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Journal Article

INHIBITORY EFFECTS OF TONGKAT ALI (*Eurycoma longifolia*) EXTRACT ON LIGHTING STRESS-INDUCED SLEEP DISORDERS IN PIGS

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SUMMARY

Tongkat Ali (*Eurycoma longifolia*), traditionally used as an herbal medicine in Southeast Asia, is known to have a variety of effects, including stress reduction. The authors divided 5-month-old pigs (three males and three females) into four groups consisting of a control group, which was not administered Tongkat Ali extract (TAE), and three groups administered TAE at 0.25%, 0.50%, or 0.75% of their feed, respectively, and observed the pigs for a period of eight weeks under conditions of disturbed sleep due to prolonged illumination. In terms of observation items, the pigs' vitality, appetite, fecal characteristics, respiration, emaciation, anxiety, restlessness, body temperature, body weight, and salivary amylase were measured as clinical symptom scores, and the intestinal microbiota in their feces was analyzed using next-generation sequencing (NGS). Although members of the control group showed anxiety and restless behaviors; such stress behaviors were clearly inhibited in the groups administered with TAE. Groups of pigs administered with TAE at the ratios of 0.25% and 0.50% showed a decrease in salivary amylase concentration ($p<0.05$) whereas the results from the control and 0.75% group were not significant. Analyses of intestinal bacteria using NGS showed that *Lachnospiraceae*, a family of bacteria known for producing butyric acid, increased significantly in the group administered with TAE at 0.50%, whereas it was not changed in the other groups. Based on the above findings, administration of TAE to fattening pigs under high levels of lighting-induced stress is expected to be effective and administration of TAE at 0.25% of the feed intake in particular, has been shown to effectively inhibit behaviors associated with stress.

Keywords: pig, illumination, induced-sleep disorder, stress behavior, Tongkat Ali (Eurycoma longifolia) extract

INTRODUCTION

Tongkat Ali, an ingredient in herbal medicine, is a plant belonging to the family Simaroubaceae and known by the scientific name *Eurycoma longifolia*. It grows naturally in lowland forests in Malaysia, Indonesia, and other areas in Southeast Asia. The roots, stems, and leaves of Tongkat Ali are used locally as ingredients in traditional folk medicine. The most well-researched effects of Tongkat Ali extract (TAE) are its applications in treating sexual dysfunction and male infertility (Mohd Tanbi *et al.,* 2010). It has been reported that the sperm counts of rats administered with standardized methanol extract of Tongkat Ali in doses of 200 mg/kg increased by 99.2% in comparison with that of controls ($p<0.01$) (Chan *et al*., 2009). It is also reported that administering TAE to estrogen-treated rats for 14 consecutive days increased spermatogenesis and sperm count, and that there exist latent drug actions that reverse the effects of estrogen in male rats (Wahab *et al*., 2010).

In addition, when testing the anxiety conditions of mice, administering TAE was found to be as effective as the sedative diazepam, which was used as a control (Ang *et al.,* 1999). Administering TAE to people under moderate stress was found to decrease cortisol and to increase testosterone in saliva (Talbott *et al.,* 2013). It has been reported that sleep deprivation induces hypertension, cardiovascular disease, and diabetes in humans because it increases sympathetic nervous system activity. It has been reported that sleep deprivation and intestinal bacteria are closely related to the onset of stress and disease in living organisms (Spiegel *et al.,* 1999, Nagai *et al.,* 2010). Although sleep duration in advanced nations such as the Netherlands, the United Kingdom, and New Zealand exceeds 7.5 hours per day, it is the longer in comparison to Japan and South Korea, at less than 6.5 hours per day (Tozer, 2018). The length and quality of human sleep is a major issue in modern society, and people are eager to achieve deep sleep as soon as they go to bed. Sleep problems and dysbiosis of the gut microbiome can lead to metabolic disorders in human beings (Neroni *et al.,* 2021). Intermittent hypoxia-exposed mice showed a higher abundance of *Firmicutes* and a smaller abundance of *Bacteroidetes* and *Proteobacteria* phyla than controls (Moreno-Indias *et al.,* 2015).

