ISSN : 2350-0743

www.ijramr.com

International Journal of Recent Advances in Multidisciplinary Research
Vol. 09, Issue 11, pp.8136-8141, November, 2022

RESEARCH ARTICLE

REVERSIVE EFFECT OF 6-SHOGAOL PROTECT AGAINST ETHANOL WITHDRAWAL INDUCED ANXIETY LIKE BEHAVIOR IN MICE

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ARTICLE INFO

Article History:
Received 28th August, 2022
Received in revised form
29th September, 2022
Accepted 17th October, 2022
Published online 29th November, 2022

Key words:

New Media, Promoting,
Yemeni Women,
Positive effects,
Negative effects.

ABSTRACT

Alcoholism is a widespread behavioral disorder with excessive consumption of alcohol, resulting in alcohol dependence with aversive symptoms upon alcohol withdrawal. Depending on various modulating factors such as genetic predisposition, provocative environmental experiences, social context, pharmacological history and others alcohol consumption can become compulsive, and finally an addictive behavior might evolve. Withdrawal from chronic ethanol exposure has been associated with heightened anxiety and severe physical symptoms, such as tremors, nausea, sweating, increased heart rate, and increased risk of convulsions. Ethanol withdrawal has been postulated to be associated with specific molecular mechanisms and neuroadaptive changes that may lead to an increased and persistent anxiety state. The present study set out to investigate the effects of 6-Shogaol in ethanol-dependent mice using Fluoxetine as a control. Measures made in this model were consistent with literature data in that a daily ethanol consumption ranging from 24 to 30 g/kg yielding ethanol blood level close to 2 g/L (43 mM) produced the emergence of symptoms such as hyperexcitability and heightened anxiety due to ethanol treatment cessation in mice. This report shows that ethanol-withdrawal on chronic administration decreases the no. of entries of mice in the light area, and acute as well as chronic treatment with 6-Shogaol dose dependently reverses their response.

INTRODUCTION

Alcoholism is a widespread behavioral disorder with excessive consumption of alcohol, resulting in alcohol dependence with aversive symptoms upon alcohol withdrawal [1]. Depending on various modulating factors such as genetic predisposition, provocative environmental experiences, social context, pharmacological history and others alcohol consumption can become compulsive, and finally an addictive behavior might evolve [2]. Alcohol withdrawal syndrome is the set of symptoms seen when an individual reduces or stops alcohol consumption after prolonged periods of excessive alcohol intake. Excessive use of alcohol leads to tolerance, physical dependence, and an alcohol withdrawal syndrome. The withdrawal syndrome is largely due to the central nervous system being in a hyper-excitable state. The withdrawal syndrome can include seizures and delirium tremens and may lead to excito-neurotoxicity.[3] Withdrawal from chronic ethanol exposure has been associated with heightened anxiety and severe physical symptoms, such as tremors, nausea, sweating, increased heart rate, and increased risk of convulsions [4]. Anxiety generated by ethanol withdrawal may be a significant contributor to relapse and, thus, may negatively influence treatment prognosis for alcoholics [5].

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In addition to the physical symptoms observed in rats, which are similar to those seen in humans [6], ethanol withdrawn rats also display increased anxiety-like behavior in a variety of tests, such as the elevated plus maze, social interaction test, and acoustic startle test [7]. Ethanol withdrawal has been postulated to be associated with specific molecular mechanisms and neuroadaptive changes that may lead to an increased and persistent anxiety state [8] The central nervous system is markedly affected by acute alcohol consumption. Alcohol causes sedation and relief of anxiety and, at higher concentrations, slurred speech, ataxia, impaired judgment, and disinhibited behavior, a condition usually called intoxication or drunkenness (Table -1).

MATERIALS AND METHODS

Animals: Adult male albino Swiss mice (22–25 g) were group housed (n=6–10) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55–65%). Mice were purchased from National Institute of Nutrition, Hyderabad, India. The animal studies were approved by the Institutional Animal Ethics Committee (Reg. No. 831/BC/04/CPCSEA), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India. Animals were naive to drug treatment and experimentation at the beginning of all studies. Each experimental group was comprised of six mice.

