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Synergistic sleep-promoting effects of magnesium and apigenin in normal and insomnia mouse models

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Insomnia is a prevalent condition affecting sleep quality and overall health. While apigenin and magnesium individually exhibit sleep-promoting properties, their combined potential remains unexplored. This study investigates the combinatory effects of apigenin and magnesium (A + M) on sleep. Results demonstrated that A + M significantly enhanced sleep duration in normal mice (44%, $p < 0.005$), caffeine-induced sleep disturbed mice (32%, $p < 0.001$), and *p*-chlorophenylalanine (PCPA)-induced insomnia mice (37%, $p < 0.05$), surpassing individual treatments of apigenin or magnesium. Mechanistically, A + M treatment suppressed systemic and hypothalamic TNFα levels in PCPA-induced mice. We further demonstrated A + M treatment could inhibit microglial activation, potentially *via* suppressing NFκB signaling. These findings demonstrate the combinatory efficacy of A + M in restoring sleep architecture and suggest its potential as a natural, effective intervention for insomnia management.

1. Introduction

Insomnia is a sleep disorder characterized by difficulties in initiating sleep, maintaining sleep, as well as early morning awakening. To qualify, such symptoms must occur at least three times per week for at least three months.[1] Despite affecting 28.3% of adults in the Asian population, insomnia is not immediately life-threatening and may therefore be under-diagnosed and under-treated.[2] Moreover, insomnia rarely exists in isolation and frequently appears alongside metabolic dysfunctions, including cardiovascular disease, obesity, and diabetes,[3] as well as psychological conditions including anxiety and depression.[4]

Currently, subjective complaints about sleep quality and duration contribute significantly to disease prognosis.[5] Even with the aid of polysomnographic assessment, which provides a more objective evaluation of sleep impairment, high costs of time and equipment, as well as brain wave variations across individuals remain significant barriers to insomnia diagnosis. For clinical management, cognitive behavioural therapy for insomnia (CBT-I) remains the first-line treatment due to its non-invasive nature. When CBT-I proves insufficient, medications such as benzodiazepine, gamma-aminobutyric acid (GABA), and melatonin receptor agonists are used in conjunction with CBT to enhance sleep outcomes.[6]

Increasing evidence suggests that there is bi-directional communication between sleep and the immune system, linking insomnia with neuroinflammation.[7] Increased pro-inflammatory cytokine levels have been identified in patients with chronic insomnia,[8] and insomnia may increase the risk of inflammatory diseases.[9,10] Treatment of inflammatory diseases with anti-inflammatory therapeutics has led to improved sleep duration and quality,[11–13] hinting at the developmental potential of anti-inflammatory therapeutics for insomnia.

Apigenin, a naturally occurring flavonoid found abundantly in chamomile, exhibits antidepressant effects;[14,15] it has also been shown to improve insomnia in mouse models of *p*-chlorophenylalanine (PCPA)-induced sleep disorders.[16] In addition to its ability to improve sleep, apigenin has also exhibited anti-inflammatory effects *in vivo*.[17] Together, these indicate the high potential of apigenin for development as a hypnotic agent.

Magnesium is the fourth most abundant ion in the body. As it participates in numerous enzymatic reactions, magnesium is important for proper bodily function, including synaptic activity.[18] In addition, magnesium has been suggested to play an important role in sleep, by regulating neurotransmitters such as GABA and the enzyme *N*-acetyltransferase, which converts 5-hydroxytryptamine (5-HT) into *N*-acetyl-5-hydroxytryptamine and then to

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melatonin.[19,20] Low GABA and melatonin levels have been implicated in sleep disorders,[21,22] and magnesium supplementation can lead to improved sleep outcomes, including increased sleep duration and better overall sleep quality.[23] Collectively, these findings underline magnesium's key role in sleep regulation and its therapeutic value in addressing sleep deficiencies.

Here, we aimed to evaluate the sleep-promoting effects of apigenin and magnesium in combination. Using multiple mouse models of sleep disturbance, we assessed its impact on sleep duration and investigated the underlying mechanisms involved.

2. Materials and methods

2.1 Animals

Male C57BL/6J wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). An inbred colony was maintained in the Centralised Animal Facilities at The Hong Kong Polytechnic University. Mice were kept under constant temperature and humidity (22 ± 2 °C; 65 ± 5%), and on a 12-hour light/dark cycle. Food and water were provided *ad libitum*. All animal experiments were approved by the Animal Subjects Ethics Sub-Committee (ASESC) of The Hong Kong Polytechnic University and adhered to the guidelines of Institutional Guidelines and the Animal Ordinance of the Department of Health, Hong Kong S.A.R.

2.2 Animal treatment and pentobarbital-induced testing

8 weeks-old male C57BL/6J mice were randomly assigned to control (water), vehicle (water) apigenin (A; 4 mg kg^{-1}), magnesium (M; 40 mg kg^{-1}), low-dose apigenin + magnesium (A + M (LD); 10 mg kg^{-1} magnesium + 1 mg kg^{-1} apigenin), medium-dose apigenin + magnesium (A + M (MD); 20 mg kg^{-1} magnesium + 2 mg kg^{-1} apigenin), high-dose apigenin + magnesium (A + M (HD); 40 mg kg^{-1} magnesium + 4 mg kg^{-1} apigenin) and diphenhydramine hydrochloride (DIPH; 20 mg kg^{-1}) treatment groups. The chemicals were gifts from Telford International Company Ltd, Hong Kong.

For the pentobarbital-induced sleep test, mice were fasted 24 hours prior to sleep testing, unless specified otherwise. 40 mg kg^{-1} sodium pentobarbital was administered intraperitoneally 30 minutes after treatments, as previously described.[24] After induction, mice were transferred to individual cages for observation. Sleep duration (the period of time from falling asleep to exhibiting stereotactic reflection) was measured as an indication of sleep status. Mice that did not exhibit any signs of stereotactic reflection loss 15 minutes after induction with pentobarbital were excluded from the test.[25] On the day after the sleep test, mice were sacrificed and blood was collected by cardiac puncture. Mouse organs, whole brains, and the hypothalamus were removed and weighed. Plasma and hypothalamus samples were stored at −80 °C for further analysis.

2.2.1 Longitudinal treatment. 8 weeks-old male C57BL/6J mice were randomly assigned to (i) control, (ii) A, (iii) M, (iv) A + M (HD), or (v) DIPH treatment groups (n = 7 per group). Mice received daily treatment *via* oral gavage for 21 days. For the DIPH group, mice received oral administration of water during the treatment period, and DIPH was administered 60 minutes before the pentobarbital-induced sleep test. Sleep tests were performed as described above.

2.2.2 Caffeine-induced sleep disturbance mice treatment. 8 weeks-old male C57BL/6J mice were randomly assigned to (i) control, (ii) vehicle, (iii) A, (iv) M, (v) A + M (HD), or (vi) DIPH treatment groups (n = 10 per group). Mice were fasted 24 hours prior to sleep testing. On the test day, mice were administered a single dose of their respective treatment *via* oral gavage. After 30 minutes, the mice were fed with 15 mg kg^{-1} caffeine, while control mice were fed water only. Pentobarbital-induced sleep test was performed 30 minutes after caffeine treatment.

