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Neuroprotective role of a monoterpene (thymol) on diazepam induced withdrawal symptoms in rats

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Abstract: Benzodiazepine administration is known to be related to tolerance and a withdrawal syndrome on sudden cessation. Thymol possesses multiple biological properties especially in the pathogenesis of different brain disorders. However, to the best of our knowledge there is no study that relates the use of thymol to benzodiazepine induced withdrawal symptoms. Therefore the aim of the current study was to investigate the usefulness of thymol in the treatment of benzodiazepine withdrawal syndrome in rats. Animals were divided into four groups, thymol (40 mg/kg/ml), diazepam (4 mg/kg), thymol + diazepam and vehicle control group. The treatment was given for 14 days, and then suddenly ceased. After 24 h animals were tested in different behavioral paradigms such as physical signs for withdrawal, marble burying test, inverted screen test, elevated plus maze, passive avoidance test and open field activity. The results of the present study revealed that co-administration of thymol significantly reduced the withdrawal symptoms induced by diazepam. Our results further suggest that administration of thymol not only ameliorates rebound anxiety associated with diazepam withdrawal but also improves motor and memory impairment in rats.

Keywords: Thymol, diazepam, withdrawal, memory, anxiety.

INTRODUCTION

Benzodiazepines are some of the most usually recommended medications in the world and one of the most misused and abused medications by patients, in parallel with opioids. They not only act as a sedative and are used for anxiety disorders and sleeping problems, but are also linked to a variety of adverse effects upon dosage reduction or discontinuation (Guina *et al*., 2018). One of the proposed mechanisms of action of benzodiazepines is through increasing the effect of a GABA (gamma amino butyric acid), a well-known brain chemical. GABA is responsible for reducing brain activity in different brain regions responsible for rational thoughts (Kim *et al*., 2020). Biological dependence on benzodiazepines along with withdrawal syndrome is usually categorized by irritability, sleep disturbance, anxiety, increased tension and panic attacks, sweating, hand tremor, dry retching and nausea, difficulty in concentration, some weight loss, palpitations, muscular pain and stiffness headache, and a number of conceptual changes (Yatham *et al*., 2018, Petrusson 1994). Benzodiazepine withdrawal may also precipitate catatonia in vulnerable individuals such as those with mood disorders (Mader *et al*., 2020). Benzodiazepines withdrawal appears to cause an overall rise in the risk of suicidal attempts. Increased rebound or withdrawal symptoms, aggression and toxicity due to overdose are the possible reasons behind parasuicidal effects (Dodds, 2017; Fluyau *et al*., 2018). In last few years a large number of nutraceuticals have been identified and many of them selected as alternative means of therapies to treat anxiety. Thus manufacture of safe and powerful drug preparation from conventional herbs may produce a better means to improve the efficacy and reduce the side effects. One such recently investigated drug is thymol. Thymol is a natural monoterpenoid phenol (5-methyl-2-isopropylphenol and 2-isopropyl-5-methylphenol), which is colourless and crystalline with a distinguishing odor. It is an active ingredient of extracted oil from the species thyme (Thymus vulgaris) (Kuete, 2017; Meeran *et al*., 2017). Its characteristic antimicrobial, anti-inflammatory, anti-oxidant, cicatrizing activities are the most investigated and reported pharmacological studies (Escobar *et al*., 2020). Thymol plays a critical role in enhancing the bioavailability of nutrients, productive and reproductive performance, immunity and general health of livestock as well as in reducing problems of many animal syndromes (Alagawany *et al*., 2021). No known adverse effects have been identified with respect to thymol when used in humans and animals (Meeran *et al*., 2017). Considering the unwanted and hazardous side effects of benzodiazepine uses on world's population and health benefits of thymol, the present study was designed to monitor the impact of thymol on benzodiazepines induced withdrawal symptoms in rats.

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MATERIALS AND METHODS

Thirty-two male Albino-Wistar rats weighing 180-200 gm were bought from Dow University of Health Sciences (DUHS), Pakistan. Animals were housed separately to minimize the influence of social interaction, with access to standard rodent diet (Bocarsly *et al*., 2012), tap water under controlled parameters (room temperature 22 ± 2◦C; 12:12 h light/dark cycle). Animals were subjected to 3 days acclimatization period and to several trials to reduce the stress of handling and novelty before experimentations. All experimentations were conducted in a balanced design (n=8 rats/group). The experimental procedures were made in strict agreement with National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, reviewed 1985) and approved by the institutional ethics and animal care committee (BASR/No./03202/Sc.).

