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Research Article
Combination of Valsartan and Melatonin to Treat Non-Dipping Hypertension Rats via Circadian Clock System

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Abstract

Background and Objective: Researches have reported that valsartan or melatonin bedtime administration showed a positive therapy to treat non-dipping Blood Pressure (BP), respectively. However, the combined effect of these two drugs in the treatment of non-dipping BP is unknown. Thus, this work aimed to evaluate the combined effects of valsartan and melatonin in the treatment of Spontaneous Hypertension Rats (SHR) with non-dipping BP and to explore its potential mechanism. **Materials and Methods:** A total of 28 male Wistar-Kyoto rats (WKY) was utilized as a non-disease control. A total of 144 male SHRs were treated with valsartan (10 mg/kg/day) combined with or without melatonin (20 mg/kg/day) after light onset. The 24 hrs BP, ANG II, ET-1, NO, *Per1* and *Per2* mRNA levels were detected. Nonlinear regression was used to calculate parameters of circadian BP rhythm. Inter-group means were compared using a One-Way Analysis Of Variance (ANOVA). **Results:** Valsartan administration with or without melatonin can significantly reduce active BP, restored circadian BP rhythm. Moreover, combination therapy reduced plasma levels of ANG II, ET-1 and NO, restored circadian rhythm ANG II in SHR rats and down-regulated the expression of *Per1* mRNA in the aorta abdominalis. **Conclusion:** Melatonin combined with valsartan significantly reduced BP and restored circadian BP rhythm than a single drug. The mechanism may associates with decreased Per genes expression and recover circadian rhythm of plasma ANG II.

Key words: Melatonin, valsartan, circadian rhythm, non-dipping hypertension, angiotensin, BP rhythm, hypothalamus, mRNA expression

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hypertension is a high-risk factor of stroke and Cardiovascular Disease (CVD), which can cause damage to target organs, including the heart, brain, kidney and aorta. Therefore, the prevention and treatment of hypertension have always been an important research topic in the global medical community[1]. The purpose of anti-hypertensive drugs in hypertension patients is to control BP, protect important organs and ultimately improve the quality of life and reduce mortality. Currently, anti-hypertensive drugs mainly include six categories: Calcium Channel Blockers (CCB), Angiotensin-Converting Enzyme Inhibitors (ACEI) and Angiotensin Receptor Blockers (ARB), beta receptor blockers and diuretic. However, single-use of anti-hypertensive drugs has a poor anti-hypertensive effect. Clinical trials have shown that patients need two or more anti-hypertensive drugs in most cases to achieve good anti-hypertensive effects[2]. Although clinicians are already using combined therapy strategies to treat hypertension, the existing treatment methods cannot solve all the clinical anti-hypertensive problems. Thus, it is necessary to explore the potential of combined therapy for refractory patients.

With the in-depth study of hypertension, it is found that BP show 24 hrs rhythmicity. Specifically, BP shows regular fluctuations with a period of 24 hrs and there are two peaks during the day, respectively at 6:00-8:00 and 16:00-18:00, the lowest BP occurred between 0:00 and 2:00. According to the BP dipper value, the BP pattern can be divided into the following four models: dippers (10-20%); non-dippers (0-10%); extreme dippers (>20%); Anti-dipper (<0)[3]. Except for the dipper pattern, the others are abnormal circadian rhythms. In recent years, it has been reported that the aberrant circadian BP rhythm of hypertension patients may be closely related to the occurrence of cardiovascular and cerebrovascular risk events[4]. With the application of dynamic BP monitoring in clinical practice, a large number of clinical studies have found that sleep means BP is more predictive of cardiovascular disease risk than the awake or 24 hrs mean BP[5-7]. Studies have confirmed that giving hypertension drugs at night has important clinical significance in reducing BP[8]. Compared with awake administration, ARBs and ACEI can improve BP at night before going to bed[9-10]. Therefore, the time therapy of hypertension should fully consider the regulation of abnormal circadian BP rhythm.

Circadian BP rhythm is controlled by a clock system. Circadian rhythms are orchestrated by the Suprachiasmatic Nucleus (SCN) located in the hypothalamus, which is an endogenous molecular pacemaker. The clock system is commonly regulated by transcription-translation feedback loops that consist of positive and negative clock components. Positive components include Circadian Locomotor Output Cycles Kaput (CLOCK) and brain and muscle Arnt-like protein 1 (Bmal1). Whereas three periods (Period 1 [*Per1*], Period 2 [*Per2*] and Period 3 [*Per3*]) and two cryptochromes (Cry1 and 2) molecules represent examples of negative clock components. Numerous studies have demonstrated that clock genes control circadian BP rhythm[11-13]. But few studies have focused on that drugs might regulating the circadian clock system, which in turn regulates circadian blood pressure rhythm.

Melatonin is an important amine hormone and is regulated by circadian rhythm[14]. Previously, numerous studies have shown that oral administration of melatonin could regulate clock gene expression[15]. Furthermore, recent studies have found that melatonin can not only regulate the secretion of renin and aldosterone through the hypothalamic-pituitary-adrenal axis but also reduce blood pressure may also act directly on the cerebral blood pressure centre through its high-affinity receptor[16]. However, few types of research focus on the pharmacological effects of melatonin on circadian blood pressure rhythm.

Recently, our research has suggested that chronotherapy of valsartan regulates 24 hrs blood concentration of the Renin-Angiotensin System (RAS) and circadian clock gene expression[17-18].

The purpose of this study was to investigate the effect of melatonin combined with valsartan on the circadian rhythm of BP in SHRs and its possible mechanism. We recorded 24 hrs Mean Arterial Pressure (MAP), Systolic BP (SBP) circadian rhythm and parameters changes and in each group. Effect of circadian rhythm of Angiotensin (ANG II), Endothelin-1 (ET-1), Nitric Oxide (NO) in plasma of rats in each group and circadian rhythm expression of clock gene *Per1*, *Per2* in the hypothalamus of rats in each group. Our study demonstrated that the combination of valsartan and melatonin is effective in reducing elevated BP and abnormal circadian BP patterns.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of pharmacology, The first affiliated hospital of Wannan medical college, Anhui, China from February, 2018-January, 2019.