2.2.3 PCPA-induced insomnia mice treatment. 6 weeks-old male C57BL/6J mice were randomly assigned to (i) control, (ii) vehicle, (iii) A, (iv) M, (v) A + M (HD), or (vi) DIPH treatment groups (n = 7 per group). Insomnia was induced in mice by injecting 400 mg kg^{-1} PCPA intraperitoneally for 2 consecutive days. Control mice were injected with PBS only. 24 hours after the last injection of PCPA, mice that showed hyperactivity, increased aggressiveness, and development of dull and dishevelled hair, together with continuous daytime movement and reduced sleep when compared with control mice were considered successfully induced with insomnia.[26] Mice were then treated orally with their respective treatments, daily for 7 days. At the end of the treatment period, mice were fasted for 24 hours and the pentobarbital-induced sleep test was performed.

2.3 Bliss independence model analysis

Bliss independence was calculated as previously reported by Petraitis *et al.* and Duarte *et al.*, using the equation $E_{IND} = E_A + E_M - E_A \times E_M$, where E_{IND} is the expected effect of A + M (HD), and E_A and E_M are respectively the promoting effects of apigenin and magnesium treatment on mouse sleep duration.[27,28] The interaction between apigenin and magnesium is represented by the difference between the expected effect and experimentally observed effect of A + M (HD) (E_{OBS}, $\Delta E = E_{OBS} - E_{IND}$). If ΔE and its 95% confidence interval (CI) were <1, synergy was concluded.

2.4 ELISA

TNFα, IL-1β, and IL-6 levels in plasma were measured by commercially available ELISA kits (BioLegend, Cambridge, UK). Procedures were performed according to the manufacturer's instructions.

Briefly, diluted standards and plasma samples were added to 96-well plates coated with TNFα, IL-1β, or IL-6 antibodies and incubated overnight. After incubation, detection antibodies and Avidin-HRP solution were added, and the colour intensity was measured using a Multiskan SkyHigh Microplate Spectrophotometer (ThermoFisher Scientific, USA). The concentrations of TNFα, IL-1β, and IL-6 were calculated relative to their corresponding standard curves.

2.5 Quantitative polymerase chain reaction (qPCR) assay

DNA was extracted from approximately 10 mg of hypothalamic tissues using the TGuide Smart Blood/Cell/Tissue RNA kit (TIANGEN BIOTECH, Beijing, China), according to manufacturer's instructions. Extracted DNA was quantified using a Nanodrop One spectrophotometer (Thermo Scientific) and subsequently stored at −80 °C until use in qPCR analysis.

qPCR reaction mixtures were prepared as follows: 10 μl of 2× PowerUp SYBR Green Master Mix (Applied Biosystems, USA), 400 nM of each forward and reverse primer (Table 1), and 2 μl of sample DNA at a concentration of 2.5 ng μl^{-1}, resulting in a total reaction volume of 20 μl. Amplification was performed on a QuantStudio 7 system (Applied Biosystems) under the following conditions: an initial incubation at 50 °C for 2 minutes, followed by an activation step at 95 °C for 2 minutes, and then 40 cycles of denaturation at 95 °C for 1 second and annealing/extension at 60 °C for 30 seconds. Relative expression of each target was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6 Western blotting

Western blot analysis was conducted as previously described.[29] In brief, hypothalamus samples were lysed in RIPA buffer and cellular debris removed by centrifugation. Protein concentration of supernatants was quantified using the DC protein assay (Bio-Rad, Hercules, CA, USA) and samples were then solubilized in Laemmli sample buffer. Equal protein amounts were resolved by SDS-PAGE and transferred onto PVDF membranes (Advansta Inc., CA, USA). Blots were then blocked in 5% non-fat skim milk and probed with primary antibodies followed by their corresponding secondary antibodies. Protein bands were visualized using WesternBright ECL HRP substrate (Advansta Inc.) and a ChemiDoc Imaging System (Bio-Rad). Protein expression levels were quantified using Image Lab software (Bio-Rad). Antibodies used were as follows: β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Iba1 (Fujifilm, Osaka, Japan), NF-κB, and phospho-NF-κB (Cell Signaling Technology, Danvers, MA, USA).

Table 1 qPCR primers

Target	Forward primer 5′–3′	Reverse primer 5′–3′
TNFα	GGTGAGGAGCACGTAGTCGG	TCCCAGGTTCTCTTCAAGGGA
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

2.7 Statistical analysis

Data were analyzed using GraphPad Prism 10.3 (GraphPad Software, USA). Results are presented as mean ± SD and were analyzed by either one-way ANOVA with Dunnett's multiple comparison test or two-way ANOVA with Tukey's comparison test. *P* values < 0.05 were considered statistically significant.

3. Results

3.1 Apigenin and magnesium treatment exhibited hypnotic effects in mice

Previous studies have shown that individually, apigenin and magnesium exhibit sleep improving properties,[30,31] however, their combinatory effects have yet to be explored. Therefore, we adopted a pentobarbital-induced sleep mouse model to evaluate their potential combined hypnotic effect. Mice were fed orally with the corresponding treatments 60 minutes before pentobarbital-induced sleep testing. The treatment scheme is illustrated in Fig. 1A.

As shown in Fig. 1B, mice receiving treatment with either apigenin or magnesium alone showed no improvement in sleep duration when compared to control, however, a dose-dependent hypnotic effect was observed when they were used in combination. A + M (MD) and A + M (HD) significantly increased the sleep duration of pentobarbital-induced mice by

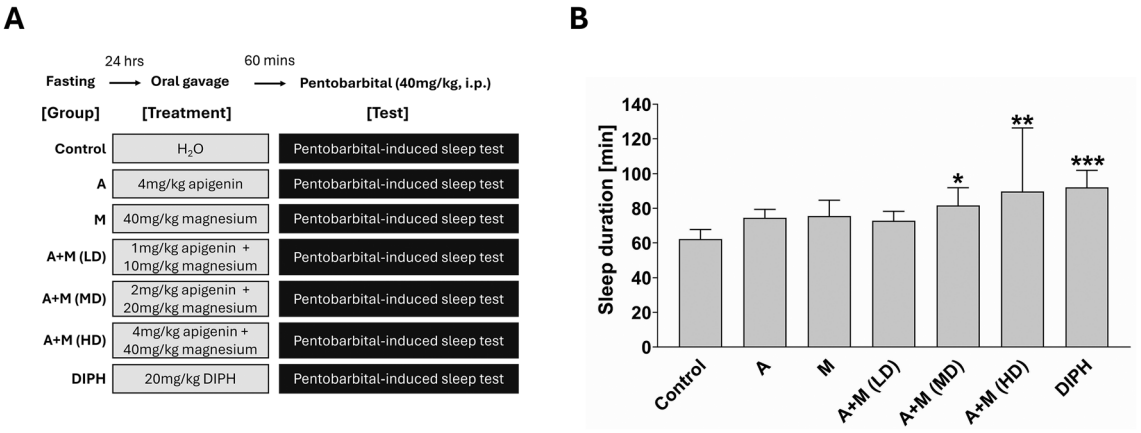

Fig. 1 Effect of A + M on sleep in mice. Mice were treated with apigenin and magnesium, alone and in combination at various doses, *via* oral gavage 1 hour before assessment *via* pentobarbital-induced sleep test. (A) Experimental outline. (B) Mouse sleep duration among different treatment groups. A: apigenin, M: magnesium, A + M (LD): A + M low dose, A + M (MD): A + M medium dose, A + M (HD): A + M high dose, DIPH: diphenhydramine. Data are expressed as mean ± SD; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 *vs.* control group.

