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2,2,2-Trichloroethanol lengthens the circadian period of *Bmal1*-driven circadian bioluminescence rhythms in U2OS cells

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ABSTRACT

2,2,2-Trichloroethanol (TCOH) is responsible for the pharmacological actions of chloral hydrate (CH), and is a major metabolite of trichloroethylene. Human exposure to TCOH is known to be increasing. Recently, it was reported that TCOH causes a significant phase delay of *Per2* expression in mouse liver when injected daily over the course of several days. However, it is not clear whether TCOH directly modulates the molecular clock. In the present study we used a cell-based assay system to test this possibility. We found that the daily oscillation period of *Bmal1* was lengthened to 3 h following treatment with 1.5 mM TCOH, and increased to 5 h with 3 mM TCOH treatment. However, low concentrations of TCOH had no noticeable effects. The effect of TCOH on *Per2* oscillation was marginal. Interestingly, serum from rats anesthetized with CH also modulated *Bmal1* period, suggesting that exposure to anesthesia should be taken into consideration for circadian rhythm studies. In summary, our study reveals a direct regulation of TCOH on molecular clock.

1. Introduction

2,2,2-Trichloroethanol (TCOH) is believed to be responsible for the pharmacological actions of chloral hydrate (CH), which is used clinically as a sedative in pediatric patients, and as an anesthetic in laboratory animals. Once administered, CH is rapidly metabolized to TCOH within minutes [1]. TCOH is also a major metabolite of trichloroethylene (TCE), which is used as a metal degreaser and dry cleaning agent in many industrial processes [2]. The commercial uses of TCE are so widespread that it has become a common environmental pollutant found in air emissions and in discharge into water and soil [3]. Therefore, the possibility for human exposure to TCOH is increasing.

Recently, it was reported that tribromoethanol, a compound with a structure similar to TCOH, can regulate the expression of a variety of clock genes, such as *Per2* expression in the liver. TCOH was reported to cause a significant phase delay of *Per2* expression in mouse liver when injected intraperitoneally daily over the course of 4 days [4]. These findings suggest that TCOH might be a regulator of molecular clock. However, it is not clear whether the regulation is direct or indirect.

The molecular clock machinery consists of autoregulatory transcriptional and translational feedback loops that have both positive and negative elements. BMAL1 and CLOCK are transcription factors that form a heterodimer and bind to the E-box enhancer and activate transcription of the *Per* and *Cry* genes. The resultant proteins PER and CRY then repress transcription through a negative feedback mechanism by interacting with the BMAL1-CLOCK complex. REV-ERB orphan nuclear receptors, retinoid-related orphan receptor, and BMAL1 form an additional feedback loop to make the molecular clock function more precisely [5]. Since the molecular clock machinery resides at the cellular level, cell-based assay systems have been developed and have become popular, especially for screening circadian clock modulators [6,7]. In general, U2OS or fibroblast cell lines stably expressing the *Bmal1-dluc* or *Per2-dluc* reporter gene are treated with potential clock modulators, and the reporter activity is examined in an automated system. These cell-based assay systems have enabled the determination of direct effects of chemicals on the circadian clock, and cell-based systems overcome the problem of *in vivo* lethality.

In the present study, we used a cell-based assay system to determine if TCOH directly modulates the molecular clock, and to what extent TCOH regulates circadian period.

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2. Materials and methods

2.1. Animals, anesthesia, and serum preparation

Male F344 rats (2–3 months old) were individually housed in a controlled environment at a constant temperature of 23 °C and humidity of 50% with food and water available *ad libitum*. The animal room was on a 12/12 h light/dark cycle (lights on at 07:00; lights off at 19:00). Rats were allowed 6 days of habituation to the animal colony. At 12:00 of day 7, five rats were anesthetized by intraperitoneal injections of CH (Sigma, St Louis, MO, USA) in phosphate-buffered saline at a dose of 400–500 mg kg^{-1}. The other five rats were housed and anesthetized in a plastic box supplied with CO_2 (>70%) for 1 min. All the rats were sacrificed immediately; blood samples were collected, incubated 30 min at room temperature, and then centrifuged 10 min at 2000 × *g*. Serum was harvested and stored at −20 °C until used. Care of the rats was in accordance with the Institutional Animal Care and Use Committee guidelines at the Capital Medical University.

