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The protective effect of cycloastragenol on aging mouse circadian rhythmic disorder induced by D-galactose

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Abstract

Aging process in mammals is associated with a decline in amplitude and a long period of circadian behaviors which are regulated by a central circadian regulator in the suprachiasmatic nucleus (SCN) and local oscillators in peripheral tissues. It is unclear whether enhancing clock function can retard aging. Using fibroblasts expressing *per2::lucSV* and senescent cells, we revealed cycloastragenol (CAG), a natural aglycone derivative from astragaloside IV, as a clock amplitude enhancing small molecule. CAG could activate telomerase to antiaging, but no reports focused on its effects on circadian rhythm disorders in aging mice. Here we analyze the potential effects of CAG on D-galactose-induced aging mice on the circadian behavior and expression of clock genes. For this purpose, CAG (20 mg/kg orally), was administered daily to D-galactose (150 mg/kg, subcutaneous) mice model of aging for 6 weeks. An actogram analysis of free-running activity of these mice showed that CAG significantly enhances the locomotor activity. We further found that CAG increase expressions of *per2* and *bmal1* genes in liver and kidney of aging mouse. Furthermore, CAG enhanced clock protein BMAL1 and PER2 levels in aging mouse liver and SCN. Our results indicated that the CAG could restore the behavior of circadian rhythm in aging mice induced by D-galactose. These data of present study suggested that CAG could be used as a novel therapeutic strategy for the treatment of age-related circadian rhythm disruption.

KEYWORDS
aging, circadian clock, cycloastragenol

1 | INTRODUCTION

As the human lifespan increased, the number of elderly population will over 800 million by the year 2025.[1] Aging is a complex process resulting in the molecular, cellular, and organic dysfunction.[2] In human, the physiological events with a period of approximately 24 hours is an endogenous oscillator that is controlled by an internal circadian clock system.[3] The master pacemaker of this system is located in the suprachiasmatic nucleus (SCN) of the hypothalamus in the brain. In addition, the circadian clock also controls the same molecular components in the peripheral tissues.[4] In mice, the mutation or loss of clock genes leads to the disruption of homeostasis and results in metabolic syndrome, cancer, and cardiovascular disease.[5] Moreover, many aging-associated diseases such as cancer, metabolic disorder, and

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deregulated immune responses[6,7] are effected by the circadian disruption.

The molecular regulation of circadian rhythms is an interlocking transcriptional-translational feedback loop involving a family of clock genes.[8] In brief, the aryl hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*), and circadian locomotor output cycle kaput (*Clock*) drive the transcription of the repressors period (*per1* and *per2*) and cryptochrome (*cry1* and *cry2*), which can feedback to inhibit the activity of BMAL1:CLOCK.[9] The mammalian circadian clock could be reset or changed by input signals such as light and food.[10] Hence, the modification of these signals results in the clock gene disruption.

Like most physiological processes, the circadian clock is also affected by aging. It is reported that the aging process significantly affected the circadian system in C57BL/6 mice.[11] In addition, several studies have examined the effects of aging on the clock genes expression in the SCN. For example, the expression PER1, PER2, and CRY1 in the SCN significantly decreased in aged mice.[12] Similarly, a study found the free-running circadian period was shorter in aged animals.[13] Therefore, these evidence suggested that aging impacts the expression of the clock gene and behavioral rhythmicity.

Cycloastragenol (CAG) (Figure 1A) is the aglycone derivative from astragaloside IV, which is a major comment extracted from the root of *Astragalus membranaceus*.[14] CAG was identified as an activator of telomerase by the Telomere Repeat Amplification Protocol (TRAP) assay.[15] Meanwhile, the use of CAG as a treatment in female mice leads to an improvement of certain healthspan without significantly increasing global cancer incidence.[16] The effects of this molecule on circadian clock in cells and mice have not been previously studies; however, considering that the benefits of CAG are associated with the effect of a telomerase activator, CAG may be a useful small molecule in avoiding the circadian disruption caused by aging.

Thus, the purpose of the present study was to analyze the potential effect of CAG on aging mice induced by D-galactose.

