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Uncovering the Penile Clock: Expression of Molecular Clock Proteins in Human Penile Cavernous Tissue

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Purpose: To evaluate the expression of core molecular clock genes/proteins in penile cavernous tissue from healthy male subjects and to determine whether their expression has circadian variation.

Materials and Methods: Corpus cavernosum biopsy samples were obtained from 10 healthy males with penile deviation or fracture who underwent surgical intervention during the day and night. The daytime group (n=5) underwent corpus cavernosum tissue sampling during zeitgeber time (ZT) 8–12, while the nighttime group (n=5) underwent sampling during ZT 20–24. The expression and localization of BMAL1, CLOCK, PER1, PER2, PER3, CRY1, and CRY2 proteins were analyzed using immunohistochemistry and quantified using H-score analysis. RT-qPCR analysis was performed to assess the expression of core molecular clock genes in the corpus cavernosum tissue of 5 additional daytime patients.

Results: The expression of core molecular clock proteins was detected in vascular endothelial cells (VECs) and smooth muscle cells (SMCs) in corpus cavernosum during daytime and nighttime. BMAL1 exhibited the most significant nuclear expression during daytime in both cell types, whereas its expression decreased significantly at night. In VECs, a significant decrease in the nuclear expression of CRY1 was observed at night. In SMCs, a significant decrease in the cytoplasmic expression of PER3 was observed at night. The expression patterns of the core molecular clock genes were ascertained through a RT-qPCR analysis.

Conclusions: Our research provides compelling evidence that core molecular clock genes are distinctly expressed in penile tissue in humans. Furthermore, we observed the expression of molecular clock proteins within the VECs and SMCs of the corpus cavernosum, with BMAL1 being the most prominently expressed. The discovery of core molecular clock genes in penile tissue, as well as proteins within the SMCs and VECs of the corpus cavernosum, introduces the potential significance of the molecular clock mechanism in the physiology of penile erection.

Keywords: Circadian clocks; Circadian rhythm; Endothelium, vascular; Penis

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INTRODUCTION

The circadian rhythm is a physiological process that regulates the rhythmic changes of complex molecular and biochemical processes within the organism to align with a 24-hour solar day [1]. The molecular clocks driving these intrinsic rhythmic changes are based on interlocked transcription-translation feedback loops that integrate with different environmental and metabolic cues to generate an internal 24-hour timing [2]. The circadian outputs are triggered by the photic cue transmitted from the retina, which is processed in the suprachiasmatic nucleus (SCN) and initiates an oscillatory process [3]. The central oscillation generated by the SCN synchronizes various physiological processes in the brain and peripheral organs [4]. These processes can be regulated directly by neuronal and hormonal signaling [1]. The molecular clocks in the neurons of the SCN and throughout peripheral tissues share a similar architecture and can generate sustained circadian rhythms [5]. The core clock genes are the transcriptional activator; Brain and muscle arntl-like1 *(BMAL1)* and its binding partner; Circadian locomotor output cycles kaput (*CLOCK*), a histone acetyltransferase that drives expression of several genes, including the repressors Period (*Per1,2,3*) and Cryptochrome (*Cry1,2*). PER and CRY in a negative feedback loop act as potent repressors of *BMAL1: CLOCK*-dependent transcription [6,7]. This self-sustaining negative feedback loop allows the expression of clock genes to oscillate in a day-night cycle. This finely tuned system enables cells to adapt to the demands of their environment and maintain homeostasis [6,7].

Recent studies have shown that disruptions in the molecular clock system are associated with several diseases, including obesity, type 2 diabetes mellitus, several types of cancer, depression, and neurodegenerative diseases [8,9]. The expression of molecular clock genes and proteins (MCGs/MCPs) has been observed in the testes of rats, mice, and hamsters [10,11]. Studies in mice have also revealed rhythmic expression of these genes in other male reproductive tissues, such as the epididymis, vas deferens, seminal vesicles, and prostate gland [10,12]. More recently, there has been accumulation of evidence suggesting a relationship between disruptions in circadian rhythm and erectile dysfunction (ED) [13,14]. Rodriguez et al [15] investigated the relationship between shift work and shift work sleep disorder (SWSD) with ED in male shift workers. They discovered that males with SWSD had a greater incidence of ED, with a particularly significant association observed in those working night shifts. Lee et al [16] demonstrated that acute sleep deprivation in rats results in alterations in the penis, characterized by decreased expression of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), which leads to significant impairment in intracavernosal pressure and ED. However, there is a need to identify of these genes in human penile erectile tissue, and to investigate their potential role in relation to circadian rhythm disruption and ED development.

Therefore, the aim of this study was to evaluate the expression of core MCGs/MCPs in penile cavernous tissue from healthy male subjects and to determine whether there is a circadian variation in their expression with the goal of shedding light on the potential role of circadian rhythm disruption in the development of ED.

MATERIALS AND METHODS

1. Ethics statement

The present study protocol was reviewed and approved by the Institutional Review Board of İstanbul Prof. Dr. Cemil Taşcıoğlu City Hospital (approval. No. 207). Informed consent was confirmed by the IRB.

2. Patient selection

This multicenter prospective study was conducted at three teaching hospitals between July 2021 and August 2023. The study population comprised ten healthy males with congenital penile deviation or pe-

Table 1. Patient characteristics of the daytime and nighttime groups

	Day group[a]	Night group[b]	p-value
Age, y	27.80±4.82	30.40±3.96	0.402
IIEF-5 total score (22–25)	23.80±0.45	23.40±0.89	0.439
Testosterone (2.8–8.0), ng/mL	4.52±0.56	4.16±1.40	0.251
Sleep and Night Work Questionnaire Score (9–17)	13±2	12.80±1.92	0.75

Values are presented as mean±standard deviation.
IIEF: International Index of Erectile Function.
[a]Patients with penile deviation (corpus cavernosum tissue samples were obtained at daytime (ZT 8–12, n=5). [b]Patients with penile fracture (corpus cavernosum tissue samples were obtained at nighttime (ZT 20–24, n=5).