2.2. Cell culture

U2OS wild type cells and U2OS cells stably expressing *Bmal1-dluc* and *Per2-dluc* were generous gifts from Prof. E.E. Zhang [8]. All U2OS cells were grown in regular Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics, and 1% GlutaMAX (Life Technologies, CA, USA). Prior to commencing the real-time reporter assay cells were synchronized with high serum medium (DMEM supplemented with 50% FBS, 1% antibiotics, 1% GlutaMAX, 45 mM $NaHCO_3$) for 2 h. During the real-time reporter assay, cells were grown in counting medium (DMEM supplemented with 10% FBS, 1% antibiotics, 1% GlutaMAX, 1% luciferin, 4.5 mM $NaHCO_3$, 10 mM HEPES (pH 7.2)). Various concentrations of TCOH (Sigma) were added into counting medium as indicated. To examine whether rats anesthetized with CH and CO_2 have altered serum factors regulating circadian clock, serum from the anesthetized animals was used to replace FBS in counting medium. To study the short-term effects of TCOH, cells were first synchronized with high serum medium and real-time reporter assays were conducted for 2 days, then the indicated concentrations of TCOH were added into the counting medium. Two hours later, counting medium was washed out and replaced with counting medium without TCOH, and real-time reporter assays were conducted for another 5 days.

2.3. Cell viability

For cell counting, Kit-8 (DoJindo, Shanghai, China) was used to measure the cytotoxic effects of TCOH on U2OS cells. Briefly, 0.5×10^4 cells were seeded into each well of 96-well plates. Twenty-four hours later, cells were synchronized and then incubated with counting medium containing the indicated concentrations of TCOH for 5 days. To examine cell viability, 20 μl CCK-8 was added into each well and incubated at 37 °C for 2 h. The plate was read on a microplate spectrophotometer (Thermo, NY, USA), using a detecting wavelength of 450 nm and a reference wavelength of 650 nm. Results are presented as percent viability relative to the control values.

2.4. Real-time reporter gene assays

Real-time reporter gene assays were conducted as previously described [9]. Stable reporter cells were synchronized with high serum medium for 2 h and then incubated with counting medium. Bioluminescence was measured in LumiCycle (Actimetrics, IL, USA) for 5–7 days. To obtain circadian period length, we used the LumiCycle Analysis program to analyze bioluminescence data. Briefly, raw data were initially fitted to baseline, and baseline-subtracted data were fitted to a sine wave from which the parameters were determined. Due to high transient bioluminescence upon medium change, the first cycle of data was excluded from analysis.

2.5. Impact of TCOH on the transcription of clock genes in U2OS

U2OS cells (5×10^5) were seeded into each 35 mm dish, 24 h later the indicated concentrations of TCOH were added into the medium, and then the cells were harvested at the indicated time-points. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), according to the user manual, and then was reverse-transcribed using PrimeScript RT Master Mix (Takara, Dalian, China). cDNA was then amplified on a Lightcycler 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) using the SYBR Premix Ex Taq II (Takara) to quantify the amount of *Bmal1*, *Per1*, *Per2* and *Rev-Erbα*. Each sample was analyzed in triplicate to ensure accuracy. *Gapdh*, a housekeeping gene, was used to normalize the differences in RNA sample content. Primer sequences for polymerase chain reaction (PCR) amplification are as follows: *Bmal1* forward, CTCCAGCCCATTGAACATC; *Bmal1* reverse, GCCTCATCATTACTGGGACT; *Per1* forward, TTCCTGACGGGCCGAAT; *Per1* reverse, CGCTTGCAACGCAGCA; *Per2* forward, CGTGCCAAGCAGTTGACTTA; *Per2* reverse, CAGCAAGGCTCAACAAATCA; *Rev-Erbα* forward, CTGGGAGGATTTCTCCATGA; *Rev-Erbα* reverse, TCACTGTCTGGTCCTTCACG; *Gapdh* forward, GTCAGTGGTGGACCTGACCT; *Gapdh* reverse, TGCTGTAGCCAAATTCGTTG.