16% ($p < 0.05$) and 44% ($p < 0.01$) respectively. Notably, the increase in sleep duration after A + M (HD) treatment was comparable to DIPH, an antihistamine drug commonly used as a sleep aid[32] and here employed as a positive control, which exhibited a 48% ($p < 0.001$) increase in sleep duration after administration. Thus, we identified a potential dose-dependent hypnotic effect for the combination of A + M. The high-dose (HD) group showed the greatest sleep improving efficacy and was therefore selected for downstream experiments.

3.2 Apigenin and magnesium treatment ameliorated caffeine-induced sleep disturbance in mice

Nocturnal use of caffeine may lead to sleep disruption and sleeplessness, as caffeine antagonizes adenosine receptors in the brain and interferes with the sleep–wake cycle.[33,34] To further investigate the sleep improving properties of A + M, the caffeine-induced sleep disturbance mice model was adopted. Mice were fed with the corresponding treatments 30 minutes before caffeine induction, and pentobarbital-induced sleep tests were performed 30 minutes after caffeine induction. The treatment scheme is shown in Fig. 2A.

In comparison with uninduced control, sleep duration was significantly reduced by 21% ($p < 0.001$) in caffeine-induced mice, indicating successful induction of sleep disturbance (Fig. 2B). Apigenin or magnesium alone did not significantly improve sleep duration, but A + M (HD) treatment lead to a significant increase in sleep duration. A + M (HD)-treated caffeine-induced mice showed a 32% increase in sleep duration when compared to vehicle control ($p < 0.001$). Again, the improvement in sleep duration of A + M (HD)-treated mice was comparable to DIPH, which also exhibited a significant increase of 32% ($p < 0.001$) when compared to vehicle control. In addition, we identified a synergistic effect of A + M (HD) in caffeine-induced mice (Table 2). These results further supported the potential hypnotic effect of the apigenin and magnesium combination.

3.3 Apigenin and magnesium treatment exhibited hypnotic and anti-inflammatory effects in mice

To further explore its potential as a dietary sleep supplement, we tested if A + M induced effects of sleep improvement after continuous treatment. As shown in Fig. 3A, mice received 21 consecutive days of treatment, and pentobarbital-induced sleep tests were performed to evaluate the hypnotic effects of continuous treatment.

Daily treatment of apigenin, magnesium, or A + M (HD) did not alter the body weight, or food and water consumption of mice when compared to control (Fig. 3B–D, SI Fig. 1). Next, we examined the sleep duration of mice. In order to mimic actual conditions, we did not fast the mice in this experiment. As shown in Fig. 3E, mice exhibited comparable sleep durations before treatment in the pentobarbital-induced sleep test, and control mice did not show alterations in sleep duration after the treatment period. No sleep improving effect was observed in mice treated with apigenin or magnesium, but significant sleep improving effects were observed in the A + M (HD) group. After 21 days of treatment, a significant increase in sleep duration of 22% ($p < 0.05$) was observed in mice treated with A + M (HD) when compared with control mice.

Table 2 Bliss independence analysis of A + M in caffeine-induced mice

	Fractional effect				
	Observed			Expected	
Sleep duration (% increase)[a]	A	M	A + M (HD)	A + M (HD)	ΔE
	37 ± 15	34 ± 8	82 ± 16	53 ± 14	29* (56–2)[b]

[a] Data are presented as mean ± SE (% increase) compared to vehicle group. [b] 95% confidence interval. *$p < 0.05$.

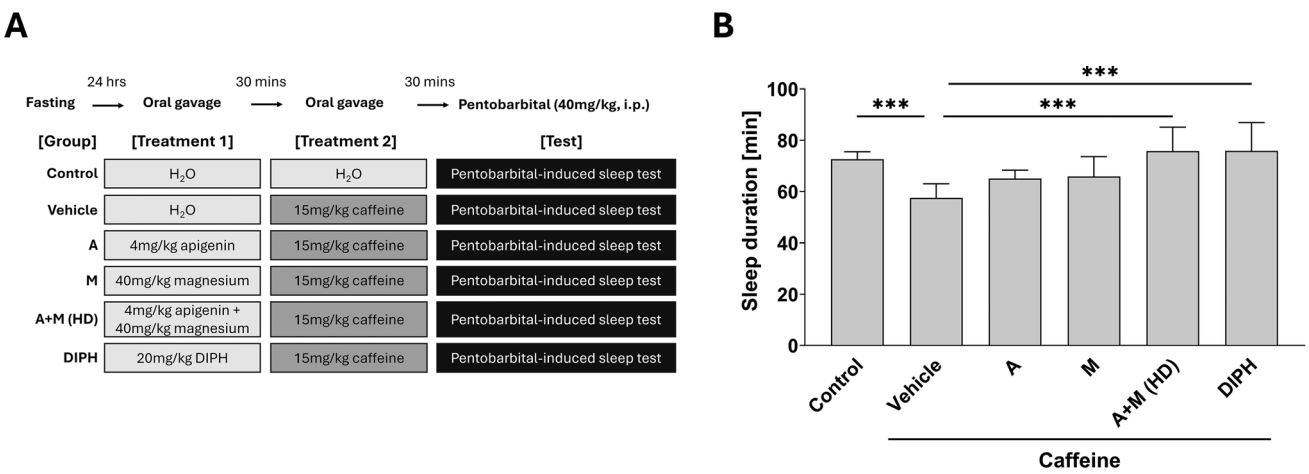

Fig. 2 Effect of A + M on sleep in caffeine-induced mice. Mice were treated with apigenin and magnesium, alone and in combination, *via* oral gavage, 30 minutes before induction with caffeine, followed by assessment *via* pentobarbital-induced sleep test. (A) Experimental outline. (B) Mouse sleep duration among different treatment groups. Data are expressed as mean ± SD; ***$p < 0.001$ *vs.* vehicle group.