nile fracture, who underwent surgical intervention. Biopsy samples of corpus cavernosum (CC) tissue were obtained at two different zeitgeber times (ZT): daytime (ZT 8–12, n=5) and nighttime (ZT 20–24, n=5). Patients in the daytime group underwent surgery for penile deviation, whereas those in the nighttime group underwent surgery for penile fractures. The demographic data of the participants are presented in Table 1. A detailed history of the risk factors for ED was obtained from the patients, and a physical examination was performed before surgery. Informed consent was obtained by all subjects when they were enrolled. The validated version of the International Index of Erectile Function 5-question (IIEF-5) [17] and The Sleep and Night Work Questionnaire (SNWQ) forms [18] were completed, scores were recorded, and morning testosterone levels were measured. For a more in-depth analysis, five patients diagnosed with penile deviation were selected for additional evaluation using the real-time quantitative polymerase chain reaction (RT-qPCR) method, specifically conducted to assess the expression levels of the core MCGs within the CC tissue during daytime. The demographic data pertaining to these additional participants are presented separately in Table 2.

Table 2. Patient characteristics of the daytime group assayed by qRT-PCR

Parameter	Value
Age, y	28.60±11.78
IIEF-5	23.60±0.55
Testosterone (2.8–8.0), ng/mL	4.46±0.67
Sleep and Night Work Questionnaire	13.00±1.58

Values are presented as mean±standard deviation.
Patients with penile deviation (corpus cavernosum tissue samples were obtained at daytime (ZT 8–12, n=5).
IIEF: International Index of Erectile Function.

3. Exclusion criteria

The present study excluded participants who reported a history of ED, a symptom score of less than 22 on the IIEF-5, type 1 diabetes, uncontrolled hypertension, a history of atherosclerotic vascular disease, use of phosphodiesterase type-5 inhibitors within the past month, morning testosterone levels (2.8–8.0 ng/mL) below the reference range, and physical examination results demonstrating evidence of hypogonadism. The SNWQ form was used to assess sleep quality and exclude participants with scores >17 to minimize potential confounding effects of sleep disorders on the expression of MCGs/MCPs in the CC.

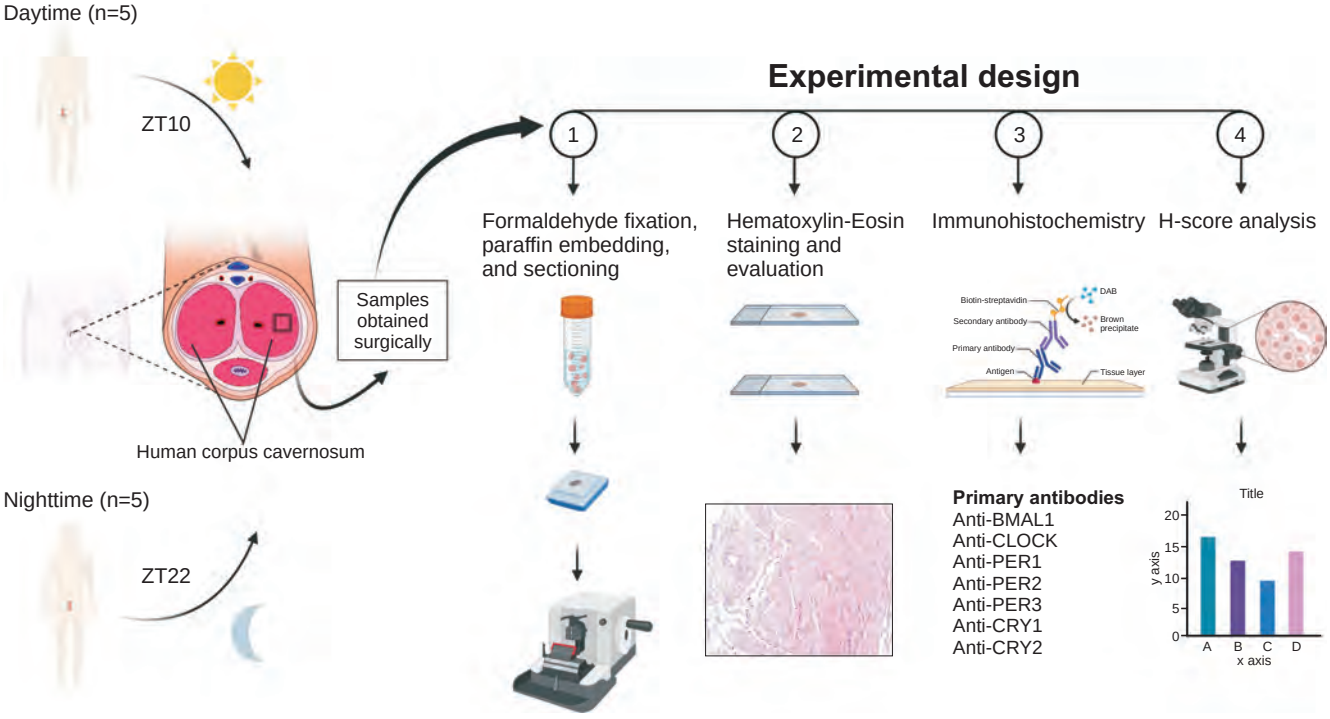


Fig. 1. Study design. This figure has been created utilizing BioRender Programme (BioRender Company). ZT: zeitgeber time.

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4. Sampling of CC tissue biopsy

The Nesbit technique has been used to repair congenital penile deviation. Subtunical CC tissue (0.5 mm) was excised from the excised tunica albuginea and tissue samples were collected in formalin. The surgical procedure for patients with penile fractures was performed according to standard protocols. After tissue debridement of traumatized cavernous tissue, a 0.5 mm sample of normal-appearing CC tissue was collected in formalin before the tear in the tunica was repaired. The experimental design is illustrated in (Fig. 1).

5. Paraffin embedding and tissue preparation

Human CC tissues were processed using the routine formalin-fixed paraffin-embedded tissue preparation protocol. The process involved immersion in formalin solution for 24 hours at room temperature, dehydration in ethanol, clearance in xylene, and embedding in paraffin. The resulting paraffin blocks were cut into 5-μm-thick serial sections and mounted on Superfrost Plus glass slides for subsequent analysis. H&E staining was used to evaluate the histological structures of human CC tissues under a bright-field microscope.

