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PROTECTIVE EFFECT OF *WITHANIA SOMNIFERA* DUNAL ROOT EXTRACT AGAINST PROTRACTED SOCIAL ISOLATION INDUCED BEHAVIOR IN RATS

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Abstract : This study investigated the effect of *Withania somnifera Dunal (WS)* root extract and diazepam in social isolation induced behavior such as anxiety and depression in rats. Rats were isolated for 6 weeks and the assessment of changed behavior were done on elevated plus maze (EPM) and forced swim test (FST). Isolation reared rats spent less time into the open arms on EPM and significantly increased immobility time in FST compared to group housed rats. WS (100, 200 or 500 mg/kg, oral) and diazepam (1 or 2 mg/kg, ip) dose dependently increased the time spent and entries into the open arms on EPM test and showed the anxiolytic activity. Subeffective dose of WS (50 mg/kg, oral) potentiated the anxiolytic action of diazepam (0.5, 1 or 2 mg/kg, ip). WS (100, 200 or 500 mg/kg, oral) also reduced the immobility time in FST, thus showed antidepressant effect in both group housed and social isolates. The investigations support the use of WS as a mood stabilizer in socially isolation behavior in Ayurveda.

Key words : *Withania somnifera* social isolation stress diazepam anxiety depression

INTRODUCTION

Stress is a term used to describe a state characterized by a broad range of physiological and behavioral changes resulting from one or more stressors that may be external or internal in origin. The use of chronic or repeated stressful events and/or social isolation/separation in rodents and primates have been used to model human affective disorders. These models include restraint stress, cold water stress, exposure to dominant males, tail suspension, as well as maternal and peer separation/ isolation (1). Of these models, social isolation has proven to be highly consistent in its ability to produce increase in anxiety and depression-like behavior (2–9).

Benzodiazepines facilitate GABAergic neurotransmission; have a number of therapeutic actions, including anxiolytic, sedative hypnotic, anticonvulsant and muscle relaxant effects and stimulation of food intake. Long term treatment with benzodiazepines, incurs the risk of cognitive dysfunction, dependence and induces withdrawal signs upon discontinuation (10).

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Herbal drugs in the recent years have gained sufficient importance because of their safety, efficacy and cost effectiveness. *Withania somnifera* (WS) Dunal (family, Solanaceae), known as ashwagandha in Ayurveda, the ancient Hindu system of medicine, has been in use for more than 2500 years. Historically, WS, or its major active principles, has been used as an antioxidant, adaptogen, anxiolytic, antidepressant, memory enhancer and antiulcerogenic agents. The active principles of WS, consisting of sitoindosides VII–X, and withaferin-A have been shown to exhibit significant anti-stress and antioxidant effect in rat brain frontal cortex and striatum (11). The experimental studies in animals have extensively demonstrated a GABA-mediated action of WS (12).

WS blocked cold restraint stress, immobilization stress, chronic foot shock induced stress and reversed swim stress in rats (13–15). Social isolation stress induced behavior, such as decrease in pentobarbital induced sleeping time, have been blocked by BR-16A (Mentat), a polyherbal psychotropic preparation, which has WS as a one of its ingredient. Changes in behavior have been postulated to be because of possible GABAergic mechanism of BR-16A (Mentat) in social isolation induced behavior (16). Socially isolated rats have been found with decrease in both the abundance of neuroactive steroids and $GABA_A$ receptor function (17). Therefore, in the present study, effects of WS in the social isolation induced behavior like anxiety and depression have been investigated in rats.

MATERIAL AND METHODS

Animals

Male, Wister albino rats weighing 150 to 180 g (90 to 110 days old) were housed under controlled light (12:12 light: dark cycle, light on at 0700 h) and temperature (25 ± 2^0C) environment and behavioral assessment was conducted during the light cycle. Food (Rat chow, Lipton, India) and water were provided *ad libitum*. They were either housed in group of 4 or 5 rats per cage ($640 \times 410 \times 250$ mm high) or individually (isolated in the same size cage) for 6 weeks before conducting the experiments (2–5, 9). All procedures were carried out under strict compliance with ethical principles and guidelines of the Institutional Animal Ethical Committee constituted as per the direction of the Committee for the Purpose of Control and Supervision of Experimental Animals; Madras (Reg. No. 870/ac/05/CPCSEA).