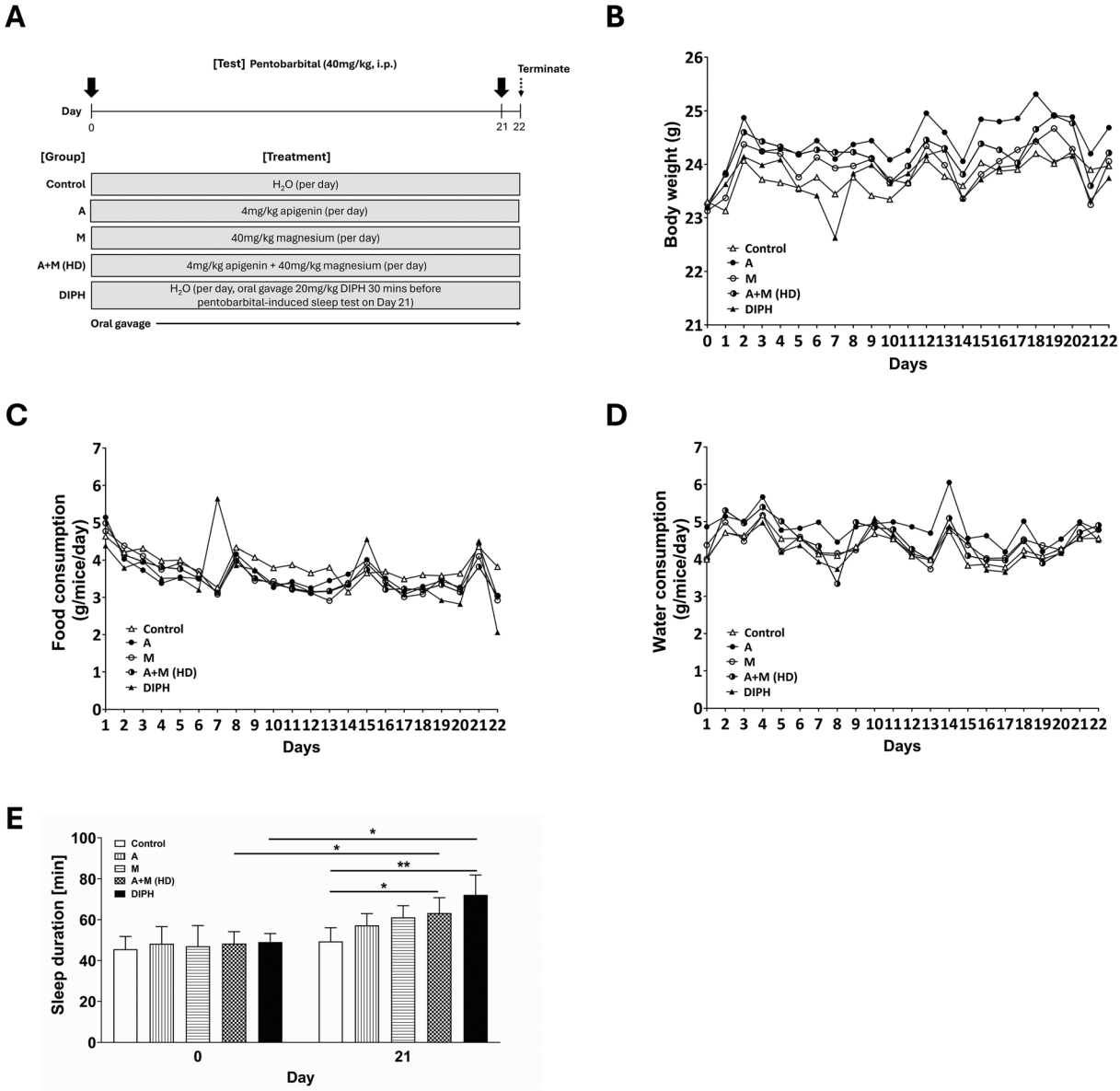

Fig. 3 Effect of A + M on sleep in mice. Mice were treated with various treatments *via* oral gavage, daily for 21 days. (A) Experimental outline. Longitudinal assessment of mouse (B) body weight, (C) food consumption, and (D) water consumption over the experimental period. (E) Sleep duration after pentobarbital-induced sleep tests conducted on days 0 and 21. Data are expressed as mean ± SD; *$p < 0.05$, **$p < 0.01$ *vs.* indicated group.

Mice were terminated after the experiment, and vital organs including kidneys, liver, lungs, heart, spleen, thymus, and brain were removed, weighed, and organ coefficients calculated (SI Fig. 2). No changes in organ gross morphology (data not shown) or organ coefficients were observed in treatment groups when compared to control.

As both apigenin and magnesium have been reported to exhibit anti-inflammatory effects,[35,36] and previous studies have linked sleeplessness to increased inflammatory status,[37,38] we hypothesized that the sleep improving effects of A + M (HD) may be mediated *via* anti-inflammatory mechanisms. Therefore, we investigated the effect of A + M (HD) treatment on systemic levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6. As shown in Fig. 4A and C, 21-day treatment of A + M (HD) significantly suppressed TNFα and IL-6 levels by 27% ($p < 0.05$) and 34% ($p < 0.001$) respectively when compared with control. In addition, apigenin and magnesium treatment alone also suppressed IL-6 levels by 29% ($p < 0.001$) and 28% ($p < 0.001$) respectively when compared with control. However, we did not observe any alterations in IL-1β in mice after the various treatments. Thus, our results indicated that the sleep improving effect of A + M (HD) was potentially mediated *via* anti-inflammatory activity.

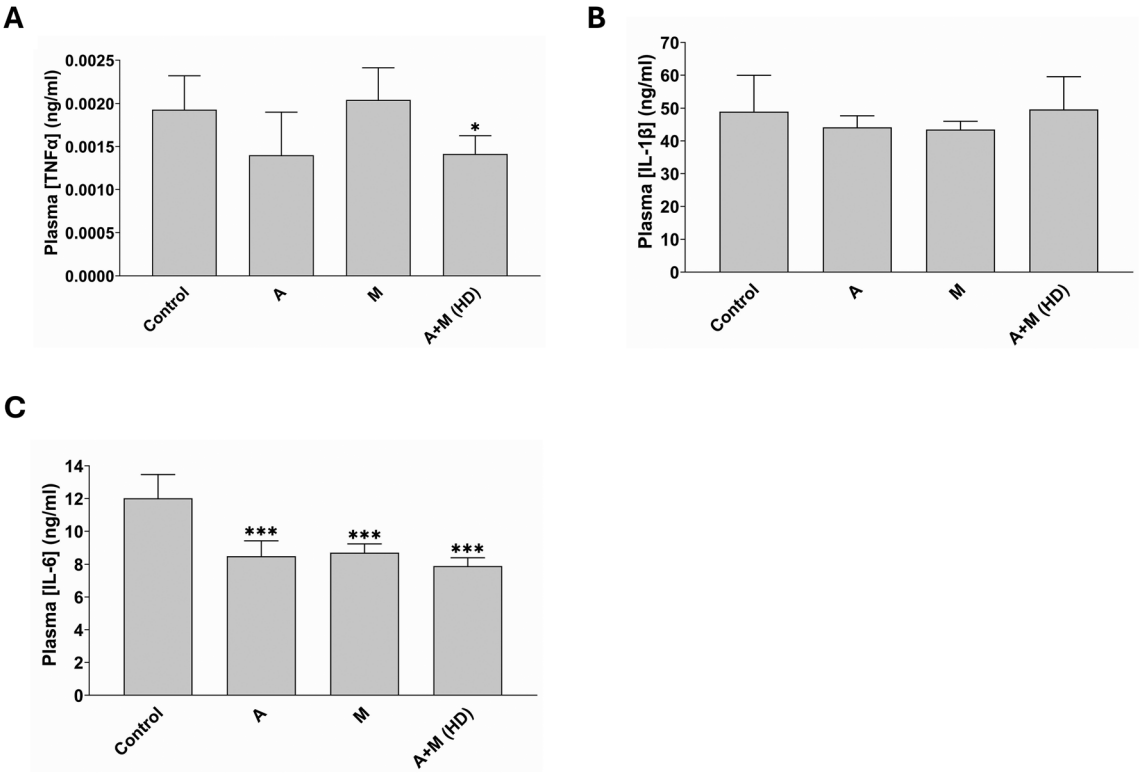

Fig. 4 Anti-inflammatory effect of A + M in mice. At termination, blood was collected and plasma levels of (A) TNFα, (B) IL-1β, and (C) IL-6 were measured by ELISA. Data are expressed as mean ± SD; *$p < 0.05$, ***$p < 0.001$ *vs.* control group.