6. Immunohistochemistry (IHC) and H-score analysis

The sections were subjected to antigen retrieval using boric acid. To ensure the specificity of clock protein expression, positive controls were included using developing mouse placental tissue samples. The sections were then treated with blocking buffer (TA-125-PBQ; Thermo Fisher Scientific) and incubated with primary antibodies against BMAL1 (1:500, ab230822; Abcam), CLOCK (1:500, ab3517; Abcam), CRY1 (1:250, sc33177; Santa Cruz), CRY2 (1:250, SAB1300079; Sigma), PER1 (1:200, ab136451; Abcam), PER2 (1:250, ab227727; Abcam), PER3 (1:500, ab224594; Abcam), and the rabbit isotype control. After rinsing and washing, the sections were incubated with a secondary antibody (1:500, ba1000; Vector) and streptavidin complex (TS-125-HR; Thermo Fisher Scientific). IHC reactions were visualized using diaminobenzidine chromogen and Mayer's hematoxylin for nuclear counterstaining. Positive control results are illustrated in Supplement Fig. 1. Three sections from each sample were subjected to staining for each respective antibody. Expression was evaluated by H-score analysis and photographed under a light microscope [19]. The intensity of cytoplasmic and nuclear expression was scored for vascular endothelial cells (VECs) and smooth muscle cells (SMCs) of the CC in the day and night groups.

Table 3. Primer sequences of the *hGAPDH*, *hBMAL1*, *hCLOCK*, *hPER1*, *hPER2*, *hPER3*, *hCRY1*, and *hCRY2* genes used for quantitative real-time PCR

Gene	Primer (5′-3′)
hGAPDH	Forward: 5′-ATGGAAATCCCATCACCATCTT-3′ Reverse: 5′-CGCCCCACTTGATTTTGG-3′
hBMAL1	Forward: 5′-TGCCACCAATCCATACACAG-3′ Reverse: 5′-TTCCCTCGGTCACATCCTAC-3′
hCLOCK	Forward: 5′-GGCACCACCCATAATAGGGTA-3′ Reverse: 5′-TGTTGCCCCTTAGTCAGGAAC-3
hPER1	Forward: 5′-CTACATTTCGGAGCAGGCAG-3′ Reverse: 5′-GAGCTCAGAGAAGCGGGTA-3′
hPER2	Forward: 5′-GCGTGTTCCACAGTTTCACC-3′ Reverse: 5′-GGCTTTTCCGGACACTGACA-3′
hPER3	Forward: 5′-GCAGGTCTATGCCAGTGTGA-3′ Reverse: 5′-ACCACCACCATTCGGTTCT-3′
hCRY1	Forward: 5′-ACAGGTGGCGATTTTTGCTTC-3′ Reverse: 5′-TCCAAAGGGCTCAGAATCATACT-3′
hCRY2	Forward: 5′-CCTGGATGCAGATTTCAGCG-3′ Reverse: 5′-AGCAGTGGAAGAACTGCTGG-3′

7. Gene expression analysis

RNA isolation was conducted using the Quick-RNA MicroPrep Kit (Zymo Research), following the manufacturer's instructions. The concentration of RNA in each sample was determined by measuring the absorbance at 260 nm using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Subsequently, 200 ng of complementary DNA (cDNA) was synthesized using the M-MLV Reverse Transcriptase (Invitrogen). For the detection and comparison of mRNA expression levels, qRT-PCR was employed using the Light Cycler 480 SYBR Green I Master. Specific primer pairs for the target mRNAs were utilized in the qRT-PCR reactions, and their sequences have been presented in Table 3. To ensure reliable results, three independent technical replicates were performed for each target gene in the qRT-PCR assay.

8. Statistical analysis

Five distinct fields were evaluated within each sample, and the mean value of these five data points was subsequently utilized for statistical analysis. Data are presented as mean±SEM. The Mann–Whitney U test was used for non-parametric group comparisons, and the GraphPad Prism 9 software was used for statisti-

cal analysis. Statistical significance was set at $p<0.05$.

RESULTS

1. Population characteristics

No statistically significant differences were observed between the daytime and nighttime groups used for the evaluation of MCPs, concerning age, blood testosterone levels, IEF-5, or total SNWQ scores (p=0.402, 0.251, 0.439, and 0.75, respectively), as delineated in Table 1. Additionally, the demographic data of the participants in the daytime penile deviation group, who underwent RT-qPCR analysis, are presented in Table 2.

2. Histological analysis

H&E staining of the human penile biopsy specimens revealed cavernous tissue composed of VECs and SMCs (Fig. 2).

3. Expression of BMAL1 AND CLOCK in VECs and SMCs in human CC tissue

In the daytime group, BMAL1 was expressed in the cytoplasm and nucleus of VECs, whereas in the nighttime group, it was expressed in the nucleus of VECs (Fig. 3A). In both groups, CLOCK expression was cytoplasmic and nuclear in VECs (Fig. 3B). BMAL1 nuclear and cytoplasmic expression and cytoplasmic expression of CLOCK decreased significantly (p=0.008 and 0.03, respectively) in VECs at night (Fig. 3C, 3D).

In the daytime group, BMAL1 expression was cytoplasmic and nuclear in SMCs (Fig. 3A). In both groups, cytoplasmic and nuclear CLOCK expression was observed in SMCs (Fig. 3B). The cytoplasmic expression of BMAL1 decreased significantly in SMCs at night (p=0.02) (Fig. 3E, 3F). The specific localization patterns of BMAL1 and CLOCK protein expression in VECs and SMCs are shown in (Fig. 3G).

