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Thymoquinone ameliorated the acquisition and expression of nicotine-induced behavioral sensitization in mice

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ABSTRACT

Recurrent methodical nicotine administration prompts locomotor hyperactivity in human and rodents, such amplification in locomotion is characterized as nicotine-induced behavioral sensitization (NIBS). Thymoquinone (a phytochemical found in *Nigella sativa* seeds) has some rudimentary reports regarding its antioxidant, anti-depressant, neuroprotective and anti-nociceptive potential with a rich religious and historical background of safety and efficacy for multiple illness. Thereof, the current study was emphasized to explore thymoquinone potential effects on acquisition and expression of NIBS. Balb-C male mice (20-30 gm) were treated with thymoquinone (10, 20 and 30 mg/kg; PO) to investigate its potential effects on acquisition and the expression of behavioral sensitization induced by repeated meticulous nicotine administration (0.5 mg/kg; IP). In both studies, locomotor activity was recorded on 1st, 7th and 11th day in activity cages. Our data revealed that thymoquinone (30 mg/kg; PO) significantly ($p< 0.001$) inhibited the acquisition of NIBS whereas thymoquinone (10, 20 and 30 mg/kg; PO) significantly ($p< 0.001$) attenuated the expression of NIBS. Concluding that the current study provided mechanistic insight to the ameliorating potential effects of thymoquinone on behavioral sensitization induced by nicotine and can be deliberated as a potential therapeutic choice for the management of nicotine addiction.

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Keywords
Nicotine,
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Thymoquinone,
Dopamine,
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INTRODUCTION

In humans, recurrent nicotine (tobacco) practice engenders hedonia, euphoria, relaxation, reduced fatigue and enhanced arousal [1]. Such effects of reinforcement play a crucial part in the initiation and unabated use of tobacco characterized by serious health risks to the individual and overall disease burden on state and society [2,3]. Nicotine and other psycho-stimulants engender behavioral influences *via* mesocorticolimbic dopaminergic system [4] in which neuro-adaptations befall in the glutamatergic, dopaminergic (DAergic) and gamma-Aminobutyric acid (GABAergic) neuronal circuitry connected to the nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala and prefrontal cortex (PFC) [5]. In rodents, central nervous system (CNS) stimulants such as amphetamine, cocaine and nicotine upon repeated exposure have ability to develop hyperactivity in locomotion [6–8]. Such amplification in locomotion is termed as drug-induced behavioral sensitization. Behavioral sensitization is a mixture of two distinct processes i.e. initiation and expression (series of actions associated with the acquisition of sensitization and persistent changes in behavior) and is mediated anatomically *via* ventral tegmental area and nucleus accumbens,

respectively [6,9,10].

There is a high density of α_7 nicotinic acetylcholine receptors (nAChRs) expressed on the presynaptic glutamatergic terminals whereas $\alpha_4\beta_2$ nAChRs are present on all VTA DAergic and GABAergic neurons. Mesolimbic DAergic neurons has been reported to be pivotal for nicotine addiction and such effect is mediated by nAChRs found in the VTA [3,11]. Literature exhibited that glutamate and dopamine are principal neurotransmitters that play role in acquisition and expression of sensitization by modulation of nAChRs expressed on the presynaptic glutamatergic terminals (α_7) and VTA DAergic and GABAergic neurons ($\alpha_4\beta_2$) [7,9]. These both nAChRs modulate this nicotine-induced hyperlocomotion and reward [3,12]. In nicotine addiction, the process of sensitization subsidizes to the process of relapse and also play a vital role in the acquisition of nicotine dependence even after prolonged abstinence [13,14]. Therefore, it is imperative to target and modulate the neural mechanisms associated with behavioral sensitization induced by abused drugs such as nicotine in order to prevent addiction.