Drugs

Commercial WS root extract (Dabur, New Delhi, India) was suspended in 0.5% w/v carboxy methyl cellulose (CMC) in distilled water and administered via oral route. The stock solution contained 100 mg/ml of WS. Diazepam (Ranbaxy, Dewas, India) was wetted with 0.5% Tween 80 and uniformly dispersed in normal saline and injected though intraperitoneal (ip) route.

Physicochemical analysis

Thin-layer chromatography (TLC) was used to identify the steroidal lactones

(withanolides) present in WS. The solvent system used was chloroform:methanol:water (64:50:10, v/v) and spots were finally identified with vanillin–phosphoric acid (18).

Elevated plus maze test (EPM) for rats

This test explains the conflict behavior between exploration to a novel area and aversion to open areas and heights. The plus maze consisted of two open arms (50 × 10 cm), and two enclosed arms (50 × 10 × 40 cm), with an open roof, arranged so that the two open and two closed arms are opposite to each other, connected by a central platform (10 × 10 cm). The maze was raised 60 cm from the ground. After drugs treatment individual rat was placed at the center of the maze, head facing an open arm. During the 5 min test period, the number of entries and time spent on the open arm as well as closed arm were measured. An entry was defined as placing all four paws of the animal on an arm (19).

Forced swim test (FST) for rats

It is a behavioral test used frequently to evaluate the potential efficacy of prospective antidepressant drugs in rodents. Individual rat was placed in a cylindrical transparent glass tank (46 cm tall × 20 cm in diameter) of 25±2°C water filled to a depth of 30 cm. Swimming session was conducted with an initial 15 min pretest followed 24 h by a 5 min test. At this depth, rat was prevented from supporting itself by touching the bottom of cylinder. Immobility was scored as the time a rat remained floating in the water making only those movements necessary to keep its head above the water. Immediately after swimming session, the rat was removed from the cylinder, placed in a heated cage for 15 min, and then returned to home cage. After testing of each animal, water of glass tank was replaced with the fresh water (20).

Experimental design

Animals were protractedly isolated for 6 weeks to develop the isolation induced anxiety and depression, tested in EPM and FST. All subjects were experimentally naïve at the beginning of each study and used only once to avoid “one-trial tolerance” to anxiolytic efficiency of drugs in EPM test (21).

(a) Effect of diazepam or WS in EPM test and FST

Animals from different groups were administered with vehicle (0.5 ml of 0.5% w/v CMC in distilled water/rat, oral, n=7) or WS (50–500 mg/kg, oral, n=7 per group) and diazepam (0.5–5 mg/kg, n=7 per group). WS was administered on each day of the last 5 days (38–42 day) and 1 hour after the last dose administration individual rat was subjected to EPM test or FST. Diazepam was injected via ip route on the last day (42 day) and after 30 min individual rat was subjected to EPM test.

(b) Influence of WS on diazepam in EPM

To assess the influence of WS on anxiolytic action of diazepam, separate groups of rats were administered with subeffective dose of WS (50 mg/kg, oral), 30 min prior to different doses of diazepam (0.5, 1 or 2 mg/kg, ip, n=7 per group) treatment.

Statistical analysis

The data are presented as mean±SEM. The difference between two groups of social isolate and group housed control rats was compared by unpaired t-test. The effects of different doses of WS and diazepam were statistically analyzed by one-way repeated-measures ANOVA, and individual means were compared by Student-Newman-Keuls post hoc test. Differences were considered to be significant at $P<0.05$.