4. Expression of PER1, PER2, and PER3 in VECs and SMCs in human CC tissue

In the daytime and nighttime groups, PER1 was expressed in the cytoplasm and nucleus of VECs (Fig. 4A). In the daytime group, the expression of PER2 in VECs was cytoplasmic (Fig. 4B). In the daytime group, PER3 expression was low in the nucleus of VECs, whereas in both groups, it was expressed in the cytoplasm of VECs (Fig. 4C). In VECs of human CC tissue collected during both daytime and nighttime, there were no statistically

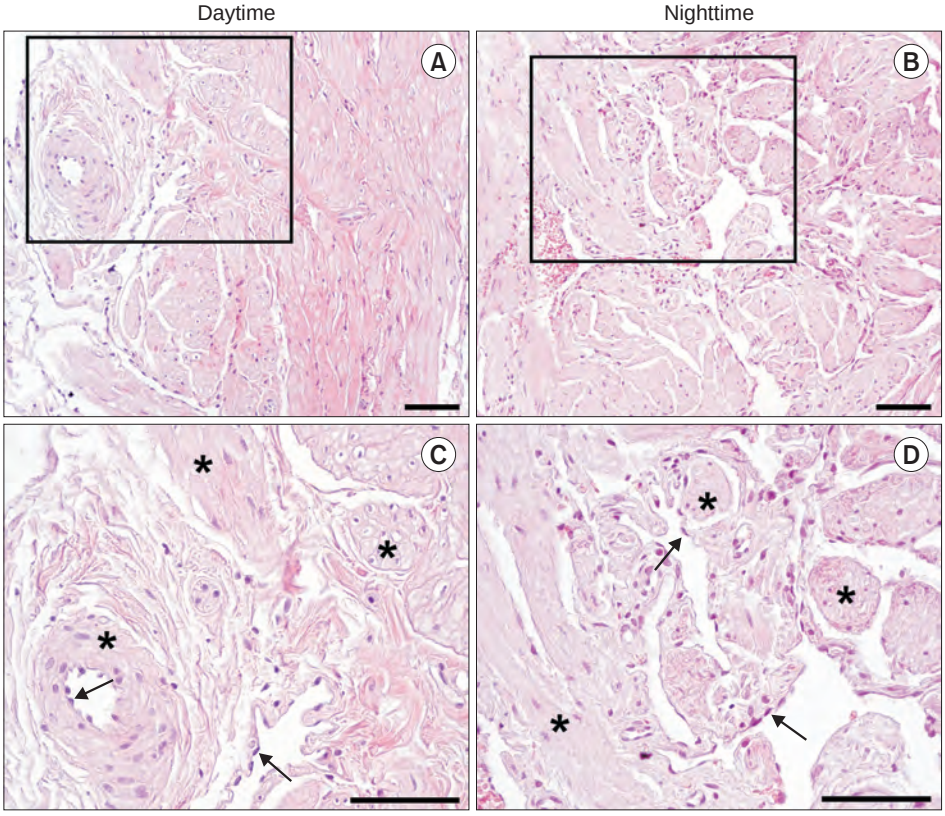
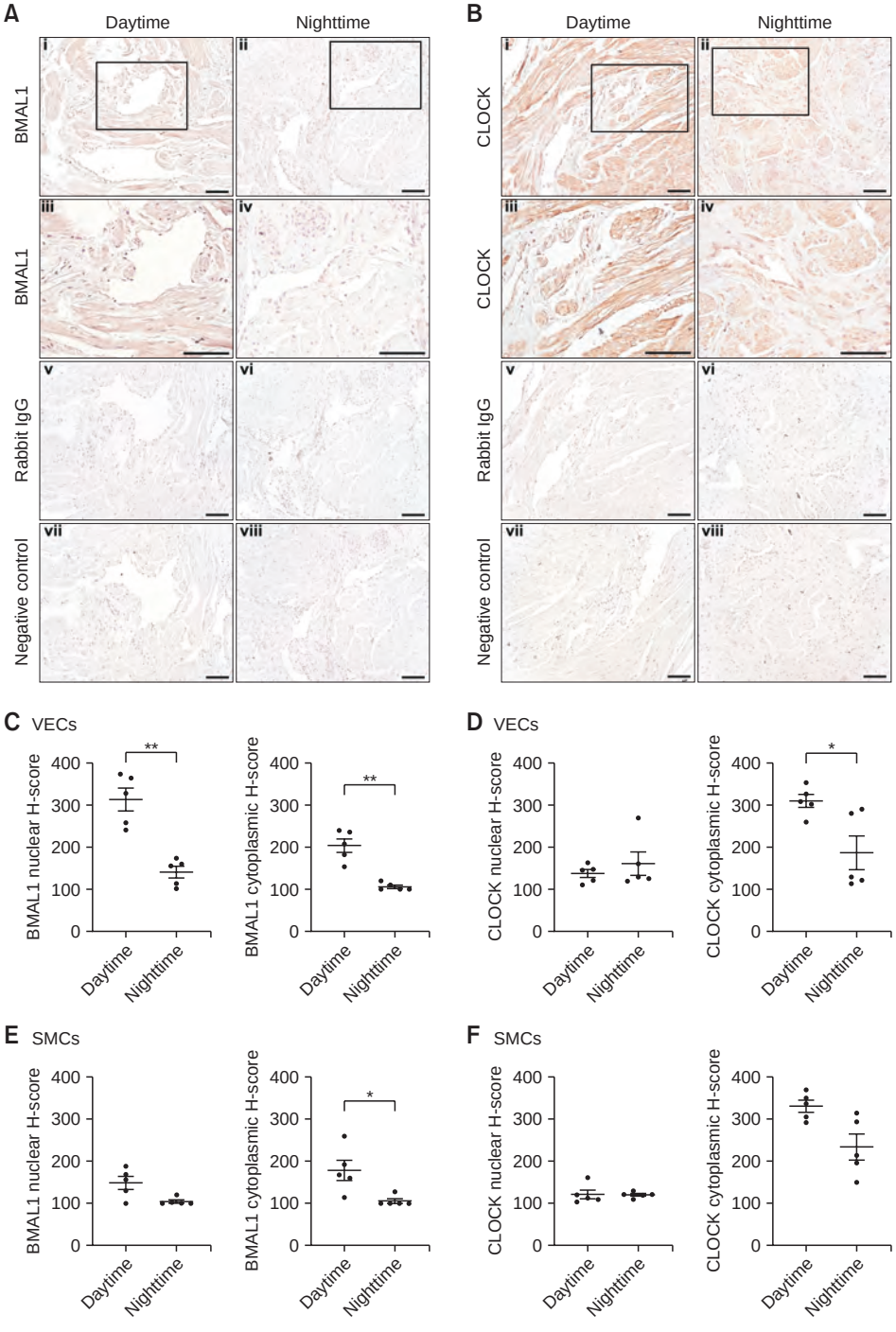


Fig. 2. H&E staining of the human corpus cavernosum (CC) tissue. (A) The CC in human penile tissue was obtained at zeitgeber time (ZT) 10. (B) The CC in human penile tissue was obtained at ZT 22. (C, D) Higher magnification of designated area with the square in A and B, respectively. Arrows indicate vascular endothelial cells; Asterisks indicate smooth muscle cells. Scala bars: 50 μm.