Animal treatment: We used eight-week-old male SHR (n = 144) and Wistar-Kyoto rats (WKY, n = 24) provided from Beijing Vital River Laboratory Animal Technology Co. Rats were

maintained under a constant environment with a controlled 12 hrs light/dark cycle (light at 7 am, Circadian Time [CT0]). All animals were restricted to diet (20 g/day) but had *ad libitum* access to water. valsartan and melatonin were dispersed in 0.5% CMC-Na. WKY rats that received 0.5% CMC-Na were used as non-disease control (WKY, n = 24). SHRs that received 0.5% CMC-Na were used as a model group. The other SHRs were randomly divided into six groups (24 per group). Three melatonin treated groups treated with melatonin (10, 20 and 40 mg/kg/day) served as the low (M10), medium (M20) and high (M40) dosage, respectively. Rats in the other two groups were treated with 10 mg/kg/day Valsartan (VAL) and the combination of Valsartan (10 mg/kg/day) and Melatonin (V+M, 20 mg/kg/day). Notably, rats are nocturnal animals, contrary to humans, the light period refers to asleep time and the dark period refers to awake time. All rats oral administrated with drugs after light onset for 8 weeks.

Measurement of BP and specimen collection: Systolic BP (SBP) and Mean Arterial BP (MAP) were constantly obtained every 4 hrs from 07:00 to the next morning 07:00 by BP-600A automatic noninvasive BP measurement system after 6 weeks of administration, each group was measured three times, analyze the circadian rhythm and continue dosing until 8 weeks. After 8 weeks of administration, each group was anaesthetized by 10% chloral hydrate at 0.3 mL/100 g in batches every 4 hrs within 24 hrs. Disinfection, quickly open the abdominal cavity, the blood sample was taken from the abdominal aorta and placed in the vacuum anticoagulant tube of heparin sodium, after being centrifuged by 2000 r min^{-1} for 15 min, plasma was stored at -80°C for further investigation. The hypothalamus was excised immediately, blotted dry and weighed, which fixed in RNA safeguard and stored at -80°C for RNA isolation and RT-PCR. Other tissue samples such as the hearts, kidneys, livers and abdominal aorta were fixed in 10% formalin and stored at -80°C for other uses.

Detection of angiotensin II, endothelin-1, nitric oxidein plasma: The concentration of ANG II, ET-1, NO were tested by an Enzyme-Linked Immune Sorbent Assay kit (ELISA) produced by Suzhou Calvin Biotechnology Co. LTD. Catalogue numbers were CK-E30668R, CK-E30591R and CK-E30097R, respectively. All procedures were strictly performed according to the manufacturer's instructions.

RNA isolation and RT-PCR: The hypothalamus was taken out from the tissue RNA safeguard, washed with physiological saline, homogenized with a tissue homogenizer. Total RNA extract from the hypothalamus via Trizol Reagent (ThermoFisher, Waltham, MA, USA). The concentration and purity of isolated total RNA were verified by ND5000 Ultraviolet-Visible Spectrophotometer (BioTek Corp., Beijing, China). Total RNA was reverse transcribed to cDNA via a PrimeScript RT reagent kit with gDNA Eraser (TAKARA, Japan). Real-time PCR reactions were performed on the Bio-Rad CFX96 system (Hercules, CA, USA). The relative number of Cq was used to calculate the relative expression of clock-related mRNAs. All miRNA primers (*Per1*, *Per2*, *Actin*) were synthesized by Guangzhou RiboBio Co., Ltd according to the Gene Bank sequence.

Statistical analysis: The 24 hrs asleep time, awake time means of arterial pressure, 24 hrs SBP data, SBP circadian rhythm parameters, the concentration of ANG II, ET-1, NO in plasma and the expression of *Per1*, *Per2* gene in the hypothalamus were provided as Mean±standard deviation. Inter-group means were compared using one-way analysis of variance, comparisons between the two groups were performed using the SNK-q test. The circadian rhythm of SBP, the concentration of ANG II, ET-1, NO in plasma and the circadian rhythm of *Per1*, *Per2* gene in the hypothalamus were fit with the following equation using Graphpad Prism:

$$Y = \text{Amplitude}*\cos(0.2618*(X-\text{PhaseShift}))+\text{Baseline}$$

Circadian rhythm parameters of plasma ANG II, ET-1, NO and *Per1*, *Per2* gene in the hypothalamus were expressed as Mean±standard error and two groups were compared using the extra sum of squares test[17].

RESULTS

BP levels: In this study, compared to control group rats, SHR group rats showed a significantly higher 24 hrs, light period and dark period MAP. Melatonin significantly reduced the 24 hrs MAP in SHR rats in a dose dosage dependent way, which was more pronounced in the light period (sleep time) (Fig. 1a-c).

After combined treatment, each drug regimen can effectively reduce the 24 hrs MAP. Melatonin (20 mg/kg/day) has a weaker anti-hypertensive effect than valsartan, while the combined treatment has a significantly better anti-hypertension effect than valsartan alone. V+M mainly reduced the MAP in the dark period (awake time) (Fig. 1d-f).

BP circadian rhythm: The SBP values obtained after measurement in each group were fitted by the cosine formula. Then the SBP fitting curve and the SBP rhythm parameters of each group were obtained. The anti-hypertension effect of

