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Fos-like immunoreactivity in rat dorsal raphe nuclei induced by alkaloid extract of *Mitragyna speciosa*

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Abstract

Mitragyna speciosa (MS) has been traditionally used for medicinal purposes especially in southern Thailand. Previously, an alkaloid extract of this plant was demonstrated to mediate antinociception, partly, through the descending serotonergic system. The present study investigated the stimulatory effect of the MS extract on the dorsal raphe nucleus and its antidepressant-like activity. The MS extract containing approximately 60% mitragynine as a major indole alkaloid was used to treat the animals. The stimulatory effect of the MS extract was determined by detecting the expression of the immediate early gene, *cfos*, in the dorsal raphe nucleus of male Wistar rats. The immunohistochemistry was used to detect Fos protein, the protein product of *cfos* gene. The present data show that a significant increase in Fos expression was observed following long-term administration of the MS extract (40 mg/kg) for 60 consecutive days. In addition, the antidepressant-like activity of the MS extract was determined by using the forced swimming test (FST) in male mice. The results show that a single injection (either 60 or 90 mg/kg doses) significantly decreased immobility time in the FST. These findings indicate that the MS extract has a stimulatory effect on the dorsal raphe nucleus and an antidepressant-like activity. Stimulation of this brain area has been known to cause antinociception. These findings suggest that the MS extract might produce antinociceptive and/or antidepressive actions partly through activation of the dorsal raphe nucleus. Moreover, the dorsal raphe nucleus may be one of site of MS action in the central nervous system.

Keywords: *Mitragyna speciosa*; Dorsal raphe; Antidepressant; Antinociception; Immunohistochemistry; Fos; Forced swimming test

Mitragyna speciosa (MS) has been used as a Thai traditional medicine especially in southern Thailand. An antinociceptive action is the most predominant property of this plant. Mitragynine, an alkaloid extracted from this plant, was demonstrated to have its antinociceptive actions by acting on supraspinal opioid receptors for suppressing mechanical and thermal noxious stimulations [16]. Later, μ and δ opioid receptor subtypes were found to mediate these actions centrally [31]. This may explain one of the reasons why MS has been mentioned for use to assist in morphine addiction treatment programs. However, antinociceptive actions produced by mitragynine and morphine were attenuated by different antagonists [31]. This indicates that the actions of mitragynine and morphine are mediated via different mechanisms in the brain. In tail-pinch and hot-plate tests, antinociceptions produced by intracerebroventricular administration of mitragynine were suppressed by intrathecal injection of an α_2-adrenoceptor antagonist and a serotonin receptor antagonist [15]. These findings indicate that antinociceptive actions of the MS are mediated via the descending noradrenergic and serotonergic pathways to alleviate peripheral pain. Until now, no specific site of action of the MS extracts has been detected.

The dorsal raphe nucleus is part of the descending pathway that mediates antinociception by inhibiting noxious inputs that are transferred through the spinal cord [32]. The majority of serotonergic fibers originate in the dorsal raphe nucleus and some of these fibers project to the dorsal horn of the spinal cord. Various kinds of noxious stimuli were found to release serotonin in the dorsal horn [32–34]. This response is believed to modulate the pain sensation process.

Since mitragynine exhibits central antinociception via the descending serotonergic system, it was hypothesized that mitragynine activates the dorsal raphe nucleus to exert

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antinociceptive effect and antidepressant like activity. However, 7-hydroxymitragynine, another novel indole constituent alkaloid from MS, also shows a potent antinociceptive effect even when orally administered [14]. In order to determine whether the dorsal raphe nucleus is activated by alkaloids extracted from the MS, we used whole alkaloid extract from the plant to treat animals. This extract contains mitragynine as the major alkaloid component and other alkaloids in minor quantities [20]. Detection of neuronal cells in the dorsal raphe nucleus activated by MS alkaloid would provide guidance on the site of MS action in the rat brain.

In this study, Fos expression was examined to evaluate the stimulatory effect of the MS extract in the dorsal raphe nucleus. Fos is the protein product of the proto-oncogene *c-fos*. Activation of this gene by various stimuli results in an increase in the Fos protein. This is used as a marker of neuronal activation [6,7,26,5]. Using an immunohistochemical technique, Fos expression was detected in the dorsal raphe nucleus of rat following acute and long-term administration of the MS extract. In addition, the antidepressant-like activity of the MS extract was examined by using the FST, the standard behavioral model for determining antidepressant-like effect of classical antidepressant drugs [4,23] and extracts of some medicinal plants that possess antidepressant-like activities [1,9,35].