Thymoquinone, a major phytochemical obtained from *Nigella sativa* seeds, has a rich historical and religious background of efficacy and safety for multiple illnesses, worldwide [15]. In 1963, it was separated from the essential oil of *Nigella sativa* for the very first time. Later on, it was obtained from various other plant species like *Eupupatorium ayapana*, *Nepeta distans*, *Thymus vulgaris* and essential oils of *Calocedrus decurrens* and *Saturaga* species [16,17]. Literature exhibited some rudimentary reports regarding thymoquinone potential as neuroprotective, antioxidant and anti-depressant compound [15]. Intracerebroventricular administration of thymoquinone, in rats, subdues epileptic seizures and these effects elicited has been proposed due to increase in GABAergic tone mediated by an opioid receptor [18]. Thymoquinone also depicted to possess anti-nociceptive and anxiolytic activity [19–21]. In addition, it has been reported to exhibit anti-addictive profile in morphine dependence and withdrawal [22], has potential to ameliorate spontaneous nicotine withdrawal [23] and development and expression of alcohol induced behavioral sensitization [24].

Taking everything into account, the current study was designed to explore thymoquinone potential effects on acquisition and expression of nicotine induced behavioral sensitization.

MATERIALS AND METHODS

Animals

Balb/c mice (male) were obtained in the weight range of 20-30 g from the Animal Care Facility of the CIIT, Abbottabad. Animals were provided with standard environmental conditions such as temperature, i.e. 25 ± 1 ºC, with a 12 hour normal circadian cycle. Food and water *ad libitum* were also provided. The experiments were performed according to the guidelines of the "Ethical Care Committee for Animal Research" at the CIIT, Abbottabad that conformed to the guidelines of "Animals Scientific Procedure Act, 1986 UK".

Chemicals

(-)-Nicotine hydrogen tartrate was purchased from Wako chemicals USA, Inc. Thymoquinone was obtained from Santa Cruz Biotechnology, USA. Nicotine was dissolved in 0.9% normal saline solution whereas pure thymoquinone suspension was prepared using 1% CMC, i.e. carboxymethylcellulose (Sigma Aldrich, USA). The solutions and suspension were prepared freshly on daily basis and were administered in a volume of 0.1 mL/10 gm body weight of mice. Nicotine was administered in a dose of 0.5 mg/kg/day *via* intraperitoneal (IP) injection. Thymoquinone suspension was prepared in three doses, i.e. 10, 20 and 30 mg/kg, for oral (PO) administration.

Apparatus

In order to access locomotor activity, activity boxes were used. Mice were acclimatized in the activity boxes having a dimension of 1.5 x 1.5 feet (45.6 x 45.6 cm) divided into four equal quadrants (22.8 x 22.8 cm). Activity boxes were swabbed and cleaned between trials and locomotor activity was recorded by an installed digital camera 300 cm above the boxes and was scrutinized on a PC by utilizing a VideoLAN client (VLC) media player.

Treatment Protocols

On day 0, i.e. the day prior to the experimental day, Balb/c male mice (20-30 g) were acclimatized to the test environment and apparatus for 1 hour. This step was followed by the injection of normal saline (10 ml/kg) to acquaint the animals to testing stress and handling. Activity boxes were swabbed and cleaned between trials to avoid conditioned preference. In order to examine the effects of thymoquinone (TQ), animals were divided into different groups (n= 8/group) and experiments were performed such as:

Induction of Behavioral Sensitization (BS) by Nicotine

Balb/c male mice (20-30 g) were divided into two groups (n= 8/group); Group 1: normal saline (10 ml/kg) (N/S) and Group 2: nicotine (0.5 mg/kg; IP). On test day 1, after administration of a normal saline (10 ml/kg) injection (IP) to animals, they were acclimatized for 60 minutes to the test apparatus. After a gap of 60 minutes, group 1 was injected with normal saline (10 ml/kg) whereas group 2 was injected with nicotine (0.5 mg/kg; IP) and immediately after administration, locomotor activity was recorded for 30 minutes. The same procedure was iterated for both groups for seven consecutive days. Following abstinence period of three days, i.e. on day 11, group 1 was treated with normal saline, intraperitoneally whereas group 2 with 0.5 mg/kg nicotine challenge dose, intraperitoneally and locomotor activity was recorded for 30 minutes, immediately after administration [24,25].