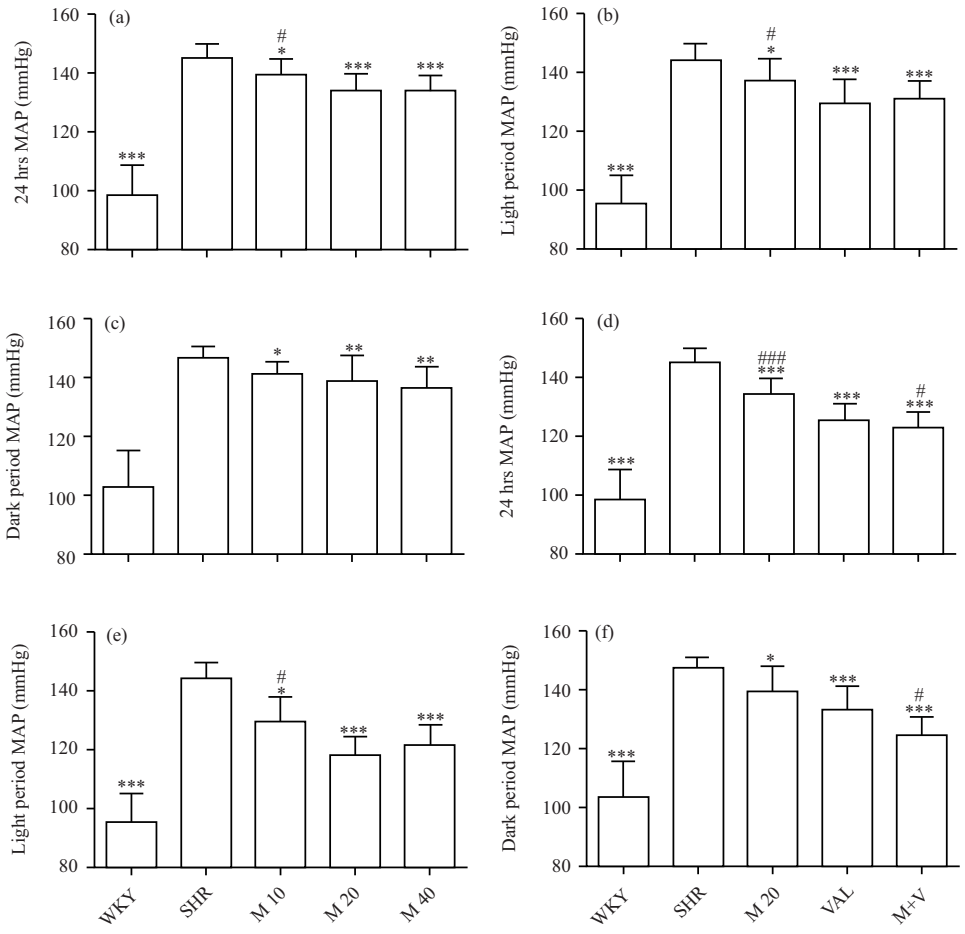

Fig. 1(a-f): Effect of melatonin administration, (a-c) Without and (d-f) With valsartan on MAP of rats in each group through 6 weeks, N = 24

Control group (WKY) rats and model group (SHR) rats were treated with 0.5 CMC-NA. Melatonin treated group (M10 , M20 and M40 mg) rats were treated with 10, 20 and 40 mg/kg/day melatonin respectively, the other two groups (VAL 10 mg and V+M) rats were treated with 10 mg/kg/day valsartan and the combination of 10 mg/kg/day valsartan and 20 mg/kg/day melatonin. The time period of 07:00-19:00 means light period (asleep time) of rats, the time period of 19:00-07:00 means dark period (awaking time) of rats. Data were represented by Mean±SEM for 168 independent experiments. (a-c) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. M20 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M10 mg group and (d-f) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. Val 10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

Table 1: Effect of valsartan with or without melatonin on circadian blood pressure rhythm in SHR among each group ($\bar{x}$±SE, n = 24)

ZT (hrs)	WKY	SHR	M10 mg	M20 mg	M40 mg	VAL10 mg	M+V
0	113.87±7.64***	178.12±7.29	164.55±8.00***$^{\#}$	158.27±6.18***$^{\#\#\#}$	156.67±10.89***	133.68±9.04***	126.92±8.63***$^{\#\#\#}$
4	109.81±5.54***	161.67±6.27	152.41±9.12***	148.36±9.10***$^{\#}$	149.20±7.06***	132.18±11.58***	124.01±6.74***$^{\#\#\#}$
8	102.59±7.97***	160.53±10.26	148.46±12.02	149.89±8.98$^{\#\#\#}$	150.29±9.33	120.28±11.94***	127.72±5.28***$^{\#\#\#}$
12	101.84±11.28***	153.52±6.67	155.16±12.92	155.31±8.42$^{\#\#\#}$	155.67±10.79	130.71±6.15***	137.94±5.23**$^{\#\#\#}$
16	114.67±11.10***	166.92±8.57	163.67±10.64	161.32±11.36$^{\#}$	155.21±10.61*	146.14±11.52**	142.69±6.12***$^{\#\#}$
20	122.54±9.29***	171.72±8.44	162.17±8.34*	163.79±7.8$^{\#}$	162.04±10.94*	154.06±4.41***	143.83±6.85***$^{\#\#\Delta}$
24	125.92±7.05***	163.60±6.28	163.85±9.83	164.27±8.89$^{\#\#\#}$	160.45±6.06	149.07±5.09***	143.13±4.31***$^{\#\#\#}$

*p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. VAL10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

valsartan with or without melatonin were showed in Fig. 2a-b. The anti-hypertensive effect of melatonin mainly occurs at CT0, CT4, CT16 and CT20 but valsartan has a significant anti-hypertensive effect within 24 hrs. The combined drug showed a significant higher anti-hypertensive effect on SBP than valsartan at CT0, CT4, CT20 and CT24 (Table 1).

In terms of SBP mean, M20 and M40 showed an anti-hypertension effect and partly revert the sleep/awake ratio of SBP (Fig. 2c-d). The combination showed a significant reduction than the single drug (Fig. 2e). In addition, valsartan better restored the sleep/awake ratio of SBP than that of M20 significantly. However,