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	VECs				SMCs			
BMAL1	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	++	±	+	±	±	±	+	±

	VECs				SMCs			
CLOCK	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	±	++	+	±	±	+++	+

Fig. 3. Immunohistochemical (IHC) analysis of BMAL1 and CLOCK protein expression in VECs and SMCs of the corpus cavernosum (CC) in human penile tissue obtained at daytime and nighttime. (A, B) BMAL1 and CLOCK IHC for the daytime and nighttime groups, respectively. (i) The CC of human penile tissue obtained in the daytime. (ii) The CC of human penile tissue obtained nighttime. (iii, iv) Higher magnification of designated area with the square in (i) and (ii), respectively. (v, vi) Negative control section where rabbit IgG was as the isotype control. (vii, viii) Negative control section where the primary antibody was omitted. (C–F) H-score results for VECs and SMCs. (G) The distribution of VECs and SMCs of BMAL1 and CLOCK protein expression. Scale bars: 50 μm. VEC: vascular endothelial cell, SMC: smooth muscle cell. Asterisks indicate significant differences (*p<0.05 and **p<0.01).

significant differences observed in the expressions of the PER1, PER2, and PER3 proteins (Fig. 4D–4F).

PER1 was expressed in the cytoplasm and nucleus of SMCs in both groups (Fig. 4A). In both groups, PER2 expression was observed only in the cytoplasm of SMCs (Fig. 4B). In the daytime group, the expression of PER3 was present only in the cytoplasm of SMCs, whereas in the nighttime group, its expression was absent (Fig.