Pigs were used as a model of obstructive sleep apnea-hypopnea syndrome for humans (Kim *et al.,* 2019, Liu *et al.*, 2019). Exposure to 20 hours of illumination per day at 300 lux increases body temperature and spontaneous movements (Takeishi *et al.,* 2018), which is thought to cause induced-sleep disorders. TAE, an herbal medicine, is reported to have anxiolytic effects on rats and humans, and it is expected to be effective in treating sleep disorders, but there are no reports of such effects. In this study, the authors created an induced-sleep-disorder model in which commercial pigs were subjected to prolonged periods of

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high illumination and reported the results of a comparative study on the effects of TAE administration in terms of intestinal microbiota, stress marker (salivary amylase), and clinical symptoms, including stress behaviors.

MATERIALS AND METHODS

Test animals and test conditions

A total of 24 healthy, 5-month-old LWD crossbred pigs were used as test animals. The test period consisted of a one-week acclimatization period and an eight-week test period. The test pigs were fed standard feed for testing, namely SDS No. 4 (Feed One Co., Ltd., Yokohama, Japan), twice per day at 8:00 a.m. and 4:00 p.m., and the amount of feed was limited to 2% of their body weight. The pigs had free access to drinking water using a waterer. The TAE (Physta, Biotropics Malaysia Berhad, Malaysia), extracted with high pressure water (Sambandan et al., 2006), was blended into the standard pig feed for testing in ratios of 0 %, 0.25 %, 0.50 %, and 0.75 %, and fed to the pigs in accordance with their body weight, the mean values of which at the start were 73.8 kg, 71.5 kg, 71.9 kg and 72.7 kg, respectively. Six pigs (three castrated males and three females) were assigned to each group, and the animals were housed individually in an open pigsty (4 m^2).

This experimental test was conducted from September to December 2020, with mean temperatures ranging from 30°C to 15°C.

Induction of sleep-disorders in pigs

A 450W floodlight (Toa Tsusho Company Limited, Osaka, Japan) was used as lighting to induce sleep disorder. During the one-week acclimation period, all four groups were exposed to light at 300 lux for 12 hours a day (Light: 6:00 a.m. to 6:00 p.m.; Dark: 6:00 p.m. to 6:00 a.m.). Once the test period commenced, sleep disorder was induced in the pigs in all four groups by exposing them to light at 300 lux for 20 hours a day (Light: 6:00 a.m. to 2:00 a.m.; Dark: 2:00 a.m. to 6:00 a.m.) (Takeishi et al., 2018).

Observation items and sampling

Clinical symptom scores were recorded daily, with a score of 0 (normal) and scores of 1, 2, 3 as abnormal. Anxiety was defined as nervous behaviours, such as being startled when touched or making abnormal noises. Restlessness was defined as behaviours such as pacing around the barn for no reason. Rectal body temperature was measured at 4 pm-5 pm according to the feeding time every four days for a total of 15 times, and body weight was measured once a week for a total of nine times. Saliva, for use in measuring amylase concentration measurement, and faeces, for use in analysing intestinal microbiota, were collected in tubes containing guanidine hydrocholoride twice in total, at the beginning and end of the test period (Ribeiro *et al*., 2018). The saliva was collected by centrifuging it out of ropes given to the pigs for chewing and individually measured using a salivary amylase monitor (Nipro Corporation, Osaka, Japan). The fecal matter was collected in a fixative, and the intestinal microbiota was analysed using a next-generation sequencer (MiSeq, Illumina, Inc., San Diego, USA).

Statistical analysis

The sums of clinical scores from day 0 through day 56 (daily) were analysed using the Steel-Dwass test; body temperature using the Tukey-Kramer test; and the rate of decrease in salivary amylase concentration using Fisher's exact test. Bacteria showing characteristic time-series variations before and after usage of TAE were identified using the Wilcoxon test. In addition, this test was conducted at a testing facility in Fukuchiyama City, Kyoto Prefecture, and in accordance with the animal testing regulations (ET209017) set forth by Kyodoken Institute.

RESULTS

The pigs in the control group began exhibiting anxious and restless behaviors from around the 15th day after the commencement of testing, and these behaviors were found to last until the final day of testing (56 days after the commencement of administration of TAE). There was no difference in anxious and restless behaviors pertaining to gender difference. In contrast, in the 0.25% TAE group, anxious and restless behaviors were barely observed during the eight-week test period. In the 0.50% TAE group, similar to the 0.25% group, few abnormal clinical symptoms were observed, but a temporary decrease in appetite was observed due to the bitter taste of the herbal medicine. In the 0.75% TAE group, three out of six pigs showed a decrease in appetite due to the bitter taste of the herbal medicine, showing a decrease in appetite of two males and one female. One of these pigs was excluded