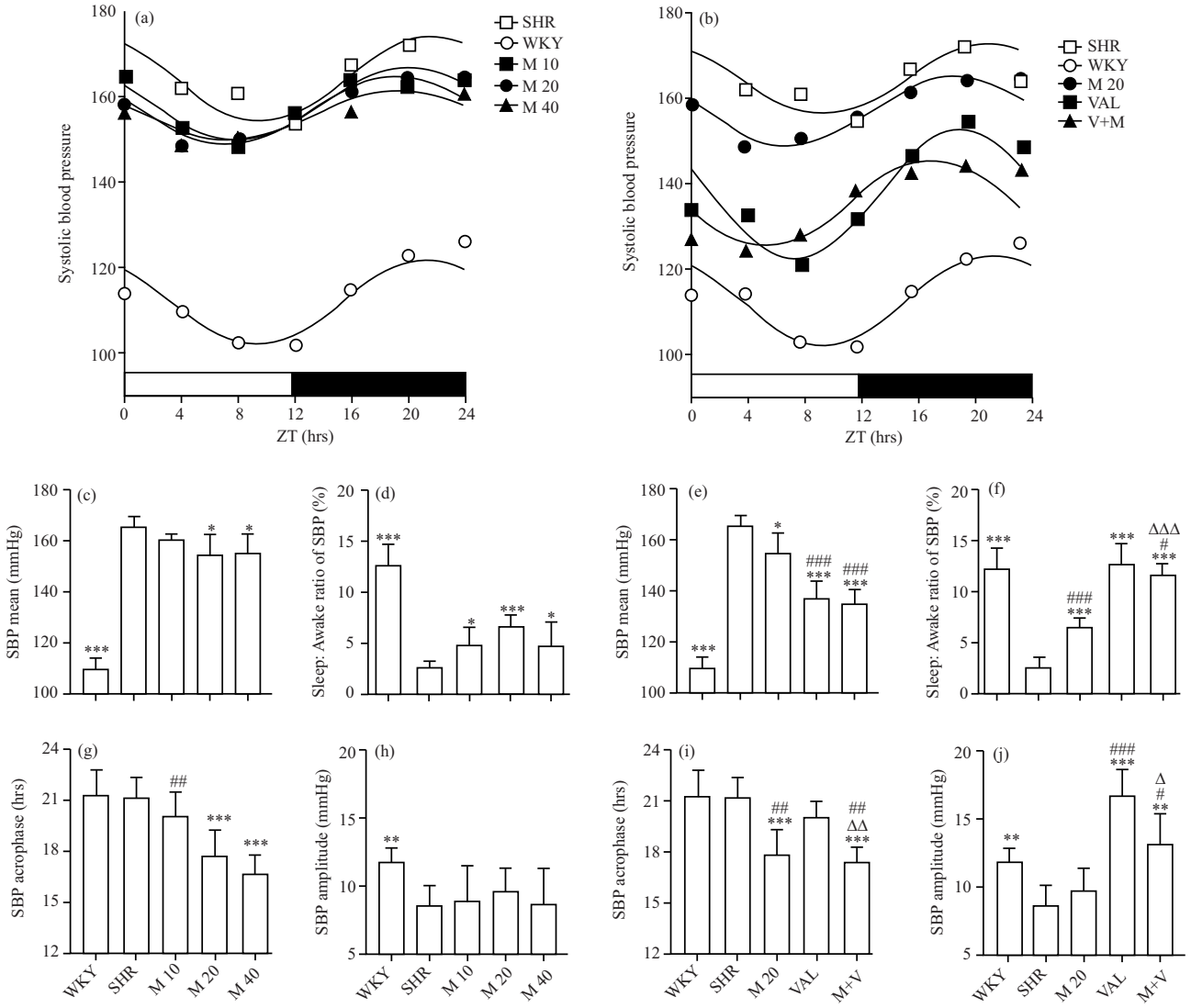

Fig. 2(a-j): Effect of melatonin administration with or without valsartan on the circadian rhythm of SBP circadian rhythm parameters of SBP and Sleep Awake ratio of SBP (%) of rats in each group through 6 weeks, N = 24. (a) Effects of different doses of melatonin on SBP circadian rhythm in SHR rats, (b) Effect on SBP circadian rhythm when combined with valsartan, (c, e) SBP mean of rats treated by melatonin with or without valsartan, (d, f) Sleep: Awake ratio of SBP (%) with two treatment methods and (d-j) The changes of circadian blood pressure parameters in each group

Data were represented by Mean±SEM for 168 independent experiments. (c-d, g-h) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, #p<0.05, ##p<0.01, ###p<0.001 vs. M20 mg group, Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.001 vs. M10 mg group and (e-f, i-j) *p<0.05 **p<0.01, ***p<0.001 vs. SHR group, #p<0.05 ##p<0.01, ###p<0.001 vs. Val10 mg group, Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.001 vs. M20 mg group

compared to combined therapy, valsartan alone was more effective in up-regulating the sleep/awake ratio of SBP (Fig. 2f).

There was no significant difference in acrophase and SBP amplitude between SHR and WKY group rats. Melatonin significantly shifts forward SBP acrophase in a dose-dependent way but had no significant effect on the abnormal SBP amplitude of SHR (Fig. 2g-h). M+V rather than valsartan alone could significantly shift forward SBP acrophase (Fig. 2i). But compared to combined therapy, valsartan alone was more effective in increasing the SBP amplitude (Fig. 2j).

In general, these results suggested that melatonin or valsartan is, to some extent, effective in restoring BP rhythms. But combination therapy showed a lower effect in restoring BP rhythms compare to valsartan alone.

