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Treatment of GABA from Fermented Rice Germ Ameliorates Caffeine-Induced Sleep Disturbance in Mice

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Abstract

γ-Aminobutyric acid (GABA), a major inhibitory neurotransmitter in the mammalian central nervous system, is involved in sleep physiology. Caffeine is widely used psychoactive substance known to induce wakefulness and insomnia to its consumers. This study was performed to examine whether GABA extracts from fermented rice germ ameliorates caffeine-induced sleep disturbance in mice, without affecting spontaneous locomotor activity and motor coordination. Indeed, caffeine (10 mg/kg, i.p.) delayed sleep onset and reduced sleep duration of mice. Conversely, rice germ ferment extracts-GABA treatment (10, 30, or 100 mg/kg, p.o.), especially at 100 mg/kg, normalized the sleep disturbance induced by caffeine. In locomotor tests, rice germ ferment extracts-GABA slightly but not significantly reduced the caffeine-induced increase in locomotor activity without affecting motor coordination. Additionally, rice germ ferment extracts-GABA *per se* did not affect the spontaneous locomotor activity and motor coordination of mice. In conclusion, rice germ ferment extracts-GABA supplementation can counter the sleep disturbance induced by caffeine, without affecting the general locomotor activities of mice.

Key Words: Sleep, γ-Aminobutyric acid, Rice germ ferment extracts, Caffeine, Hyperactivity, Anxiety

INTRODUCTION

There is a growing interest in investigating the effect of inhibitory neurotransmission by γ-aminobutyric acid (GABA), which hints a potential benefit in counteracting the sleep disruption induced by various conditions such as stress, diseases and caffeine consumption among many others. GABA is a non-protein, four-carbon amino acid ubiquitously existing in living organisms. It functions as a major inhibitory neurotransmitter in the mammalian central nervous system (Watanabe *et al*., 2002) and is synthesized through the catalysis of glutamic acid decarboxylation by glutamic acid decarboxylase (GAD). One of the major external sources of GABA is in plants as a bioactive component (Narayan and Nair, 1990). In particular, GABA was found to accumulate from water-soaking of rice germ (Saikusa *et al*., 1994). GABA production was then enhanced by various techniques including glutamic acid and calcium solution, or soaking and gaseous treatment of germinated brown rice (Oh, 2003; Komatsuzaki *et al*., 2007). Interestingly, reports have suggested GABA to possess functional health benefits, showing potential therapeutic effects on blood pressure, stress, cancer, and inflammatory diseases (Nakamura *et al*., 2000; Oh and Oh, 2004; Akama *et al*., 2009; Nakamura *et al*., 2009; Tian *et al*., 2011).

Several lines of evidences suggest the role of GABA mechanisms on sleep (Gottesmann, 2002). Studies suggest that GABA receptor agonists and uptake inhibitors alleviate sleep disturbance and help regulate circadian rhythm (Turek and Losee-Olson, 1986; Mathias *et al*., 2001). GABAergic neurons in the thalamus is also thought to play an important role in the regulation of sleep (Juhász *et al*., 1989). Since GABA could be readily available in a dietary formula, finding evidences for its therapeutic benefits in a wide variety of health conditions would be valuable.

For decades, caffeine, or 1,3,7-trimethylxanthine, has been one of the most widely used psychoactive substances due to its worldwide high consumption of soda, coffee, tea, energy drinks, and cocoa, among others (Barone and Roberts, 1996;

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Frary *et al*., 2005; Beckford *et al*., 2015). It has been claimed that 90% of adults in the US take at least a meal or beverage every day which contains caffeine (Frary *et al*., 2005), while the consumption of caffeinated drinks in children and adolescents have increased to up to 48% since three decades ago (French *et al*., 2003). In the US, the average daily consumption of caffeine from beverages is 165 ± 1 mg (>2 years of age) while the 90th percentile consumes up to 380 mg/day (Mitchell *et al*., 2014), which is almost similar with the UK population, including food intake (Fitt *et al*., 2013).