Table 1. Blood Alcohol Concentration (BAC) and Clinical Effects in Non tolerant Individuals

BAC (mg/dL)	Clinical Effect
50–100	Sedation, subjective "high," increased reaction times
100–200	Impaired motor function, slurred speech, ataxia
200–300	Emesis, stupor
300–400	Coma
> 500	Respiratory depression, death

Testing was carried out in a counterbalanced order with respect to the treatment conditions in the noise free room.

Drug and chemicals: The 6-Shogaol (a fine white crystalline powder, >95% purity by HPLC, mol. wt. 470.61, Lot no: T7K047) was purchased from Natural Remedies Ltd., Bangalore, India stored at 20°C, Fluoxetine was purchased from Cadila Pharmaceuticals and Analytical grade ethanol was purchased from Merck India. Fresh solution was prepared before each experiment.

Dosage: Based on our first round studies with different dosages (10 mg/kg, 20 mg/kg and 30 mg/kg) of this 6-shogaol, it was found that 30 mg/kg body wt dosage produced significant effect on the ethanol withdrawling syndrome. Hence 30 mg/kg body wt dosage was considered for this study.

Ethanol-Withdrawal State: During the first time period, all singly housed animals received a liquid diet (40 ml/day at 08:00 a.m.) for 7-10 days ad libitum to habituate them to these sole food and fluid sources. The liquid diet consisted of chocolate milk supplemented with 5 g/L of minerals and vitamin mixture (Profeed; Syncom health care Ltd., Mumbai). Mice consumed 900-1100 g/kg/day over this period. There were no differences in the weights of animals at the end of this habituation period. During the second time period, the ethanol administration procedure described by verley et al.,[49] with slight modifications was used. Briefly, ethanol-treated mice received a diet containing 3% (Vol/Vol) ethanol for 8 days then a diet containing 4% (Vol/Vol) ethanol for 7 days. Control mice received the same chocolate diet. No extra chow or water was supplied over this period and all animals had unlimited access to the diet. At day 15 at 08:00 a.m., alcohol chocolate diet was replaced by the non-alcohol diet until use of animals in the different experiments. Separate groups of mice were used for each set of experiments.

Behavioral Activity

Measurement of the weight of mice before and after ethanol-withdrawal: Before the starting to experiment first of measure weight of all animals then after the administration of ethanol on experimental groups observe continuously the weight of all the animals, and note down the weight of each animal continuously as per record.

Influence of ethanol-withdrawal on behavioral activity in mice: Light and dark test, and Elevated Plus Maze (EPM) was assessed at 0, 6, 24, 48, and 96 h time interval after ethanol-withdrawal. The time interval at which mice exhibited light area was recorded in experimental (ethanol diet) group. The locomotor activity was recorded simultaneously.

Light and Dark Test: The light and dark paradigm was according to the design by Verley et al. (2009)[49] with slight modifications. This test makes use of rodent's natural aversion to bright areas compared to darker ones. In the two-compartment light and dark box, rodents prefer the small dark area and hesitate to enter the brightly lit, open area. The apparatus is a Perspex rectangular box (46×27× 30 cm), divided into a small area (18×27 cm) and a large area (27×27 cm) with an opening door (7.5× 7.5 cm) located in the center of the partition at floor level. The close-topped small compartment is painted black and illuminated by a dim red light 60 W (4 lux), whereas the open-topped large compartment is painted white and brightly illuminated by a 60 W (400 lux) light source. The compartments are equipped with infrared beam sensors enabling the detection of locomotion in each zone, latency of the first crossing from one compartment to the other and shuttle crossings between both compartments. The test was conducted in a sound-attenuated room, under a light intensity of 400-500 lux. Mice were placed individually in the middle of the light area facing the opening. A 5-min test was given during which the latency to enter the brightly lit area with all four paws, the number of crossings in the white compartment, and the number of transitions between the two compartments were recorded. The floor of each box was cleaned with 10% ethanol between sessions. 6-Shogaol (10 and 30 mg/kg) and Fluoxetine (10 and 30 mg/kg) were administered p.o. 30 min, respectively before the test for Acute study and twice daily for chronic study. Control animals received an equivalent volume of corresponding vehicle.