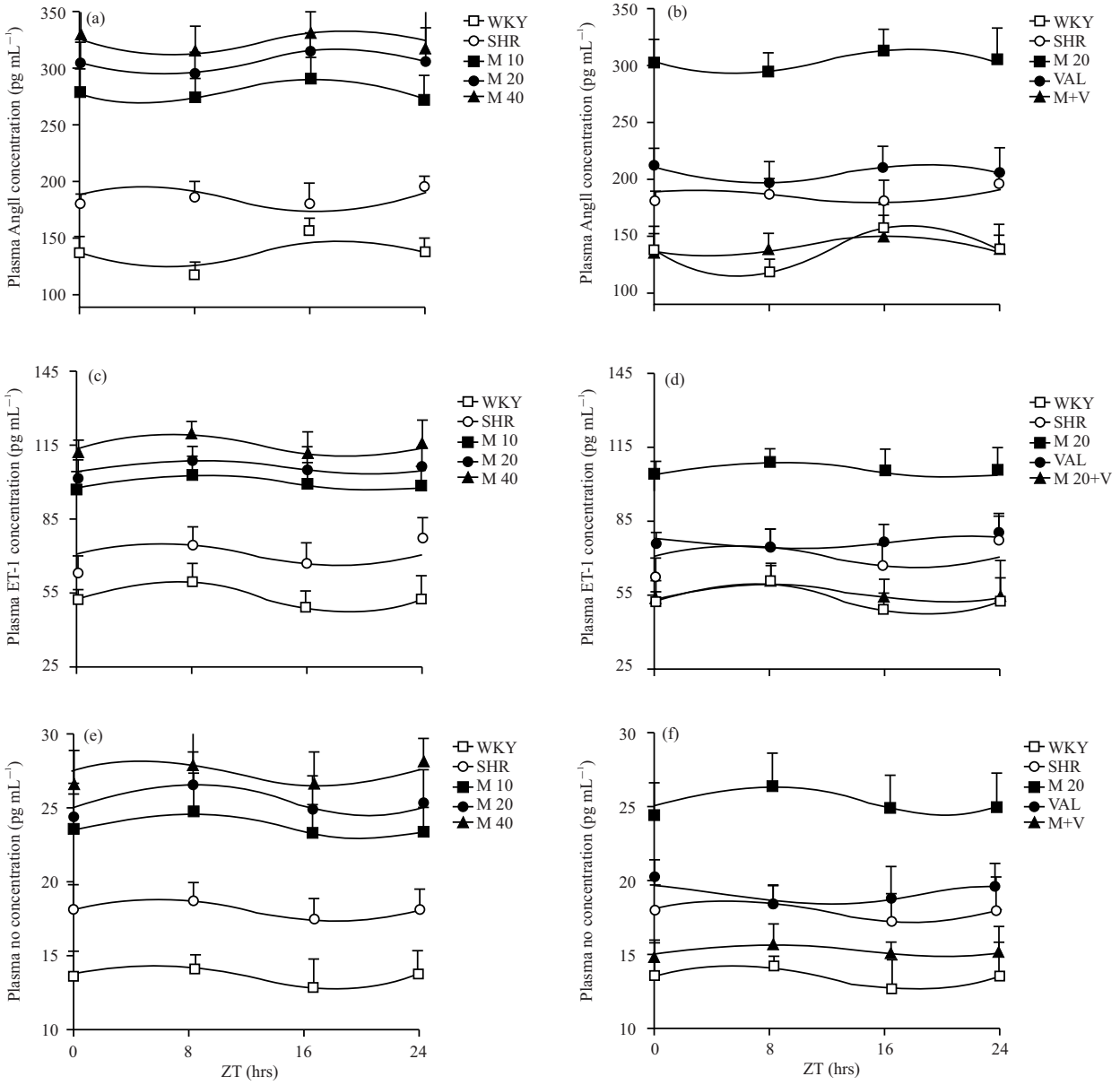

Fig. 3(a-f): Effect of melatonin with or without valsartan administration on circadian rhythm of (a-b) Ang II, (c-d) ET-1 and (e-f) NO in plasma of rats in each group through 6 weeks, N = 24

Figure including the concentration of Ang II, ET-1, NO in plasma of rats treated by melatonin with or without valsartan and the circadian plasma Ang II, ET-1, NO concentration were showed during the light and dark period, respectively. The period of 07:00-19:00 means the light period (sleep time) of rats, the period of 19:00-07:00 means the dark period (awake time) of rats. All data are expressed as the Mean±SEM. (a, c, e) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. M20 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M10 mg group and (b, d, h) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. Val10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

Concentration and circadian rhythm of ANG II, ET-1, NO in plasma: Compared with the WKY group rats, SHR group rats showed an increased and reverse acrophase of plasma ANG II concentration, which may be related to the abnormal BP rhythm of SHR group rats. Melatonin not only increased plasma ANG II concentration but also shift forward acrophase in a dose-dependent way (Fig. 3a).

Valsartan slightly increased the ANG II concentration and restored the acrophase of ANG II. Melatonin combined with valsartan can not only improve the abnormal rhythm of ANG II but also reduced the concentration of ANG II in plasma (Fig. 3b) (Table 2).

As shown in the picture (Fig. 3c-f), the diagram expressed the 24 hrs profile of plasma ET-1 and NO concentration of each

Table 2: Circadian parameters of Ang II in plasma of rats in all groups ($\bar{x}\pm SE$, n = 24)

Parameters	WKY	SHR	M10 mg	M20 mg	M40 mg	VAL10 mg	M+V
A (pg mL^{-1})	22.48±4.09**	4.907±4.19	10.92±6.42	11.23±7.22	8.21±9.61	7.67±6.43	9.01±7.08
M (pg mL^{-1})	137.4±2.62***	184.9±2.89	279.8±4.38***$^{\#\#\#}$	304.8±4.66***$^{\#\#\#}$	322.8±6.13***$^{\#}$	205.50±4.19***	141.6±5.00***$^{\#\#\#}$
Φ1 (hrs)	18.10	3.48	16.16	18.00	18.53	19.45	16.14
Φ2 (hrs)	6.10	15.48	4.16	6.00	6.53	7.45	4.14

Amplitude (A): Change in expression at high and low points relative to the corresponding baseline, Mesor (M): A measurement of the average level around which the curve oscillates over time, Peak phase (Φ_1 (hrs)): Time when the fit curve was highest and Troughs phase (Φ_2 (hrs)): Time to reach the lowest point of the fitted curve. All replicates of M and A were expressed as Mean±standard error in each group. The p-values were derived through comparison of any two groups of curve-fitting using the extra sum of squares test.*p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. VAL10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

Table 3: Circadian parameters of ET-1 in plasma of rats in all groups ($\bar{x}\pm SE$, n = 24)

Parameters	WKY	SHR	M10 mg	M20 mg	M40 mg	VAl 10 mg	M+V
A (pg mL^{-1})	6.43±2.63	4.08±3.03	3.14±2.35	2.87±2.35	4.56±2.47	2.63±2.21	3.38±3.10
M (pg mL^{-1})	53.24±1.67***	70.56±1.94	99.87±1.66***$^{\#}$	105.60±1.63***$^{\#\#\#}$	114.80±1.61***$^{\#\#\#}$	78.21±1.40**	55.60±2.10***$^{\#\#\#}$
Φ1 (hrs)	6.90	6.00	9.00	8.63	7.00	22.06	8.13
Φ2 (hrs)	18.90	18.00	21.00	20.63	19.00	10.06	20.13

Amplitude (A): Change in expression at high and low points relative to the corresponding baseline, Mesor (M): A measurement of the average level around which the curve oscillates over time, Peak phase(Φ_1 (hrs)): Time when the fit curve was highest and Troughs phase(Φ_2 (hrs)): Time to reach the lowest point of the fitted curve. All replicates of M and A were expressed as Mean±standard error in each group. The p-values were derived through comparison of any two groups of curve-fitting using the extra sum of squares test.*p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. VAL10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

Table 4: Circadian parameters of NO in plasma of rats in all groups ($\bar{x}\pm SE$, n = 168)

Parameters	WKY	SHR	M10 mg	M20 mg	M40 mg	VAl 10 mg	M+V
A (μmol mL^{-1})	0.79±0.74	0.73±0.57	0.81±0.79	1.06±0.79	0.79±0.75	0.60±0.49	0.38±0.43
M (μmol mL^{-1})	13.46±0.46***	17.95±0.37	23.67±0.49***$^{\#}$	25.39±0.50***$^{\#\#\#}$	27.15±0.50***$^{\#}$	18.93±0.39***	15.23±0.29***$^{\#\#\#}$
Φ1 (hrs)	5.48	5.36	7.50	7.91	4.52	23.61	8.39
Φ2 (hrs)	17.48	17.36	19.50	19.91	16.52	11.61	20.39

Amplitude (A): Change in expression at high and low points relative to the corresponding baseline, Mesor (M): A measurement of the average level around which the curve oscillates over time, Peak phase (Φ_1 (hrs)): Time when the fit curve was highest and Troughs phase (Φ_2 (hrs)): Time to reach the lowest point of the fitted curve. All replicates of M and A were expressed as Mean±standard error in each group. The p-values were derived through comparison of any two groups of curve-fitting using the extra sum of squares test.*p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. VAL10 mg group $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

group. Compared to the WKY group rats, SHR group rats showed a significantly increased plasma concentration of ET-1 and NO but there was no significant difference in amplitude and acrophase of ET-1 or NO between WKY and SHR group rats. Melatonin significantly up-regulated the plasma concentration, slightly shift the acrophase of both ET-1 and NO. No significant difference was found in amplitude between melatonin and SHR group rats.