3.4 Apigenin and magnesium treatment improved sleep duration in PCPA-induced insomnia mice

To further understand the potential of A + M on sleep improvement, we adopted a commonly used insomnia mouse model to investigate the effects of A + M. PCPA is a selective inhibitor of tryptophan hydroxylase (TPH), the key and rate-limiting enzyme in 5-HT biosynthesis.[39] Administration of PCPA leads to suppression of 5-HT production, subsequent disturbance of sleep rhythms, and insomnia-like symptoms.[26,40] To induce insomnia, mice were injected with PCPA for two consecutive days before daily treatment with apigenin, magnesium, A + M (HD), or water for 7 consecutive days (Fig. 5A). As shown in Fig. 5B–D and SI Fig. 3, no alterations in mouse body weight, or food and water consumption were identified when comparing treatment groups with vehicle during the experimental period.

At the end of the treatment period, pentobarbital-induced sleep testing was performed to evaluate the potential sleep-improving effect of the treatments. In comparison with control, sleep duration was reduced by 33% ($p < 0.001$) in vehicle-treated mice, indicating successful induction of insomnia after PCPA induction (Fig. 5E). Although no improvements in sleep were observed in PCPA-induced mice receiving apigenin or magnesium treatments alone, A + M (HD) treatment significantly increased sleep duration in mice by 37% ($p < 0.05$). A synergistic effect was observed in PCPA-induced mice treated with A + M (HD) (Table 3).

At termination, no significant alterations were observed among organ coefficients (kidney, liver, lung, heart, spleen, thymus, and brain) of mice in treatment groups (SI Fig. 4).

3.5 Apigenin and magnesium treatment exhibited anti-inflammatory effects in PCPA-induced insomnia mice

As we observed anti-inflammatory effects of apigenin, magnesium, and A + M (HD) in the previous mouse models, we next examined whether the treatments also exhibited anti-inflammatory effects in PCPA-induced mice. As shown in Fig. 6A–C, plasma levels of TNFα, IL-1β, and IL-6 were significantly increased in PCPA-induced mice when compared with control mice, confirming an inflammatory state. No anti-inflammatory effects were observed in PCPA-induced mice treated with magnesium alone, whereas apigenin treatment in PCPA-induced mice led to a significant suppression of plasma IL-6 levels by 21% ($p < 0.05$). Treatment with A + M (HD) significantly suppressed the plasma levels of TNFα, IL-1β, and IL-6 by 37% ($p < 0.01$), 31% ($p < 0.05$), and 23% ($p < 0.01$) respectively when compared with vehicle.

The hypothalamus plays a critical role in regulating various aspects of the sleep–wake cycle, and sleep deprivation can lead to neuroinflammation.[41,42] Since A + M (HD) treatment reduced plasma levels of pro-inflammatory cytokines in PCPA-induced mice, we asked if the treatment also exhibited anti-neuroinflammatory effects. Based on the importance of the hypothalamus in sleep cycle regulation, we focused on this