Assessment of Thymoquinone on Acquisition of

Nicotine-Induced Behavioral Sensitization (NIBS)

Male mice (20-30 gm) were divided into five groups (n=8/group); Group 1: Saline (10 ml/kg), Group 2: Nicotine (0.5 mg/kg; IP), Group 3: Nicotine (0.5 mg/kg; IP) + TQ1 (10 mg/kg; PO), Group 4: Nicotine (0.5 mg/kg; IP) + TQ2 (20 mg/kg; PO), Group 5: Nicotine (0.5 mg/kg; IP) + TQ3 (30 mg/kg; PO). On test day 1, animals were acclimatized for 1 hr. (7- 8 am) to the test apparatus. After a gap of 60 minutes (9 am), groups 1 and 2 received saline injection; (10 ml/kg) IP and groups 3/4/5 were treated with 10, 20 and 30 mg/kg; PO dose of thymoquinone, respectively. After a gap of 60 minutes (10 am), group 1 was administered with saline (10 ml/kg) whereas groups 2/3/4/5 were injected with nicotine (0.5 mg/kg; IP). Immediately after administration, the locomotor activity was recorded for 30 minutes. The same procedure was repeated for all the groups for seven consecutive days. Following abstinence period of three days, on day 11, group 1 was administered with saline (10 ml/kg) whereas groups 2/3/4/5 were administered nicotine challenge dose (0.5 mg/kg; IP) and immediately after administration, locomotor activity was recorded for 30 minutes [24,25].

Assessment of Thymoquinone on Expression of Nicotine-Induced Behavioral Sensitization (NIBS)

Balb/c male mice (20-30 g) were divided into five groups (n = 8/group); Group 1: Saline (10 ml/kg), Group 2: Nicotine (0.5 mg/kg; IP), Group 3: Nicotine (0.5 mg/kg; IP) + TQ1 (10 mg/kg; PO), Group 4: Nicotine (0.5 mg/kg; IP) + TQ2 (20 mg/kg; PO), Group 5: Nicotine (0.5 mg/kg; IP) + TQ3 (30 mg/kg; PO). On day 1st, mice were acclimatized to the locomotor cages for 60 minutes (7-8 am) and then all groups were treated with saline; IP (10 ml/kg). After a gap of 60 minutes (9 am), group 1 was administered with saline (10 ml/kg) whereas the remaining groups were injected with nicotine, i.e. 0.5 mg/kg; IP, once daily. Immediately after administration, locomotor activity was recorded for 30 minutes. The same procedure was repeated for all the groups for seven consecutive days. Following abstinence period of three days (on day 11th, 9 am), groups 1 and 2 received an injection of saline whereas groups 3/4/5 were treated with 10, 20 and 30 mg/kg; PO, dose of thymoquinone once daily, respectively. After a gap of 60 minutes (10 am), group 1 was administered with saline (10 ml/kg) whereas groups 2/3/4/5 were injected with nicotine challenge dose (0.5 mg/kg; IP). Immediately after administration, locomotor activity was recorded for 30 minutes [24,25].

Statistical Analysis

For statistical analysis, Graphpad prism 5 was utilized. All the data were manifested as Mean ± SEM. One way ANOVA followed by *post hoc* Dunnett's test and the student's t-test were utilized to analyse the data. Data were considered only significant if *p < 0.05, **p < 0.01 and ***p < 0.001.

RESULTS

Induction of Behavioral Sensitization by Nicotine

Analysis using student's t-test revealed that there was a significant (p< 0.001) increase in locomotion, on day 1, in nicotine (0.5 mg/kg; IP) treated animals in comparison to the saline-treated group (Fg. 1). Following 7 days of consecutive nicotine (0.5 mg/kg; IP) administration, a gradual significant (p< 0.01) enhancement in locomotion in the nicotine-treated group was observed, using student's t-test, on day 7 as compared to saline-treated group shown in Figure 1. After 3 days of abstinence period, i.e. on day 11, a marked (p< 0.001) increase in the locomotion was observed in nicotine-treated group compared to saline-treated group using student's t-test depicted by Figure 1.