Although, valsartan alone had slightly up-regulated the content of ET-1 and NO in the plasma (p<0.01), it altered the rhythm of ET-1 and NO to abnormal. Compared to monotherapy, the combination of melatonin and valsartan can not only reduce the concentration of ET-1 and NO in the plasma to returned to normal (p<0.001) but also does not have any adverse effects on its normal rhythm (Table 3 and 4).

Relative expression and circadian rhythm of *Per1*, *Per2* mRNA in the hypothalamus: The circadian rhythm of BP is regulated by the central system rhythm, which is mainly controlled by the suprachiasmatic nucleus. Therefore, in this study, we have explored the internal relationship between clock genes in the hypothalamus and BP rhythm. As shown in the picture (Fig. 4a-b), the circadian rhythm of relative expression of *Per1* mRNA in the hypothalamus, there was a difference in the expression rhythm of *Per1* gene in the hypothalamus between WKY rats and SHR rats and this difference was consistent with the plasma ANG and BP rhythms in comparison between WKY rats and SHE rats. Therefore, it is considered that the abnormal BP rhythm is related to the abnormal expression rhythm of the circadian clock gene *Per1*. However, in the comparison of expression rhythm of hypothalamic clock gene *Per2* between SHR rats and WKY rats (Fig. 4c-d), there was no significant difference in expression rhythm, the only significant difference in amplitude and the median value. Therefore, it can be considered that the abnormal BP rhythm is not significantly related to the expression of the circadian clock gene *Per2* in the hypothalamus.

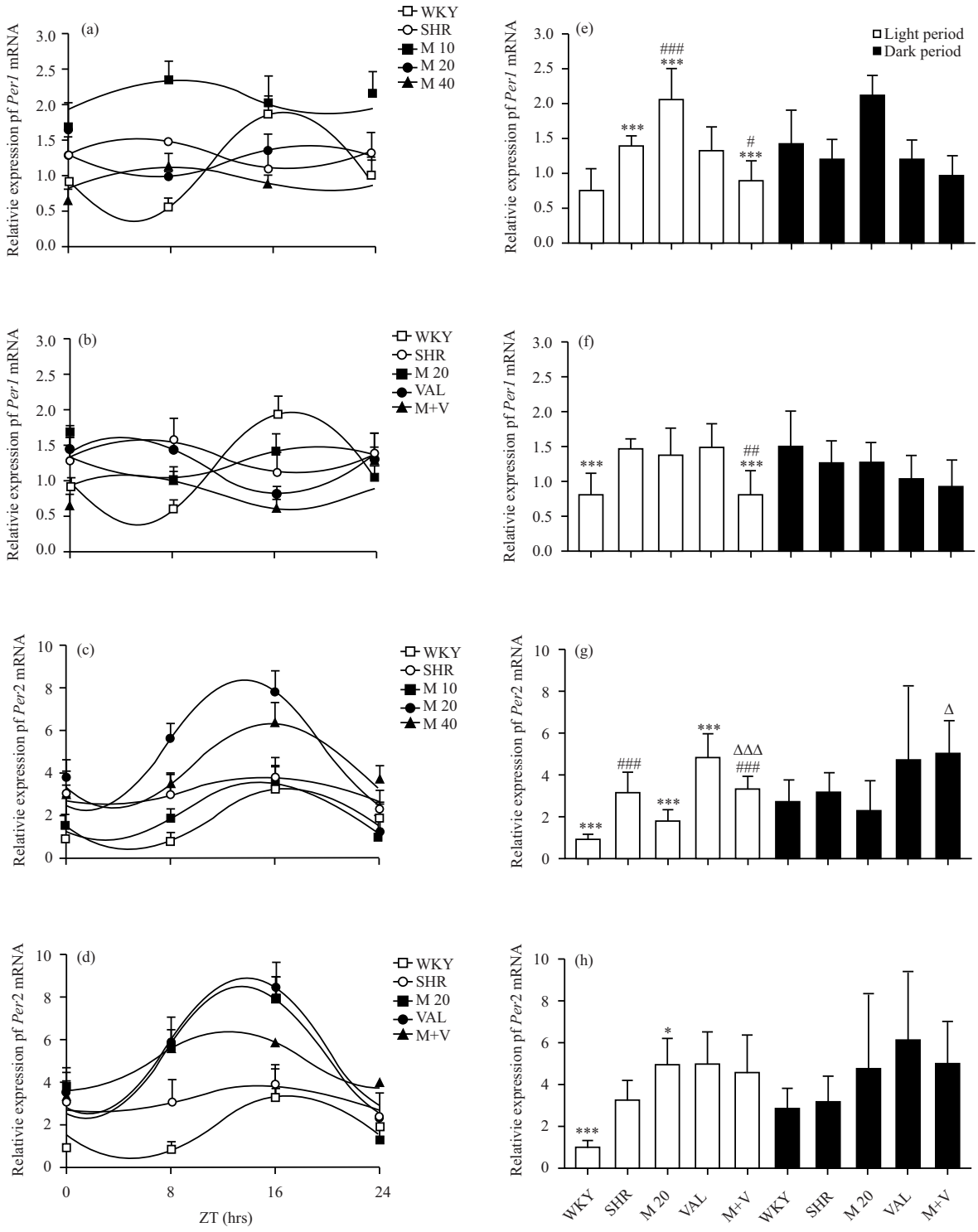

Fig. 4(a-h): Effect of melatonin with or without valsartan administration on the circadian rhythm of (a-b, e-f) *Per1* and (c-d, g-h) *Per2* gene in the hypothalamus of rats in each group through 6 weeks, N = 24

Circadian hypothalamus (a-b) *Per1* and (c-d) *Per2* concentration profile in each group. The circadian hypothalamus (e-f) *Per1* and (g-h) *Per2* concentration were showed during the light and dark period, respectively. The time period of 07:00-19:00 means light period (asleep time) of rats, the time period of 19:00-07:00 means dark period (awake time) of rats. All data are expressed as the Mean±SEM. (e, g) *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05 $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. M20 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M10 mg group and (f, h) *p<0.05 **p<0.01, ***p<0.001 vs. SHR group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. Val10 mg group, $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 vs. M20 mg group

Table 5: Circadian parameters of *PER1* gene in the hypothalamus of rats in all groups (x̄±SE, n = 168)

