Regular Article

Polyherbal Formulation Enhancing Cerebral Slow Waves in Sleeping Rats

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Received March 27, 2020; accepted June 12, 2020

Polyherbal medicines are composed of multiple herbs and have traditionally been used in East Asian countries for the remedy of physiological symptoms. Although the effects of polyherbal formulations have been investigated at the molecular and behavioral levels, less is known about whether and how medicinal herbs affect the central nervous system in terms of neurophysiology. We introduced a novel blended herbal formulation that consisted of 35% linden, 21% mulberry, 20% lavandin, 20% butterfly pea, and 4% tulsi. After intraperitoneal administration of this formulation or saline, we simultaneously recorded epidural electrocorticograms (ECoGs) from the olfactory bulb (OB), primary somatosensory cortex (S1), and primary motor cortex (M1), along with electromyograms (EMGs) and electrocardiograms (ECGs), of rats exploring an open field arena. Using the EMGs and OB ECoGs, we segmented the behavioral states of rats into active awake, quiet awake, and sleeping states. Compared to saline, herbal medicine significantly shortened the total sleep time. Moreover, we converted the ECoG signal into a frequency domain using a fast Fourier transform (FFT) and calculated the powers at various ECoG oscillation frequencies. In the sleeping state, a slow component (0.5–3 Hz) of S1 ECoGs was significantly enhanced following the administration of the formulation, which suggests a region- and frequency-specific modulation of extracellular field oscillations by the polyherbal medicine.

Key words electrocorticogram; herbal medicine; primary somatosensory cortex; open field; rat; slow wave

INTRODUCTION

The effects of polyherbal formulations on brain functions, such as memory and learning, have been widely documented at the molecular and behavioral levels. For example, Abana, an Ayurvedic herbomineral preparation, reduces brain cholinesterase activity.¹⁾ Another polyherbal formulation, Bramhi Ghrita, enhances memory retention.²⁾ In addition, there are other polyherbal formulations enhancing learning and memory.³⁾ However, most of these studies have examined the effects of formulations solely in molecular and behavioral experiments. Therefore, it remains almost unknown how polyherbal medicines exert neurophysiological impacts on central activity.

As one example of a polyherbal formulation, we focused on a mixture of five medicinal herbs, linden (*Tilia* spp.), mulberry (*Morus alba* L.), lavandin (*Lavandula × intermedia*), butterfly pea (*Clitoria ternatea* L.), and tulsi (*Ocimum gratissimum*, also known as holy basil). Of these medicinal herbs, linden and tulsi have anxiolytic effects.^{4–6)} Mulberry and lavandin have antioxidant effects,^{7–9)} while butterfly pea facilitates memory retention.¹⁰⁾ Therefore, we reasoned that their mixture would have a central impact. To assess the effects of these herbs on central functions, we developed the novel herbal formulation TuMBuLLa, named after the abbreviation of tulsi, mulberry, butterfly pea, linden, and lavandin, and we intraperitoneally administered the formulation in rats and monitored their behaviors and the neuronal activity of the cerebral cortex.

MATERIALS AND METHODS

Animal Ethics Animal experiments were performed with

the approval of the Animal Experiment Ethics Committee at the University of Tokyo (approval number: P29-7) and according to the University of Tokyo guidelines for the care and use of laboratory animals. These experimental protocols were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006) and the Guidelines on the Method of Animal Disposal (Prime Minister's Office, Notice No. 40 of 1995). All efforts were made to minimize animal suffering.

Animal Preparation Male 8- to 10-week-old Wister rats (Japan SLC, Shizuoka, Japan) with a preoperative weight of 300–450 g were individually housed under conditions of controlled temperature and humidity (22 ± 1°C, 55 ± 5%) and maintained on a 12/12-h light/dark cycle (lights off from 07:00 to 19:00) with *ad libitum* access to food and water. Rats were habituated to an experimenter by daily handling for 2 d before the experiments.