2 | MATERIALS AND METHODS

2.1 | Cell lines and high-throughput screen

Adult mouse ear fibroblast cells from *per2::lucSV* reporter mice and mouse embryonic fibroblast (MEF)

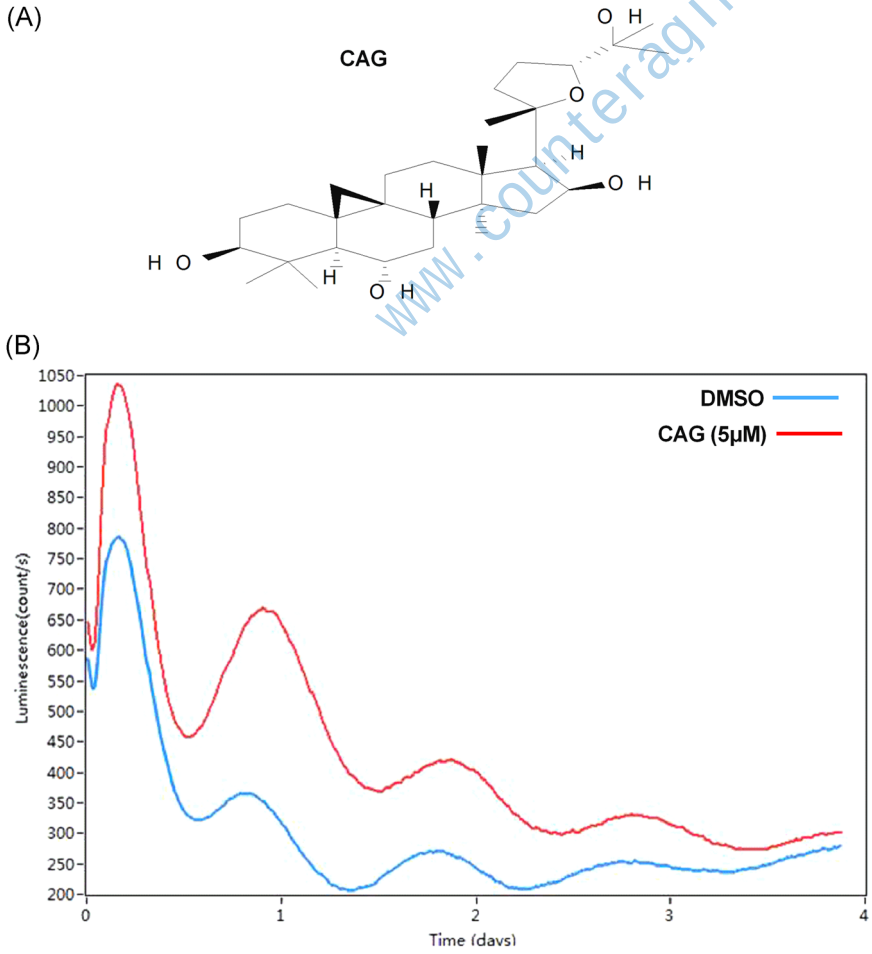

FIGURE 1 CAG enhances the amplitude of circadian rhythms. A, Chemical structure of CAG. B, CAG enhanced the amplitude of Per2::LucSV reporter rhythm. CAG, cycloastragenol; DMSO, dimethyl sulfoxide

cells were obtained from Dr. Zheng Chen Laboratory for use in these experiments.[17] Briefly, cells were grown to full confluency in 30 mm plates, and treated with 5 μM forskolin for 1 hour. Then the media was aspirated and add 2 mL reporter media to dishes with or without CAG. The edge of dishes were applied with vacuum gel and subjected to LumiCycler Reader (Perkin Elmer, Waltham, MA). The date were recorded and then measured for period, phase, amplitude, and damping rate.

2.2 | Senescence-associated β-galactosidase staining of WT-MEFs

WT-MEFs were passaged five times at 20% O_2 to induce senescence then seeded at 2.5×10^5 cells per well in six-well plates. Following the senescence-associated β-galactosidase (SA-β-gal) staining, the MEFs were incubated for 24 to 48 hours at 20% O_2. Subsequently, SA-β-gal activity was measured in three independent experiments, as previously described.[18]

