In Vivo Characterization of Sedative Activities of Fossilia Mastodi Ossis

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Received January 4, 2006; accepted March 16, 2006

Fossilia Mastodi Ossis, which is a skeletal fossil of a Mastodon, an ancient mammal, has been found to have anxiolytic, sedative and anticonvulsant activities in Oriental medicine. In this study, in vivo characterization of the sedative activities of Fossilia Mastodi Ossis was performed in order to obtain basic information for the development of a putative natural sedative. The 80% methanol extract of Fossilia Mastodi Ossis given per os at a dose of 3 g/kg in mice showed anxiolysis, potentiation of pentobarbital sleeping time, reduced locomotor activity, and anticonvulsant activity. Fossilia elicited GABA_A receptor-mediated anxiolysis. The data obtained suggest that the 80% methanol extract of Fossilia Mastodi Ossis contains some biologically active principles with sedative activity.

Key words Fossilia Mastodi; natural sedative; γ-aminobutyric acid (GABA_A) receptor; fossil

Enhancement of the inhibitory effect of γ-aminobutyric acid (GABA) acting on its type A receptor in the mammalian brain explains the pharmacological and therapeutic actions of benzodiazepines which bind to a specific site in the GABA_A receptor. However, the usefulness of benzodiazepines as anxiolytics, sedatives, anticonvulsants and muscle relaxants is compromised by the occurrence of several adverse effects such as ataxia, amnesia, alcohol intolerance, and residual sedation, as well as the related problems of tolerance and dependence after chronic use. Molecular biology studies have established the heterogeneity of GABA_A receptors and the pharmacological and electrophysiological study of the different subtypes has allowed the search of new ligands with improved selectivity which eventually may lead to the development of safer drugs.1—3) Natural materials including flavonoids have recently attracted interest as new chemical entities with activity on the central nervous system. Many researchers have demonstrated that some naturally-occurring substances possess a selective and relatively mild affinity for the central benzodiazepine binding site in GABA_A receptors, and exert selective activities and sedative but not adverse effects.4—10)

Fossilia Mastodi Ossis, which is a skeletal fossil of an ancient, extinct mammal, has been reported to have anxiolytic, sedative and anticonvulsant activities in Oriental medicine. It is known to be composed of calcium carbonate, calcium phosphate, trace amounts of Fe, Al and Mg, and 0.03% uranium, and is usually collectable from Shandong Province and surrounding across in China.11) In this study, in vivo characterization of the anxiolytic-sedative activities of Fossilia Mastodi Ossis was carried out in order to obtain basic information on a putative natural sedative.

MATERIALS AND METHODS

Chemicals and Animals Diazepam was kindly donated by Roche (Switzerland) and sodium pentobarbital was purchased from Han-Rim Pharmaceuticals (Korea). Other assay reagents and chemicals including picrotoxin were purchased from Sigma (St. Louis, MO, U.S.A.). Male ICR mice weighing 35—40 g were used. Animals were housed in a regulated environment (temperature: 21±1 °C, humidity: 50±5%) with a 12 h light–dark cycle.

Preparation of Extract Fossilia Mastodi Ossis, a Chinese herbal powder, was purchased from Electronic Kyung-Dong Market (EKD, Seoul, Korea), which guarantees the quality of the herb according to the guidelines of the Korean Pharmacopoeia (KP). Dried powder was extracted two times with 80% methanol for 3 h. The solvent was then removed at low temperature under reduced pressure. The extracts were resuspended in normal saline and stored at −20 °C for pharmacological studies.

Elevated Plus Maze Test This test has been validated to measure anxiety in rodents.12,13) The maze is a horizontal cross made by two open platforms of 25 cm×5 cm crossed by two platforms of the same dimensions, closed by walls 35 cm high. The maze is suspended 50 cm from the room floor. Mice are placed on the central part of the cross facing a walled arm. The number of entries and the time spent in open and closed arms are counted during 5 min using the plus maze monitoring program (Elevated Plus maze, Vatican Production, Inc). A selective increase in the parameters corresponding to open arms indicates an anxiolytic effects.

percent time in open (%) = time in the open arms × 100
time in the open arms + time in the closed arms

percent open crosses (%) = number of entries into the open arms × 100
total number of entries

Experiment 1: Effect of extract or diazepam in the elevated plus maze: The extract or diazepam (0.5 mg/kg, i.p.) was administered to independent groups of mice (n=8, each) and tested in the elevated plus maze. In this case, diazepam dissolved in propylene glycol was used as a positive control group. Control mice for the extract-treated group were administered saline per os, and control mice for the diazepam-treated group were given propylene glycol intraperitoneally. Control data from both groups of mice were not significantly different.