RESULTS

Social isolates in EPM and FST

We observed significant reduction in the time spent [Unpaired t-test; t=3.556, df=12, P=0.0039] and the number of entries into open arms [Unpaired t-test; t=2.449, df=12, P=0.0306] in protracted socially isolated rats for 6 weeks as compared to the group-housed rats when observed in EPM test (Table I). The closed arms entries were also markedly increased in socially isolated rats [Unpaired t-test; t=6.582, df=12, P<0.0001] (Table I). Similarly, socially isolated rats showed significant increase in the immobility time [Unpaired t-test; t=9.692, df=12, P<0.0001] (Table IV), as compared to group-housed rats in FST. Thus, it indicates the development of marked anxiety and depression in protracted social isolated rats.

TABLE I : Activity profile of group housed and social isolated rats in elevated plus maze test.

Groups	*Time spent in open arm (sec)*	*Number of open arms entries*	*Number of closed arms entries*
Group housed	22.57±3.88	0.86±0.14	3.71±0.29
Social isolates	7.43±1.76**	0.29±0.18*	6.29±0.29***

All values are Mean±SEM; n=7; Unpaired t-test. *P<0.05, **P<0.01, ***P<0.0001 as compared with group-housed value.

Effect of diazepam or WS in EPM test

In social isolates, Diazepam (1 or 2 mg/kg, ip, n=7 per group) showed significant increase in the time spent ($F_{(10,60)}$=109.60; P<0.001), the number of entries ($F_{(10,60)}$=26.514; P<0.001) into open arms and decreased number of entries ($F_{(10,60)}$=9.190; P<0.001) into closed arms (Table III). The same doses of diazepam significantly increased the time spent ($F_{(10,60)}$= 44.435; P<0.001) and number of entries ($F_{(10,60)}$= 17.248; P<0.01, P<0.001) into open arms in group housed rats (Table II). Insignificant change in number of closed arms entries ($F_{(10,60)}$=0.9733; P=0.4759) were observed irrespective of the treatment when compared with control, in group housed rats (Table II). However, the lower dose of diazepam (0.5 mg/kg, ip) did not show any significant change in time spent and number of entries in open arms as compared to vehicle in both group housed and social isolates (Table II & III). Diazepam (0.5 mg/kg, ip) significantly decreased number of entries in closed arms as compared to vehicle in social isolates (Table III) while insignificant change was observed in group housed rats (Table II). The higher dose of diazepam (5 mg/kg, ip) resulted in sedation and loss of all locomotor activity in both group housed and social isolates.

WS (100, 200 or 500 mg/kg, oral, n=7 per group) dose dependently increased the time spent ($F_{(10,60)}$=109.60; P<0.001), number of entries ($F_{(10,60)}$=26.514; P<0.05, P<0.001,

TABLE II: Effect of diazepam and WS in group housed rats in elevated plus maze test.

Treatment (mg/kg)	*Time spent in open arm (sec)*	*Number of open arms entries*	*Number of closed arms entries*
Control	22.57±3.88	0.86±0.14	3.71±0.29
WS (50)	28.86±5.47	1.14±0.14	3.43±0.20
WS (100)	40.43±3.49***	2.00±0.22*	3.57±0.37
WS (200)	46.71±2.53***	2.29±0.29*	3.43±0.20
WS (500)	51.71±3.08***	3.14±0.26***	3.143±0.26
DZ (0.5)	30.29±1.36	1.43±0.20	2.86±0.26
DZ (1)	42.14±1.65***	2.43±0.37**	3.29±0.18
DZ (2)	54.71±1.63***	3.14±0.26***	3.00±0.31
WS (50) + DZ (0.5)	53.00±1.99[c]	3.00±0.38[b]	3.14±0.14
WS (50) + DZ (1)	73.29±1.82[c]	4.14±0.40[b]	3.43±0.30
WS (50) + DZ (2)	93.71±4.29[c]	4.86±0.40[c]	3.29±0.18

All values are Mean±SEM; n=7; *P<0.05, **P<0.01, ***P<0.001 as compared with control; [b]P<0.01, [c]P<0.001 as compared with respective diazepam control; DZ = Diazepam.