2.3 | Real-time quantitative polymerase chain reaction

For quantitative polymerase chain reaction (qPCR) analysis, nonsenescent and senescent cells were split into six-well plates at a density of 2.5×10^5 cells and incubated for 2 days before 5 μM forskolin synchronization followed by CAG treatment. Total RNA was extracted from cells at a different time by using TRIzol Reagent (Invitrogen, Carlsbad, CA), and 500 ng total RNA was reverse-transcribed into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster, San Francisco, CA). The SYBR Green assay was used for reverse-transcription quantitative polymerase chain reaction (qRT-PCR) with the MaxPro3000 Thermocycler (Agilent, Santa Clara, CA). The following primers were used: per2 forward, 5′-CCCAG CTTTACCTGCAGAAG-3′ and per2 reverse, 5′-ATG GTCGAAAGGAAGCCTCT-3′; Bmal1 forward, 5′-CC AAGAAAGTATGGACACAGACAAA-3′ and Bmal1 reverse, 5′-GCATTCTTGATCCTTCCTTGGT-3′; and GAPDH forward, 5′-CTCCATCCTGGCCTCGCT-3′ and GAPDH reverse, 5′-GCTGTCACCTTCACCGTTCC-3′. Real-time system using a program consisting of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Data analysis was performed using the following equations: $\Delta C = C_t(\text{sample}) - C_t(\text{endogenous control})$; $\Delta\Delta C_t = \Delta C_t(\text{sample}) - C_t(\text{untreated})$; and fold change = $2^{-\Delta\Delta C_t}$.

2.4 | Animal models and compound treatment

C57BL/6 mice (aged 2 months) were obtained from the experimental animal center of University of Texas Health Sciences Center at Houston. All animals were raised under a 24-hour light-dark (LD) cycle with 12 hours of light and 12 hours of darkness. After acclimation for 2 weeks, the mice were randomly divided into three groups (control group, aging group, and CAG treatment group; $n = 6$). In aging and CAG treatment group, mice were injected subcutaneously with 150 mg/kg D-galactose (Sigma-Aldrich, Milwaukee, WI) daily for 6 weeks. Mice in the control group were injected with the same volume of vehicle (0.9% saline). At the same time, the mice were treated with vehicle (CMC-Na) or CAG (20 mg/kg orally, Sigma-Aldrich) via oral gavage daily, in the time window of (ZT8-ZT10; zeitgeber time [ZT]), throughout the experimental period.

2.5 | Circadian locomotor activity

Control group, aging group, and CAG treatment group mice were used for circadian locomotor activity experiments. Briefly, mice were housed in individual cages equipped with a running wheel. Wheel-running activity was tested by an online PC using the Chronobiology Kit (Stanford Software Systems, Naalehu, HI). After vehicle or CAG treatment for 6 weeks in a 24-hour LD cycle, then released into the 24 hours darkness, free-running condition. The mice were following maintained in constant darkness for another 2 weeks. The data of wheel-running condition was downloaded as VitalView data files and analyzed with the ActiView and ActogramJ program.[19]

2.6 | Western blot analyses

After 2 weeks of circadian locomotor activity test, all the mice were killed with CO_2 at ZT = 2 and ZT14. Livers were treated in lysis buffer. Each amount of the protein extracts were denatured by boiling at 95°C for 10 minutes in sample buffer (Bio-Rad Laboratories, Inc, Hercules, CA). The total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The level of BMAL1 was detected using mouse BMAL1-specific antibody (Santa Cruz Biotechnology, Dallas, TX). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) was provided as a loading control.