2.6. Statistical analysis

Experiments were conducted in triplicate and repeated three times. Data are expressed as mean ± standard deviation (SD). Comparisons of clock gene expression levels and period length between two study groups were performed by independent sample two-tailed t-tests. Any difference was considered statistically significant if $p \leq 0.05$.

3. Results

To examine whether TCOH directly affects the molecular clock, U2OS cells stably expressing the *Bmal1-dluc* reporter were treated with different concentrations of TCOH. Results showed that the low concentrations of TCOH (≤0.6 mM) did not alter *Bmal1* oscillation. However, treatment with high concentrations (≥1.5 mM) TCOH significantly lengthened the period of *Bmal1* transcriptional oscillation. The period for control cells (without TCOH) was 23.6 ± 0.1 h, whereas that for cells treated with 1.5 mM and 3 mM TCOH was 26.8 ± 1.0 and 28.8 ± 0.2 h, respectively (Fig. 1A and B). To determine the cytotoxicity of TCOH, cells were treated with various concentrations of TCOH for 5 days, and cell viabilities were examined using the CCK-8 method. A dose-dependent increase in cytotoxicity was observed. Significant inhibition of cell growth was evident, even with the 0.3 mM concentration of TCOH. Relative cell viability was 64% and 42% at 1.5 mM and 3 mM TCOH, respectively (Fig. 1C).

To determine whether short-term treatment of TCOH could lengthen the *Bmal1* oscillation period, U2OS cells stably expressing the *Bmal1-dluc* reporter were synchronized with high serum medium, then the cells were grown in regular counting medium for 2 days. Subsequently, cells were treated with various concentrations of TCOH for 2 h and then the counting medium containing TCOH was washed out and replaced with counting medium without