Experimental Protocol

Animals were indiscriminately divided into four groups, vehicle control (VC), thymol (THY), diazepam (DZ) and THY + DZ. An oral dose of thymol (40 mg/kg/ml in slightly warm water) and diazepam i.p. (4 mg/kg/ml) was administered daily for 14 days to the groups assigned as THY and DZ respectively. The vehicle control group was given water and saline. THY + DZ animals were co-administered with thymol and diazepam with the same doses. Doses of thymol and diazepam were selected on the basis of earlier reported studies (Saravanan and Pari, 2016; Begg *et al*., 2005) respectively. The treatments were abruptly terminated after 14 days and different behavioral tests were performed to assess the withdrawal symptoms after 24 hours.

Behavioral Analysis

To avoid the order effect all activities were monitored in a balanced design.

Open Field Activity

Open field test is used for the evaluation of ambulatory performance and examination in a new atmosphere. It is simple assessment of behaviors that requires no animal training. To determine locomotor activity, the animal was positioned in the central of the open field arena and allowed to explore the area. The total of squares crossed by animal with its 4 paws was noted for 5 min as reported earlier (Haider *et al*., 2004).

Elevated Plus Maze

EPM is employed to access learning and memory related to spatial cues in animals. The method is same as described by Mutlu *et al*. (2011). The test comprised of two sessions (acquisition and retention). In the acquisition session rat was positioned at the corner of open arm in a way that its face was away from the central area and the transfer latency to come into one of the closed arms with all four paws was monitored. The rat was further permitted to move for 10s in the maze irrespective of closed and open arms when it entered in the closed arm. After 24 h retention session was performed and transfer latency was again monitored.

Passive avoidance test

To assess memory and learning function in rats, passive avoidance test was also performed. The methodology was basically identical as defined by Tabassum *et al*. (2017). The time consumed by the rat to move in the dark compartment was monitored (step-through latency) for 3 minutes (cut off time). The test consisted of two sessions; training session and testing session. The animal was positioned in lightened box during training session. The rat received a foot shock (1.5 mA) through the grid floor to its paws for 5 sec once it entered into the dark compartment, after which it instantly moved back to the lightened compartment. Retention of passive avoidance was assessed after 90 minutes of training. In the testing session the rat was positioned in the bright compartment again for a maximum of 5 minutes and the step-through latency time that is the time lapsed before the rat entered the dark compartment was noted.

Physical signs of Diazepam Withdrawal

Physical signs for diazepam withdrawal were monitored for 10 minutes, 24 hours after cessation of diazepam treatment. Changes in scratching, shaking, chewing (swallowing and licking), grooming (including paw licks, paw tremors and genital licking), and mouthing (burst of rapid jaw movements) behaviors, which are classically used to measure withdrawal symptoms of addictive drugs were observed (Perez and Biasi, 2015; MacRae and Siegel, 1997; Toki *et al*., 1996). During each withdrawal test session, single rat was placed in an operant box and withdrawal signs were monitored. Each rat received 10 minutes observation period for each test.

Marble Burying Test

The marble burying test is widely used to explore obsessive compulsive or repetitive like behaviors and anxiety in rodents (Brouwer *et al*., 2018; Dixit *et al*., 2020). Defensive burying is known as a typical rodent behavior in which the animals mess up saw dust vigorously with their forepaw in treading like movements and their heads in shovelling movements directed towards noxious stimuli (Kedia and Chatteiji, 2014). Twenty glass marbles were placed in a cage having sawdust. The marbles were positioned in a regular grid pattern and rats were allowed to explore the cage for 20 minutes. The total number of marble buried in bedding was counted. The percentage of marbles buried under the saw dust was the main outcome.

Inverted screen test

In 1964, Kondziela proposed the inverted screen test for monitoring the muscular strength of all four limbs. The

test is used to assess rodents with neuromuscular disorders in order to monitor motor coordination and neuromuscular impairment. Most rodents are enthusiastic to perform the test as their natural behaviors do not want these animals to fall off the screen. In this study, untrained rats were placed on top of screen. The screen was then inverted 180° so that rat was on the bottom of the screen. The latency to fall off was recorded over 1 min testing session.