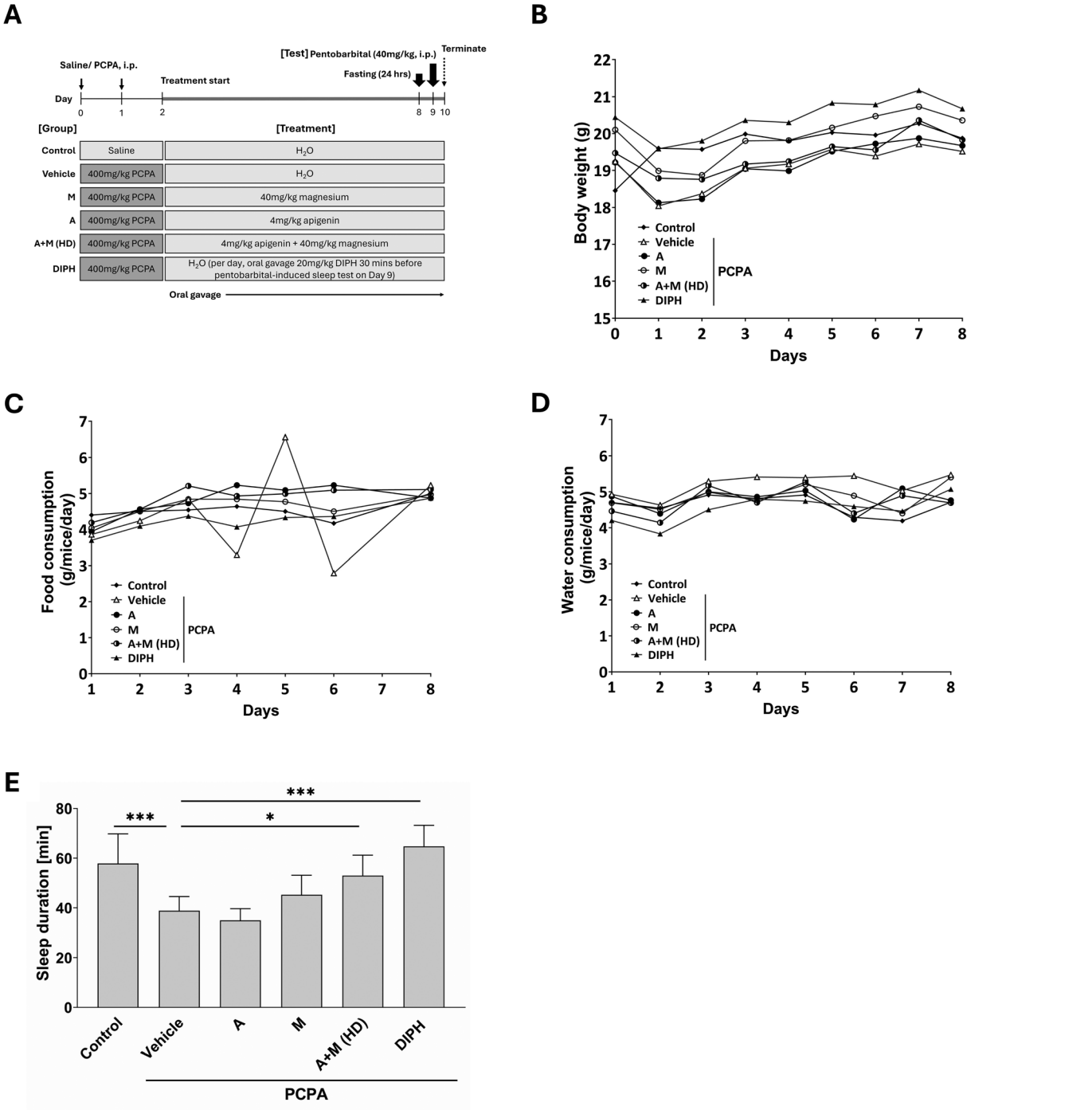

Fig. 5 Effect of A + M on sleep in PCPA-induced mice. Mice were induced with PCPA for two consecutive days before the commencement of a 7-day treatment period. Pentobarbital-induced sleep test was conducted at the end of the treatment period. (A) Experimental outline. Mice (B) body weight, (C) food consumption, and (D) water consumption over the treatment period. (E) After 7 days treatment, mice were induced with pentobarbital and sleep duration was assessed. Data are expressed as mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* vehicle group.

brain region in subsequent experiments. Previous studies have suggested that hypothalamus size may be associated with sleep disturbance, as reductions in hypothalamic volume were observed in neurodegenerative subjects with sleep dysfunction.[43] Therefore, we examined the hypothalamic weights of mice. As shown in Fig. 6D, PCPA-induced mice exhibited a significant reduction of 44% ($p < 0.001$) in hypothalamus coefficient when compared with control. A + M (HD) treatment rescued this hypothalamic deficiency by 63% ($p < 0.01$) when compared with vehicle-treated mice. No significant effects were identified in mice treated with apigenin or magnesium alone. Next, we examined the expression of TNFα, IL-1β, and

Table 3 Bliss independence analysis of A + M in PCPA-induced mice

Sleep duration (% increase)[a]	Fractional effect				
	Observed			Expected	
	A	M	A + M (HD)	A + M (HD)	ΔE
	−20 ± 11	25 ± 9	75 ± 7	23 ± 11	52** (86–18)[b]

[a] Data are presented as mean ± SE (% increase) compared to vehicle group. [b] 95% confidence interval. **$p < 0.01$.

IL-6 in the hypothalamus. A significant increase in hypothalamic TNFα expression was observed in PCPA-induced mice when compared with control mice (Fig. 6E). Notably, mice treated with apigenin, magnesium, or A + M (HD) showed significant suppression of TNFα. We did not observe alteration of IL-1β or IL-6 expression in the hypothalamuses of PCPA-induced mice (data not shown).

Given that microglial activation can lead to increased inflammatory cytokine expression in the hypothalamus, and that PCPA-induced insomnia leads to microglial activation,[44–46] we examined if the treatments could alter the activation of microglia, resulting in the reduced expression of inflammatory cytokines. As shown in Fig. 7A, a significant increase in the expression of Iba1, a commonly used microglial activation marker, was observed in the hypothalamus of PCPA-induced mice when compared with control, indicating microglia activation. However, apigenin, magnesium, and A + M treatments significantly suppressed the expression levels of Iba1. It has been shown that activation of NFκB can lead to increased expression of pro-inflammatory cytokines, including TNFα, in microglial cells.[47] To further explore the anti-inflammatory mechanism of the treatments, we evaluated the activation of NFκB in the hypothalamic tissues of the mice. We observed activation of NFκB in PCPA-induced mice when compared with control, while A + M (HD) could significantly suppress this activation (Fig. 7B). In summary, we showed that the hypnotic effect of A + M (HD) was potentially mediated *via* suppression of NFκB and microglial activation.

4. Discussion

CBT-I is widely used in conjunction with sleep medications for the treatment of insomnia.[6] However, long-term usage of hypnotic agents has been linked to the development of tolerance and reduction of therapeutic efficacy,[48–50] and studies have demonstrated that prolonged administration of pharmacological treatments for insomnia is associated with a range of adverse effects, including impairments in psychomotor performance, cognitive function, and memory. DIPH is a common antihistamine used to treat allergic reactions, motion sickness, and insomnia. It acts as an inverse agonist by binding to histamine 1 (H1) receptors in the central nervous system (CNS), thereby inhibiting the effects of histamine, an excitatory neurotransmitter that primarily facilitates alertness and wakefulness. Hence, blocking H1 receptors leads to increased drowsiness and sleep promotion.[51] Mice with histamine-deficiency exhibit obesity, increased visceral adiposity, hyperleptinemia, and hyperinsulinemia when fed a high-fat diet.[52,53] Notably, we did not observe similar effects in apigenin, magnesium, or A + M (HD)-treated mice, highlighting their advantage as sleep aids.

The naturally occurring flavonoid apigenin has been previously demonstrated to have sleep-promoting effects.[54] The anti-inflammatory and TNFα-suppressing effects of apigenin have also been demonstrated *in vitro* and *in vivo* in different animal models.[55–59] Apigenin has been shown to suppress activated NFκB in LPS-stimulated macrophages, as well as in microglia cells.[55,60] Mechanistically, apigenin has been shown to inhibit the phosphorylation and degradation of IκB, which functions to sequester NFκB in the cytoplasm and prevents it from entering the nucleus and regulating the expression of pro-inflammatory genes.[60] On the other hand, magnesium is an essential ion required for proper bodily functioning as well as regulation of immunity.[18] Magnesium deficiency has been linked to activation of the immune system and increased risk of various diseases including Alzheimer's disease, cardiovascular disease, stroke, and type 2 diabetes mellitus.[61,62] In endothelial cells, low magnesium leads to redox imbalance, increased production of free radicals, and upregulation of the NFκB pathway.[63] Similar effects were also observed in immune cells, where neutrophils and macrophages produced higher levels of free radicals when they were maintained in low magnesium conditions.[62] Further, a previous study has demonstrated the anti-inflammatory effect of magnesium, where treatment of LPS-activated microglia cells with magnesium sulfate could suppress the activity of NFκB.[64] In addition to the inflammatory response, magnesium is also involved in the regulation of circadian rhythms, and magnesium intake has been correlated with improved sleep quality and increased sleep duration,[23,65] indicating its potential as a supplement for sleep. Therefore, the combination of apigenin and magnesium may simultaneously target IκB and NFκB of the NFκB signaling pathway, providing a more substantial suppressive effect on NFκB and downstream TNFα expression and offering a more comprehensive approach to improving sleep. Moreover, the effective dose used in our study, equivalent to 19.4 mg apigenin and 194.4 mg magnesium for a 60 kg human, is well below doses previously shown to be well-tolerated in clinical trials (23.4 mg and 225 mg for apigenin and magnesium respectively),[66,67] underscoring the translational potential of this combination.

