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Effect of *Withania somnifera* Dunal root extract against pentylenetetrazol seizure threshold in mice: Possible involvement of GABAergic system

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Withania somnifera (ashwagandha) is a widely used herb in the Ayurvedic system of medicine. The objective of the present study was to elucidate the effect of *W. somnifera* root extract (*Ws*) alone or in combination with exogenous γ-amino butyric acid (GABA), a GABA receptor agonist or with diazepam, a GABA receptor modulator against pentylenetetrazol (PTZ, iv) seizure threshold in mice. Minimal dose of PTZ (iv, mg/kg) needed to induce different phases (myoclonic jerks, generalized clonus and tonic extension) of convulsions were recorded as an index of seizure threshold. *Ws* (100 or 200 mg/kg, po) increased the PTZ seizure threshold for the onset of tonic extension phase whereas a lower dose (50 mg/kg, po) did not show any effect on the seizure threshold. Co-administration of a sub-effective dose of *Ws* (50 mg/kg, po) with a sub-protective dose of either GABA (25 mg/kg, ip) or diazepam (0.5 mg/kg, ip) increased the seizure threshold. The results suggested that the anticonvulsant effect of *W. somnifera* against PTZ seizure threshold paradigm involved the $GABA_A$ergic modulation.

Keywords: Diazepam, GABA, Pentylenetetrazol, Seizure threshold, *Withania somnifera*.

Withania somnifera Dunal (family Solanaceae) also known as Indian ginseng (Ashwagandha in Sanskrit) is an ancient Indian herbal drug which finds popular use as a convenient home remedy for many ailments[1]. Roots of this plant have been extensively used in Ayurvedic and Unani systems of medicine over the years in geriatric tonics for its potent aphrodisiac, rejuvenative and life prolonging properties[2]. Antiinflammatory, anticancer, antistress, adaptogenic, endocrine and cardiovascular activities of *W. somnifera* root extract (*Ws*) are reported[3-6]. The active constituents of plant (Withaferin A, Sitoindosides VII–X) are also reported to have an antioxidant activity which may contribute at least in part to these therapeutic properties[7,8]. *W. somnifera* has also been shown to be effective in alleviating several central nervous system (CNS) disorders like epilepsy, anxiety, depression, catalepsy, morphine tolerance, tardive dyskinesia, and sleep[8-13]. Even though the exact mechanism(s) of action involved in the CNS activities are not fully understood, *Ws* preparations modulate various neurotransmitter receptors particularly the γ-amino-butyric acid (GABA) system in the central nervous system[14].

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Unlike other animal models, pentylenetetrazol (PTZ) seizure threshold is an effective tool for screening the efficacy of antiepileptic drugs where their effect on the seizure threshold is determined against a constant rate of PTZ iv infusion. This model enables to specifically determine the effect of these drugs on different phases of clonic convulsions and thereby the efficacy of these drugs on seizure generation and propagation can be separately scrutinized in different seizure types in a small number of rodents with negligible intra-animal variation[15].

The inhibitory $GABA_A$ receptor is the major site of drug action for a variety of anticonvulsant drugs like benzodiazepines. Diazepam, a $GABA_A$ receptor modulator facilitates the effect of GABA mediated Cl^- conductance and thus results in CNS inhibitory activities. Antiepileptic activity of *Ws* in various models of acute and chronic epilepsy has been reported[16]. The antiepileptic activity of *Ws* has also been studied using radio-ligand binding techniques[17] to demonstrate the involvement of $GABA_A$ receptor mediation in action. The present study investigated the effect of *Ws* against PTZ (iv) seizure threshold paradigm in mice and also to further establish its interaction with GABA (GABA receptor agonist) or diazepam (a GABA receptor modulator), the ligands of the inhibitory neurotransmission.

Materials and Methods

Animals—Male Albino mice (Laka strain) weighing between 22-30 g bred in Central Animal House facility of Panjab University, Chandigarh were used. The animals were housed under standard laboratory conditions maintained under a natural light and dark cycle, and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. In order to minimise the circadian changes and inter group variations all the experiments were carried out between 0900 and 1500 hrs. In all experimental protocols animals were properly randomized based on their body weights. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the Indian National Science Academy (INSA) guidelines for the use and care of experimental animals.