STATISTICAL ANALYSIS

The statistical evaluation was done by one-way ANOVA and student's t-test while post-hoc analysis was done by Tukey's test via SPSS (Version 20). Results are stated as the mean ± SD and $p<0.05$ was considered as significant.

RESULTS

Effect of Thymol on Diazepam induced physical withdrawal symptoms

Data analysis by ANOVA (one-way) revealed a significant effect of treatment on different withdrawal symptoms such as shake [$F(3, 28) = 60.762$, $p<0.01$)], scratch [$F(3, 28) = 25.220$, $p<0.01$)], chew [$F(3, 28) = 82.177$, $p<0.01$)], groom [$F(3, 28) = 36.068$, $p<0.01$)] and mouthing [$F(3, 28) = 79.653$, $p<0.01$)] 24 hours as shown in fig. 1.

Post-hoc analysis showed significant ($p<0.01$) decrement in withdrawal symptoms (shake, scratch, chew, groom and mouthing) observed in rats co-administered with THY + DZ as compared to the DZ administered rats. A significant ($p<0.01$) decline in groom behavior was also observed in rats treated with THY as compared to their respective controls. However a nonsignificant effect was observed in other withdrawal symptoms such as shake, scratch, chew and mouthing in THY treated animals as compared to their controls.

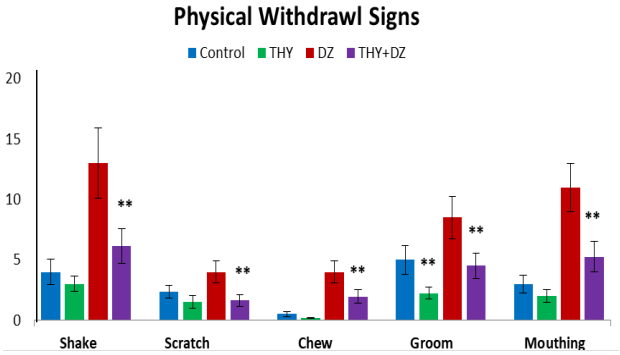

Fig. 1: Effect of Thymol on physical withdrawal symptoms induced by abrupt discontinuation of Diazepam after 24 hours. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by Tukey's test. **p<0.01 verses respective controls.

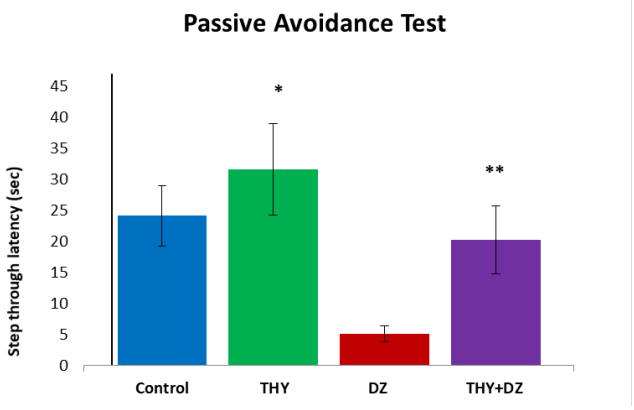

Fig. 2: Effect of Thymol on memory impairments induced by abrupt discontinuation of Diazepam after 24 hours in PAT. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by Tukey's test. *p<0.05; **p<0.01 verses respective controls.

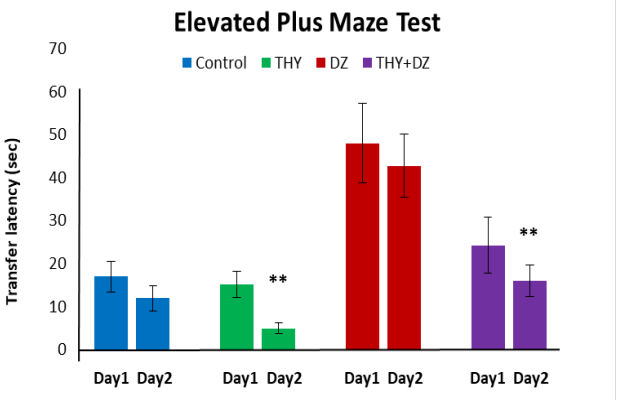

Fig. 3: Effect of Thymol on memory impairments induced by abrupt discontinuation of Diazepam after 24 hours in EPM. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by student's t-test. **p<0.01 verses respective controls.