Caffeine, generally known to antagonize adrenergic receptors that modulates an inhibitory effect on neuronal processes (Dunwiddie and Masino, 2001), promotes brain excitation. It is widely known to maintain wakefulness while enhancing alertness and performance in many energy-exhausting tasks (Smit and Rogers, 2002). Moreover, there is a vast majority of literature citing and suggesting the beneficial effects of caffeine to human health, extending its preventive role in metabolic syndromes such as obesity (Westerterp-Plantenga *et al*., 2006; Hino *et al*., 2007). On the other end, many researchers identified evidences of the adverse effects of high caffeine consumption, especially in relation to cardiovascular problems (Heckman *et al*., 2010). In addition, while individuals consume caffeine to enhance their daily performance, they consequently experience insomnia and decreased overall sleep quality (Shirlow and Mathers, 1985; Landolt *et al*., 2004). The proposed benefits of caffeine are seemingly disparaged by its side effects, affecting the quality of life of its consumers. In addition to the use of caffeine as a model system to induce sleep disturbance in animals and human in laboratory settings, an agent to neutralize the sleep-disrupting properties of caffeine should be beneficial in everyday life as well.

In this study, we evaluated the attenuating effect of GABA extract obtained from rice germ ferment (rice germ ferment extracts-GABA, RFE-GABA) to caffeine-induced sleep disruption. We hypothesized that the sleep onset delay and sleep reduction in mice caused by caffeine may be counteracted by GABA. Furthermore, it is our aim to show that RFE-GABA can supplement caffeine to improve sleep without affecting the overall locomotor performance in mice.

MATERIALS AND METHODS

Animal care and treatment

Male ICR mice weighing 24-30 g were obtained (Hanlim Animal Co., Hwasung, Gyeonggi-do, Korea) and used in the experiments. The animals were maintained in cage groups in a room with automated systems for lights on and off cycle every 12 h (7AM: 7PM) and a constant temperature (22 ± 2^{o}C) and humidity (55 ± 5%). They were given food and water *ad libitum*, except for the night before experiments when food was removed. The number of animals used in every experiment and the extent of their sufferings were carefully minimized. Treatment and maintenance of mice were done in accordance to the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985)(National Institute of Health, 1985, Guide for the care and use of laboratory animals) and the Animal Care and Use Guidelines of Sahmyook University, Korea (SYUIACUC2014-017).

Materials

Rice germ ferment extracts containing 15% GABA (w/w) (RFE-GABA) obtained from Biovan Co. Ltd (Chunchen, Korea) was dissolved in distilled water just before the experiment, while the caffeine powder (Sigma-Aldrich, St. Louis, MO, USA) is prepared in saline. Pentobarbital sodium was obtained from Hanlim Pharm. Co., Ltd. (Seoul, Korea) and diazepam from Samjin Pharm. Co., Ltd. (Seoul, Korea). Maltodextrin (Nutriose) which was used as vehicle of RFE-GABA was purchased from Roquette Freres (Lestrem, France).

Treatments with RFE-GABA and caffeine

Forty-five minutes prior to pentobarbital-hypnosis test and 1h before open field and rotarod tests, mice were treated with RFE-GABA extracts (10, 30, 100 mg/kg, p.o.), maltodextrin extracts (100 mg/kg), or distilled water (vehicle). To induce sleep disruption, caffeine (10 mg/kg) (Huang *et al*., 2005) was given intraperitoneally (i.p.) 30 min before the above-mentioned tests. The caffeine dosage was selected after our preliminary test yielded a result demonstrating 10 mg/kg significantly induced sleep disruption over 5 and 15 mg/kg.

Pentobarbital-induced sleep

Pentobarbital-induced sleep was conducted according to previously established methods with slight modifications (Ojima *et al*., 1995). Forty-five minutes after RFE-GABA treatment and 30 minutes after caffeine administration, sodium pentobarbital (42 mg/kg) (Ma *et al*., 2009) was administered intraperitoneal in each mouse. The time elapsed from the administration to the loss of righting reflex (sleep onset) and the time from the loss of righting reflex to its return (sleep duration) were measured in seconds.

Open field test

To evaluate locomotor activity (Vuillermot *et al*., 2011), mice were gently placed in an open-field arena made of a square Plexiglas container (42×42 cm) with its field bordered with 42 cm sidewalls. A 2 min habituation was done before the actual test to remove the bias of novelty. Distance travelled and movement duration were observed and measured for 10 minutes using Ethovision (Noldus Information Technology, Wageningen, Netherlands) system.

Rota-rod test

Motor control, balance, and coordination were tested using rota-rod test. Mice were trained a day prior to the experiment through placing them on a rotating rod at 36 r.p.m. over a period of 3 min. During the actual test, latency to fall and falling frequency were observed and recorded for 10 min.