Effect of Thymoquinone on Acquisition of Nicotine-Induced Behavioral Sensitization (NIBS)

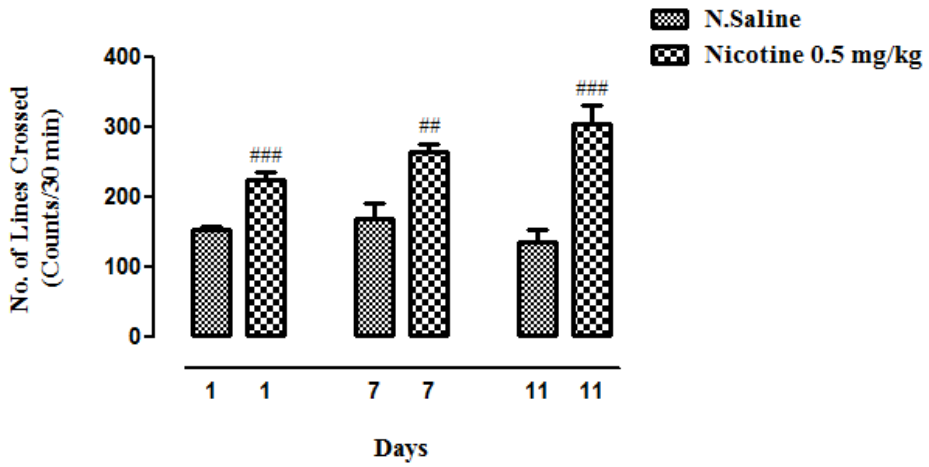

Figure 1. Bar graph illustrates the effect of nicotine administration to locomotor activity in the activity boxes. Nicotine (0.5 mg/kg; IP) treatment to the drug naïve mice showed significant increase in locomotion on days 1, 7 and 11 in comparison to the saline-treated group. The bar depicts mean + SEM of locomotion counts/30 min. ##p < 0.01 and ###p < 0.001 treatment was significantly distinct in comparison to saline-treated group. (n = 8).

There was a significant induction of locomotor sensitization by nicotine (0.5 mg/kg; IP) as compared to saline treated group when student's t-test was applied ($p< 0.05$) on day 1, 7 and 11 depicted by Figure 2. One-way ANOVA followed by *post hoc* Dunnett's test revealed that the locomotor activity induced by nicotine (0.5 mg/kg; IP) was significantly reduced by thymoquinone at doses 10 mg/kg ($p< 0.05$) and 30 mg/kg ($p< 0.001$), on day 1 (Fig. 2). Upon repeated treatment, thymoquinone significantly ($p < 0.001$) inhibited the nicotine-induced locomotor sensitization (NILS) on day 7 and 11 as shown in Figure 2. Thus, signifying that thymoquinone has potential to inhibit the development of behavioral sensitization induced by nicotine at dose 30 mg/kg.

Effect of Thymoquinone on Expression of Nicotine-Induced Behavioral Sensitization (NIBS)

Analysis using student's t-test showed that there was a significant development of locomotor sensitization on day 1, 7 and 11 by nicotine (0.5 mg/kg; IP) in comparison to saline treated group (Fig. 3). One-way ANOVA followed by *post hoc* Dunnett's test revealed that there was a significant