Drugs and treatment—The following drugs were used: *Withania somnifera* root extract (*Ws*, Himalaya Drugs Co. Bangalore, India), diazepam (Ranbaxy Research Laboratories, India), GABA (Sigma, MO, USA) and pentylenetetrazol (Sigma, MO, USA). Pentylenetetrazol was prepared in normal saline (0.9% w/v) at a concentration of 0.5% w/v solution and was infused intravenously. The *Ws* was suspended in 0.25% w/v CMC solution and administered, po 45 min before PTZ iv infusion. GABA or diazepam was dissolved in distilled water and was administered ip 30 min before measuring the PTZ seizure threshold. The doses of the drugs used in the present study were selected based on the previous experiments conducted in our laboratory[8,18].

Determination of convulsive parameters and seizure threshold—The threshold for different phases, i.e., myoclonic jerks, generalized clonus and tonic extensor of PTZ-induced seizures was determined by infusing PTZ solution via a 27Gx3/4" butterfly needle inserted into the lateral tail vein of mouse. The needle was secured to the tail vein by an adhesive tape and the animal was permitted to move about freely inside an inverted glass beaker (2l) with free aeration from the top. The PTZ solution was infused into the tail vein at a constant rate of 0.3 ml/min using a Hamilton microsyringe mounted to infusion pump (Harvard Apparatus, model '11' plus syringe pump, USA) and connected to the "butterfly" needle by polyethylene tubing. Infusion was stopped at 3 min or at the onset of extension phase, whichever occurred first[18,19].

In the present experiment, the following endpoints were used for seizure threshold determination: (1) the initial myoclonic jerk; (2) the onset of generalized clonus with loss of righting reflexes; and (3) the onset of tonic extension phase. Latencies were measured from the start of PTZ infusion to the onset of all these three stages. Termination of PTZ infusion was coincident with the occurrence of tonic seizure in mice. From this latency, rate of drug delivery and weight of the animal, the seizure threshold for these stages was computed by determining the amount of PTZ in mg/kg needed to produce the first apparent signs occurring at the begin of each stage of these convulsion.

Statistical analysis—One specific group of mice was assigned to one specific drug treatment condition and each group comprised 6–10 mice. All the values were expressed as mean±SE. The data were analyzed by using one way analysis of variance (ANOVA) followed by Tukey's test. In all tests, the criterion for statistical significance was $P < 0.05$.

Results

Effect of Ws on pentylenetetrazol seizure threshold—Fig. 1 shows the effect of acute administration of different doses of *Ws* (50-200 mg/kg, po) on the seizure threshold for the onset of myoclonic jerks, generalized clonus and tonic extension produced by 0.5% w/v PTZ iv infusion in mice. *Ws* (100 or 200 mg/kg, po) showed a significant increase in seizure threshold for the onset of tonic extension ($F=17.54$, $P<0.001$) as compared to the vehicle treated control group. However, a lower dose of the extract (50 mg/kg, po) was ineffective in increasing the seizure threshold in any of the convulsive phases induced by PTZ iv infusion. This

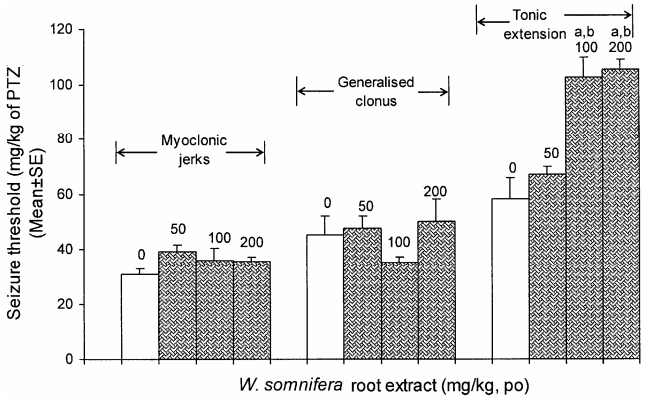

Fig. 1—Effect of *W. somnifera* root extract (50-200 mg/kg, po) on the seizure threshold for the onset of myoclonic jerks, generalized clonus and tonic extensor induced by 0.5% w/v PTZ iv infusion in mice. $P<0.05$ as compared to [a]control group and [b]*Ws* (50 mg/kg) treated group (ANOVA followed by Tukey's test).

sub-convulsive dose i.e. 50 mg/kg, which failed to induce any anticonvulsant effect, was chosen for further experiments to explore the possible mechanism of action in the anticonvulsant effect of the extract.