Elevated Plus-Maze Test (EPM): The elevated plus maze test was performed as previously described.[50, 51] The apparatus comprised two open arms (30×5 cm) and two closed arms (30×5×15 cm) that extended from a common central platform (5×5 cm). A small raised lip (0.5 cm) around the edges of the open arms helped prevent mice from slipping off. The apparatus was constructed from polypropylene and Plexiglas, with a white floor and clear walls, and elevated to a height of 38 cm above floor level. After dosing, the mouse was placed on the center square facing an open arm and allowed to freely explore the apparatus under a light intensity of 200 lux for 5 min. The apparatus was cleaned with 70% ethanol solution between subjects. Behaviors scored were open and closed arm entries (an arm entry was defined as all four paws into an arm) and the time spent in the open arms.

RESULTS

Effect of 6-Shogaol on mice behavior after the withdrawal of acute and chronic ethanol

Measurment of the weight of mice before and after ethanol-withdrawal: The records of the weight measurement are shown that, weight of animals are raised very fast after the ethanol withdrawal as compared to the normal weight, but 6-Shogaol (30 mg/kg, p.o.) and Fluoxetine(30 mg/kg, p.o.) prevent the excessive weight increment of mice. Two-way ANOVA followed by Bonferroni test revealed that in the ethanol-withdrawal state, the weight variation was significantly higher at 1, 5, 10 and 15th day interval compared to control (sucrose diet) group but test drug controlled it. [F (3, 80) = 30.66, p< 0.0001].

Influence of ethanol-withdrawal on behavioral activity in mice: Two-way ANOVA followed by Bonferroni test revealed that in the ethanol-withdrawal state, the light and dark test was significantly higher at 6, 24, 48 and 96 h time interval compared to control (sucrose diet) group with its peak at 24 h

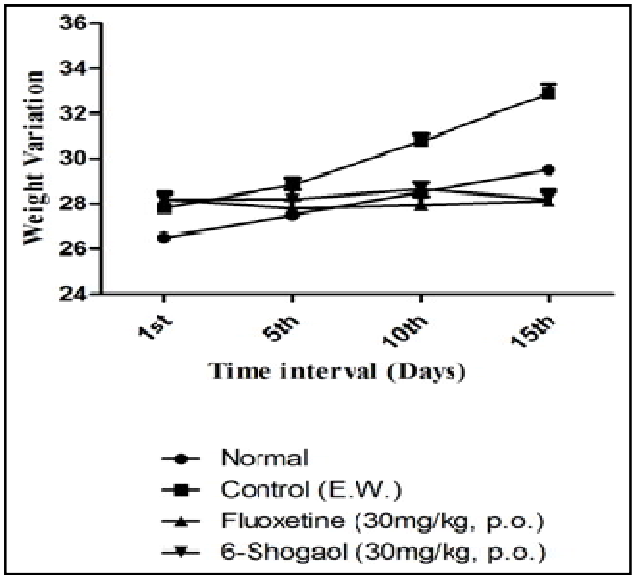
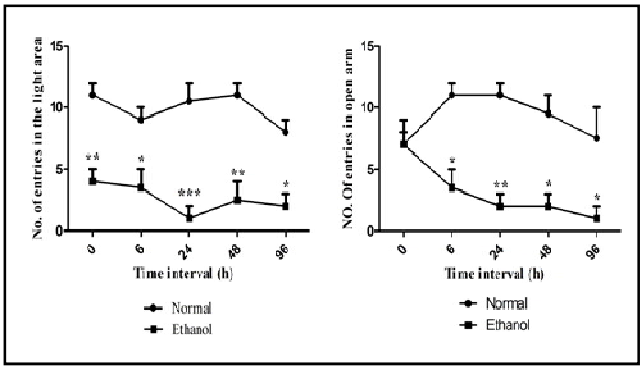