TABLE III: Effect of diazepam and WS in social isolated rats in elevated plus maze test.

Treatment (mg/kg)	*Time spent in open arm (sec)*	*Number of open arms entries*	*Number of closed arms entries*
Control	7.43±1.757	0.29±0.18	6.29±0.29
WS (50)	12.57±1.31	0.57±0.20	5.43±0.37
WS (100)	30.43±1.62***	1.14±0.14*	4.86±0.40*
WS (200)	41.14±2.47***	1.71±0.18***	4.14±0.51***
WS (500)	48.71±1.63***	2.14±0.14***	3.86±0.34***
DZ (0.5)	13.86±1.14	0.861±0.26	4.86±0.26**
DZ (1)	37.00±2.51***	2.00±0.22***	3.51±0.20***
DZ (2)	52.29±2.28***	2.43±0.20***	3.86±0.26***
WS (50) + DZ (0.5)	29.57±1.93[c]	1.71±0.18[a]	3.71±0.29
WS (50) + DZ (1)	53.57±1.89[c]	2.86±0.26[a]	3.43±0.20
WS (50) + DZ (2)	81.71±2.28[c]	3.86±0.26[c]	3.14±0.26

All values are Mean±SEM; n=7; *P<0.05, **P<0.01, ***P<0.001 as compared with control; [a]P<0.05, [c]P<0.001 as compared with respective diazepam control; DZ = Diazepam.

P<0.001) into open arms and decreased number of entries ($F_{(10,60)}$=9.190; P<0.05, P<0.001, P<0.001) into closed arms in social isolates (Table III). The same doses of WS significantly increased the time spent ($F_{(10,60)}$=44.435; P<0.001) and number of entries ($F_{(10,60)}$=17.248; P<0.05, P<0.05, P<0.001) into open arms in group housed rats (Table II). The closed arms entries in group housed ($F_{(10,60)}$=0.9733; P=0.4759) did not significantly change irrespective of the treatment given (Table II). However, the lower dose of WS (50 mg/kg, oral) did not show any significant change in time spent and number of entries in open as well as closed arms as compared to vehicle in both group housed and social isolates (Table II & III).

Influence of WS on diazepam in EPM

Prior treatment with subeffective dose of WS (50 mg/kg, oral) significantly increased anxiolytic effect of diazepam (0.5, 1 or 2 mg/kg, ip), by increasing the time spent ($F_{(10,60)}$=109.60; P<0.001), number of entries ($F_{(10,60)}$=26.514; P<0.05, P<0.05, P<0.001) into open arms and insignificantly changed number of entries (P>0.05) into closed arms as compared to corresponding diazepam given alone in social isolates (Table III). The same dose of WS prior to diazepam (0.5, 1 or 2 mg/kg, ip) significantly increased the time spent ($F_{(10,60)}$=44.435; P<0.001) and number of entries ($F_{(10,60)}$=17.248; P<0.01, P<0.01, P<0.001) into open arms in group housed rats (Table II). The closed arms entries in group housed ($F_{(10,60)}$=0.9733; P>0.05) did not significantly change irrespective of the treatment given (Table II).

Effect of WS in FST

WS (100, 200 or 500 mg/kg, oral, n=7 per group) showed decrease in the immobility time in both group housed ($F_{(4,24)}$=160.08; P<0.001) and social isolates ($F_{(4,24)}$=147.06; P<0.001) in FST (Table IV).

TABLE IV : Effect of WS in group housed and social isolated rats in forced swim test.

Treatment (mg/kg)	*Immobility time in group housed*	*Immobility time in social isolates*
Control	59.71±1.02	86.14±2.53#
WS (50)	55.29±1.38	80.86±2.42
WS (100)	38.86±1.71*	56.71±2.65*
WS (200)	23.86±2.37*	35.71±2.20*
WS (500)	10.57±1.25*	19.14±2.06*

All values are Mean±SEM; n=7; *P<0.001 as compared with control.
[Unpaired t-test. #P<0.0001 as compared with control group-housed value]

Physicochemical analysis

Upon physicochemical analysis, TLC showed the presence of four blueish violet spots (R_f value: 0.8–0.9).