Statistical analysis

All results were presented as means and standard error of the mean (± S.E.M.). One way analysis of variance (ANOVA) was applied to analysis statistical significance followed by Dunnett's post hoc test which compare the effects of each group versus the vehicle group. All statistical analyses were conducted using GraphPad Prism Version 4.01 software (California, USA).

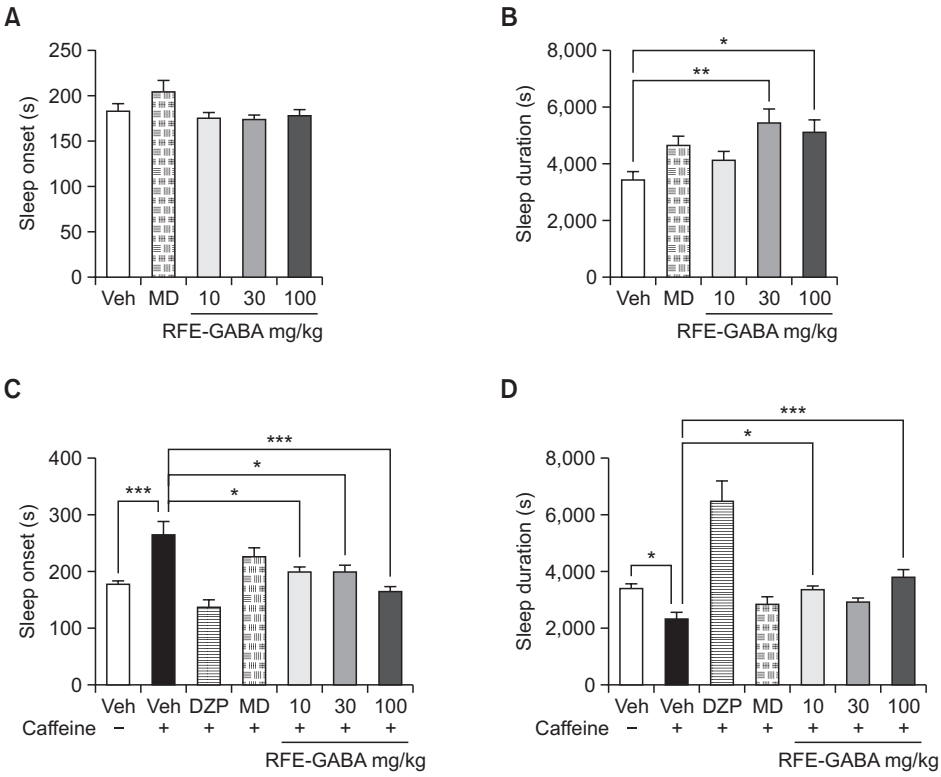

Fig. 1. Effects of RFE-GABA treatment on onset (A) and duration (B) of sleep in pentobarbital-induced sleeping test. Caffeine was introduced 30 min prior to the test to induce sleep disruption in mice. Figures 1C and 1D show the effects of RFE-GABA in pentobarbital-induced sleeping test after caffeine administration. Each bar represents the mean ± S.E.M. and statistics were analyzed using one-way ANOVA. **p*<0.05, ***p*<0.01, and ****p*<0.001. Abbreviations: Veh: vehicle; MD: maltodextrin; DZP: diazepam; RFE-GABA: rice germ fermented extract of γ-Aminobutyric acid (Each group, n=10).

RESULTS

RFE-GABA treatment improves sleep duration from caffeine-induced sleep disturbance model

Test materials were administered 45 minutes prior to pentobarbital-induced sleeping test. Under this condition, RFE-GABA did not show any differences in the onset of sleep (Fig. 1A) compared to its vehicle groups using one-way ANOVA [$F_{(4,49)}$=2.150, p<0.0901]. However, RFE-GABA exhibited a significant effect on sleep duration of mice compared to the vehicle treatment [$F_{(4,43)}$=4.709, p<0.0034], especially at 30 and 100 mg/kg dosages, but not at 10 (Fig. 1B).

