the AW, QS, PS, and total sleep stages (Figure 3A, B, $P < 0.01$ in male, $P < 0.05$ in female) when compared with those at baseline, respectively. During baseline of all SHRs, the RR intervals were significantly prolonged at the QS and PS stages when compared...
with those at the AW stage (Figure 3A, B, all \( P < 0.01 \)). After estrogen supplementation in all SHRs, the RR intervals at the QS and PS stages were also significantly longer than those at the AW stage (Figure 3A, B, all \( P < 0.01 \)).

In all SHRs, the MAP value exhibited no significant difference between baseline and after estrogen supplementation at the AW, PS, QS, and total sleep stages (Figure 3C, D), respectively. In all SHRs during baseline, the MAP was significantly decreased at the QS and PS stages when compared with that at the AW stage (Figure 3C, D, all \( P < 0.01 \)). In all SHRs after estrogen supplementation, the MAPs of the QS and PS stages were also significantly lower than those of the AW stage (Figure 3C, all \( P < 0.01 \); Figure 3D, all \( P < 0.05 \)).

In all SHRs, the BLF expressed no significant change between baseline and after estrogen supplementation among all stages of sleep, respectively (Figure 3E, F). In all SHRs during baseline, the BLFs at the QS and PS stages were significantly decreased when compared with that at the AW stage (Figure 3E, \( P < 0.05 \), \( P < 0.01 \); Figure 3F, all \( P < 0.01 \)). In all SHRs after estrogen supplementation, the BLFs at the QS and PS stages were also significantly decreased when compared with that at the AW stage (Figure 3E, all \( P < 0.01 \), \( P < 0.05 \); Figure 3F, all \( P < 0.01 \)). In all SHRs during baseline and after estrogen supplementation, the BLF exhibited no difference between the QS and PS stages (Figure 3E, F).

**Analyses of cardiovascular variability:** In all SHRs, the LF/HF revealed no difference between baseline and after estrogen supplementation in total sleep, AW, QS, and PS stages (Figure 4A, B). During the QS stage at baseline, the LF/HF was significantly lower than that at the AW and PS stages (Figure 4A, B, all \( P < 0.01 \)). The LF/HF at the QS stage after estrogen supplementation was also significantly lower than that at the AW and PS stages (Figure 4A, B, all \( P < 0.01 \)).

In male SHRs, the HFs and LFs were significantly increased at all sleep stages after estrogen supplementation when compared with those at sleep stages at baseline (Figure 4C, E, all \( P < 0.01 \)). In addition, the HFs at the QS and PS stages during baseline in male SHRs were significantly increased when compared with that at the AW stage (Figure 4C, all \( P < 0.05 \)). The HFs at QS and PS stages after estrogen supplementation in male SHRs were also significantly higher than that at the AW stage (Figure 4C, all \( P < 0.05 \)).

In female SHRs, the HFs of the total sleep and AW stages were significantly increased after estrogen supplementation when compared with that at baseline (Figure 4D, all \( P < 0.05 \)), whereas the QS and PS stages exhibited a trend of higher HF after estrogen supplementation (Figure 4D). During baseline in female SHRs, the HFs of the QS and PS stages were significantly increased when compared with that at the AW stage (Figure 4D, all \( P < 0.05 \)). The HFs of the QS and PS stages after estrogen supplementation in female SHRs were also significantly higher than that at the AW stage (Figure 4D, all \( P < 0.05 \)). After estrogen supplementation in female SHRs, the LFs at the QS and PS stages were significantly higher than those at baseline (Figure 4F, all \( P < 0.01 \)).

**Analyses of sleep stages:** In all SHRs, the accumulation time revealed no difference between baseline and after estrogen supplementation at the AW, QS, and PS stages, respectively (Figure 5A, B). In all SHRs during baseline, the accumulation time of the QS stage was significantly longer than that of the AW stage (Figure 5A, \( P < 0.05 \); Figure 5B, \( P < 0.01 \)). In all SHRs after estrogen supplementation, the accumulation time of the QS stage was also significantly longer than that of the AW stage (Figure 5A, \( P < 0.05 \); Figure 5B, \( P < 0.01 \)). During baseline and after estrogen supplementation in female SHRs, the accumulation time of the PS stage was significantly shorter than that of the AW stage (Figure 5B, all \( P < 0.01 \)).