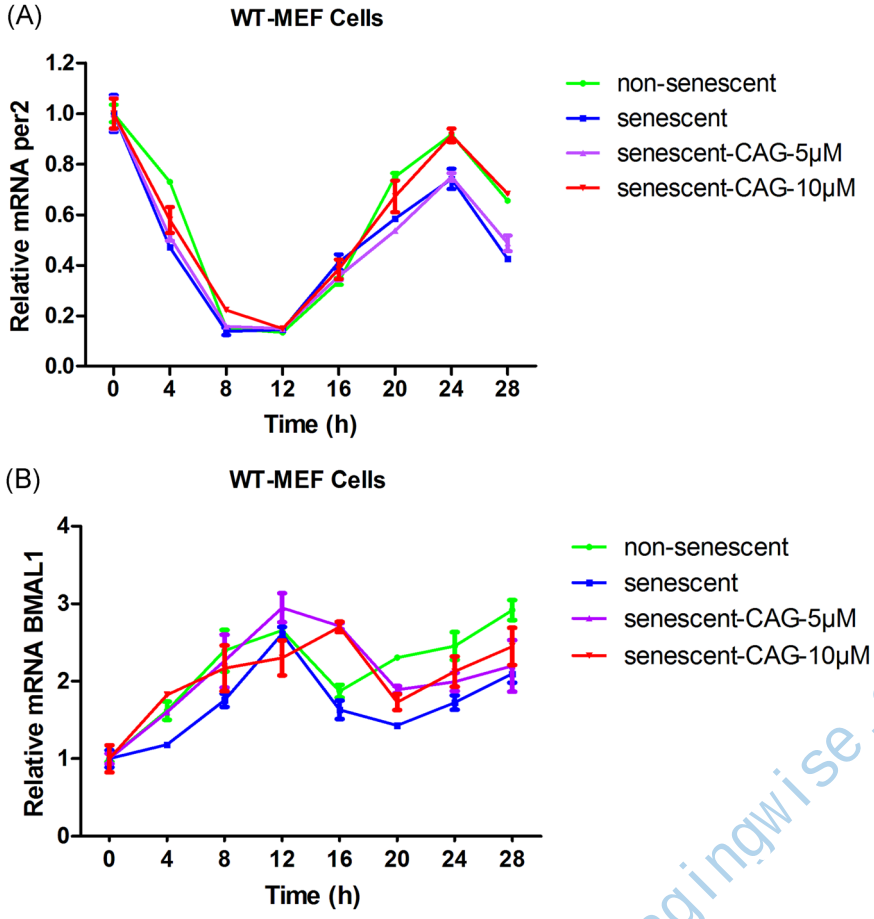

FIGURE 2 Effect of CAG on Clock genes expression in senescent WT-MEF cells. A, The expressions of *per2* mRNA expression by CAG treatment. B, The expressions of *Bmal1* mRNA expression by CAG treatment. CAG, cycloastragenol; MEF, mouse embryonic fibroblast; mRNA, messenger RNA

2.7 | Immunohistochemistry analysis

After 2 weeks of circadian locomotor activity test, all the mice were killed with CO_2 at ZT = 2 and ZT14. Liver, kidney, and brains were fixed in 10% formaldehyde solution overnight, embedded in paraffin, and cut into 8 mm sections. The endogenous peroxidase activity was inhibited using H_2O_2. The mPER2 polyclonal antibody was incu Vector Laboratories bated overnight at 4°C. The primary antibody was detected with the secondary antibodies according to the standard protocols provided by the manufacturer and treatment with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate and hematoxylin counterstain (Vector Laboratories, Burlingame, CA). The tissues were examined using an inverted microscope at ×200 magnification (Eclipse TS100; Nikon, Japan).

2.8 | Statistical analyses

Statistical differences were evaluated using the two-tailed the Student *t* test and analysis of variance followed by *q* test, considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

3 | RESULTS

3.1 | CAG enhanced the amplitude of *Per2::LucSV* reporter rhythm

To identify the circadian clock enhancing small molecules, we screened 25 small molecules using the *per2::LucSV* reporter cell. Among these molecules, CAG robustly enhanced the amplitude of *per2::LucSV* reporter rhythm (Figure S1 and Figure 1B). To further confirm the active effect of CAG on per2 expression, we identified the per2 expression in CAG treatment WT-MEF cells. Data are not shown, CAG has no effects on the messenger RNA (mRNA) expression of per2 in WT-MEF.

3.2 | CAG increased the mRNA expression of *per2* and *Bmal1* in senescent WT-MEF cells

Several studies suggested an antiaging role of CAG by activating telomerase.[15,20] However, its role as a modulator of the circadian clock was previously unknown. Therefore, we first induce the senescent cell model as shown in Figure S2. Further data showed that CAG did

not increase the per2 expression in nonsenescent WT-MEF cells, but significantly increase the mRNA level of *per2* in senescent WT-MEF cells (Figure 2A). Consistent with the expression of *per2*, the expression of other core clock genes *Bmal1* which was a transcriptional activator of *per2* also altered by CGA (Figure 2B). The enrichment of *per2* and *Bmal1* in senescent cells suggested that CAG might affect the circadian rhythms in aging mice.

3.3 | Effect of CAG on circadian rhythms

To address whether the increasing of *per2* and *bmal1* by CAG in senescent cells associated with a clock function, we tested the circadian rhythms. We first used D-galactose-induced aging mouse model using young WT C57BL/6 mice. Consistent with previous results,[11] old mice showed significantly slower than young counterparts. As shown in Figure 3A, we detected a significant delay in the time of activity onset in old mice compared with the control group when the mice were transferred to a dark:dark (DD) cycle, while CAG remarkably reversed this delay. The statistical results show that the D-galactose-induced old mice significantly decrease wheel-running activity levels compared with the control group. In contrast, CAG greatly increased wheel-running activity levels in old mice (Figure 3B). Most encouragingly, CAG did not affect mice body weight (Figure 3C) or cause other observable side effects relative to control. Together, these results demonstrate a circadian associated efficacy of CAG against aging.