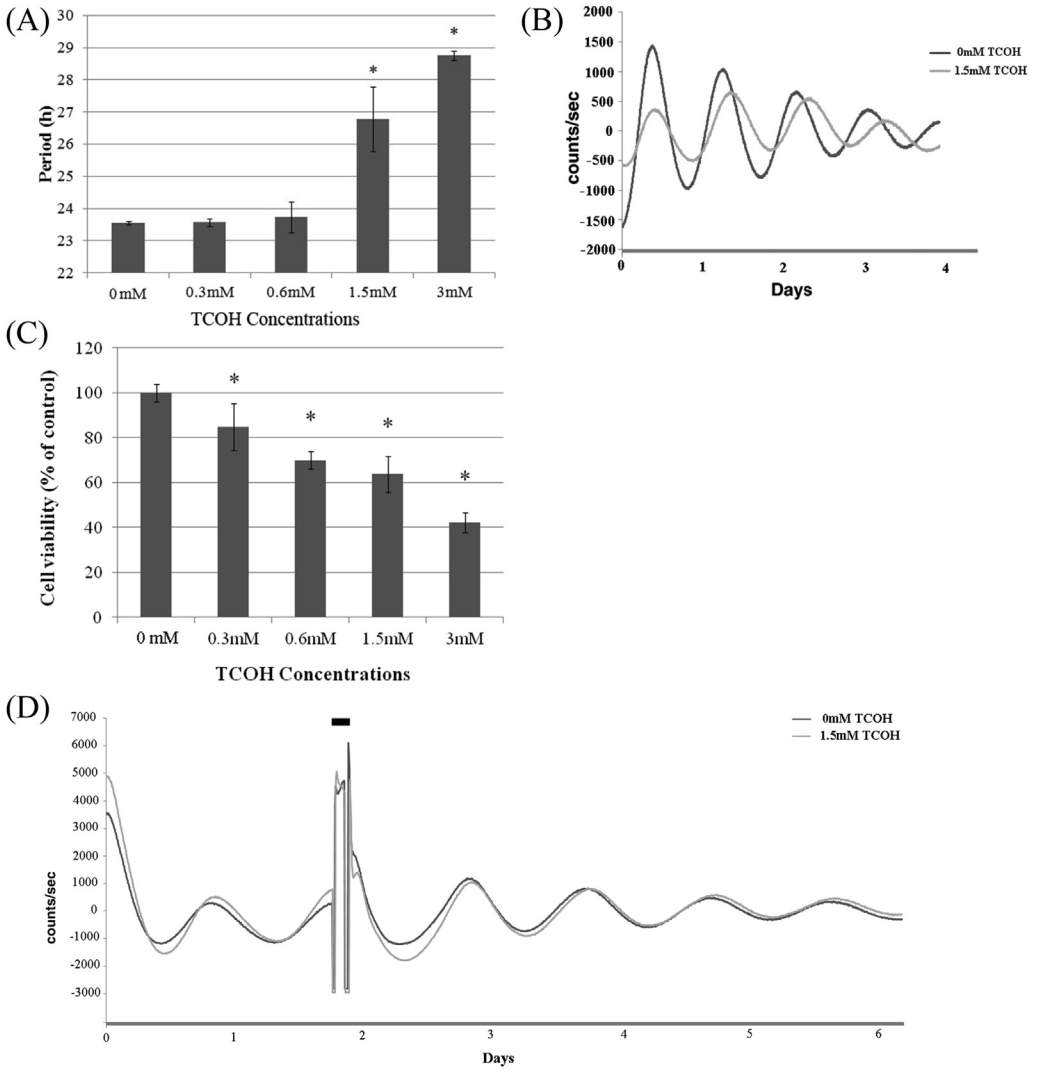

Fig. 1. TCOH lengthens the *Bmal1* oscillation period. (A) Circadian period of *Bmal1* in response to TCOH treatment. Indicated concentrations of TCOH were added into counting medium and bioluminescence was examined using LumiCycle. Circadian period was calculated from fit curve data. Values are mean ± SEM of triplicate assays. * indicates $p < 0.05$. (B) Bioluminescence from cells treated with (grey) or without (black) TCOH. Results are representative of triplicate experiments. (C) Cytotoxic effects of TCOH on U2OS cells. U2OS cells were treated with different concentrations of TCOH for 5 days and cytotoxicity was determined by CCK-8. U2OS cells without treatment served as controls. Values are expressed as mean ± SEM of triplicate assays. * indicates $p < 0.05$. (D) Transient TCOH treatment failed to lengthen *Bmal1* oscillation period. U2OS cells were synchronized with high serum for 2 h, and bioluminescence was measured for 2 days, then TCOH was added into the medium and incubated for 2 h. Cells were washed and the culture medium was replaced with counting medium without TCOH. Black horizontal bar on the top indicates the 2 h period for TCOH treatment. Grey and black lines represent 1.5 mM TCOH treatment or no treatment, respectively. Fit curve data are representative of triplicate experiments.

TCOH. Cells were grown in counting medium without TCOH for another 5 days, and the real-time reporter assays were carried out. We found that *Bmal1* period was equivalent among cells with and without short-term TCOH treatment, indicating that short-term treatment is not sufficient to lengthen the circadian period (Fig. 1D).

We next investigated whether TCOH could modulate the transcriptional oscillation of *Per2*, another key clock gene. U2OS cells stably expressing the *Per2-dluc* reporter were also treated with 1.5 mM TCOH. The results showed that although 1.5 mM TCOH lengthened *Bmal1* period, there was no evident effect on *Per2* daily variation (Fig. 2). The period length of *Per2* in cells incubated with (1.5 mM) and without TCOH were 24.4 ± 0.6 and 23.7 ± 0.1 h, respectively, which showed only marginal, but not statistically significant, difference ($p = 0.08$).