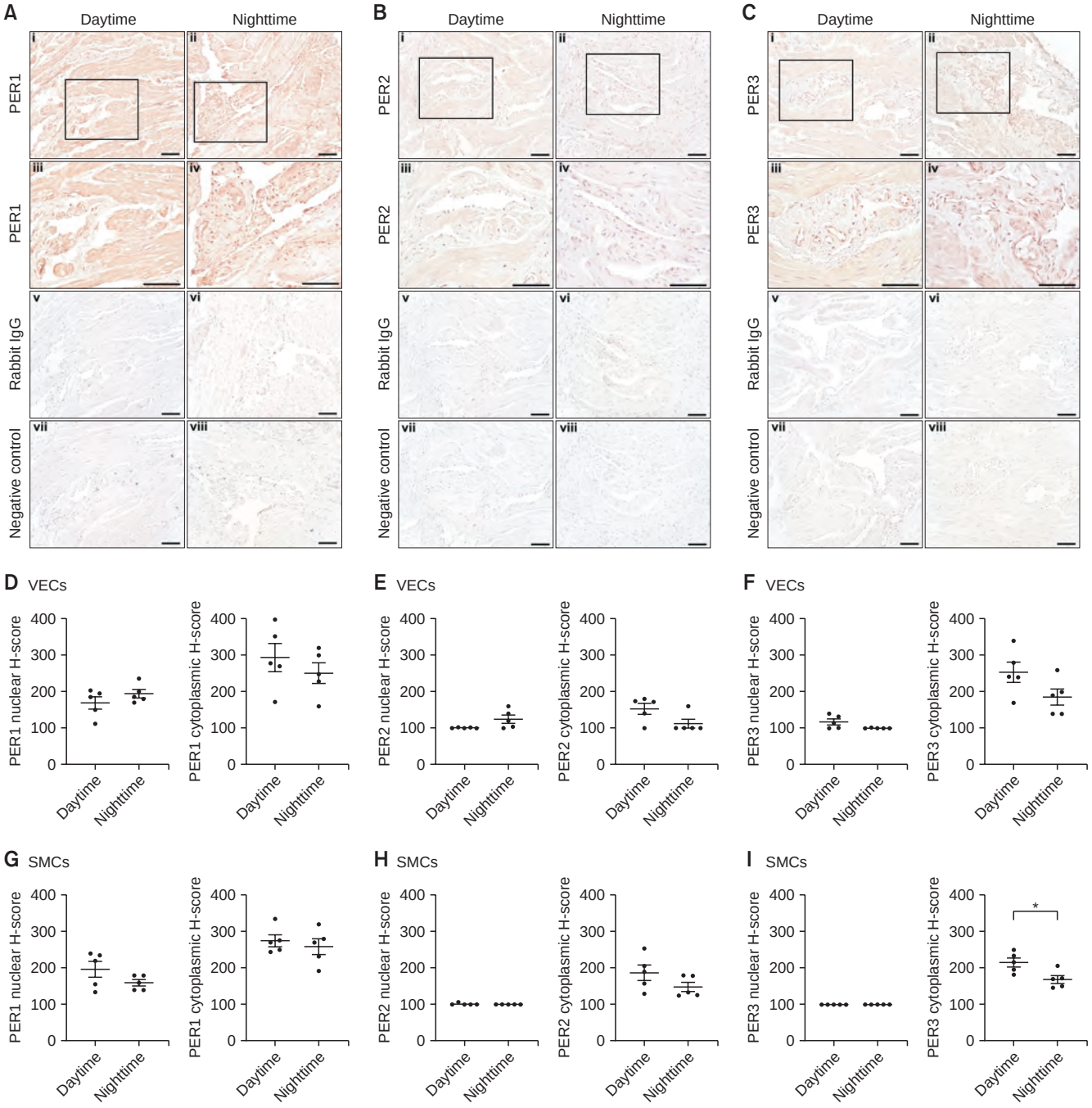


Fig. 4. Immunohistochemical (IHC) analysis of PER1, PER2, and PER3 protein expression in VECs and SMCs of the corpus cavernosum (CC) of human penile tissue obtained at daytime and nighttime. (A–C) PER1, PER2, and PER3 IHC for the daytime and nighttime groups, respectively. (i) The CC of human penile tissue obtained in the daytime. (ii) The CC of human penile tissue obtained nighttime. (iii, iv) Higher magnification of designated area with the square in (i) and (ii), respectively. (v, vi) Negative control section where rabbit IgG was as the isotype control. (vii, viii) Negative control section where the primary antibody was omitted. (D–I) H-score results for VECs and SMCs. (J) The distribution of VECs and SMCs of PER1, PER2, and PER3 protein expression. Scale bars: 50 μm. VEC: vascular endothelial cell, SMC: smooth muscle cell. Asterisks indicate significant differences (*p<0.05).

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PER1	VECs				SMCs			
	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	+	++	++	+	±	++	++

PER2	VECs				SMCs			
	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	±	±	±	±	±	±	±

PER3	VECs				SMCs			
	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	±	++	+	±	±	±	±

Fig. 4. Continued.

4C). In the SMCs of the nighttime group, there was a significant decrease in the cytoplasmic expression of PER3 (p=0.03) (Fig. 4G–4I).

The specific localization patterns of PER1, PER2, and PER3 protein expression in VECs and SMCs are shown in (Fig. 4J).

5. Expression of CRY1 and CRY2 in VECs and SMCs in human CC tissue

In the daytime group, CRY1 was weakly expressed in the cytoplasm and nucleus of VECs, whereas it was not expressed in the nighttime group (Fig. 5A). CRY2 expression was observed in the cytoplasm and nucleus of VECs in both the groups (Fig. 5B). In the VECs of the nighttime group, the nuclear expression of CRY1 decreased significantly (p=0.02) (Fig. 5C, 5D).

In both groups, CRY1 was slightly expressed in the cytoplasm of SMCs (Fig. 5A) and CRY2 was expressed in the cytoplasm and nucleus of SMCs (Fig. 5B). There was a significant decrease in the cytoplasmic expression of CRY2 in the SMCs of the nighttime group (p=0.03) (Fig. 5E, 5F). The specific localization patterns of CRY1 and CRY2 protein expression in VECs and SMCs are shown in (Fig. 5G).

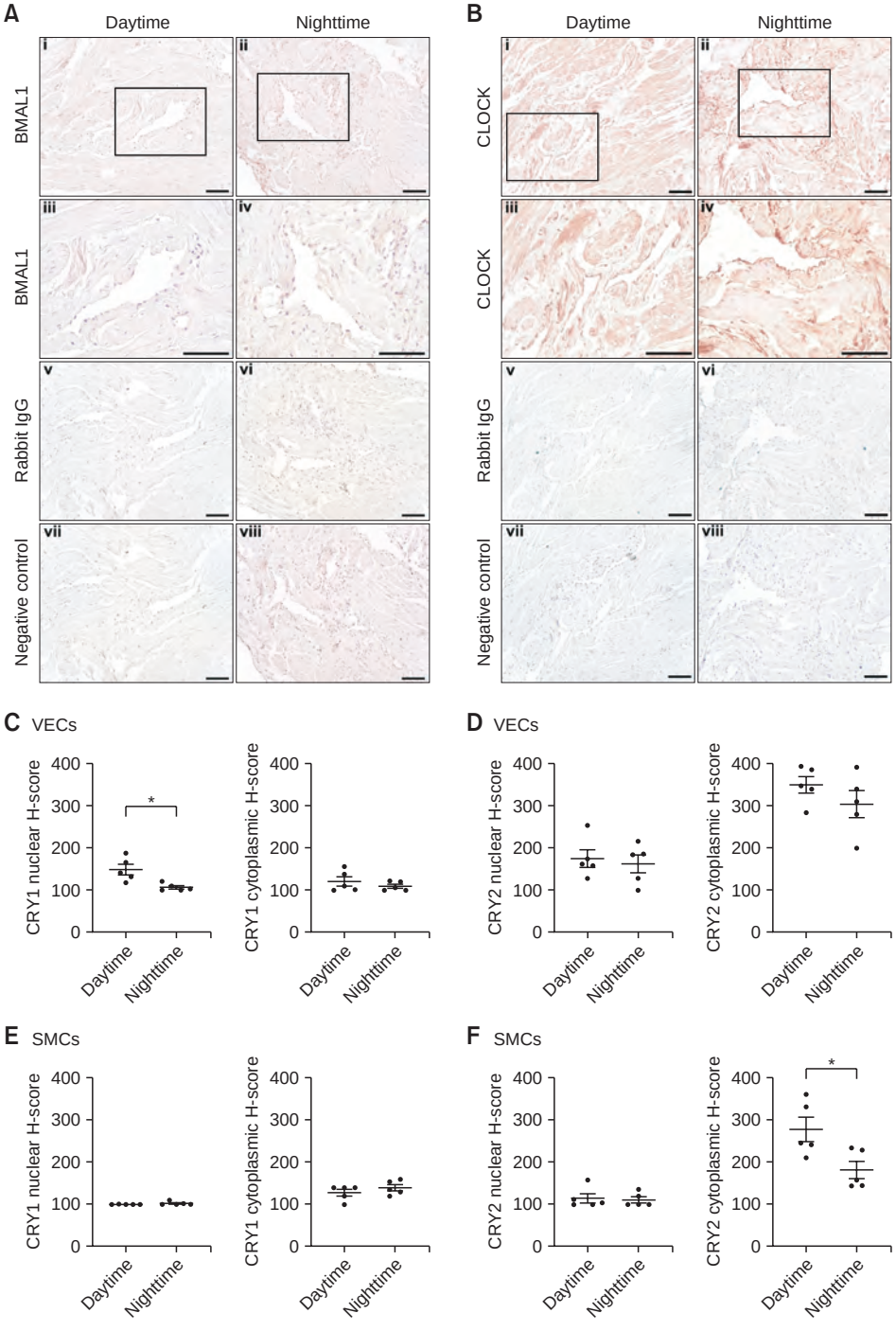
6. RT-qPCR analysis of CC in the daytime penile deviation group

The RT-qPCR analysis validated the expression of the core MCGs (BMAL1, CLOCK, PER1, PER2, PER3, CRY1, and CRY2), previously observed at the protein level through IHC. The findings are depicted in Fig. 6. RT-qPCR electrophoresis results are presented in Supplement Fig. 2.