Surgery After a rat was anesthetized with 2–3% isoflurane gas, two wire electrodes (stainless-steel wires, 15 cm long, 0.147 mm in diameter; AS633, Cooner Wire Company, CA, U.S.A.) were implanted in both the left and the right pectoral muscles to record electrocardiograms (ECGs), and one wire electrode (AS633) was implanted in the trapezius to record electromyograms (EMGs).¹¹⁾ The scalp was then removed with a surgical knife. An approximately 1.0-mm diameter craniotomy was performed using a dental drill. Stainless-steel screws (1.4 mm in diameter, 3 mm in length) were used

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to record electrocorticograms (ECoGs) from the primary somatosensory cortex (S1) and the primary motor cortex (M1),¹²⁾ whereas a smaller screw electrode (1.0 mm in diameter, 4 mm in length) was used to record ECoGs from the olfactory bulb (OB). The three screw electrodes were stereotactically implanted into the left S1 (2.16 mm posterior and 2.8 mm lateral to bregma), the left M1 (3.24 mm anterior and 3.0 mm lateral to bregma), and the left OB (10.00 mm anterior and 1.0 mm lateral to bregma). In addition, another two stainless-steel screws were implanted in the bone above the cerebellum as ground and reference electrodes. The recording device and the electrodes were secured to the skull using stainless-steel screws and dental cement. Following surgery, each rat was individually housed in transparent Plexiglass cages with free access to water and food for a week. After 4 d of recovery from surgery, the weight of each rat was reduced to 85% of their *ad libitum* weight through limited daily watering, while food was readily available.

Drug The polyherbal formulation TuMBuLLa was provided by an industrial source (HTT-B, ikoa Ltd., Tokyo, Japan) and was composed of 35% linden, 21% mulberry, 20% lavandin, 20% butterfly pea, and 4% tulsi. TuMBuLLa was extracted with 100°C distilled water at 2.2% (w/v) for 5 min. NaCl (0.9% (w/v)) was then added to the extracted solution.

Apparatus The open field used in this study measured 40 cm in width, 40 cm in depth, and 40 cm in height. The walls were made of black-painted wood. For habituation, each rat was placed in the open field arena and was allowed to freely explore for 4 h. This habituation procedure was repeated for approximately 4 d.

Electrophysiology Following familiarization with the open arena, either saline or the herbal extract (15 mL/kg for both) was intraperitoneally administered to each rat. Immediately after administration, each rat was placed in the open

field, and the recording electrode assembly was connected to a digitally programmable low-noise amplifier (C3324, Intan Technologies, CA, U.S.A.). The output of the head stage was conducted through an SPI cable (C3216, Intan Technologies) and a commutator to the acquisition board (Open Ephys, MA, U.S.A.).¹³⁾ We recorded ECoGs, EMGs, and ECGs for 4 h. The electrophysiological signals were amplified and digitized at 2 kHz.

The moment-to-moment behavior of the rat was monitored using a USB camera above the open arena. The videos were recorded at 20 frames per second. At every moment capturing an image, a transistor-transistor logic pulse was sent to a desktop computer *via* the acquisition board to synchronize the video clips with the electrophysiological signals.

Data Analysis The data were analyzed using custom-made Python routines. The summarized data are reported as the mean $\pm$ the standard error of the mean (S.E.M.). $p < 0.05$ was considered statistically significant unless otherwise specified.

The ECoG signals were offline filtered between 0.1 and 500 Hz and were denoised through a notch filter that rejected the humming-noise frequencies between 49.38 and 50.62 Hz. We categorized the behavioral states into active behaving and immobile (including quiet awake and sleeping) states using video clips and EMGs. We utilized DeepLabCut,¹⁴⁾ a marker-less tracking system (Fig. 1A), to track the rats' moment-to-moment positions and calculate the time spent in the center (*i.e.*, 12 $\times$ 12 cm) of the open arena.¹⁵⁾ Trajectories taken by rats were calculated by differentiating the *x* and *y* coordinates of the head and were manually defined using a threshold for discriminating between the active behaving and immobile states; note that large and small displacements in the coordinates were basically categorized into the behaving and immobile states, respectively, together based on the EMG amplitudes.