Based on the biopharmaceutics classification system, apigenin has been classified as a class II drug, indicating it possesses poor aqueous solubility but high intestinal permeability.[70] Though apigenin has been shown to cross the blood–brain barrier and act on the CNS through the circulatory system,[71] the oral bioavailability of apigenin remains low due to its low water solubility,[72,73] hindering its widespread use as a functional food. In order to improve the aqueous solubility, dissolution, and *in vivo* bioavailability of apigenin, different

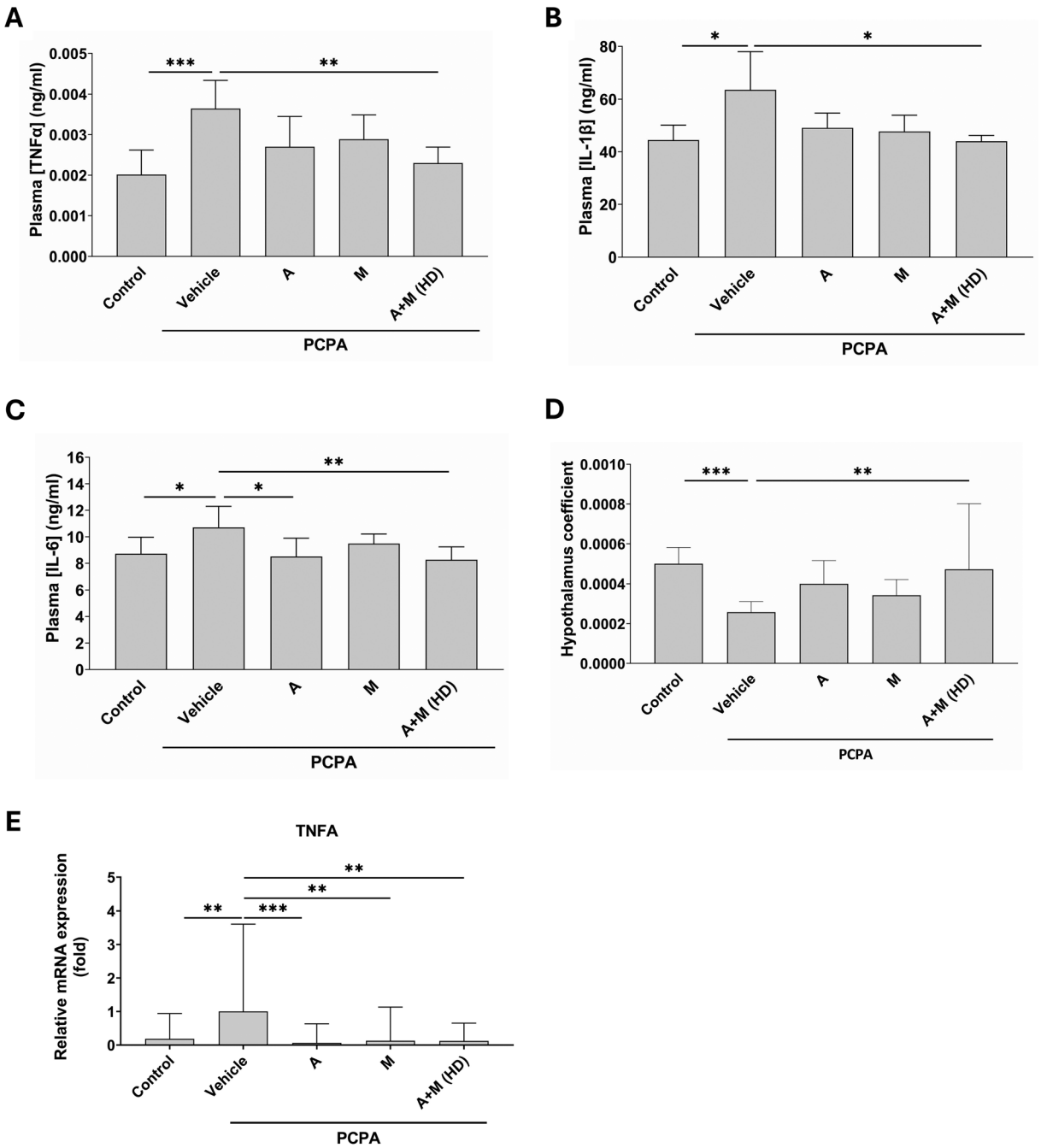

Fig. 6 Anti-inflammatory effect of A + M in PCPA-induced mice. At termination, blood was collected and plasma levels of (A) TNFα, (B) IL-1β, and (C) IL-6 were measured by ELISA. Hypothalamus were isolated and (D) hypothalamus coefficient (hypothalamus weight/whole brain weight) was calculated. (E) Hypothalamic TNFα mRNA levels. Data are expressed as mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* vehicle group.

nanoformulations have been proposed, including apigenin-phospholipid phytosome, apigenin-loaded polylactic-*co*-glycolic-acid *meso*-2,3-dimercaptosuccinic acid nanoparticles, and hydrogel encapsulated apigenin.[74–76] However, these nanoformulations of apigenin are in the experimental stage and require further development for practical application. In contrast, different forms of magnesium compounds are currently available as dietary supplements, though comparisons of their bioavailability and efficacy are limited. A previous report by Uysal *et al.* showed that among five tested magnesium compounds (magnesium sulfate, magnesium oxide, magnesium acetyl taurate, magnesium citrate, and magnesium malate), magnesium acetyl taurate was the most rapidly absorbed into the brain and exhibited considerable effects on anxious behaviours.[68] In another study, Kappeler *et al.* demonstrated that magnesium citrate exhibited higher bioavailability than mag-