To test whether RFE-GABA can counteract the sleep disrupting effect of caffeine, we added the administration of caffeine 10 mg/kg i.p. and diazepam 10 mg/kg i.p. (positive vehicle) in another set of animals following the above-mentioned procedure. Using one-way ANOVA, significant differences were seen among the test materials in the sleep onset time (Fig. 1C) of mice [$F_{(6,105)}$=6.976, p<0.0001]. In the post hoc comparisons, sleep onset time of caffeine-treated group was significantly increased as compared to the vehicle group (p<0.001). Sleep onset time of RFE-GABA-treated group at 10, 30 and 100 mg/kg were dose-selectively normalized to vehicle sleep onset time (p<0.05, p<0.05, p<0.001, respectively). Sleep duration (Fig. 1D) analysis of one-way ANOVA also showed significant differences among test materials [$F_{(6,101)}$=17.36, p<0.0001]. Post-hoc analysis revealed reduced level of sleep duration in caffeine-treated group compared to vehicle group (p<0.05). Interestingly, sleep duration of RFE-GABA-treated groups at 10 and 100 mg/kg were normalized to vehicle levels (p<0.05 and p<0.001, respectively vs caffeine), but not at 30 mg/kg. The overall result suggests that caffeine affects the sleep quality of mice and treatment of RFE-GABA could counteract this caffeine-induced sleep disruption.

Effects of RFE-GABA treatment on the hyper-locomotive activity of caffeine in mice

RFE-GABA was assessed for ameliorating effects on caffeine-induced increase in spontaneous locomotor activity in mice. RFE-GABA and their vehicles were administered 1h prior to the experiments while caffeine was administered intraperitoneally, 30 minutes after RFE-GABA was given. One-way ANOVA analysis revealed no significant difference among groups in the distance moved [$F_{(5,48)}$=1.174, p<0.3377], but an effect was observed in the movement duration [$F_{(5,48)}$=3.751, p<0.0066] (Fig. 2A, 2B). Post hoc analysis of movement duration parameter showed a significant increase in caffeine and caffeine+MD treated groups compared to vehicle group (both p<0.01). It must also be noted that groups administered with RFE-GABA have slightly lowered levels in both parameter, though not significant.

In the center area analysis (Fig. 2C, 2D), the duration of stay parameter [$F_{(5,48)}$=3.066, p<0.0183] revealed an increase in time spent in the center area of the caffeine-treated group