Parameters	WKY	SHR	M10 mg	M20 mg	M40 mg	VAl 10 mg	M+V
A (pg mL^{-1})	0.77±0.09***	0.23±0.07	0.25±0.12	0.23±0.12	0.18±0.10	0.39±0.10	0.23±0.10
M (pg mL^{-1})	1.13±0.06*	1.29±0.05	2.11±0.09***###	1.21±0.08	0.95±0.07***##	1.17±0.07	0.81±0.07***###
Φ1 (hrs)	17.13	5.65	8.69	19.28	8.57	4.23	4.95
Φ2 (hrs)	5.13	17.65	20.69	7.28	20.57	16.23	16.95

Amplitude (A): Change in expression at high and low points relative to the corresponding baseline Mesor (M): A measurement of the average level around which the curve oscillates over time, Peak phase (Φ_1 (hrs)): Time when the fit curve was highest and Troughs phase (Φ_2 (hrs)): Time to reach the lowest point of the fitted curve. All replicates of M and A were expressed as Mean±standard error in each group. The p-values were derived through comparison of any two groups of curve-fitting using the extra sum of squares test. *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, #p<0.05, ##p<0.01, ###p<0.001 vs. VAL10 mg group, ^p<0.05, ^^p<0.01, ^^^p<0.001 vs. M20 mg group

Table 6: Circadian parameters of *Per2* gene in the hypothalamus of rats in all groups (x̄±SE, n = 168)

Parameters	WKY	SHR	M10 mg	M20 mg	M40 mg	VAl 10 mg	M+V
A	1.47±0.23*	0.65±0.30	1.40±0.19*##	3.07±0.37***	1.95±0.25***#	3.14±0.33***	1.36±0.44
M	1.90±0.18***	3.18±0.21	2.25±0.13**###	5.33±0.28***	4.40±0.17***##	5.65±0.26***	4.97±0.34***
Φ1 (hrs)	17.06	14.86	15.06	13.63	15.73	13.81	12.56
Φ2 (hrs)	5.06	2.86	3.06	1.63	3.73	1.81	0.56

Amplitude (A): Change in expression at high and low points relative to the corresponding baseline, Mesor (M): A measurement of the average level around which the curve oscillates over time, Peak phase (Φ1 (hrs)) Time when the fit curve was highest and Troughs phase (Φ2 (hrs)): Time to reach the lowest point of the fitted curve. All replicates of M and A were expressed as Mean±standard error in each group. The p-values were derived through comparison of any two groups of curve-fitting using the extra sum of squares test. *p<0.05, **p<0.01, ***p<0.001 vs. SHR group, #p<0.05, ##p<0.01, ###p<0.001 vs. VAL10 mg group, ^p<0.05, ^^p<0.01, ^^^p<0.001 vs. M20 mg group

After drug treatment, the abnormal *Per1* expression rhythm was significantly restored in the M20 group, while the effect was not obvious in other dose groups. For the relative expression level of the *Per1* gene, melatonin has a dose-effect on reducing the expression level of the *Per1* gene in both the resting period and active period (Fig. 4e) and has a similar effect on reducing MAP. Compared with the SHR model group, valsartan had no significant effect on the expression rhythm and relative expression of the *Per1* gene. The combination therapy reversed the recovery effect of melatonin on the expression rhythm of the *Per1* gene, which may be related to the antagonistic effect after the addition of valsartan. But the combination can significantly reduce the relative expression of the *Per1* gene during rest and restored it to normal levels (Fig. 4f and Table 5). Moreover, this restorative effect is consistent with that of ANG in plasma. As for the *Per2* gene in the hypothalamus, compared with WKY rats, the amplitude of the *Per2* gene in the hypothalamus in SHR rats was relatively small and the median value was relatively large. After administration of the drug, the median value of each group showed different degrees of improvement. In terms of amplitude, the M 10 group, M 40 group and M+V group can effectively recover abnormal amplitude, while the M 20 group and VAL group can increase amplitude more. Our research data showed that M 10 could significantly reduce the relative expression of the *Per2* gene in SHR rats at light period (Fig. 4g-h and Table 6).

DISCUSSION

With a deeper understanding of the pathogenesis of hypertension and the progress of modern medical technology, people have realized that controlling BP level is only one aspect to reduce the harm of hypertension. The weaken or disappearance of circadian BP rhythm in hypertension patients may be closely related to the damage of target organs such as the heart, kidney, brain and blood vessels[19]. A 10-20% drop in BP at night is considered to be a mode of BP drop. There was a significant increase in the risk of cardiovascular outcomes because of increased BP at night. Further clinical trials have shown that melatonin not only improves sleep but also has significant implications for reduces BP and restores BP circadian rhythm[20]. For valsartan, also plays an important role in controls BP levels and regulates BP rhythm patterns. However, there is currently no study on the combination of melatonin and valsartan in the treatment of hypertension and the mechanism of combined treatment of hypertension is still unclear. Therefore, our study examined the effects of melatonin combined with valsartan on BP rhythm and found a partly potential circadian molecular mechanism. The major findings were as follows: (i) Melatonin combined with valsartan significantly reduced 24 hrs MAP and SBP mean compared to monotherapy. (ii) Combination of melatonin and valsartan or valsartan alone can effectively restore the abnormal circadian rhythm of blood pressure. (iii) The over-expression and abnormal expression rhythm of

Per1, may cause abnormal ANG content and rhythm in peripheral vascular tissues. The combination of melatonin and valsartan can restore the rhythm and content of ANG in plasma by reduced the overexpression of the *Per1* gene, thereby reduced BP and restored abnormal BP rhythm.

The BP dipper value is also known as sleep-time relative BP decline (mainly refers to Systolic BP (SBP), also can be expressed as Sleep: Awake ratio of SBP), defined as resting BP relative to the active period percentage of BP drop, calculated as (active BP- resting blood pressure)/active BP×100%, Therefore, the BP circadian rhythm pattern is divided into four categories: (i) Dippers (10-20%), (ii) Non-dippers (0-10%) (iii) Extreme-dippers (>20%) (iv) Anti-dipper (<0)[21]. The BP dipper value is also an important predictor of cardiovascular event morbidity and mortality. The results of our present study showed that before administration, the BP dipper value was between 10 and 20% in a normal control group, which showed the circadian rhythm pattern of dippers blood pressure. The BP dipper value was less than 10% in the model group and the dosing group, which showed the circadian rhythm pattern of non-dippers blood pressure. During the medication, valsartan alone and valsartan combined with melatonin can restore the BP rhythm pattern from non-dippers pattern to dippers pattern, with the former is slightly stronger than the latter. The reason for this may be due to the combination of melatonin and valsartan was more effective in reducing the active BP than valsartan alone. This suggests that the combination of melatonin and valsartan may be more effective in improving the extreme-dippers BP rhythm. In the comparison of amplitude, the combination of melatonin and valsartan was significantly lower than valsartan, which was closer to the normal control group, which means that the combination drug is more conducive to control the abnormal fluctuation of blood pressure. In terms of peak phase (acrophase), we have found that melatonin can significantly advance the phase parameters of BP rhythm and no anti-hypertensive drugs have been reported to significantly improve the phase of blood pressure. Studies suggest that the regulation of circadian BP rhythm is closely related to the rhythmic changes of the clock genes *Per1* and *Per2* and the rhythmic changes of the Renin-Angiotensin System (RAS) but the specific mechanism needs further study[22].