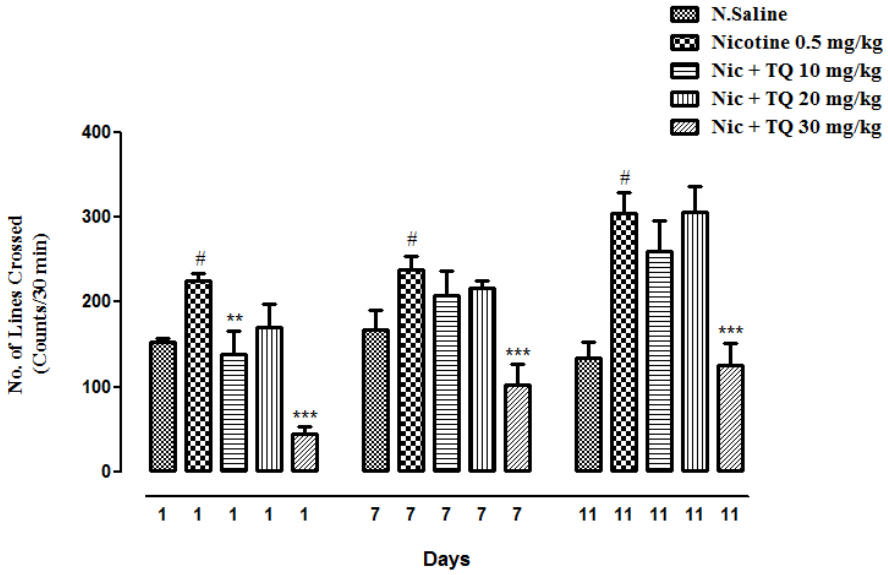

Figure 2. Bar diagram showed the locomotor activity (Mean + SEM, counts/30 min) in the activity boxes after thymoquinone administration sub-acutely, at doses 10, 20 and 30 mg/kg; PO, 1 hour before the nicotine's administration, 0.5 mg/kg; IP, on days 1 and 7. Day 11, depicts the locomotor activity in the activity boxes after nicotine challenge dose, 0.5 mg/kg; IP, in the sub-acute thymoquinone + nicotine pre-treated mice following a 3 days withdrawal. #$p < 0.05$ treatment was significantly distinct in comparison to saline-treated group. *$p < 0.05$ and ***$p < 0.001$ treatment was significantly distinct in comparison to nicotine-treated group. ($n = 8$).

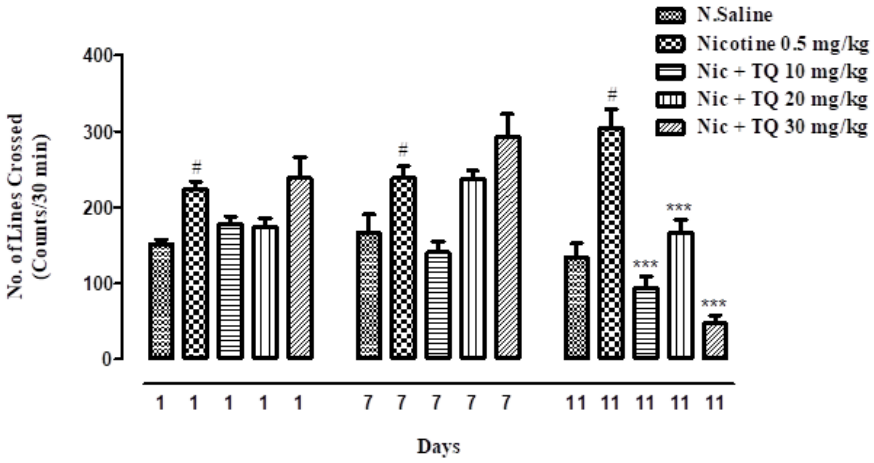

Figure 3. Bar graph depicts locomotor activity (Mean + SEM, counts/30 min) in the activity boxes after thymoquinone administration acutely, at doses 10, 20 and 30 mg/kg; PO, 1 hour before the nicotine challenge dose, 0.5 mg/kg; IP, on day 11. #$p < 0.05$ treatment was significantly distinct in comparison to saline-treated group. ***$p < 0.001$ treatment was significantly distinct in comparison to nicotine-treated group. ($n = 8$).

($p<0.001$) alleviation in locomotor hyperactivity with all doses of thymoquinone (10, 20 and 30 mg/kg) in comparison to nicotine-treated group as illustrated in Figure 3. Thus, signifying that thymoquinone has potential to inhibit the expression of behavioral sensitization induced by nicotine.