To determine whether TCOH resets transcription of clock genes, U2OS cells treated with 1.5 mM TCOH were harvested at different time-points and the transcription of four key clock genes, i.e., *Bmal1*, *Rev-Erbα*, *Per1* and *Per2*, were analyzed using real-time reverse-transcription (RT)-PCR assay. We found that short-term treatment (2 h) of TCOH had no noticeable effect on clock gene expression. However, TCOH significantly activated *Bmal1* and *Rev-Erbα* expression after 24 h (Fig. 3).

Since TCOH is a major metabolite of CH, and CH is an anesthetic widely used for laboratory animals, we hypothesized that serum from animals anesthetized with CH may contain TCOH and might be able to modulate molecular clock. To test this hypothesis, rats were anesthetized either with CH or with CO_2 at noon (12:00), and then sacrificed immediately. Ten percent FBS, or serum from rats anesthetized with CH or CO_2 was added into the counting medium for the real-time reporter assay. In U2OS cells stably expressing the *Bmal1-dluc* reporter, serum from CH-treated rats resulted in period elongation, while serum from CO_2-treated rats did not. In U2OS cells stably expressing the *Per2-dluc* reporter, serum from both CH and CO_2-treated rats did not change period (Fig. 4).

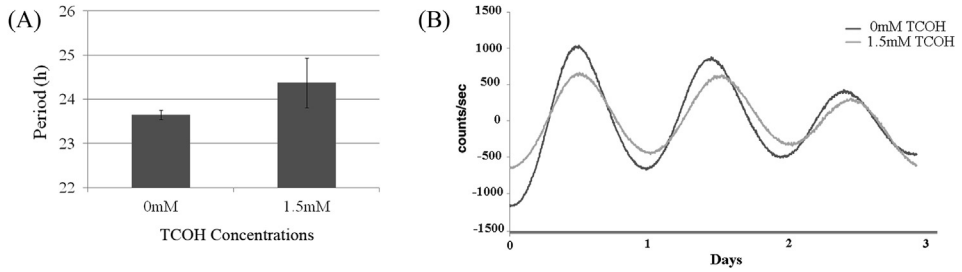

Fig. 2. TCOH has no noticeable effect on *Per2* oscillation period. (A) Circadian period of *Per2* in response to TCOH treatment. Indicated concentrations of TCOH were added and bioluminescence was examined. Circadian period was calculated from fit curve data. Values are mean ± SEM of triplicate assays. * indicates $p < 0.05$. (B) Bioluminescence from cells treated with (grey) or without (black) TCOH. Results are representative of triplicate experiments.