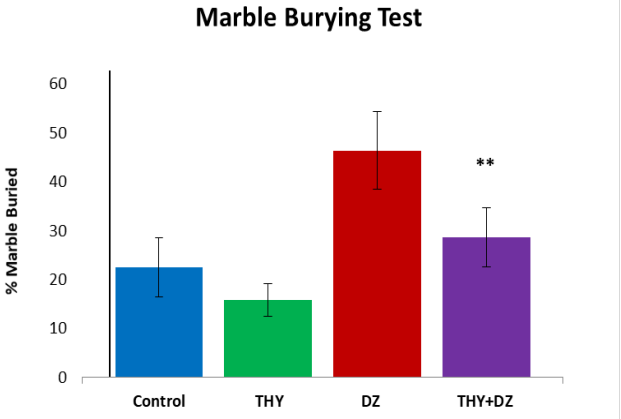

Fig. 4: Effect of Thymol on rebound anxiety induced by abrupt discontinuation of Diazepam after 24 hours in MBT. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by Tukey's test. **p<0.01 verses respective controls.

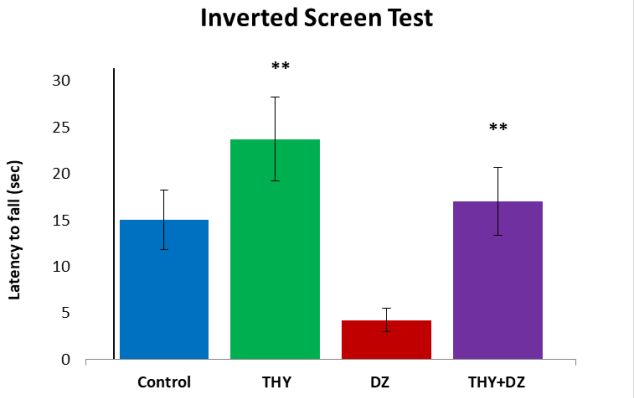

Fig. 5: Effect of Thymol on motor dysfunction induced by abrupt discontinuation of Diazepam after 24 hours in IST. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by Tukey's test. **p<0.01 verses respective controls.

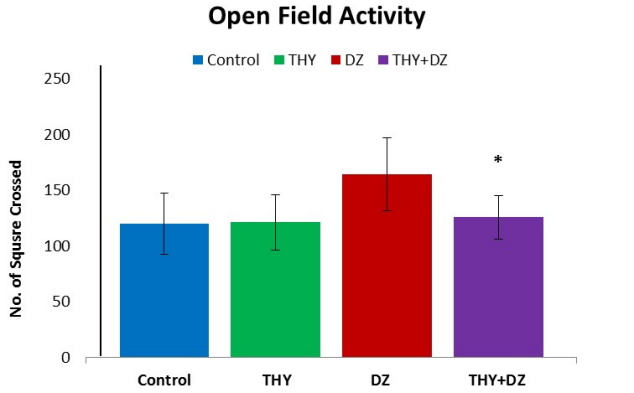

Fig. 6: Effect of Thymol on increased ambulatory activity induced by abrupt discontinuation of Diazepam after 24 hours in OFT. Data represented as mean ± SD; (n=8) rats per group. Significance difference was done by Tukey's test. *p<0.05 verses respective controls.

Effect of Thymol on Diazepam induced memory impairments in PAT and EPM

Data analysis by ANOVA (one-way) revealed a significant [F (3, 28) =36.023, p<0.01)] effect of treatments on retention of memory in PAT after 24 hours shown in fig. 2. Post-hoc analysis showed significant improvement in cognitive abilities in rats treated with THY (p<0.05) as compared to VC. Moreover the rats co-treated with THY + DZ also exhibited a significant (p<0.01) increase in memory retention as compared to DZ treated rats which was evident by an increase in the latency time by rats to enter into the punishable compartment.

Data for transfer latency in EPM was analyzed by student's *t*-test after 24 hours of treatment as shown in fig. 3. Significant (p<0.01) decline in transfer latency was observed on 2nd day (retention session) in THY and THY +DZ groups as compared to their respective 1st day (acquisition session).

