Author’s response to reviews

Title: Sedative and hypnotic effects of Compound Anshen essential oil inhalation for insomnia

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1. Euthanasia/sacrifice methods
   Methods
   Cervical dislocation
   Rationale

   This method is the most commonly used method for killing mice by dislocating the cervical vertebra and breaking the spinal cord. During the operation, the experimentator grasped the root of the mouse tail with his right hand and lifted it, placed it on the cage cover or other rough surface, and pressed
down on the head and neck with his left thumb and index finger. With his right hand, he grasped the root of the mouse tail and pulled it backward and upward, resulting in cervical dislocation, spinal cord and brain stem breaking, and the experimental animal died immediately.

2. The timeline of the animal treatment/euthanasia

Animals
ICR mice aged 6-8 weeks, male and female, weighing 25-35g, provided by Jiangsu Jicui Yaokang Biotechnology Co., Ltd. License number: SCXK (su) 2018-0008. During the whole experiment, the animals could get enough food and water. The temperature of the feeding environment was 21 °C ± 1 °C, the humidity was 55% ± 5%, the light and dark alternate for 12 hours, and the test was started after 1 week of adaptive feeding.

Establishing Mice Models of Insomnia
Experimental animals except for the blank group were given intraperitoneal injection of PCPA to induce animal insomnia. PCPA is an inhibitor of tryptophan hydroxylase (TDH), and TDH is the limit of the sleep neurotransmitter serotonin (5-HT). Speed enzyme, so PCPA deprives sleep by blocking 5-HT. Therefore, PCPA is used to deprive sleep by blocking 5-HT. Each mouse was accurately weighed to PCPA according to the amount of 300 mg/kg, and was mixed with weak alkaline saline to prepare a suspension. After continuous injection for 2 days, after the first intraperitoneal injection for 28-32 hours, the circadian rhythm disappeared and the day and night activities continued, indicating successful modeling.

Grouping and Treatment
ICR mice were used, male and female, and mice were randomly divided into groups of 8 animals. This experiment consisted of 1 blank group, 1 model group, 1 diazepam group, and 3 Compound Anshen essential oil groups (low-dose group, medium-dose group, high-dose group). After successful modeling, the model group did not implement inhalation essential oil intervention. The low-dose, medium-dose, and high-dose groups were daily aromatherapy, and the daily concentration of low, medium, and high doses of Compound Anshen essential oil was 1x10-3, 2x10-3, 4x10-3 for 7 consecutive days. The essential oil was diluted with 1% tween 80 solution and inhaled continuously for 60min. The inhalation time was set at 8:00-16:00 daily. Diazepam was prepared into a solution of 0.1ml/10g with distilled water. The diazepam group was given diazepam solution by gavage, and the dose was 1mg/kg (the dose was converted into the dose of mice by body surface area according to the adult dose). The control group, model group, and each Compound Anshen essential oil groups were given the same volume of distilled water by gavage.

Pentobarbital-Induced Sleeping
Mice in each group were intraperitoneally injected with a threshold dose of pentobarbital sodium of 45mg/kg after the final aromatherapy or 30 min of administration. After the administration, the righting reflex of the mice disappeared for 1 min as the index of falling into sleep. The time from the injection of the drug to the disappearance of the righting reflex was latency of sleeping time, the time that the righting reflex disappeared until the recovery was duration of sleeping time, and latency of sleeping time and duration of sleeping time were recorded. At the end of the experiment, the mice were sacrificed by cervical dislocation.

Analysis of Brain Neurotransmitters
After the open-field test was completed, the mice were fasted for 6 hours. The mice in each group were sacrificed by cervical dislocation, the skull was removed, brain tissue was exposed, and the brain tissue was completely removed. After separating the brain, the blood and tissues were washed in ice-cold PBS
buffer and weighed with an electronic balance. The mouse brain was placed in a centrifuge tube, and a certain amount of PBS buffer was added thereto, thoroughly homogenized, centrifuged (3000 r/min, 20 min), and the supernatant was taken. After treatment with the mouse 5-HT and GABA ELISA kits, the 5-HT and GABA contents were measured at 450 nm on a microplate reader.