In male SHRs, the mean duration of the QS and PS stages at baseline was significantly shorter than that of the AW stage (Figure 5C, all \( P < 0.05 \)). After estrogen supplementation in male SHRs, the mean duration of the QS and PS stages was also significantly shorter than that of the AW stage (Figure 5C, \( P < 0.05 \)). In male SHRs, the mean duration of the QS stage was significantly decreased after estrogen supplementation when compared with that of baseline (Figure 5C, \( P < 0.05 \)). In female SHRs, the mean duration of the QS stage was also significantly shorter than that of the AW stage (Figure 5C, \( P < 0.05 \)).

In all SHRs, the frequency of QS and PS stages was significantly increased after estrogen supplementation, when compared with that of baseline (Figure 5E, \( P < 0.01 \), \( P < 0.05 \)). During the baseline in all SHRs, the frequency of QS and PS stages was significantly higher than that of the AW stage (Figure 5E, \( P < 0.01 \), \( P < 0.05 \)). After estrogen supplementation in all SHRs, the frequency of QS and PS stages was also significantly higher than that of AW stage (Figure 5E, \( P < 0.01 \)).

**Analyses of brain waves:** In all SHRs during total sleep, the percentage of delta waves at baseline was significantly increased in comparison with the theta, alpha, and beta waves (Figure 6A, B, \( P < 0.01 \)). After estrogen supplementation, the percentage of delta waves in all SHRs was significantly higher than those of theta, alpha, and beta waves (Figure 6A, B, all \( P < 0.01 \)). In male SHRs during total sleep, all brain waves exhibited no difference between the baseline and after estrogen supplementation (Figure 6A). In female SHRs during total sleep, the percentage of delta waves after estrogen supplementation was significantly increased when compared with that at baseline (Figure 6B, \( P < 0.01 \)).

In all SHRs, the QS and PS stages before and after estrogen supplementation were analyzed individually and exhibited no difference in the percentage of delta, theta, alpha, and beta waves (Figure 6C-F, respectively). In all SHRs during the QS stage, the percentage of delta waves at baseline and after estrogen supplementation was significantly higher than those of theta, alpha, and beta waves (Figure 6C, D, all \( P < 0.01 \)). In female SHRs, the percentage of delta wave at the QS stage was significantly increased after estrogen supplementation when compared with that at baseline at the QS stage (Figure 6D, \( P < 0.01 \)).
Figure 4. Comparison of heart rate variability parameters between baseline and after estrogen supplementation during total sleep, AW, QS, and PS stages in male and female SHRs. A, B: Low-frequency and high-frequency ratio (LF/HF); C, D: high frequency (HF); E, F: low frequency (LF). *P < 0.05, **P < 0.01 compared with the same category at baseline. +P < 0.05, ++P < 0.01 compared with AW stage at baseline. #P < 0.05, ##P < 0.01 compared with QS stage at baseline, $P < 0.01 compared with AW and PS stages at baseline, $$P < 0.01 compared with AW and PS stages after estrogen supplementation.

Discussion

Major findings: In this study, the main results of estrogen supplementation in SHRs were as follows: (1) increased HF and prolonged RR interval demonstrated significant elevated parasympathetic tone in all SHRs; (2) LF/HF revealed no difference, suggesting that no significant sympathetic disturbance was found in all SHRs; (3) decreased mean duration of QS stage in male SHRs, which indicated a significant reduction of deep sleep (Figure 5C), whereas the female SHRs exhibited no reduction of the QS stage (Figure 5D); (4) increased frequency of switching between QS and PS stages (Figure 5E) in male SHRs indicated a significant arousal during sleep, whereas the female SHRs exhibited no arousal during sleep (Figure 5F); and (5) increased percentage of delta wave at the total sleep and QS stages in female SHRs indicated increased deep sleep (Figure 6B, D). These findings, after estrogen supplementation resulted in an elevated parasympathetic tone without changing sympathetic activities in SHRs, suggested less vulnerability to arrhythmogenesis and CV events. Furthermore, the estrogen supplementation increased sleep arousal in male SHRs, whereas the female
SHRs exhibited increased deep sleep.