Experiment 2: Analysis of the participation of the GABA_A receptor complex in the anxiolytic-like actions of extract in the elevated plus maze: For this experimental series, independent groups of mice (n=5—24, each) were
used. In the first part of this experiment, the effects of picrotoxin or muscimol alone were analyzed in the elevated plus maze. In the second part of this experiment, the GABA<sub>A</sub> receptor antagonist picrotoxin (0.5 mg/kg) was simultaneously administered with extract to mice tested in the elevated plus maze. Finally, in the third part, possible synergism of the anxiolytic-like actions of extract with muscimol (0.5 mg/kg) and of extract simultaneously administered to mice was analyzed in the elevated plus maze.

**Sodium Pentobarbital-Induced Sleeping Time** In this test the time, in minutes, elapsing from the loss to the regaining of the righting reflex after drug administration is recorded and referred to as sleeping time. The hypnotic effect of extract in combination with sodium pentobarbital was evaluated. For this purpose, mice were divided into three groups (n = 8, each). The first group served as control and was injected with the vehicle, while the 2nd group received the extract 30 min before the administration of an effective dose of sodium pentobarbital (42 mg/kg, i.p.). The last group was administered diazepam (1.0 mg/kg) 30 min before the administration of pentobarbital. This last group served as a positive control. In the second part of the experiment, mice were treated with the extract or diazepam (1.0 mg/kg, −30 min) before the administration of a subthreshold dose of pentobarbital (30 mg/kg).

**Assay for Locomotor Activity** Mice were divided randomly into 3 groups of 8 animals each. One group was given the extract at a single dose of 3 g/kg per os. Mice in the 2nd and 3rd groups received diazepam (5 mg/kg) and normal saline, respectively. Thirty minutes after administration, the animals were transferred to individual cages. Activity counts were measured for 60 min using video tracking (Smart, Panlab s.l., Barcelona, Spain).

**Picrotoxin-Induced Clonic Convulsion** The effects of compounds on picrotoxin-induced clonic convulsions were evaluated. Picrotoxin (7.5 mg/kg) was administered intraperitoneally to mice 30 min after extract or vehicle administration. The latency (min) of seizure onset and percent lethality were calculated.

**Statistical Analysis** Data are presented as the mean±S.E. Intergroup comparisons of data were made by Student t-test (Systat, Intelligent Software, Evanston, IL, U.S.A.).

**RESULTS**

Mice administered an 80% methanol extract of *Fossilia Mastodi Ossis* given per os at a dose of 3 g/kg showed anxiolysis. As shown in Fig. 1, in the extract-treated mice, open crosses (%) were significantly (p<0.05) increased to 53.0±1.5 from the control value of 45.2±1.6 and time in the open (%) was significantly (p<0.05) increased to 48.9±2.8 from the control value of 36.5±3.5. In a preliminary study, the optimal dose of the extract for this study could be obtained.

As shown in Fig. 2, extracts reduced spontaneous motor activity in mice, the distance travelled (%) was significantly (p<0.05) decreased to 52.5±2.6 from 100% in control mice but was significantly (p<0.05) higher than the value (41.1±3.2) in the 5 mg/kg of diazepam treated mice. In a preliminary study, separate dose of diazepam suitable for each experiment could be obtained.