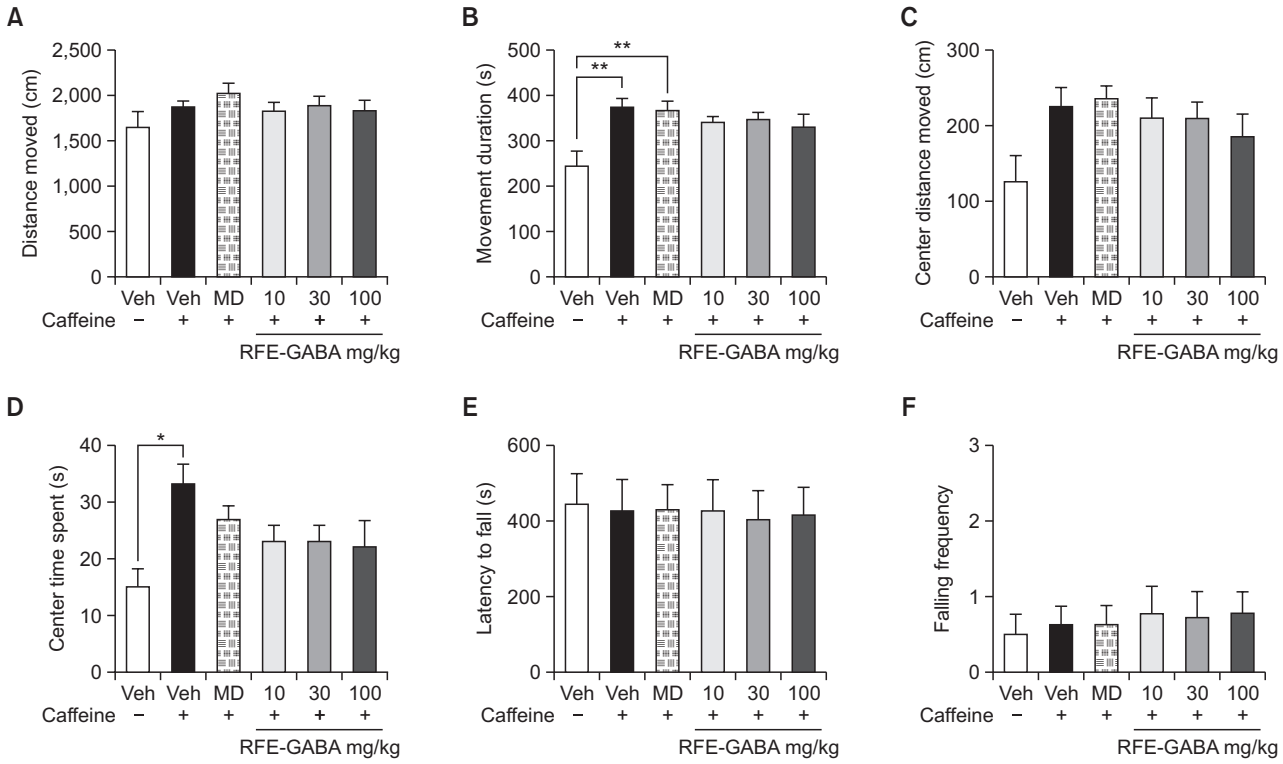

Fig. 2. Effects of RFE-GABA treatment on caffeine-induced locomotor activity and coordination of mice. Open field test was performed measuring the distance moved (Fig. 2A), movement duration (Fig. 2B), distance moved in the center (Fig. 2C), and time spent in the center (Fig. 2D) parameters. Fig. 2E and 2F demonstrate the effects of RFE-GABA on motor coordination and balance of mice under caffeine treatment by counting the latency to fall and falling frequency through rotarod test. Each bar represents the mean ± S.E.M. and statistics were analyzed using one-way ANOVA. *p<0.05 and **p<0.01. Abbreviations: Veh: vehicle; MD: maltodextrin; DZP: diazepam; RFE-GABA: rice germ fermented extract of γ-Aminobutyric acid (Each group, n=10).

as compared to the vehicle group (p<0.05). There is no significant differences between groups, however, in the distance moved [$F_{(5,48)}$=2.158, p<0.0757]. Notably, RFE-GABA administration tends to normalize the caffeine-induced increased locomotor activity and decreased anxiety of mice in the center area showing slight decreases in both parameters. But the rotarod motor performance of mice (Fig. 2E, 2F) was not affected by caffeine and RFE-GABA administrations in both the latency to fall [$F_{(5,48)}$=0.03267, p<0.9994] and fall frequency [$F_{(5,48)}$=0.1294, p<0.9849] parameters.

These findings show the movement-enhancing activity of caffeine by inducing increased spontaneous locomotor activity without affecting motor coordination and balance. Furthermore, the administration of RFE-GABA could slightly normalize the effects of caffeine on hyperlocomotion as well as on the decreased anxiety and/or impulsivity.

RFE-GABA treatment does not affect the locomotor performance in mice

To confirm that RFE-GABA does not induce sedative properties that may affect the spontaneous locomotor activity and motor coordination of mice, open field and rotarod tests were performed 1 h after designated treatments. In the open field test, one-way ANOVA revealed no decrement in the parameters of locomotor activity of RFE-GABA-treated mice (Fig. 3A, 3B) in both distance moved [$F_{(4,98)}$=0.1268, p<0.9724] and movement duration [$F_{(4,98)}$=0.1077, p<0.9796]. Center area movement analysis of anxiety and exploratory behavior (Fig. 3C, 3D) also did not differ between groups in both distance moved [$F_{(4,98)}$=0.4397, p<0.7796] and time spent parameters [$F_{(4,98)}$=0.1347, p<0.9691]. Finally, in the rotarod test assessment of balance and motor coordination (Fig. 3E, 3F), no significant effect was observed among the treatment groups in both latency to fall [$F_{(4,94)}$=0.1259, p<0.9728] and fall frequency [$F_{(4,97)}$=0.6109, p<0.6558]. These results affirm that RFE-GABA itself does not affect the spontaneous locomotor activity and motor coordination of mice under normal conditions.