Fig.1. Influence of ethanol-withdrawal on Weight variation in mice: ethanol-treated mice received a diet containing 3% (vol/vol) ethanol for 8 days then a diet containing 4% (vol/vol) ethanol for 7 days. Control mice received the same chocolate diet. On day 1st, 5th, 10th and 15th of experiment measure the weight of all mice individually. Values are expressed as mean±S.E.M (n = 6). Values are statistically significant at *p < 0.001 vs. respective control group (Two-way ANOVA followed by Bonferroni test)



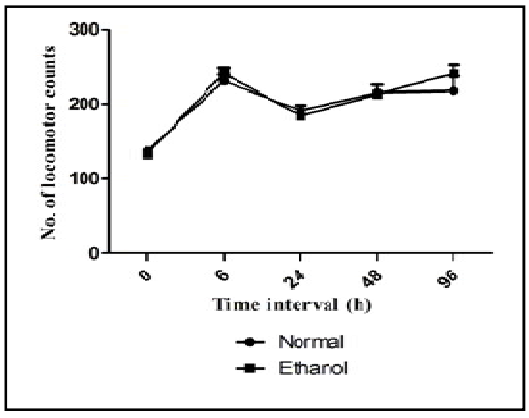


Fig.2. Influence of ethanol-withdrawal on Light & dark test and EPM in mice: ethanol-treated mice received a diet containing 3% (vol/vol) ethanol for 8 days then a diet containing 4% (vol/vol) ethanol for 7 days. Control mice received the same chocolate diet. On day 15th, ethanol was withdrawn and the Light & dark test and EPM along with locomotor activity was assessed at 0, 6, 24, 48, and 96 h intervals. Values are expressed as mean±S.E.M (n = 6). Values are statistically significant at *p <0.001 vs. respective control group (Two-way ANOVA followed by Bonferroni test).

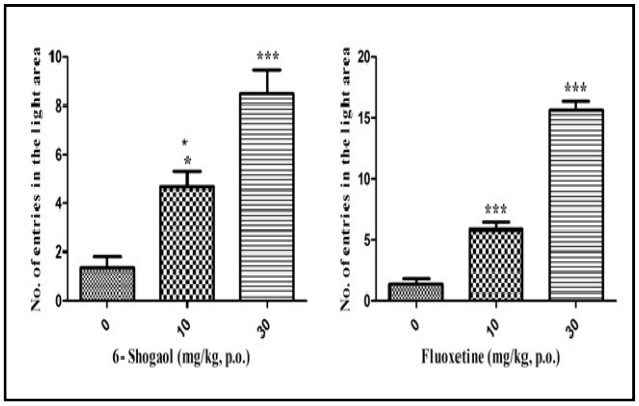


Fig.3:-Effect of acute treatment with 6-Shogaol or Fluoxetine on Light and dark test after ethanol withdrawal: On day 15, 24h after ethanol-withdrawal, experimental (ethanol diet) groups were treated with 6-Shogaol (10 and 30 mg/kg, p.o.) or fluoxetine (10 and 30 mg/kg, p.o.) or vehicle, and after 30 min, Light and dark activity of individual mouse was assessed. Values are expressed as mean±S.E.M (n = 6). Values are statistically significant at *p <0.001 vs. respective control group (One-way ANOVA followed by Bonferroni test)

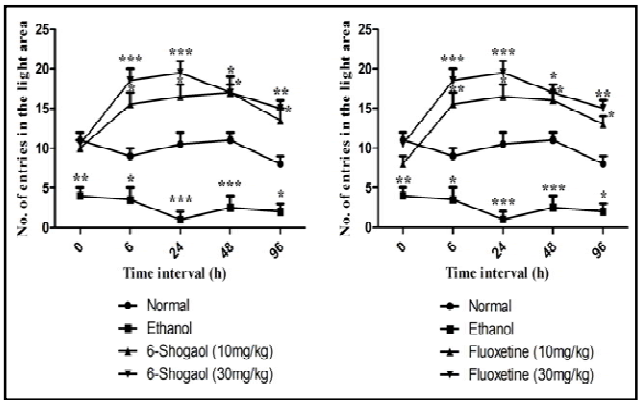


Fig.4. Effect of chronic treatment with 6-Shogaol or fluoxetine on light and dark test after ethanol-withdrawal: Experimental (ethanol diet) groups were treated with 6-Shogaol (10 and 30 mg/kg, p.o.) or fluoxetine (10 and 30 mg/kg, p.o.) or vehicle twice daily. Control group was daily treated with liquid diet (40 ml/day at 08:00 a.m.). On the 15th day, ethanol was withdrawn; light and dark test of individual group of mouse was examined at 0, 6, 24, 48, and 96 h time intervals. Values are expressed as mean±S.E.M (n = 6). Values are statistically significant at *p <0.05 vs. respective control group, p<0.05 vs. respective vehicle treated experimental group (Two-way ANOVA followed by Bonferroni test).