Table 1. Comparisons of seven clinical scores by level of TAE administration in induced-sleep-disordered pigs

	Control	0.25%	0.50%	0.75%
Vitality	0.0	0.0	2.0	1.5
Appetite	0.0 [a]	0.0 [a]	7.5 [b]	28.5 [c]
Fecal characteristics	0.0	0.0	0.3	0.0
Respiration	1.0	0.8	0.8	1.3
Emaciation	0.0	0.0	0.0	0.0
Anxiety	11.0 [d]	0.0 [e]	0.0 [e]	0.2 [e]
Restlessness	12.3 [f]	0.0 [g]	0.2 [g]	0.2 [g]
Total	24.5	0.8	10.8	22.6

Note: A score of 0 is considered normal and scores of 1, 2, 3 are considered abnormal (worse). Each value is shown as the sum of clinical scores from day 0 through day 56. P values obtained by Steel-Dwass test. Symbols with different letters (a,b,c),(d,e),(f,g) indicate mutual significant differences at $p < 0.05$.

from the test on the 26th day after the commencement of testing due to a loss of vitality resulting from an extreme loss of appetite. The average total anxiety and restlessness scores in the control group were significantly higher than those in the 0.25%, 0.50%, and 0.75% TAE groups ($p < 0.05$). In addition, the 0.75% TAE group showed significantly higher (worse) appetite scores than the control group, 0.25% TAE group, and 0.50% TAE group ($p < 0.05$) (Table 1).

Comparisons of rectal temperature trends showed no obvious difference between the groups during the experimental period, but on the 40th and 44th days, the 0.25% TAE group and 0.75% TAE group showed temporarily lower temperatures than other groups. The salivary amylase concentrations were compared before (day 0) and after (day 56) the commencement of the test (Figure 1).

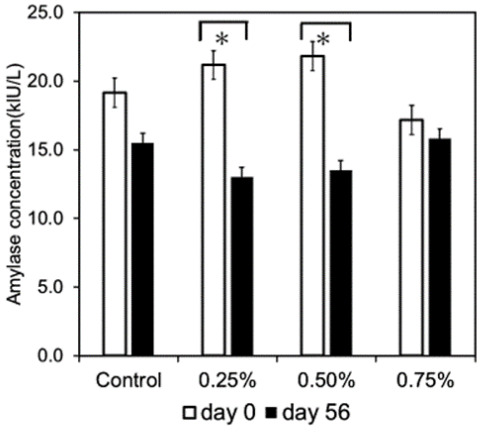

Figure 1. Comparisons of salivary amylase concentration on day 0 (open) and day 56 (solid) by level of TAE administration in induced-sleep-disordered pigs. Amylase concentration showed mean ± standard error. There was a significant difference in salivary amylase levels between day 0 and day 56 in the 0.25% TAE and 0.5%TAE groups, respectively (* $p < 0.05$).

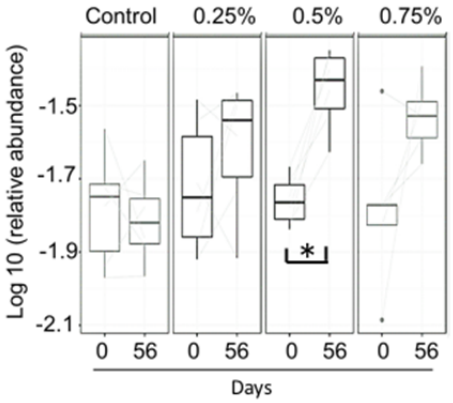

Figure 2. Comparisons of *Lachnospiraceae* in intestinal microbiota before (day 0) and after (day 56) by level of TAE administration in induced-sleep-disordered pigs There was a significant difference in amount of change of *Lachnospiraceae* between day 0 and day 56 in the 0.5 % TAE group (*$p < 0.05$), but not in the other groups.

There was a significant difference in salivary amylase levels between day 0 and day 56 in the 0.25% TAE and 0.5% TAE groups, respectively ($p<0.05$), but there was no difference in the levels between day 0 and day 56 in the control and 0.75% groups.

Analyses of changes in intestinal microbiota before (day 0) and after (day 56) administration of TAE showed significant differences in *Lachnospiraceae* family bacteria between the control group and the 0.50% TAE group on day 56 ($p<0.05$) (Figure 2).