DISCUSSION

This study presents novel findings regarding the expression of the MCPs in the VECs and SMCs of human CC, with BMAL1 exhibiting the highest expression level. This discovery sheds new light on the molecular architecture of CC (as depicted in Fig. 7A) and highlights the presence of MCPs in penile cavernous tissue, specifically in VECs and SMCs, which are directly involved in penile erection. Notably, we observed a significant decrease in the expression of core MCPs, including BMAL1, a key component of the molecular clock mechanism, at nighttime in both VECs and SMCs of the CC. To further validate the presence of MCGs and our results, we performed RT-qPCR on samples from five daytime penile deviation patients that confirmed the expression of the core MCGs observed at the protein level through IHC. The RT-qPCR data provided quantitative evidence supporting our initial findings, affirming the presence of MCGs in human penile cavernous tissue. Regrettably, due to the rarity of nighttime penile fracture cases, we couldn't collect tissue samples from these patients, hindering the demonstration of MCGs rhythmicity in human cavernous tissue at gene level.

The aim of our qRT-PCR assay was to validate IHC results for MCGs. Sampling at ZT10 suggested a low BMAL1 transcription, which might be consistent with its earlier peak activity, and could explain the observed discrepancy between BMAL1 protein and gene expressions. Meanwhile, Per and Cry transcription appeared to be relatively high, possibly reflecting BMAL1/CLOCK complex activation. However, these preliminary observations, based on a single time point, under-



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	VECs				SMCs			
CRY1	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	±	±	±	±	±	±	±

	VECs				SMCs			
CRY2	Nuclear		Cytoplasmic		Nuclear		Cytoplasmic	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
	±	±	+++	++	±	±	++	+

Fig. 5. Immunohistochemical (IHC) analysis of CRY1 and CRY2 protein expression in VECs and SMCs of the corpus cavernosum (CC) in human penile tissue obtained at daytime and nighttime. (A, B) CRY1 and CRY2 IHC for the daytime and nighttime groups, respectively. (i) The CC of human penile tissue obtained in the daytime. (ii) The CC of human penile tissue obtained nighttime. (iii, iv) Higher magnification of designated area with the square in (i) and (ii), respectively. (v, vi) Negative control section where rabbit IgG was as the isotype control. (vii, viii) Negative control section where the primary antibody was omitted. (C–F) H-score results for VECs and SMCs. (G) The distribution of VECs and SMCs of CRY1 and CRY2 protein expression. Scale bars: 50 μm. VEC: vascular endothelial cell, SMC: smooth muscle cell. Asterisks indicate significant differences (*p<0.05).

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score the need for advanced techniques and continuous monitoring, such as tissue culture, to fully unravel the complex temporal relationships, rapid changes, and degradation patterns in these clock genes.

BMAL1 is a critical component of the molecular clock mechanism, responsible for regulating circadian rhythms in various tissues and organs, including the reproductive system [20]. Perturbations to the circadian clock, such as alterations in BMAL1 expression, have been associated with male infertility [20]. Studies conducted on *Bmal1* gene knockout mice have demonstrated that they are infertile, presenting with impairments in reproductive organs and gametes [20]. Endothelial dysfunction is widely considered to be a vascular disorder and, in the CC, it is associated with reduced nitric oxide (NO) production and activity, which can contribute to the development of ED [21,22]. In mice, disruption of *Bmal1* or *Clock* genes leads to decreased NO signaling, resulting in endothelial dysfunction and vascular injury [23,24]. Furthermore, studies with cultured aortic rings show that *Clock* mutation, *Per1* mutation, or *Bmal1* deletion leads to reduced endothelium-dependent vascular relaxation, suggesting a potential role for the clock genes in regulating vascular function [24,25]. *Bmal1* deficient mice show reduced eNOS activation, which leads to decrease NO production and increased superoxide levels in the endothelium [24,25]. Conversely, *Per2* mutant mice exhibit elevated COX

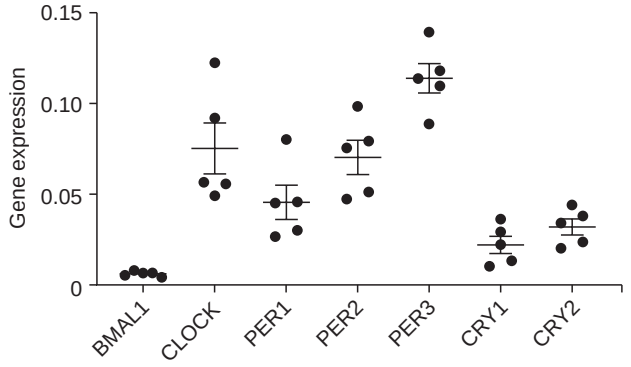


Fig. 6. Validation of molecular clock genes in human penile cavernous tissue via RT-qPCR. The relative expression levels of core molecular clock genes (BMAL1, CLOCK, PER1, PER2, PER3, CRY1, and CRY2) were assessed using RT-qPCR in penile tissue samples. Tissue samples were obtained from five distinct male participants during daylight hours. For each participant, measurements were conducted in triplicate. Error bars represent the standard error of the mean.