DISCUSSION

In this study, we demonstrated administration of GABA from rice germ ferment extracts counteracts caffeine-induced sleep disturbance such as sleep onset delay and decreased sleep reduction in mice. Our results showed increased sleep duration from RFE-GABA administration, which remarkably neutralized the sleep disrupting effects of caffeine. In addition, RFE-GABA treatment did not influence the general locomotor performance of mice. More interestingly, RFE-GABA showed a tendency to mitigate the caffeine-induced increase in locomotor activity of mice. Furthermore, RFE-GABA slightly, albeit not significant, neutralized the caffeine-induced decreased anxiety-like behavior. To our knowledge, this is the first study showing the prospect of RFE-GABA supplementation in allevi-

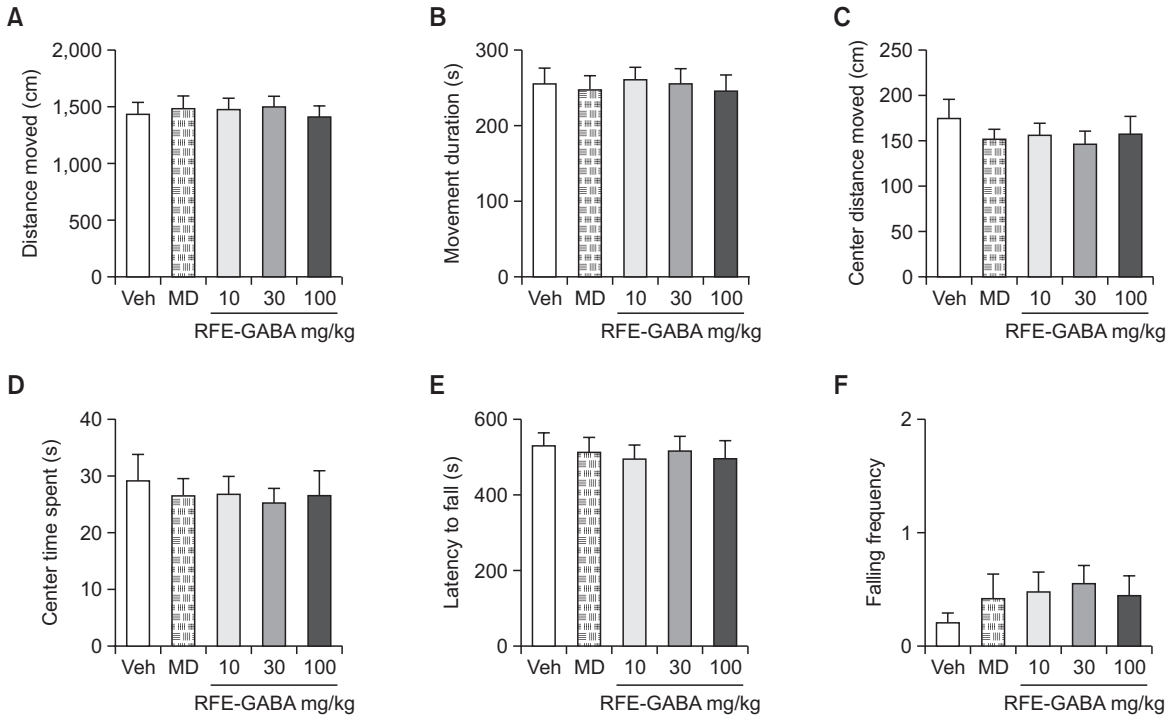

Fig. 3. Effects of RFE-GABA treatment on spontaneous locomotor activity and coordination of mice. Open field test was performed measuring the distance moved (Fig. 3A), movement duration (Fig. 3B), distance moved in the center (Fig. 3C), and time spent in the center (Fig. 3D) parameters. Fig. 3E and 3F demonstrate the effects of RFE-GABA on motor coordination and balance of mice by counting the latency to fall and falling frequency through rotarod test. Each bar represents the mean ± S.E.M. and statistics were analyzed using one-way ANOVA. No significance was observed. Abbreviations: Veh: vehicle; MD: maltodextrin; DZP: diazepam; RFE-GABA: rice germ fermented extract of γ-Aminobutyric acid (Each group, n=10).

ating sleep interference induced by caffeine.

While it is well understood that caffeine mainly antagonizes the adrenergic receptors to induce excitatory states (Dunwiddie and Masino, 2001), the relationship of GABA supplementation and sleep enhancement, and how GABA improves sleep under caffeine induction are still to be explored. A study performed by Kardos and Bandl demonstrated an allosteric inhibitory effect of caffeine to $GABA_A$ receptors (Kardos and Blandl, 1994). In addition, caffeine may directly affect the levels of GABA neurotransmitters in yet unknown pathways (Fredholm *et al*., 1999). Another study revealed that caffeine administration increased the levels of 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP or allopregnanolone), a strong positive allosteric modulator of $GABA_A$ receptors in the brain (Concas *et al*., 2000). In this regard, we can postulate that caffeine's modulatory action to $GABA_A$ receptors could be counteracted by the increased availability of GABA neurotransmitters to be utilized by GABAergic neurons.