RAS plays an important role in the pathophysiology of hypertension and renal disease. Studies have shown that this regulatory system is a regulatory cascade that plays an essential role in the development of cardiovascular disease by regulating blood pressure, electrolytes, water balance and inflammation[23-25]. Recent research evidence indicates that the RAS circulatory system exhibits a circadian rhythm with the highest and lowest concentration of angiotensin appear in the morning and late evening, respectively[26]. Wang *et al.*[27] and our previous study have found that abnormal circadian BP rhythm was related to ANG II and reduce the ANG II level can restore abnormal BP rhythm in patients with primary hypertension[18]. Our results showed that although melatonin alone could restore the abnormal rhythm of ANG II, there was a dose-effect of melatonin on ANG II plasma increase in SHR, which was contrary to the anti-hypertensive effect. However, when the combination of melatonin and valsartan were used, the rhythm of ANG II was restored and the concentration of ANG II in plasma was reduced to a normal level. Although valsartan alone recovered the ANG II rhythm, the ANG II concentration increased. Other studies have found that ACE2 converts ANG II-ANG1-7 and these downstream products have a protective effect on the cardiovascular system[28]. Under conditions of hypertension, ventricular remodelling and heart failure, ANG1-7 has resistance to ANG II-induced vasoconstriction, inflammation and cell growth signalling at the cardiac and cardiovascular levels[29-31]. However, the content of these downstream products is much lower than ANG II[32]. Therefore, we believe that the increase of ANG II content caused by melatonin also induces part of ANG II to be converted into ANG 1-7, to achieve the effect of BP reduction and protect target organs. Certainly, this needs further verified in this experiment. In the detection of ET-I and NO in plasma, we found that the expression rhythm of ET-1 and NO in plasma is normal, which means the abnormal circadian rhythm of BP may be unrelated to the rhythm of ET-1 and NO in plasma. A combination of melatonin and valsartan can reduce the concentration of both in plasma compared with single drug use.

The circadian rhythm of the organism is very essential to the circadian BP rhythm. Circadian rhythm is regulated by internal central clock genes, peripheral clock genes and humoral factors through a transcription-translation-post-translational processing feedback loop. This negative feedback loop activates the cycle of circadian gene transcription, forming a circadian rhythm of approximately 24 hrs. It was found that the circadian rhythm of mice was abnormal regardless of a single mutation of the *mPer1* gene, a single mutation of the *mPer2* gene, or a double mutation of *mPer1/mPer2* gene[33]. Our present research found that the *Per1* gene expression rhythm was significantly abnormal in the SHR model group compared with the WKY control group,

while the *Per2* gene expression was not significantly different but the rhythm parameter amplitude (A) decreased significantly ($p<0.05$) and Mesor (M) increased significantly ($p<0.001$). Moreover, the difference in the expression of the *Per1* gene between WKY and SHR rats was consistent with the plasma ANG II and SBP rhythms and the combination of melatonin and valsartan reduced the expression level of *Per1* to normal level. The expression rhythm of the *Per2* gene was not significantly increased after administration but the amplitude was significantly increased. Nonaka *et al.*[34] found that transient administration of ANG II caused strong expression of the *mPer2* gene, which subsequently caused simultaneous periodic oscillations of other components of the circadian clock. Our study found that when the content of ANG II in plasma increased, the amplitude of *mPer2* is significantly increased.

Numerous studies have reported that relative to awake time administration of anti-hypertension drugs asleep time administration not only decreases the blood pressure but also restore blood pressure rhythm. However, the underlying mechanism is not clear for now. In addition, melatonin has an anti-hypertension effect and consider as an endogenous biomarker of the clock system. The combination of the two drugs might show a more obvious therapy effect compare to monotherapy. In our work, we found the combination of melatonin and valsartan treatment significantly reduced BP in SHR rats and ameliorated the abnormal circadian BP rhythm. The mechanism may be that the combination of drugs can reduce the expression of the clock gene *Per1* in the central clock system, thus ameliorated the ANG II content and circadian rhythm in the peripheral blood vessels and finally reduced BP and ameliorated the circadian rhythm of blood pressure. Our work shed light on the potential combination therapeutic strategy and partly provided evidence for the treatment of non-dipping hypertension by taking hypertensive drugs before bedtime. However, although our work has evaluated the efficacy of the combination therapy on circadian BP rhythm, the deeper cellular and molecular mechanisms have not been explored. Thus, We will further verify the deeper mechanism in future studies.

CONCLUSION

In summary, our present study indicates that, compared to the single-drug treatment, the combination of melatonin and valsartan treatment significantly reduced BP in SHR rats and partly restore the circadian BP rhythm. This may be that the combination of drugs can reduce the expression of the clock gene *Per1* in the central clock system, thus decreased the ANG II content and circadian rhythm in the peripheral blood vessels and finally reduced BP and ameliorated the circadian rhythm of blood pressure. Our work shed the light on the possibility of combination usage of valsartan and melatonin. We also indicate the advantages and disadvantages of this combination therapy in the treatment of hypertension or BP rhythm disorder.

SIGNIFICANCE STATEMENT

Our present study indicates that, compared to the single-drug treatment, the combination of melatonin and valsartan treatment significantly reduced BP in SHR rats and ameliorated the abnormal circadian BP rhythm. The mechanism may be that the combination of drugs can reduce the expression of the clock gene *Per1* in the central clock system, thus ameliorated the ANG II content and circadian rhythm in the peripheral blood vessels and finally reduced BP and ameliorated the circadian rhythm of blood pressure. Our work shed light on the potential combination therapeutic strategy and partly provided evidence for the treatment of non-dipping hypertension by taking hypertensive drugs before bedtime.

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