DISCUSSION

Chronic nicotine administration (≥ 7 subsequent days) directs neurophysiological changes in the circuitry of glutamatergic, dopaminergic and GABAergic neurons connected to the VTA, NAc, amygdala and PFC [26–28] which can be manifested by the hyperactivity in locomotion [6–8]. Such amplification in locomotion is termed as drug-induced behavioral sensitization. In similarity with existing reports, our data revealed that intermittent nicotine administration, for consecutive seven days, effectuated locomotor sensitization and after a three days of drug-free period, i.e. on day 11, when nicotine challenge dose was administered, it induced the expression of sensitization (Fig. 1). In addition, our data also exhibited that sub-acute thymoquinone treatment (30 mg/kg; PO) significantly attenuated the acquisition of NIBS (Fig. 2) and this effect is significant on day 1 at a dose of 30 mg/kg; PO in contrast with nicotine-treated group (Fig. 2) whereas acute thymoquinone administration at all doses (10, 20 and 30 mg/kg; PO) significantly inhibited the expression of NIBS (Fig. 3). In existing reports, such depreciation of behavioral sensitization was also reported with the first dose of clozapine and risperidone on day 1, during sub-acute treatment [29,30].

For neuronal mechanism behind sensitization induced by nicotine, it has been documented that nicotine engender behavioral influences *via* mesocorticolimbic dopaminergic system [31]. The soma of DAergic neurons, in the mesocorticolimbic DAergic pathway, are situated in the VTA and their axons are projected to the NAc and mPFC. Moreover, glutamatergic projections are indirect from mPFC to the VTA *via* LDT/PPT and are direct from mPFC itself and from BLA to the NAc [9] whereas GABAergic projections are from VTA to the NAc and mPFC and from NAc to VTA and VP and then from VP to the thalamus and VTA [32]. A dynorphin (Dyn) input from the NAc core to the VTA has also been reported [33]. Briefly, glutamate and dopamine are principal neurotransmitters that are found to play a pivotal role in acquisition and expression of sensitization by modulation of nAChRs expressed on the presynaptic glutamatergic terminals (α_7) and VTA DAergic and GABAergic neurons ($\alpha_4\beta_2$) [7,9]. These both nAChRs ($\alpha_4\beta_2$ and α_7) modulate nicotine-induced hyperlocomotion and reward [3,12].

Recurrent nicotine habit engender desensitization of such receptors [34]. Desensitization or deficits in α_7 nAChRs enhances motivation to nicotine self-administration or nicotine-seeking behavior and thus contributes to the nicotine addiction. Such effects can be masked by the enhanced expression of α_7 nAChRs or by the local administration of α_7 nAChRs agonist which play a role in reducing the motivation to nicotine self-administration or nicotine-seeking behavior [35]. In addition, Varenicline (α_7 nAChRs agonist) has been reported to ameliorate the development and expression of NIBS [25]. Literature illustrated that thymoquinone has potential to enhance the expression of α_7 nAChRs [36]. Thereof, the thymoquinone potential to attenuate the acquisition and expression of NIBS might be attributed towards its known potential of enhancing the expression of α_7 nAChRs. Also, it has been reported that GABA has a role in induction of hyperlocomotion. Following acute nicotine administration, depression in locomotion was observed due to enhanced GABA levels while chronic administration leads to the decrease in GABA levels due to desensitization of receptors and thus hyperlocomotion is observed due to enhanced DA levels in NAc [37,38]. In earlier reports, Thymoquinone was reported to enhance the levels of GABA [39], also it was reported to inhibit sensitization through an opioid receptor-mediated increase in GABAergic tone [24]. Such known property of thymoquinone to enhance GABA levels might contribute to its inhibitory effect on locomotion by decreasing the DA levels in NAc. Taking everything into account, the ability of thymoquinone to alleviate the acquisition and expression of nicotine induced behavioral sensitization might be attributed towards its known potential of enhancing the expression of α_7 nAChRs and enhancing the levels of GABA.

Additionally, it is pertinent to mention that LD_{50} of thymoquinone via oral route was reported to be 870.9 mg/kg [40]. In a pilot studies, thymoquinone was found to be safe in children at a dose of 1 mg/kg [41] whereas in adults, 75 mg/day to 2600 mg/day was well tolerated . It has also been reported that thymoquinone at a dose of 1000 mg/day for 5 months, 2600 mg/day for one week or 400 mg/day for 2 weeks did not yield any adverse effects [42]. So, the drug can safely be used in high doses for more prominent effects.

Keeping this in mind, it can be deduced that thymoquinone has potential to ameliorate the effects of behavioral sensitization induced by nicotine and can be used as a potential therapeutic choice for the management of nicotine addiction.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any institute.

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