time interval [F (4, 10) = 96.89, p< 0.0001] (Fig.-2). And the EPM test was also significantly higher at 6, 24, 48 and 96 h time interval compared to control group with its peak at 24 h time interval [F (4, 10) = 44.83, p< 0.0001]. However, locomotor activity in the ethanol - withdrawal state was unaffected. Two-way ANOVA revealed is an insignificant ethanol-withdrawal effect [F (4, 10) = 0.38, p= 0.5500].

Light and Dark test: One-way ANOVA followed by Bonferroni test revealed that acute treatment with 6-Shogaol (10 and 30 mg/kg, p.o.), dose dependently peak increase in the light and dark model in ethanol-withdrawal state [F (2, 15) = 23.07, p< 0.0001] as shown in Fig.-8. Fluoxetine (10 and 30 mg/kg, p.o.) had a similar effect [F (2, 15) = 153.5, p< 0.0001]. Two-way ANOVA followed by Bonferroni test revealed that cronic treatment with 6-Shogaol (10 and 30

mg/kg, p.o.) to experimental (ethanol diet) group, significantly (p<0.05) increased the no. of entries in light area evident at 6, 24, and 48h time interval after ethanol-withdrawal. Two-way ANOVA revealed a significant effect of 6-Shogaol treatment [F (3, 20) = 106.89, p< 0.0001] (Fig. 4). Fluoxetine (10 and 30 mg/kg, p.o.) had also a significant effect of light and dark test [F (3, 20) = 116.64, p< 0.0001].

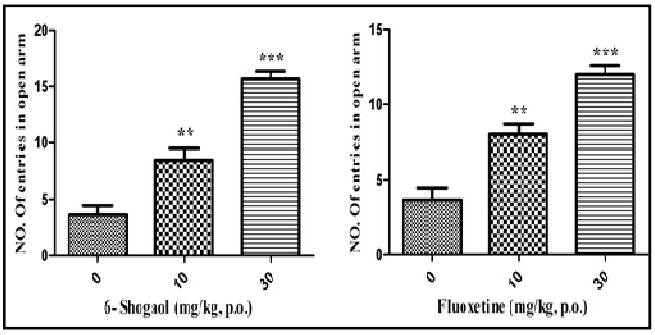


Fig.5 :- Effect of acute treatment with 6-Shogaol or Fluoxetine on EPM test after ethanol withdrawal: On day 15, 24h after ethanol-withdrawal, experimental (ethanol diet) groups were treated with 6-Shogaol (10 and 30 mg/kg, p.o.) or fluoxetine (10 and 30 mg/kg, p.o.) or vehicle, and after 30 min, EPM activity of individual mouse was assessed. Values are expressed as mean±S.E.M (n= 6). Values are statistically significant at *p <0.001 vs. respective control group (One-way ANOVA followed by Bonferroni test)