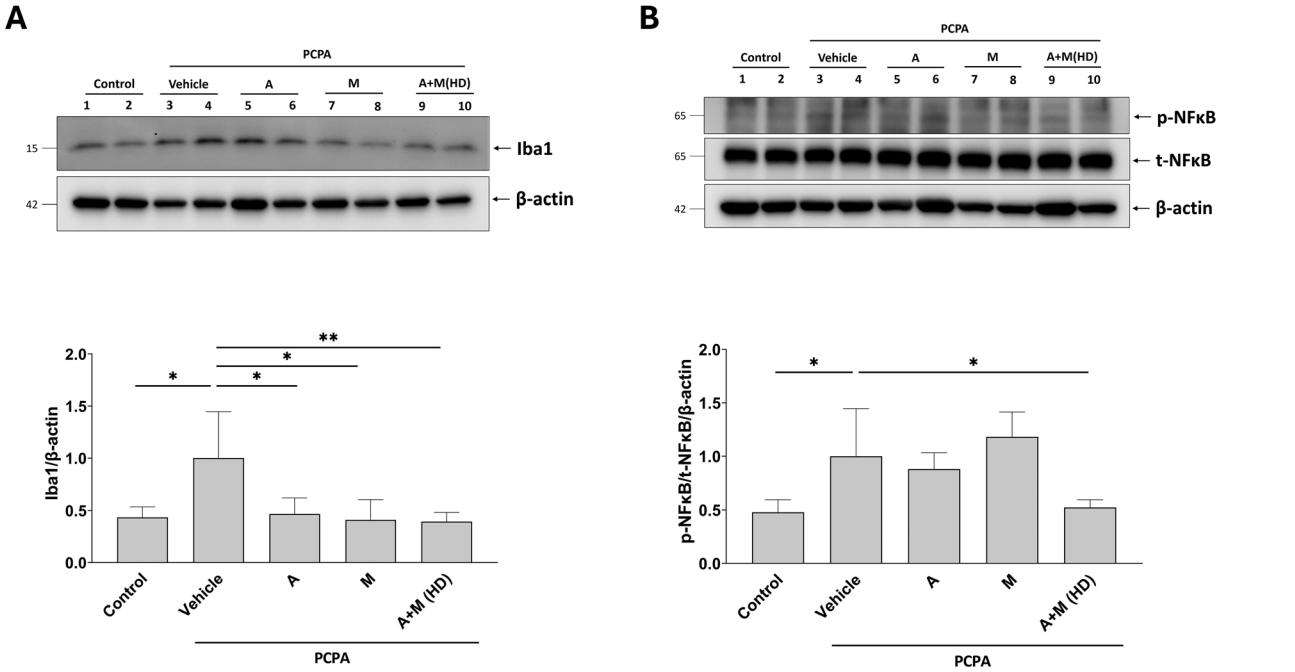

Fig. 7 A + M suppressed neuroinflammation in PCPA-induced mice. Hypothalamic protein expression of (A) Iba1 and (B) phospho-/total NFκB. β-actin was used as loading control. Representative immunoblot results and their quantifications are shown. Data are expressed as mean ± SD; $*p < 0.05$, $**p < 0.01$ *vs.* vehicle group.

nesium oxide in humans.[69] Although the selection of magnesium compounds varies across different studies, these findings may provide some insight into the selection of magnesium compound when composing the A + M formulation.

The hypothalamus plays a vital role in regulating sleep functions through its complex interactions with various neurotransmitter systems and neuropeptides. It is central to both homeostatic and circadian regulation of sleep, integrating signals that influence sleep–wake cycles.[41,77] The hypothalamus is a key component of the hypothalamic–pituitary–adrenal (HPA) axis, which modulates stress responses that can affect sleep quality. Activation of the HPA axis typically results in lighter sleep and increased nocturnal awakenings, while insufficient sleep can heighten HPA activity, creating a feedback loop that exacerbates insomnia.[78] Hypothalamic dysfunction, such as that induced by PCPA, disrupts the balance of sleep–wake regulation, destabilizing the HPA axis and amplifying stress–sleep feedback loops.

Chronic sleep deprivation or insomnia may contribute to increased pro-inflammatory cytokine activity through hyperactivation of the HPA axis and sympathetic nervous system pathways.[79] TNFα, a major pro-inflammatory cytokine, has been implicated in sleep regulation. While previous animal studies have demonstrated increased TNFα levels in the brains of PCPA-induced insomnia rodents,[46] most human studies are restricted to systemic TNFα levels. Various diseases with elevated levels of TNFα have been reported to be associated with sleeplessness, and treatment with TNFα inhibitors not only alleviated disease symptoms, but also restored sleep patterns.[80] Although it is still unclear how systemic TNFα communicates with the brain in humans, it has been shown that systemic TNFα can induce brain expression of TNFα mRNA in animal studies.[80] In our study, we found increased plasma and hypothalamic levels of TNFα in PCPA-induced mice, indicative of an inflammatory status. However, A + M (HD) treatment suppressed TNFα levels in both the plasma and hypothalamus, an effect that could not be achieved by treatment with apigenin or magnesium alone, and which may contribute to the enhanced sleep-promoting effect of the combination.

The significance of reducing pro-inflammatory cytokines extends beyond sleep regulation; TNFα, and IL-6 which we demonstrated to be downregulated by A + M treatment, are also established mediators of stress, anxiety, and depression. A dysregulated inflammatory response is a core feature linking these conditions and studies have consistently identified increased levels of TNFα and IL-6 in anxious and depressed subjects when compared to healthy controls.[81,82] This creates a mechanistic bridge to the well-documented, bidirectional relationship between sleep disturbance and mood disorders, where poor sleep exacerbates inflammatory signaling and emotional dysregulation, while anxiety and depression drive sleep-disrupting hyperarousal.[83] The capacity of A + M to suppress TNFα and IL-6 thus positions it as an intervention capable of targeting this shared neuroinflammatory pathway. This is supported by evidence showing that inhibiting these cytokines has mood-stabilizing effects: inhibition of TNFα in mouse models ameliorates depressive-like behaviours,[84] and patients with inflammatory diseases treated with TNFα or IL-6

inhibitors show significant improvements in depressive and emotional measures.[85,86] Thus, the intake of apigenin and magnesium could potentially alleviate anxiety and depression not only *via* restoration of sleep but also through direct anti-inflammatory activity.

Microglia are the resident phagocytes in the CNS, playing a critical role in maintaining homeostasis and defence against invasion. Microglial activation is a hallmark of neuroinflammatory responses and has been implicated in sleep regulation through modulation of cytokine release.[46] Since chronic sleep deprivation has been shown to activate microglia,[87] the observed reduction of Iba1 in the hypothalamus of PCPA-induced mice after A + M (HD) treatment indicates suppression of microglial activation, further supporting the anti-inflammatory effect of the combination. Collectively, these findings support a model in which the sleep-promoting effects of apigenin and magnesium are mediated, at least in part, by the suppression of neuroinflammation, as evidenced by the reduction in microglial activation within the hypothalamus.

Our study primarily utilized pentobarbital-induced sleep tests, a well-established model commonly used for research on sleep-promoting therapies. While the model measures sleep duration *via* the behavioural endpoint of loss of righting reflex, it cannot provide direct information on changes in the architecture of natural sleep such as transitions between wakefulness, rapid eye movement (REM), and non-REM (NREM) sleep stages. Furthermore, our study focussed on pro-inflammatory mediators in circulation and in the brain rather than sleep-associated neuropeptides. For example, changes in the levels of orexin affect the sleep–wake cycle through its promotion of wakefulness, while melatonin is a well-known regulator of the circadian rhythm.[88,89] Future studies employing electroencephalography and electromyography assessments would enable the quantification of specific sleep parameters such as REM and NREM sleep latency, duration, bout continuity and fragmentation.[90] Measurement of neuropeptides and inflammatory markers such as orexin, melatonin, and cortisol in the cerebrospinal fluid would provide further insight into the potential involvement of central nervous system signaling and central inflammation. Together, this multimodal-approach would provide a deeper understanding of the sleep-promoting mechanism of A + M.

5. Conclusion

In this study we showed that the A + M combinatory treatment led to improvements in sleep duration under normal sleep conditions and in insomnia models. Mechanistic analyses showed that A + M modulated systemic and hypothalamic TNFα levels and suppressed activation of microglia in the hypothalamus through suppression of NFκB. These findings highlight the potential of A + M as a natural intervention to restore sleep architecture and mitigate neurophysiological deficits associated with insomnia, positioning it as a promising alternative to pharmacological agents.

Author contributions

Wing-Yan Wong: writing – original draft, writing – review & editing, investigation, conceptualization. Brandon Dow Chan: writing – original draft, writing – review & editing, investigation. Hengshen Zhou: investigation. Tsz Chun Leung: investigation. William Chi-Shing Tai: conceptualization, writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interests in the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in the published article.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo04538j.

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