Effect of pretreatment of Ws (50 mg/kg, po) with sub-effective dose of GABA (25 mg/kg, ip) or diazepam (0.5 mg/kg, ip) against PTZ seizure threshold—The extract (50 mg/kg, po) *per se* did not show any protection against PTZ seizure threshold. However, when the sub-effective dose of the extract (50 mg/kg, po) was co-administered with a sub-protective dose of GABA (25 mg/kg, ip) or diazepam (DZP, 0.5 mg/kg, ip) it enhanced the seizure threshold for the onset of generalized clonus [diazepam ($F=6.73$, $P<0.003$)] and tonic extension phase [GABA ($F=17.09$, $P<0.001$); diazepam ($F=13.30$, $P<0.001$)] induced by PTZ i.v. infusion, respectively (Fig 2 & Fig 3).

Discussion

PTZ iv seizure threshold is a well acknowledged animal model used for screening anticonvulsant effects of various chemical entities. In comparison to the sc or ip injection of a fixed dose of PTZ in laboratory animals, determination of seizure threshold by iv infusion has an advantage because threshold for clonic and tonic phases can be separately determined in the same animals, thus providing a sensitive test for separate evaluation of drug effects in different seizure types in a small number of rodents[15]. PTZ-induced convulsions represent the petit-mal type of seizures and this has been primarily utilized as animal model to evaluate antiepileptic drugs. PTZ is known to block the postsynaptic $GABA_A$ receptor mediated Cl^- conductance and thus produce seizures[20].

In the present study, administration of the *Ws* showed anticonvulsant action by increasing the PTZ seizure threshold for the onset of tonic extension phase in mice. Further, a sub-effective dose of the extract potentiated the actions of GABA (γ-gamma amino butyric acid), a GABA receptor agonist, and diazepam, a GABA receptor modulator against the PTZ iv seizure threshold. Besides monitoring the changes in latency of different phases of seizures, the computation of the PTZ dose (in mg/kg) required by the animal made a precise assessment of the role of different doses of *Ws* in attenuating the seizures. Higher doses of the extract increased the PTZ seizure threshold for the onset of tonic extensor phase. This establishes the role of the root extract in preventing seizure propagation. Antiepileptic drugs which increase the threshold for the onset of myoclonic jerks and tonic extensor are known to prevent seizure generation and propagation, respectively[15].

The leaves and the root extract of *W. somnifera* have long been used in Indian system of medicine for inflammations, arthritis, asthma, hypertension, rheumatism, tumors, conjunctivitis and other bacterial infections[14]. The chemical investigations of *W. somnifera* extract contained several withanolides as

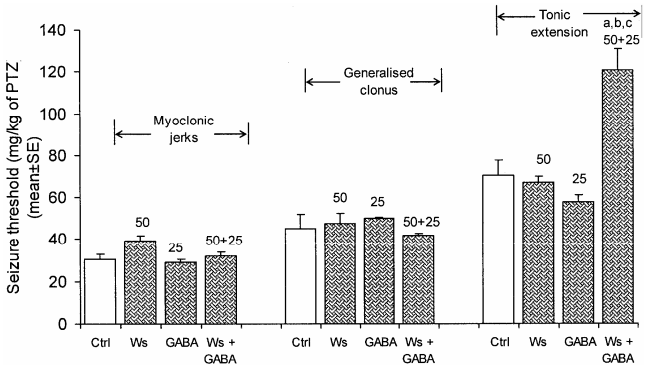

Fig. 2—Effect of *Ws* (50 mg/kg, po) with sub-effective dose of GABA (25 mg/kg, ip) on seizure threshold for the onset of myoclonic jerks, generalized clonus and tonic extension induced by 0.5% w/v PTZ iv infusion in mice. GABA was administered 30 min before challenging the animals to PTZ iv infusion. *Ws* was administered 15 min before GABA treatment. $P<0.05$ as compared to [a]vehicle-treated control, [b]*Ws* (50 mg/kg, po) *per se* and [c]GABA (25 mg/kg, ip) *per se* groups (ANOVA followed by Tukey's test) [Ctrl, control (vehicle treatment); *Ws*, *W. somnifera* root extract; GABA, γ-amino butyric acid].