3.4 | Effect of CAG on circadian regulation genes and proteins expression in vivo

The expression of clock genes (*per2* and *Bmal1*) was observed in the livers and kidneys of mice at ZT2 and ZT14. (In an LD 12:12 cycle, light is turned on at ZT 0 and off at ZT 12.) As shown in Figure 4A, the mRNA

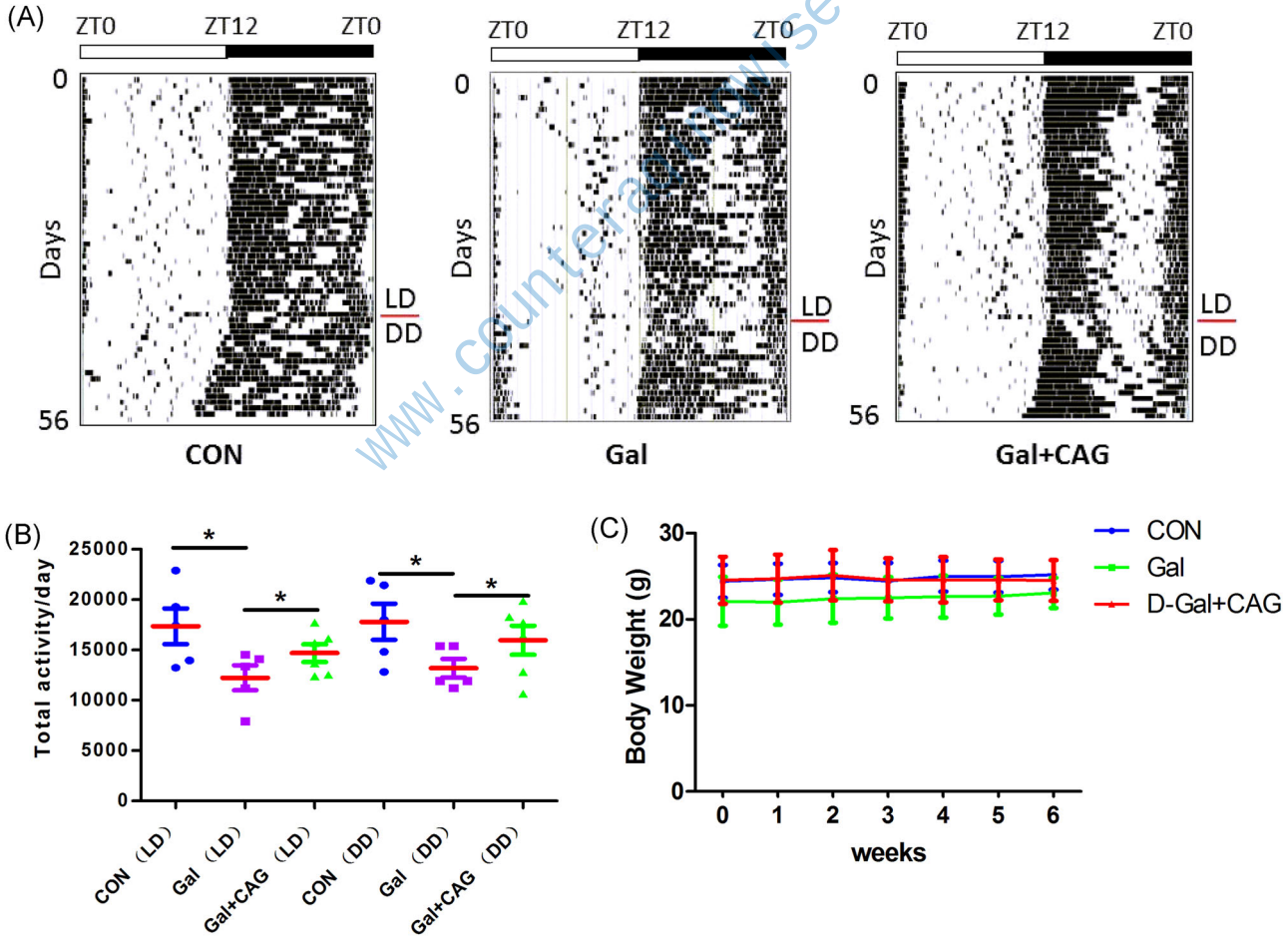

FIGURE 3 CAG enhances the circadian behavior of mice. A, Wheel-running activity of young and D-galactose-induced aging mice. B, Summary of total activity for young ($n = 6$), aging mice ($n = 6$) and CAG treated aging mice ($n = 6$) in LD and DD condition, respectively. C, The body weight of mice in a different group. CAG, cycloastragenol; CON, control; DD, dark:dark; Gal, D-galactose; LD, light:dark; ZT, zeitgeber time