Fig. 1. TuMBuLLa Reduces Sleep Time

A, Machine-learning-based behavioral analyses of rats. *Left*: A representative top view of the open arena for recording. The blue circle and the pale blue lines indicate the position of the rat's head detected by the DeepLabCut algorithm and the edges of the recording arena, respectively. *Middle*: A representative 210-min trajectory of the rat shown in the left panel. *Right*: A pseudocolor map of the time spent in each subarea of the open arena. Warmer colors indicate longer sojourn times. **B**, Behavioral analyses of rats. *Left*: Time spent sleeping in rats treated with saline and TuMBuLLa. * $p = 2.91 \times 10^{-2}$, $t_4 = 3.33$, $n = 6$ rats, Student's *t*-test. *Middle*: Distance traveled in rats treated with saline or TuMBuLLa. $p = 4.48 \times 10^{-1}$, $t_4 = 8.41 \times 10^{-1}$, $n = 6$ rats, Student's *t*-test. *Right*: Time spent in the center in rats treated with saline or TuMBuLLa. $p = 3.81 \times 10^{-1}$, $t_4 = 9.85 \times 10^{-1}$, $n = 6$ rats, Student's *t*-test. Note that the mean value (0.15) is displayed in parentheses. (Color figure can be accessed in the online version.)

Fig. 2. The Herbal Extract Increases Slow Wave Power during Sleeping

A, A top-view diagram of ECoG recording sites (OB, M1, and S1, *pale blue*) and the ground/reference sites (*black*). **B**, Representative traces of ECoGs simultaneously recorded from the OB (*top*), M1 (*middle*), and S1 (*bottom*). **C**, Time changes in the slow wave powers in the S1, M1, and OB during sleeping. * $p = 2.57 \times 10^{-2}$, $t_4 = 3.47$, $n = 3$ rats, Student's *t*-test. The *black* and *green* symbols indicate treatment with saline and TuMBuLLa, respectively. (Color figure can be accessed in the online version.)

We further manually defined another threshold for dividing the immobile states into quiet awake and sleeping states using the EMGs and the OB ECoGs.¹⁶⁾ The EMG signals from the first 60 min were used to determine the two thresholds.

The recording periods were split into multiple 30-min segments. For each segment, we detected sleeping periods in 1-min units and applied a fast Fourier transform (FFT) to each ECoG signal during the 1-min sleeping period to obtain the power spectrum. We calculated the FFT powers as areas under the spectra for frequencies of 0.5–3 Hz (slow wave), 4–8 Hz (theta), 8–16 Hz (beta), and 16–32 Hz (alpha). We repeated this procedure for the other segments (Fig. 2C).

RESULTS

To assess the effects of TuMBuLLa on behavioral neurophysiology, we intraperitoneally injected saline or TuMBuLLa into the rats and recorded the ECoGs from the S1, M1, and OB as well as EMGs and ECGs while rats freely explored an open arena for 3.5 h (Figs. 2A, B), with simultaneous top-view video monitoring of their behavior (Fig. 1A). At least 2 d after the initial experiment, we performed the same experiment with the other drug, either saline or TuMBuLLa.

The total distances traveled by the rats were not significantly different between the rats treated with saline and those treated with TuMBuLLa ($p = 0.448$, $t_4 = 0.841$, $n = 6$ rats, Student's *t*-test; Fig. 1B). The total times spent exploring the center of the arena¹⁵⁾ were also not significantly different between the two conditions ($p = 0.381$, $t_4 = 0.985$, $n = 6$ rats, Student's *t*-test; Fig. 1B). Thus, it seemed unlikely that TuMBuLLa had anxiolytic effects. Video monitoring and EMG recordings revealed that the total sleep time was shorter in TuMBuLLa-treated rats than in saline-treated rats ($p = 2.91 \times 10^{-2}$, $t_4 = 3.33$, $n = 6$ rats, Student's *t*-test; Fig. 1B).

Thus, we focused on neurophysiological signals in the sleeping state. We scrutinized the effect of TuMBuLLa on the FFT spectra at various oscillation frequencies during the sleeping state and found that the slow wave (0.5–3 Hz) power in the S1 gradually increased after TuMBuLLa administra-

tion (Fig. 2C). After 180 min, the slow wave power in the TuMBuLLa-treated rats was significantly higher than that in the saline-treated rats ($p = 2.57 \times 10^{-2}$, $t_4 = 3.47$, $n = 6$ rats, Student's *t*-test), but this effect was not observed in the slow wave power in the M1 or OB (Fig. 2C). TuMBuLLa did not affect the power at the other assessed frequencies (data not shown).