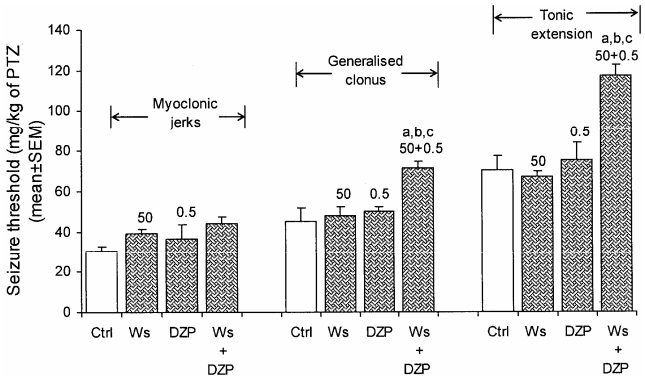

Fig. 3—Effect of *Ws* (50 mg/kg, po) with sub-effective dose of diazepam (0.5 mg/kg, ip) on seizure threshold for the onset of myoclonic jerks, generalized clonus and tonic extension induced by 0.5% w/v PTZ iv infusion in mice. Diazepam was administered 30 min before challenging the animals to PTZ iv infusion. *Ws* was administered 15 min before diazepam treatment. $P<0.05$ as compared to [a]vehicle-treated control, [b]*Ws* (50 mg/kg, po) *per se* and [c]diazepam (0.5 mg/kg, ip) *per se* groups (ANOVA followed by Tukey's test) [Ctrl, control (vehicle treatment); *Ws*, *W. somnifera* root extract; DZP, diazepam].

the major chemical constituents. These compounds are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form a δ-lactone[21]. Besides its known conventional use, studies have also revealed the effectiveness of *Ws* in a variety of central nervous systems disorders like anxiety, depression, catalepsy, morphine tolerance, tardive dyskinesia, stress, cognition and chronic fatigue syndrome[8-13,22].

Studies conducted in our laboratory have shown the anticonvulsant properties of *Ws* in acute and chronic models of epilepsy[16]. The root extract was effective against pentylenetetrazol (PTZ)-induced kindling in mice[23], amygdaloid kindling[24] and status epilepticus in rats[25], respectively. The protective effect of *Ws* in epilepsy is conceived to be through the GABAergic modulation[16].

GABA is an important endogenous inhibitory neurotransmitter widely distributed throughout the central nervous system. A reduction in GABA function in the brain is associated with several psychiatric and neurological disorders, including anxiety, depression, insomnia, and epilepsy[16]. Numerous natural and synthetic compounds interact with the $GABA_A$ receptor at distinct, yet incompletely defined sites[26]. These compounds include barbiturates, benzodiazepines, neurosteroids and picrotoxin[27,28]. The postsynaptic $GABA_A$ receptors are implicated in the inhibitory mechanisms. $GABA_A$ receptor agonists as well as drugs, which allosterically modulate the $GABA_A$ receptor channel complex, are therapeutically effective anticonvulsant agents[29].

In the present study, sub-protective doses of *Ws* elicited potent anticonvulsant effect when co-administered with sub-effective doses of exogenous GABA or diazepam. In an *in vitro* assay using mammalian spinal cord neurons, *Ws* was shown to increase $[^{36}Cl]^-$ influx in the absence of GABA[17]. The radioligand binding studies have also shown that the *Ws* inhibited the specific binding of $[^3H]$ GABA and enhanced the binding of $[^3H]$ flunitrazepam to their putative receptor sites[17].

It is concluded that the anticonvulsant effect of *Ws* in PTZ iv threshold paradigm possibly involved the $GABA_A$ receptor modulation and thus reinforces the use of *W. somnifera* preparation in reducing the seizure propagation during convulsive episodes.

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