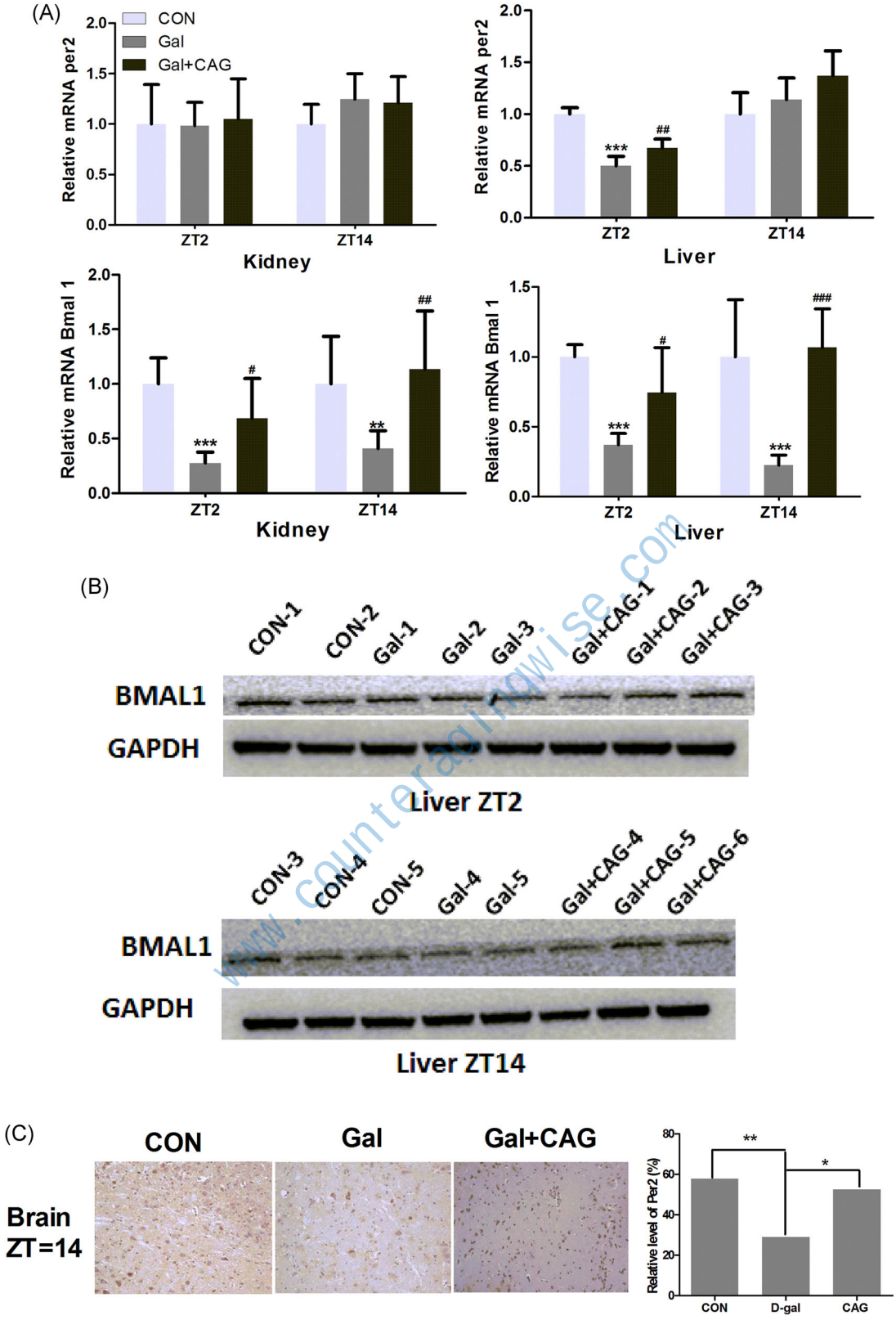

FIGURE 4 Effect of CAG on Clock genes and proteins expression in vivo. A, The expressions of *per2* and *Bmal1* mRNA expression in liver and kidney after CAG treatment at ZT2 and ZT14. B, The expressions of BMAL1 expression in liver by CAG treatment at ZT2 and ZT14. C, The expressions of PER2 expression in SCN by CAG treatment at ZT14. CAG, cycloastragenol; CON, control; Gal, D-galactose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; SCN, suprachiasmatic nucleus; ZT, zeitgeber time

expression of clock genes was decrease in liver and kidney of aging mice at ZT2 or ZT14. In particular, the clock positive regulator *Bmal1* was significantly downregulated both at ZT2 or ZT14. In contrast, these genes were upregulated after CAG treatment. Similar to gene changes, the protein expression of BMAL1 in livers was decreased in old mice. CAG also significantly increased BMAL1 expression (Figure 4B). Because the circadian system is controlled by the SCN, we further investigated whether CAG affects PER2 expression in SCN. Immunohistochemical staining of SCN sections from mice with PER2 antibody showed that PER2 expression is decrease in SCN from old mice compressed with the control group and CAG reversed these changes. Taken together, these studies demonstrate that CAG could reverse the circadian regulators downregulation in D-galactose induce old mice.

4 | DISCUSSION

A lot of evidence from human and animal studies has demonstrated that aging could change the circadian rhythm.[21,22] This is the first study to examine the effects of CAG on circadian rhythms in cells and mice and whether the circadian clock is altered by CAG treatments. In this study, we demonstrated the circadian regulate effects of CAG in *per2::LucSV* reporter cells and senescent WT-MEF cells, respectively. The results suggested that CAG could active the per2 promoter and increase the mRNA expression in senescent cells.The most novel finding of the present study was the robust disruption of circadian rhythms in D-galactose induces aging mice. The aging mice displayed a loss of locomotor activity and a longer circadian period. While most of these changes in CAG mice were restored under the same condition. The mechanism for the function in the study is not completely clear but one confounding factor may associate with the activation of *per2* and clock regulating network.