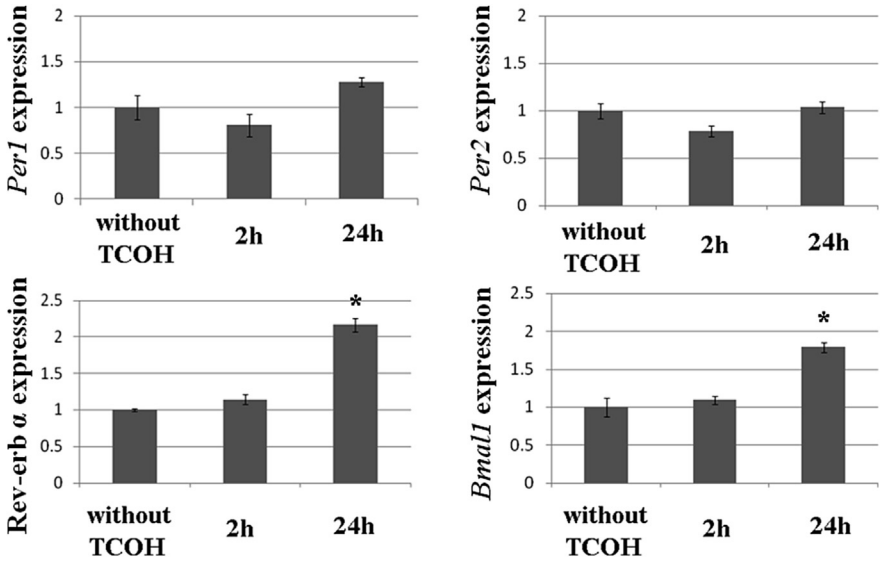

Fig. 3. Long-term TCOH treatment enhances *Bmal1* and *Rev-erbα* transcription. U2OS cells were incubated with 1.5 mM TCOH for 2 or 24 h and expression of *Per1*, *Per2*, *Bmal1* and *Rev-erbα* was analyzed by real-time quantitative RT-PCR. Levels of RNA were normalized to *Gapdh* expression. Expression levels are indicated relative to that without TCOH. Values are means ± SEM of triplicate assays. * indicates $p < 0.05$.

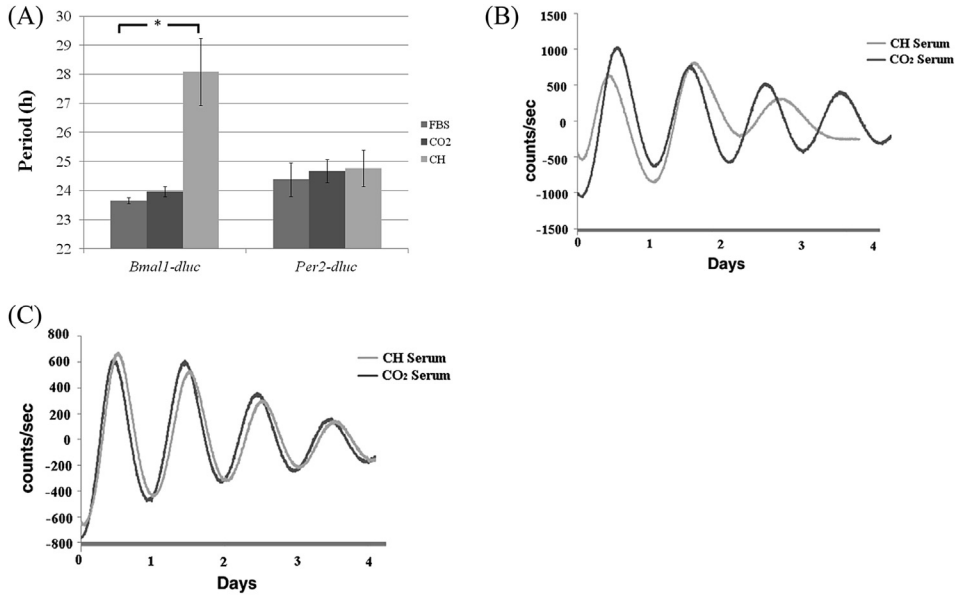

Fig. 4. Serum from rats anesthetized with CH lengthens *Bmal1* oscillation period, but has no effect on *Per2* oscillation period. (A) Period of *Bmal1* and *Per2* oscillation in U2OS cells treated with FBS (dark grey) or serum from rats anesthetized with CH (light grey) or with CO_2 (black). Values are means ± SEM of triplicate assays. * indicates $p < 0.05$. Representative traces of *Bmal1* (B) and *Per2* (C) bioluminescence rhythms in U2OS. Cells were treated with serum from rats anesthetized with CH (grey) or with CO_2 (black).