DISCUSSION

In this study, we developed TuMBuLLa, a novel herbal extract, and evaluated its effect on *in vivo* neurophysiological signs. Intraperitoneal administration of TuMBuLLa strengthened the slow wave power in the S1.

We performed an open field test to assess the anxiolytic effects of the polyherbal formulation,¹⁷⁾ which would be validated more reliably by other behavioral test batteries, such as the elevated plus-maze test, the light dark test, the zero maze test, and the social interaction test.^{18,19)} Previous reports demonstrated an anxiolytic effect of linden and tulsi,^{4,5)} which was not observed in this study. This contradiction may stem from the difference in the extraction method and the administration route. The herbal formulation used in this study was extracted with distilled water, while the previous study used nonpolar solvent, followed by chromatographic separation.⁴⁾ Thus, our extract was a mixture mainly of water-soluble polar compounds and did not contain purified nonpolar compounds. Moreover, although drugs were orally administered in some previous studies,^{4,10)} we chose intraperitoneal injection over oral administration to assess the direct effect of herbal extract. Drug effects could vary more widely for oral administration because of the individual differences in digestive systems between animals. In addition, as herbal extracts are absorbed into tissues *via* digestive organs, some compounds are decomposed. Therefore, we assumed that oral administration would mask the direct and systemic effects of the herbal formulation and thus administered herbal extracts intraperitoneally, not orally.

For decades, the two-process model has been believed to

be one of the convincing mechanisms for sleep and awakening rhythms.^{20–23)} In this model, some sleepiness-related substances produced in the body accumulate as an animal stays up until the amount of the substances reaches the sleep threshold. This sleepiness produced by the lack of sleep (*i.e.*, sleep debt) is called ‘process S.’ Process S builds up sleep pressure and drives homeostatic sleep during arousal, but it vanishes during sleep. In contrast, process S is suppressed by arousal signals, called ‘process C,’ in the daytime. The balance between process S and process C generates sleep and awakening rhythms.^{20–23)} In accordance with this model, the reduction in the sleep time caused by the herbal formulation suggests that the formulation eliminated process S more quickly than saline. Moreover, the speed of the dissipation of the sleepiness is proportional to slow wave activity.²⁴⁾ Thus, the high power of slow waves reduced the total sleep time *via* quick dissipation of the sleep need accumulated during arousal.

Consistent with the previous study,²⁵⁾ we found locally confined enhancement of the slow wave, which serves as an indicator of non-rapid eye movement sleep depth and sleep homeostasis.²²⁾ Specifically, our FFT analyses demonstrated that the slow wave power in the S1, but not in either the M1 or the OB, was significantly increased in TuMBuLLa-treated rats. This region-specific enhancement of slow oscillations may be dependent on the difference in the amount of acetylcholine release during the day (*i.e.*, when rats habitually sleep) and night (*i.e.*, when rats actively behave). When rats are likely inactive in the daytime, less acetylcholine is released in the S1 than in the M1, whereas the amounts of acetylcholine released in the S1 and M1 are almost the same when rats are behaviorally active at night.²⁶⁾ Because acetylcholine release generally decreases the power of cortical slow waves,²⁷⁾ less acetylcholine release in the S1, not in the M1, caused the region-specific enhancement of the slow wave power. These findings can explain the relationship between S1-specific enhancement of the slow wave and the reduction in total sleep time in TuMBuLLa-treated rats. Neocortical slow waves during sleep are critical in learning and memory²⁸⁾; thus, TuMBuLLa may have remedial effects on memory and learning by modulating slow waves.

Acknowledgments This work was supported by JST ERATO (JPMJER1801), JSPS Grants-in-Aid for Scientific Research (18H05525, 20K15926), and the Human Frontier Science Program (RGP0019/2016). This work was conducted partially as a program at the International Research Center for Neurointelligence (WPI-IRCN) of the University of Tokyo Institutes for Advanced Study at The University of Tokyo.

Conflict of Interest MA is an employee of ikoa Ltd. KY, NM, and YI have used experimental materials provided by the company. However, the company has had no arbitrary control over the research design, interpretation, writing, or publication of this work.

Supplementary Materials The online version of this article contains supplementary materials.

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