CAG (Figure 1A) is a natural sapogenin derived from Astragaloside, which exists in *Astragalus membranaceus*.[23] It has been reported to activate telomerase and extend lifespan in many model organisms.[24] In addition, it has been suggested that CAG could inhibit inflammation by suppressing reactive oxygen species-associated endoplasmic reticulum stress.[25] In this article, we showed the CAG intervention resulted in increased level of *per2*, and there was an improvement not only in *per2* but also in the expression of circadian clock positive regulator *Bmal1*. Thus CAG, a telomerase activator, can also be used as a circadian clock activator.

D-galactose aging model has been widely used for aging and antiaging research.[26,27] It can experimentally model the natural aging process. Animals treated with D-galactose showed decreased activity of antioxidant enzymes and displayed a shortened lifespan and poor learning and memory.[28,29] It was reported that the naturally aging mice showed a decrease in locomotor activity and an increase in the free-running period in DD (12:12).[11] However, it is unclear about the circadian rhythm in the aging mice induced by D-galactose. In the present study, we found that D-galactose aging model mice also showed a decrease of locomotor activity and an increase in the free-running period in DD. Meanwhile, the SCN and livers of old mice displayed less PER2 and BMAL1 expression relative to the young mice. In contrast, CAG not only restored several parameters of the circadian rhythm of locomotor activity and free-running period but also increase the protein expression of PER2 and BMAL1.

In summary, our results demonstrated that CAG could activate the senescent cells circadian expression of clock genes such as *Per2* and *Bmal1*. Consistent with the results in vitro, CAG could restore the behavior of circadian rhythm in aging mice induced by D-galactose. However, the circadian regulated effect of CAG on naturally aging mice is needed to further study. In addition, it has found that the human and mouse telomerase reverse transcriptase (TERT) mRNA expression oscillates with circadian rhythms and are under the control of CLOCK-BMAL1 heterodimers. Therefore, whether the circadian regulation effect of CAG is associated with telomerase activity is required to further demonstrate.

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CONFLICT OF INTERESTS

The authors declare no potential conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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