Effect of Thymol on Diazepam induced rebound anxiety in MBT

Data analysis by ANOVA (one-way) showed a significant [F (3, 28) =37.502, p<0.01)] effect of treatments on burying behavior in % MBT after 24 hours of treatment as shown in fig. 4. Post-hoc analysis showed a significant decrease (p<0.01) in % marble burring in rats co-treated with THY + DZ as compared to DZ treated rats, while rats treated with THY showed a non-significant increase in % marble burring compared to vehicle control.

Effect of Thymol on Diazepam induced motor dysfunction in IST

Data analysis by ANOVA (one-way) showed a significant [F (3, 28) = 45.906, p<0.01)] effect of treatments on motor co-ordination in IST after 24 hours of treatment as shown in fig. 5. Post hoc analysis determined a significant decrease (p<0.01) in latency to fall in groups treated with THY and co-treated with THY + DZ rats as compared to vehicle control and DZ induced withdrawal rats group respectively.

Effect of Thymol on Diazepam induced decreased ambulatory activity in OFT

Data analysis by ANOVA (one-way) showed a significant [F (3, 28) =5.083, p<0.01)] effect of treatments on ambulatory activity in OFT after 24 hours of treatment as shown in fig. 6. Post-hoc analysis revealed a significant (p<0.05) decreased in number of squares crossed/5min in rats co-treated with THY + DZ as compared to DZ withdrawal animals. However the number of squares crossed/5min in THY treated animals was comparable to the vehicle controls.

DISCUSSION

The outcomes of the present study clearly indicated that co-administration of thymol significantly attenuated physical withdrawal symptoms associated with abrupt cessation of diazepam. Previously it has been reported that abrupt cessation of diazepam produced withdrawal symptoms (Toki *et al*., 1996, Yatham *et al*., 2018). This study also showed that cessation of diazepam after 14 days produced increased shaking, mouthing, grooming, scratching and chewing like behaviors in rats as shown in fig. 1. However, rats that were co-administered with thymol showed a considerable decrease in the above mentioned withdrawal symptoms. Discontinuation from chronic benzodiazepines is associated with rebound anxiety like symptoms (Brett and Murnion, 2015; Allison and Pratt 2006). Termination of diazepam administration in the present study induced a significant increase in anxiety like behavior in rats evident by increased burying of marbles and the number of squares crossed in open field test, while co-administration of thymol significantly attenuated rebound anxiety and decreased agitation induced by diazepam cessation indicating anxiolytic potential of thymol. Current findings are consistent with

the previous works in which supplementation of thymol ameliorated anxiety like symptoms (Bhandaria and Kabra 2014, Capatina *et al*., 2020). The antidepressant-like role of thymol that may be through the enhancement of the monoaminergic response such as serotonin and noradrenaline has also been reported (Deng *et al*., 2015). Common adverse effects of diazepam uses also include anterograde amnesia (Dhaliwal *et al*., 2020; Beppe *et al*., 2020). Abrupt cessation of diazepam after 14 days significantly impaired memory in both memory assessment tests while co-administration of thymol significantly reverted this diazepam-induced memory decline as shown by decreased transfer latency and increased step-through latency in EPM and PAT respectively. Neuroprotective role of thymol on learning and memory and improved synaptic plasticity has also been reported previously (Asadbegi *et al*., 2017, 2018). Once a person develops dependency on the drug, the possibility of developing withdrawal symptoms surges. Withdrawal symptoms of benzodiazepine also include ataxia and altered mental alertness. (Kang *et al*., 2020). The results of present study depicted that abrupt cessation of diazepam significantly impaired the motor coordination evident by decrease in latency to fall in inverted screen test. However, rats that were co-administered with thymol showed considerable improvement in motor coordination as animals took more time to fall (fig. 5). Neuroprotective and antioxidant roles of thymol against neurodegenerative disorder Parkinson's have also been reported (Javed *et al*., 2019).

CONCLUSION

In conclusion results of the present study clearly establish the protective effects of thymol in managing diazepam induced withdrawal symptoms. This monoterpene not only prevents the vicious onset of rebound anxiety and depression after the discontinuation of diazepam usage, but is also helpful in improving memory and motor coordination in rats. Hence, the present study strongly suggests the role of thymol in reducing unwanted side effect especially withdrawal symptoms of chronic use of benzodiazepines.

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