DISCUSSION

In this test, anxious and restless behaviors were strongly inhibited in the groups administered TAE at ratios of 0.25 % and 0.50 % of their feed in comparison to the control group, and this is thought to be due to inhibitory effects on the sympathetic nervous system. In addition, salivary amylase levels decreased in the 0.25 % and 0.50 % TAE groups, which is thought to be due to a decrease in catecholamine. Salivary amylase is regulated by the sympathetic-adrenal medullary system and direct nerve action, and it increases with unpleasant stimuli. Thus, the rate of decrease in salivary amylase was compared between each group because a decrease in salivary amylase is an indicator of stress reduction (Fuentes *et al*., 2011, Xiao-jun *et al.*, 2016).

Pigs are considered to suffer no physiological problems under a minimum of eight hours of illumination at 40 lux. However, exposure to 20 hours of illumination per day at 300 lux increases body temperature and spontaneous movements (Neroni *et al*., 2021), which is thought to cause induced-sleep disorders. As significant anxious and restless behaviors in control group were observed under the prolonged and continuous lighting conditions of this test, it is highly probable that the induced-sleep disorders were due to sympathetic hyperactivity with shortened sleep. The authors observed decreases in body temperature of 1°C or more in mice administered TAE compared to non-administered mice (Kuroki *et al*., 2021). In addition, when a preliminary test was conducted from January to March, when mean temperatures were low (<10°C), body temperatures increased in the control group but decreased roughly one month after administration of TAE, and a significant difference of up to 1.5°C was observed between the administered and non-administered groups (unpublished). However, in this study, comparisons of rectal temperature trends showed no obvious difference between the groups during experimental period. The reason for the difference in the degree of decreases in body temperature between this test and the preliminary test is thought to be due to the weather temperatures during the test periods. In other words, inhibition effects on body temperature in the pigs given TAE was clearly found in winter, but such effects by TAE would be contradicted superficially in summer.

Stress in pigs includes social stress, such as cramped housing conditions, and environmental stress, such as heat, cold, sleep disorders, etc. It has been reported that stress caused by cramped housing conditions accompanying the increasing scale and consolidation of pig farming operations adversely affects pigs' ability to gain weight, which could result in economic losses on farms (Brumm,

1996, Johnston *et al*., 2017). Pigs are particularly susceptible to heat stress due to an absence of sweat glands, and reductions in milk production by sows during the hot season reduces piglet growth (Guo *et al*., 2018). There are reports that housing fattening pigs under high temperatures, such as 30°C, reduces feed intake and increases haptoglobin levels, which indicates inflammation, in comparison to fattening pigs housed under moderate temperatures, such as 23°C (Serviento *et al.,* 2020). The TAE used in this test has the potential to reduce heat stress in sows.

On the other hand, there was no influence on vitality and emaciation in the pigs during the experimental period, despite the decreased appetite in the 0.75% TAE group and the increased anxiety and restlessness in the control group. In addition, respiration rates and fecal characteristics in the pigs used in this study also showed no significant change. Therefore, stress levels caused by 20 hrs-lighting at 300 lux might be enough to maintain wakefulness in pigs.

A significant increase on day 56 in *Lachnospiraceae*, a family of bacteria known for producing butyric acid (Biddle *et al.,* 2013), was observed in the 0.50% TAE group. The *Lachnospiraceae* family has been reported to activate regulatory T cells (Portune *et al.,* 2017) and form tight junctions in intestinal mucosal cells (Braniste *et al.,* 2014) *Lachnospiraceae* bacterium 28-4, and *Lachnospiraceae phytofermentans* were enriched in pigs with low residual feed intake and high feed efficiency (Jiang *et al.,* 2021). These effects are due to butyric acid produced by the butyric acid-producing bacteria, and the increase in butyric acid-producing bacteria is expected to improve immunity and prevent harmful bacteria from invading the body.

TAE contains a bitter quassinoid as an active ingredient, and a significant decrease in appetite was observed in the 0.75% TAE group, and some individuals in the 0.50% TAE group also showed a decrease in appetite. As decreases in appetite among pigs affects their ability to gain weight, it is thought that the 0.75% and 0.50% doses of TAE were excessive. On the other hand, when administering 0.25% doses of TAE, no decrease in appetite was observed, and anxiety and restlessness were sufficiently inhibited. Therefore, the optimal TAE dosage for administration to pigs is thought to be 0.25% of the volume of feed.

In conclusion, administration of TAE to fattening pigs under high levels of stress is expected to be effective, as administration of TAE at 0.25% of the feed intake has been shown to effectively inhibit stress behaviors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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