There are increasing evidences of GABA targeting receptors and pathways other than the expected GABA receptors. Recently, it was found that GABA treatment in mice increases immunoreactivity and protein levels of adenosine A1 receptors (AA1R) in the circadian rhythm-controlling suprachiasmatic nucleus of the hypothalamus (Ahn *et al*., 2014). AA1R is thought to play an important role in the sleep-promoting effects of adenosine by inhibiting hypocretin/orexin neuronal activity (Liu and Gao, 2007). It is indeed interesting to note in our study that GABA treatment was able to enhance sleep under caffeine that acts as a non-selective antagonist at adenosine receptors. Thus, it is also possible that GABA targets other receptors and independent pathways which have direct involvement in sleep physiology. For example, GABA may increase the levels of melatonin and serotonin in the brain adding to its sleep-promoting properties (Kim *et al*., 2010). Indeed, there is a need for further mechanistic studies to explain the action of GABA extracts in the brain.

It is noteworthy to mention that caffeine prompted an increased locomotor activity and decreased anxiety-like behavior in mice, which could imply an induction of hyperactivity. Although statistically insignificant, RFE-GABA supplementation slightly neutralized these caffeine-induced behaviors. It can be inferred that RFE-GABA supplement may have calming properties, but would not actually affect the level of locomotor performance in mice when administered alone. Though caffeine-induced psychomotor activation had been previously found and modelled (Estler, 1979; Skoog *et al*., 1986), recent studies have introduced caffeine as a mild stimulant improving attention in individuals with attention deficit hyperactivity disorder (ADHD) (Caballero *et al*., 2011; Pandolfo *et al*., 2013; Ioannidis *et al*., 2014). Thus, it would be valuable to consider that whereas caffeine may improve attention in attention-deficient individuals, there could be a consequent increase in locomotor activity as an independent pathway in vehicle subjects as is the case in amphetamine-stimulated animals. More interestingly, it would also be of worth to test the possible effects of RFE-GABA in relation to drug-induced and strain-dependent

animal models of hyperactivity.

Caffeine has been generally correlated to be anxiogenic, but only in exceptionally high dosages, which could be rarely achieved in human average consumption. However, this study found that caffeine at a lower dosage (10 mg/kg) induced anxiolytic (hence impulsive) effect in mice, in contrast to its anxiogenic properties at higher dosages (Jain *et al*., 2005). Several researchers commonly utilized caffeine-induced model of anxiety using higher doses (Baldwin and File, 1989; Youngstedt *et al*., 1998). Interestingly, during an elevated plus maze test of anxiety in rats using different caffeine dosages, 5 and 10 mg/kg caffeine did not induce anxiogenesis while 50 and 100 mg/kg caffeine brought a remarkably increased anxiety-like behavior (Jain *et al*., 2005). Thus, at lower dose, caffeine appears to have little or no effect in anxiety or possibly even decrease anxiety levels (Smith, 2002). We can also infer that the increased time spent of caffeine-induced mice in the center of the open field may indicate increased exploratory or response behavior to a novel environment (dela Peña *et al*, 2014). Thus, the effect of caffeine in increasing center field duration of stay in mice might be related to an elevated mood or increased attentive behavior.

Finally, in showing the promising potential benefits of RFE-GABA administration in counteracting caffeine's wakeful effects in mice, advancing the investigation of GABA pharmacokinetics and mechanisms of actions at the cellular and organ level would be an essential subsequent step. Based in the scope of this study, interpretation of the results are still limited. Nevertheless, our study promotes the investigation of RFE-GABA supplementation for its concrete therapeutic benefits in few studied conditions including our newly revealed findings in alleviating caffeine-induced sleep disruption. Eventually, RFE-GABA could undergo clinical trials as a novel nutraceutical supplement for sleep. Henceforth, in addressing the sleep problems of caffeine consumers, there is a great benefit in introducing nutraceutical agents from plant resources, as they are generally considered safe and easily produced.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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