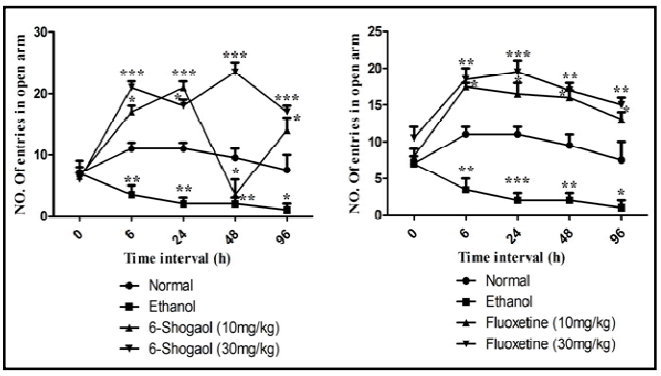


Fig. 6. Effect of chronic treatment with 6-Shogaol or fluoxetine on EPM test after ethanol-withdrawal: Experimental (ethanol diet) groups were treated with 6-Shogaol (10 and 30 mg/kg, p.o.) or fluoxetine (10 and 30 mg/kg, p.o.) or vehicle twice daily. Control group was daily treated with liquid diet (40 ml/day at 08:00 a.m.). On the 15th day, ethanol was withdrawn; EPM test of individual group of mouse was examined at 0, 6, 24, 48, and 96 h time intervals. Values are expressed as mean±S.E.M (n= 6). Values are statistically significant at *p <0.05 vs. respective control group, p<0.05 vs. respective vehicle treated experimental group (Two-way ANOVA followed by Bonferroni test)

Elevated Plus-Maze (EPM): One-way ANOVA followed by Bonferroni test revealed that acute treatment with 6-Shogaol (10 and 30 mg/kg, p.o.), dose dependently peak increase in the EPM model in ethanol-withdrawal state shows significant effect [F (2, 15) = 52.77, p< 0.0001]. Fluoxetine (10 and 30 mg/kg, p.o.) had a similar effect [F (2, 15) = 36.08, p< 0.0001] (Fig.-5) Two-way ANOVA followed by Bonferroni test revealed that cronic treatment with 6-Shogaol (10 and 30 mg/kg, p.o.) to experimental (ethanol diet) group, significantly (p<0.05) increased the no. of entries in open arm evident at 6, 24, and 48h time interval after ethanol-withdrawal. Two-way ANOVA revealed a significant effect of 6-Shogaol treatment [F (3, 20) = 83.22, p< 0.0001] (Fig.- 6). Fluoxetine (10 and 30 mg/kg, p.o.) had also a significant effect on EPM test [F (3, 20) = 96.58, p< 0.0001].

DISCUSSION

The present study set out to investigate the effects of 6-Shogaol in ethanol-dependent mice using Fluoxetine as a control. Measures made in this model were consistent with literature data [52, 53] in that a daily ethanol consumption ranging from 24 to 30 g/kg yielding ethanol blood level close to 2 g/L (43 mM) produced the emergence of symptoms such as hyperexcitability and heightened anxiety due to ethanol treatment cessation in mice. This report shows that ethanol-withdrawal on chronic administration decreases the no. of entries of mice in the light area, and acute treatment with 6-Shogaol dose dependently reverses their response. Chronic treatment with 6-Shogaol decreases the time expend in the light area on light & dark test and EPM test. In rodents, increased anxiety-like behavior during withdrawal is likely a reflection of the direct effects of ethanol exposure on neuronal functioning affecting particularly the GABAergic transmission [54, 54]. It is known that the GABAergic system plays an important role in the control of anxiety, and dysfunction of GABA A receptors in some key brain structures might underlie anxious states. As indicated in the Introduction, the physical signs and increased anxiety during ethanol withdrawal might be attributable to differential alterations in GABAA receptors subunits function and expression [56]. The present study revealed that peak increase in light & dark test and EPM was observed at 24 h time interval after ethanol-withdrawal, which later declined to normal by 96 h. The ethanol - withdrawal state is characterized by serotonin dysfunction, and hyperactivity of dopamine and glutamate [57]. Further, it was observed that acute treatment with 6-Shogaol (10-30 mg/kg, p.o.), 30 min prior to the peak, dose dependently attenuated the increased light & dark test and EPM test in the ethanol - withdrawal state. The effect of 6-Shogaol was comparable to that of fluoxetine (10-30 mg/kg, p.o.). In addition, chronic treatment with 6-Shogaol (30 mg/kg) or fluoxetine (30 mg/kg), twice daily along with ethanol diet prevented an increase in light & dark test and EPM test evident after ethanol-withdrawal.

CONCLUSION

The results of the present study revealed the inhibitory influence of 6-Shogaol in ethanol-induced motivational effects, which may be due to modulatory action on various neurotransmitters.

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