DISCUSSION

In the present study, WS, diazepam and their combination inhibited the social isolation induced behavior like anxiety and depression in both group housed and social isolates. The wealth of literature have documented the development of anxiety (6–9) and depression like (6, 8) behavior in socially isolated rodents. Social separation/isolation from either maternal or peer influence can induce a biobehavioral response in rodents and nonhuman primates seeming to mimic certain aspects of human psychopathology. For instance, the behavioral effects of social isolation in rats can include: enduring hyperactivity, aggressiveness (1, 22). The neurobiology involved has authenticated the alteration of these behaviors in protracted social isolation. The neurobiological changes that reported in the clinical state of anxiety and depression are similar with observed changes in the animals. The altered number of $GABA_A$ receptor (17), modulators of these receptors, neurosteroids (7) and endogenous substances such as inverse benzodiazepine receptor agonist, diazepam-binding inhibitor (3) have been shown to be affected in isolates. The monoamines like dopamine, serotonin (23) or noradrenaline (24) has been depleted in isolates, which directly resembles with the clinical state of depression. The neuropeptide corticotropin-releasing factor (CRF) has widely studied in social isolates. The level of CRF and its receptors have been shown

to be increased in isolation and contributed for the behavioral manifestations of isolation (25). Several other neurotransmitter systems such as cholecystokinin (26), neurokinin, substance P (27), adrenocorticoropin hormone and melanocortins (28) have been postulated to be playing a role in the neurobiology of isolation-induced symptoms. However, the system, which is particularly involved in the regulation of isolates behavior is not clear.

Social isolation-induced anxiogenic-like behavior was assessed in EPM test (2, 6, 9) with reduction in the open arm activity, based on the natural aversion of rodents for open spaces. Diazepam and WS showed significant increase in time spent and number of entries into open arms and produced anxiolytic-like effect in both group housed and social isolates. Diazepam and WS also reversed increase number of entries into closed arms in social isolates. Diazepam and WS may modulate isolation induced decreased $GABA_A$ receptor function by their GABA mimetic effect. In this study, subeffective dose of WS positively modulated the anxiolytic action of diazepam through potentiation of its response in EPM test in both group housed and social isolates. WS acts on GABA binding site and increases the binding of benzodiazepine at benzodiazepine binding site, which is allosteric to GABA binding site at $GABA_A$ receptors (12). This suggests that anxiolytic effect of diazepam may be potentated by GABA mimetic effect of WS.

WS shortened immobility time in both group housed and social isolates in FST. The enhanced 5-HT turnover ratio in the prefrontal cortex, nucleus accumbens, and hippocampus of the group-reared group was attenuated in the isolated-reared group (29). Isolation may exacerbate adaptation to stress and be related to the etiology of human depression (29). CRF has been shown to have both direct and indirect effects on dorsal raphe nucleus (DRN)-5-HT neurons and GABA is implicated as a primary mediator by which CRF and stressors alter the activity of the DRN-5-HT system (30). Therefore the neuronal complexity comprising 5-HT, norepineprine, GABA, CRF and alpha-melanocyte-stimulating hormone might play a critical role in the socially isolated subjects. The modification of these neurotransmitter(s) by WS may be responsible for the observed antidepressant activity.

The total number of arms entries has been criticized as a measure of locomotor or general activity, since it changes following anxiolytic and anxiogenic agents (31, 32). The absolute number of closed arms entries has been suggested as critical measures for locomotion in EPM test (9, 32). Moreover, WS reversed the increased entries into closed arms in social isolates. In the treatment of group housed rats with WS and diazepam, the closed arms entries were not changed significantly ($P>0.05$) and hence the involvement of the locomotor component may be ruled out.

The investigations support the use of WS as a mood stabilizer in socially isolate behavior like anxiety and depression in Ayurveda, without side effects (11) sometimes seen with medication used as anxiolytics and antidepressants.

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