Extraction and isolation of alkaloids from MS have been described in the previous study [20]. With some modifications, the major alkaloid was isolated by silica gel column chromatography (eluted with 5% methanol in chloroform), and was identified as mitragynine by the standard spectroscopic method (MS, ^{1}H-NMR and ^{13}C-NMR). According to the TLC analysis, mitragynine constituted about 60% of the crude alkaloid extract content.

Adult male Wistar rats bred at the Faculty's animal house and weighing between 180–200 g were used. They were maintained at 22 °C with a 12/12 dark/light cycle (lights on at 06:00 am). Standard commercial food pellets and filtered tap water were available *ad libitum*. Animals were handled for at least one week before the experiment. The experimental protocols for care and use of the experimental animals used in the present study were approved and guided by the Animals Ethical Committee of Prince of Songkla University.

For Fos expression study, animals were divided into control, acute and chronic groups for once a day intraperitoneal injection (i.p.) administrations. The control and chronic groups received saline and the MS extract (40 mg/kg) respectively for 60 consecutive days ($n = 7$). The acute group received saline for 59 days and followed by a single administration of the MS extract (40 mg/kg) on day 60 ($n = 7$). All administrations were made between 09:00–09:15 a.m. On the final day of treatment, animals were deeply anesthetized with sodium penthobarbitone (40 mg/kg) 2 h and 30 min after the final injection. This anesthetic does not by itself have a strong inducing effect on Fos expression in rat brain [29]. Animals were then perfused with fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Brains were removed and postfixed overnight in the same fixative solution at 4 °C. They were then transferred to sucrose solution (30% sucrose in 0.1 M PB) for cryoprotection until they sank. Brains were frozen and stored at −70 °C until processed for detection and localization of Fos protein.

Coronal sections (40 μm) were cut through the midbrain of the brainstem with a cryostat between 7.30–8.80 mm posterior to the bregma. The rat brain atlas was used for confirmation of the dorsal raphe nucleus brain area [18]. The free-floating sections were incubated with anti-Fos antibodies raised in sheep (Chemicon, CA, USA) diluted 1:500 in PBT (PB containing 2% normal donkey serum and 0.3% TritonX100 overnight at 4 °C and washed 3X 15 min with PBT. They were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-sheep immunoglobulin antibodies (Chemicon, CA, USA) for 2 h at room temperature and washed 3X 15 min with PBT. Sections were mounted on glass slides. Images were captured with an Olympus DP-50 digital camera and image files were processed using Microimage software (Olympus).

Controls for non-specific staining were performed in which either the first or secondary antibody was omitted. These controls did not show any staining.

The images were viewed with a PC computer using the Adobe Photoshop 7.0 program. Sections were coded with random numbers to prevent bias in quantification. Fos-like immunoreactive nuclei were counted in the dorsal raphe nucleus. At least ten separate sections of each rat were used for quantification. The numbers of Fos-like immunoreactive cells per section were counted bilaterally and averaged for each rat.

Forced swimming test (FST) in this study was modified from the original method described by Porsolt et al. [21]. Briefly, mice were given helplessness condition by forcing to swim individually for six min in a cylinder (30 cm in height and 15 cm in diameter) containing a column of 20 cm of water at 25 °C approximately. Animals did not receive a pretest swimming session in order to keep them without experience of available rescue at the end of the test. They were treated with a single injection (i.p.) of saline, 60 or 90 mg/kg dose, 60 min before placing them into the water individually. Each animal was judged to be immobile when it remained floating in the water with only passive and small movements to keep its head above the water without struggling. On the other hand, it was recognized to express mobility whenever it tried to escape by swimming actively. The duration of immobility was scored during the last 4 min of the 6 min period. The FST was performed between 2–4 p.m. and recorded using a digital video camera by an observer blind to the treatment that mice received. Each animal was used only once. The decrease of immobile period of test animal was calculated compared to the saline control.

Values are expressed as means (±S.E.M.) per experimental group. Overall effect of the MS extract was analyzed by one-way analysis (ANOVA). In Fos detection study, post hoc individual comparisons were made to compare the numbers of Fos-like immunoreactive cells of each treatment group with control group using t- test. In FST, post hoc individual comparisons of each dose with control saline were made using Dunnett's test. Differences with a $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ were considered statistically significant.

Sections from rat brains from control, acute and chronic groups fixed 2 h and 30 min after the final injection showed