**Sodium Pentobarbital-Induced Sleeping Time** In this test the time, in minutes, elapsing from the loss to the regaining of the righting reflex after drug administration is recorded and referred to as sleeping time. The hypnotic effect of extract in combination with sodium pentobarbital was evaluated. For this purpose, mice were divided into three groups (n = 8, each). The first group served as control and was injected with the vehicle, while the 2nd group received the extract 30 min before the administration of an effective dose of sodium pentobarbital (42 mg/kg, i.p.). The last group was administered diazepam (1.0 mg/kg, −30 min) before the administration of pentobarbital. This last group served as a positive control. In the second part of the experiment, mice were treated with the extract or diazepam (1.0 mg/kg, −30 min) before the administration of a subthreshold dose of pentobarbital (30 mg/kg).
As shown in Fig. 3, the 80% methanol extract of Fossilia Mastodi OSSIS potentiated the pentobarbital-induced sleeping time. Groups treated with a hypnotic dose of pentobarbital, 42 mg/kg, intraperitoneally, showed more potentiation than groups treated with a subthreshold dose of pentobarbital (30 mg/kg). In the 42 mg/kg pentobarbital treated group, latency of sleeping time, in the Fossilia-treated group, was significantly \( p<0.05 \) reduced to 3.5 \pm 0.2 from the control value of 8.5 \pm 0.3 and duration of sleeping time (min) was significantly \( p<0.05 \) increased to 50.5 \pm 1.6 from the control value of 44.3 \pm 1.2. 43\% of mice showed hypnosis by administration of a subthreshold dose (30 mg/kg) of pentobarbital. In the 30 mg/kg pentobarbital treated group, latency of sleeping time was significantly \( p<0.05 \) reduced to 3.4 \pm 0.2 from the control value of 8.5 \pm 0.6 and duration of sleeping time (min) was significantly \( p<0.05 \) increased to 96.3 \pm 2.6 from the control value of 52.8 \pm 1.6.

As shown in Fig. 3, diazepam also potentiated the pentobarbital-induced sleeping time. Groups treated with a hypnotic dose of pentobarbital (42 mg/kg, i.p.), showed more potentiation than groups treated with a subthreshold dose (30 mg/kg) of pentobarbital. In the 42 mg/kg pentobarbital treated group, latency of sleeping time was significantly \( p<0.05 \) reduced to 4.5 \pm 0.1 from the control value of 8.5 \pm 0.3 and the duration of sleeping time (min) was significantly \( p<0.05 \) increased to 76.7 \pm 1.2 from the control value of 44.3 \pm 1.2. In the 30 mg/kg pentobarbital treated group, latency of sleeping time, was significantly \( p<0.05 \) reduced to 1.0 \pm 0.1 from the control value of 8.5 \pm 0.6 and duration of sleeping time (min) was significantly \( p<0.05 \) increased to 116.6 \pm 4.3 from the control value of 52.8 \pm 1.6.

As shown in Fig. 4, the extract of Fossilia Mastodi OSSIS increased the latency (min) of picrotoxin-induced clonic convolution in mice. In the Fossilia-treated group, latency (min) was significantly \( p<0.05 \) increased to 10.0 \pm 0.9 from the control value of 7.5 \pm 0.6. However, the extract did not change the picrotoxin-induced lethality or myorelaxant activities in the horizontal-wire test (data not shown).

As shown in Fig. 5, the extract of Fossilia Mastodi OSSIS potentiated picrotoxin-induced anxiolysis. Single treatment of muscimol (0.5 mg/kg, i.p.) a GABA\(_a\) agonist, showed anxiolysis in mice. In the muscimol-treated mice, the response (%) in percent open crossings was significantly \( p<0.05 \) increased to 119.7 \pm 2.4 and percent time in the open was significantly \( p<0.05 \) increased to 140.6 \pm 5.3 from the control value of 100 \pm 0.0. In the group treated with both Fossilia and muscimol, response (%) in percent time in the open (%) was significantly \( p<0.05 \) increased to 152.1 \pm 1.2 from the 133.8 \pm 5.6 of the muscimol-treated group.

As shown in Fig. 5, the extract of Fossilia Mastodi OSSIS reversed picrotoxin-induced anxiolysis. As single dose of picrotoxin (0.5 mg/kg, s.c.), a GABA\(_a\) antagonist, induced anxiolysis in mice. In the picrotoxin-treated mice, response (%) in percent time in the open was significantly \( p<0.05 \) increased to 133.8 \pm 5.6 from control, however, in the group treated with both Fossilia and picrotoxin, response (%) was significantly \( p<0.05 \) decreased to 91.0 \pm 7.3 from the 133.8 \pm 5.6 of the picrotoxin-treated group.