4. Discussion

In a recent publication, it was reported that both tribromoethanol and TCOH can regulate the phase of *Per2* expression in the liver [4]. Because that study was carried out in intact animals there exists the possibility that this regulation is indirect. To determine if TCOH directly modulates molecular clock, in the present study we used a cell-based assay system. Our major finding is that TCOH regulates molecular clock directly and lengthens the period of *Bmal1* transcription. We found that TCOH regulates molecular clock in a dose-dependent manner. The period of *Bmal1* expression was elongated to 3 h with 1.5 mM TCOH, and to 5 h with 3 mM TCOH, but 0.6 mM or lower concentrations of TCOH did not result in noticeable effects on molecular clock. It was previously reported that the blood concentration of TCOH is much lower than 0.6 mM in patients taking CH [10], and our study indicated that short-term TCOH treatment has no noticeable effect on *Bmal1* oscillation and clock gene expression. Therefore, patients taking CH are not likely to have a disrupted molecular clock and circadian rhythm. By comparison, TCE-exposed workers may have higher concentrations of TCOH in their blood. In actuality cells are usually treated with 0.5–5 mM TCOH to mimic TCE exposure *in vitro* [11–13], which is comparable with the TCOH concentration used in our study. Thus, it is very likely that TCE exposure may lead to disturbance of the molecular clock and circadian disruption. Indeed, sleep problems have been reported in rats exposed to TCE, which fits within the context of our findings [14].

Expression of IL-6 and matrix metalloproteinases (MMPs) were found to be elevated in cultured cells treated with TCE, and may play an important role in TCE-induced toxicity [11,12]. Interestingly, transcription of IL-6 and MMPs was found to be under the control of molecular clock [15,16]. However, whether dysfunction of molecular clock contributes to disturbed expression of cytokines and MMPs, and therefore leads to TCE-induced toxicity warrants further study.

A growing body of evidence supports a strong association between surgery under general anesthesia and disturbances in circadian rhythms, which is likely to result in sleep problems and cognitive dysfunctions after surgery [17,18]. Specific medications used during general anesthesia are thought to play a role in these circadian disruptions. Indeed, sevoflurane and propofol have been found to influence the expression of clock genes. It is worth noting that CH is also used in general anesthesia. It would be interesting to examine the influence of anesthetics on molecular clock using a cell-based assay to elucidate whether fewer effects on molecular clock is associated with decreased severity of sleep problems and cognitive dysfunctions.

The suprachiasmatic nucleus (SCN) is the main regulator of circadian rhythm. Anzai and colleagues compared the responses of the SCN and peripheral tissues (kidney cortex and anterior pituitary gland) with sevoflurane treatment *in vitro* [19], and found that sevoflurane causes a phase delay in the *Per2* expression in SCN slice but does not affect the molecular clock in peripheral tissues. Since the molecular clock is believed to be the same for the SCN and peripheral tissues, Anzai et al. postulated that sevoflurane does not directly affect the clock machinery. Instead, sevoflurane may modulate clock machinery by blocking neuronal communications and/or by regulating GABA receptor in the SCN. SCN response to TCOH has yet to be determined; however, our study and the work by Kubo et al. [4] clearly indicate that TCOH can modulate peripheral clock *in vivo* and *in vitro*, suggesting that TCOH may regulate molecular clock with a mechanism different from sevoflurane. The underlying mechanism for this regulation warrants further study.

Another interesting finding of the present study is that serum from rats anesthetized with CH causes a lengthening of the period of *Bmal1* expression, while serum from rats anesthetized with CO_2 does not. This suggests that CH and/or related molecules present in the serum of anesthetized animals may alter the synchronization of molecular clock in peripheral tissues. It was reported that serum factors in older individuals changes substantially and is likely to be responsible for some age-related circadian dysfunction observed *in vivo* [20]. Our study indicates that the type of anesthesia should be taken into consideration when examining the effect of serum factors on molecular clock in both human and animal studies.

In summary, our study reveals a direct regulation of TCOH on molecular clock, since a malfunctioning molecular clock contributes to a variety of diseases, our findings may shed new light on understanding the effects of CH and TCE toxicity.

Conflict of interest

There is no conflict of interest among the authors.

Acknowledgments

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