Autonomic activity during sleep in SHRs with estrogen supplementation: In previous studies, hypertensive subjects were associated with enhanced sympathetic tone and poor baroreflex function.8,28) The sleep-related changes of SHRs suggested that sympathetic hyperactivity is observed during sleep but accompanied by blunt baroreflex sensitivity.1) The modulation of autonomic tone for the treatment of HTN includes estrogen supplementation, which has been proven to reduce CV events through intensified parasympathetic tone.12,29)

In the frequency domain of HRV, it is demonstrated that the LF is influenced by a mixture of cardiac and parasympathetic activity, whereas the HF only represents the parasympathetic tone.30) In this study, the finding of elevated HF indicates a dominant parasympathetic tone in all SHRs after estrogen supplementation during sleep. This finding is in agreement with those of previous studies which resulted in attenuated CV events followed by HTN and reduced risk of sudden cardiac death (SCD). In animal studies, tachyarrhythmias were often preceded by extrinsic or intrinsic cardiac nerve activities pinpointing the result of sympathetic discharges before arrhythmias.31,32) In our study, the LF/HF revealed no difference between before and after estrogen supplementation in all SHRs which demonstrated insignificant sympathetic disturbance under the influence of estrogen supplementation. This finding suggests that estrogen supplementation af-
fected the parasympathetic tone more prominently than the sympathetic tone.

As a result of this study, an insignificant difference of arterial pressure variability before and after estrogen supplementation in all SHRs suggested poor baroreflex sensitivity during sleep despite estrogen supplementation. Our findings of unchanged baroreflex sensitivity after estrogen supplementation are similar to those of other studies despite the different populations and methods. Vir- tanen and colleagues also reported unchanged baroreflex function and BP after 3 months of estrogen therapy. In randomized controlled trials, increased vascular stiffness with unchanged BP was also reported under hormone therapy. Despite the impression that baroreflex function plays an important role in arterial BP variability, other regulations, such as the renin-angiotensin system, dyslipidemia, and endothelial dysfunction, should be considered.

Finally, the MAP remained unchanged after the estrogen supplementation in our study. This finding is consistent with those of the randomized clinical trials revealing limited effect on MAP after estrogen supplementation. In the Women’s Health Initiative Randomized Control Trial, BP was also not decreased under hormone therapy.

**Sleep quality in SHRs with estrogen supplementation:**
Sleep interruption is common among HTN subjects, and estrogen supplementation has been proven to enhance bet-
ter control of BP and influence recovery from sleep deprevation.29,39 In male SHRs in this study, the percentage of the delta, theta, alpha, and beta waves revealed no difference between baseline and after estrogen supplementation at the QS and PS stages of sleep, indicating that the architecture of brain waves at the QS and PS stage has not been altered (Figure 6C, E). Despite unchanged structure of brain waves in male SHRs, sleep interruption was increased due to shorter duration of each QS stage (Figure 5C) and frequent transition between QS and PS stages causing sleep arousal (Figure 5E). In male rat studies, castration had a limited change in the sleep cycle, and estrogen supplementation resulted in increased arousal during sleep.40,41 Our findings were consistent with previous studies and may confirm the different responses from opposite gender. In many studies, sex hormone imbalance in men is linked with elevated adverse cardiac events.6,7 The main source of estrogen in men is the testosterone, and age-associated decline of testosterone level results in aromatase hyperactivity and increased adipose tissue which potentially mimics obesity causing amplified risk of CV events accompanied by poor sleep quality.2-48.

On the other hand, the female SHRs in our study revealed a significant increase of delta waves during the QS stage after estrogen supplementation, indicating increased deep sleep and lowered risk of HTN progression. This finding was consistent with those of previous animal studies in that the influence of estrogen was most prominent during the QS stage.15 In clinical studies, decreased delta wave was associated with a greater risk of future progression of HTN and elevated nighttime BP.45,46 The results from our study were compatible with those of previous studies that demonstrated improved sleep quality and less sleep disturbance after estrogen supplementation.45,46

Conclusions

The effect of estrogen supplementation in all SHRs resulted in an elevated parasympathetic tone and no sympathovagal disturbance during sleep. Estrogen supplementation in the male SHRs leads to poor sleeping quality by shorter maintenance of the QS stage and increased interruption between QS and PS stages, which suggested the potential imbalance of sex hormone. On the contrary, estrogen supplementation in female SHRs lead to improved sleep quality and lowered risk of future progression of HTN by increased delta waves representing deep sleep throughout the total sleep. In HTN subjects, estrogen plays an important role in the neuromodulation during the sleeping cycle which may lower the risk of CV events, influence the vulnerability to tachyarrhythmias, and reduce the risk of SCD. Further studies are warranted for the practice of hormone supplements in the treatment of these subjects.

Disclosure

Conflicts of interest: None.

Statement of ethics: Animal experiments conform to internationally accepted standards and have been approved by the appropriate insti-
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