**DISCUSSION**

Benzodiazepines have an established efficacy in the treatment of anxiety, insomnia and epilepsy. They produce their pharmacological effects by positively modulating the action of GABA\(_a\) receptors through an allosteric binding site called the benzodiazepine binding site. Even though benzodiazepines are relatively safe drugs when used in the treatment of pathological anxiety, they may produce untoward side effects such as sedation, muscle relaxation, memory impairment, tolerance and physical dependence. Recent work has shown that there is considerable heterogeneity of GABA\(_a\) receptors. At least 15 different subunits have been identified in the mammalian CNS (\(\alpha_1—6, \beta_1—3, \gamma_1—3, \rho_1—2 \) and \(1\)) and it is now widely acknowledged that the \(1\) subtype corresponds to GABA\(_a\) receptors containing \(\alpha_1\) subunit, while the \(\alpha_2\) subtype represents a heterogeneous population of receptors possessing \(\alpha_2, \alpha_3 \) or \(\alpha_5\) subunits.\(^{14}\)

Benzodiazepines, such as diazepam or bretazenil, act with high affinity at all receptor subtypes. However, diazepam exhibits high intrinsic efficacy, while bretazenil displays reduced efficacy and is therefore described as a partial agonist. Diazepam produces anticonvulsant, anxiolytic, muscle relaxant, and sedative effects, whereas bretazenil is mainly an
ticonvulsant and anxiolytic, but has only weak or no myorelaxant and sedative activities. Partial agonists require higher fractional receptor occupancy than full agonists to elicit a given response in vivo. In the presence of full agonists, partial agonists compete for the receptor and may attenuate the actions of full agonists, indicating that partial agonists can exhibit antagonistic effects in those circumstances where they display limited or no effect. For example, numerous studies have shown that partial agonists antagonized sedation and muscle relaxation induced by full agonists. β-Carboline abecarnil showed differences in subtype-selectivity and intrinsic efficacy. Several studies in rodents have indicated that abecarnil produced clear anxiolytic-like effects.15—17

Active ingredients isolated from several traditional sedatives, such as chrysin (5,7-dihydroxyflavone), miltron and apigenin (5,7,4′-tri-hydroxyflavone) showed partial agonistic activity to the central type of benzodiazepine receptor. Among these, apigenin and synthetic 6,3′-dinitroflavone and 6-bromoflavone have been proposed to have selective anxiolytic activities, and have been adopted by medicinal chemists to improve their activities for better clinical efficacy.13—15

In this study, mice treated with an 80% methanol extract of Fossilia Mastodi Ossis exhibited anxiolysis in the elevated plus maze like mice on diazepam, a known synthetic anxiolytic-sedative. Benzodiazepines including diazepam are known to have pharmacological activities mediated via allosteric potentiation of GABA_A receptor function. Fossilia reversed the anxiolysis induced by picrotoxin, a GABA_A receptor antagonist, however, it potentiated the anxiolysis induced by muscimol, a GABA_A receptor agonist. From these data, it can be concluded that a GABA_A receptor mediated the anxiolytic effects of Fossilia. Like diazepam, Fossilia enhanced pentobarbital-induced sleeping time, and reduced locomotor activity. However, the activities of Fossilia were not as great as those of diazepam. Furthermore, Fossilia did not show prominent anticonvulsant and myorelaxant activities. Diazepam, a full agonist of the benzodiazepine receptor, exhibits a wide spectrum of pharmacological activities, including anticonvulsion and myorelaxation activity.

From the data obtained in this study, the methanol extract of Fossilia Mastodi Ossis may be proposed as a source of natural sedative. Partial agonistic and/or subtype-selective modulation to GABAA receptors by the ingredients of Fossilia may be hypothesized from this study. Prolonged modification of organic materials of extinct mammals induced by microorganisms in the soil, could be postulated as an explanation for the production of biologically active substances. Further investigations on clarification of the action mechanism and identification of some biologically active principles with sedative activity need to be done before practical use is considered.

Acknowledgement This study was partially supported by the Kyungpook National University Research Fund, 2004.

REFERENCES