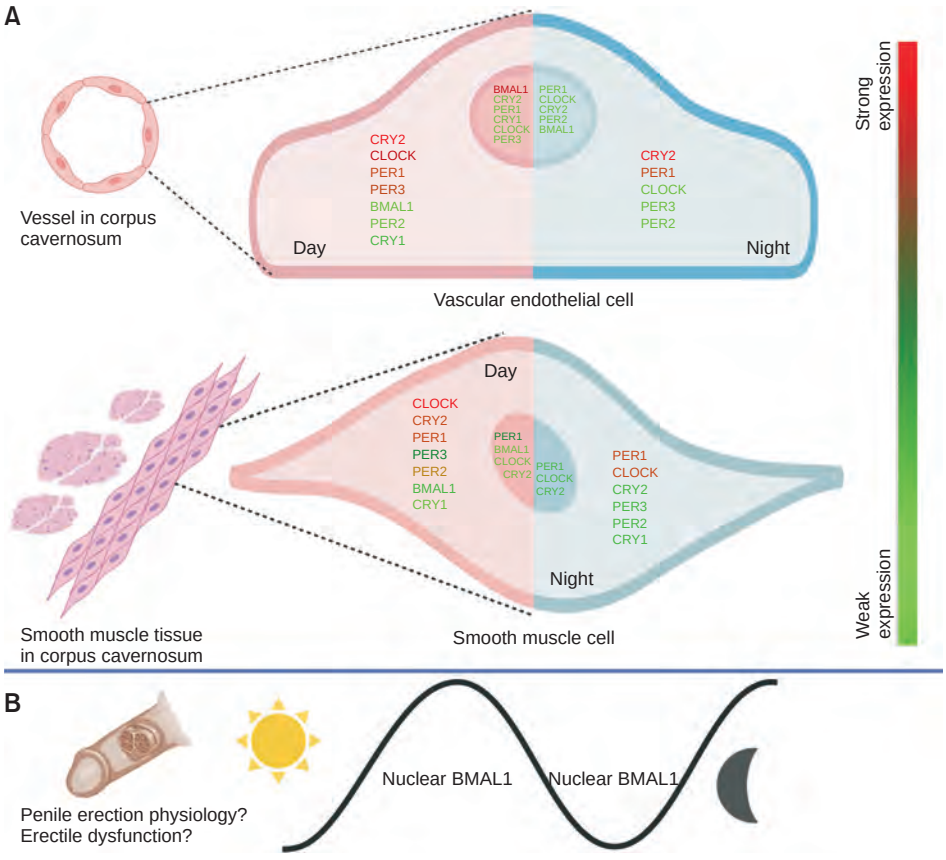


Fig. 7. (A) Summary figure schematizing that cytoplasmic and nuclear expression of core MCPs in vascular endothelial cells and smooth muscle cells in human penile tissue corpus cavernosum differs during daytime and nighttime. (B) Possible mechanism schematizing the contribution of circadian expression of BMAL1 to the mechanism of erection. This figure has been created utilizing BioRender Programme (BioRender Company).

expression, which enhances vasoconstriction of vascular endothelium [26]. Altogether, these data point to the impact of circadian rhythm disruption on endothelial cell function, leading to impaired vasodilation and increased oxidative stress.

VECs and SMCs play crucial roles in penile erection regulation. VECs regulate blood flow into the CC, whereas SMCs control the CC tone. Despite normal NO release, ED may arise from alterations in the abundance, composition, or regulation of SMCs [27]. Deletion of *Bmal1* in SMCs has been shown to negatively impact vessel contractility, leading to an increase in arterial lumen diameter and a reduction in mean arterial pressure [28]. SMCs from disease-free arteries exhibit prominent peaks and troughs in the expression of molecular clock genes, whereas the expression patterns of SMCs derived from lesioned areas of the artery are blunted and flat [29].

The vascular studies discussed in the literature provide additional evidence for the potential role of the molecular clock mechanism in regulating vascular function. Disruptions in circadian rhythms, including changes in the expression of MCPs, have been associated with endothelial dysfunction and impaired vasodilation in other vascular systems, which are also critical components of the penile erection mechanism. Our findings of a MCPs expression in penile cavernous tissue, specifically in VECs and SMCs, suggest a potential link between circadian rhythm disturbances and endothelial dysfunction in the mechanism of human penile erection. A possible mechanism of circadian rhythm-regulated penile erectile physiology and pathology by BMAL1 is presented in Fig. 7B.

Our study encountered limitations in obtaining multiple human penile CC tissue samples at different ZT times from the same individuals due to the invasive nature of tissue collection and ethical concerns. Additionally, it is worth noting that we could not completely demonstrate the rhythmicity of these genes due to the limited ZT times and the lack of RT-qPCR results from the nighttime group. Future studies may benefit from alternative methodologies, such as the P(Per2)-dLuc reporter, which enables real-time monitoring of gene expressions in cultured cells of penile VECs or SMCs, offering a more comprehensive understanding of the circadian regulation of core MCGs in human penile cavernous tissue [30].

Our study's discovery of MCPs in penile cavernous tissue, specifically in VECs and SMCs, has significant clinical implications for understanding the molecular mechanisms of penile erection and its relationship with ED. Our findings concerning the expression of MCPs in penile cavernous tissue necessitate further in-depth investigations into specific molecular pathways. This includes exploring the relationship between MCGs and eNOS-mediated NO production in the penile CC to demonstrate related pathways of MCGs in penile erection physiology. These studies are crucial for understanding the impact of the clock mechanism on penile erection regulation.

CONCLUSIONS

For the first time, we have demonstrated the expression of MCPs, specifically BMAL1, CLOCK, PER1, PER2, PER3, CRY1, and CRY2, in VECs and SMCs of the CC in humans. BMAL1 exhibits the highest expression at the protein level. Moreover, RT-qPCR analysis confirmed the expression of core MCGs. This novel finding highlights the presence of MCPs in penile cavernous tissue, particularly in VECs and SMCs, which are directly involved in penile erection, thus emphasizing the potential role of the molecular clock mechanism in penile erection physiology.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: IA, CCO. Data curation: BD, MU, MB. Formal analysis: BD, MU, MB. Funding acquisition: IA, CCO,

HLC. Investigation: CCO, BD, MU. Methodology: IA, CCO, HLC. Project administration: IA, CCO. Resources: HLC, MB, IA, CCO. Software: BD, MU, MB. Supervision: CCO, IA. Validation: CCO, BD, MU. Visualization: BD, MU, CCO, MB. Writing – original draft: IA, CCO. Writing – review & editing: all authors.

Supplementary Materials

Supplementary materials can be found via https://doi.org/10.5534/wjmh.230136.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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