Theophylline, adenosine receptor antagonist prevents behavioral, biochemical and neurochemical changes associated with an animal model of tardive dyskinesia

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Abstract:
Tardive dyskinesia is considered to be the late onset adverse effect of prolonged administration of typical neuroleptic drugs. Adenosine is now widely accepted as the major inhibitory neuromodulators in the central nervous system besides GABA. Antagonists of A₂A receptors are known to confer protection against neuronal damage caused by toxins and reactive oxygen species. The present study investigated the effect of adenosine receptor antagonist, theophylline (25 and 50 mg/kg, ip) in an animal model of tardive dyskinesia by using different behavioral (orofacial dyskinetic movements, stereotypy, locomotor activity, % retention), biochemical (lipid peroxidation, reduced glutathione levels, antioxidant enzyme levels (SOD and catalase)) and neurochemical (neurotransmitter levels) parameters. Chronic administration of haloperidol (1 mg/kg ip for 21 days) significantly increased vacuous chewing movements (VCMs), tongue protrusions, facial jerking in rats which was dose-dependently inhibited by theophylline. Chronic administration of haloperidol also resulted in the increased dopamine receptor sensitivity as evidenced by increased locomotor activity and stereotypic rearing. Further, it also decreased % retention time in elevated plus maze paradigm. Pretreatment with theophylline reversed these behavioral changes. Chronic administration of haloperidol also induced oxidative damage in all the brain regions which was prevented by theophylline, especially in the striatum. Chronic administration of haloperidol resulted in a decrease in dopamine levels which was reversed by treatment with theophylline (at higher doses). The findings of the present study suggested the involvement of adenosinergic receptor system in the development of tardive dyskinesia and possible therapeutic potential of theophylline in this disorder.

Key words:
adenosine, neuroleptic drugs, tardive dyskinesia, theophylline

Introduction

Tardive dyskinesia is a syndrome characterized by repetitive involuntary movements usually involving mouth, face and tongue. It is considered as a late onset adverse effect of prolonged administration of neuroleptic drugs [7, 23]. About 20–30% of neuroleptic-treated patients present this syndrome that can last for years and is even irreversible in some cases [14, 17].
Long-term treatment with haloperidol, a classical neuroleptic drug widely used for the treatment of schizophrenia and affective disorders can lead to tardive dyskinesia. It blocks dopamine receptors, and concomitant increase in turnover of this amine may contribute to haloperidol toxicity due to generation of free radicals and increased lipid peroxidation [3, 6, 24].

Although the hypothesis of dopamine supersensitivity has dominated conceptual approaches used to study and treat tardive dyskinesia, neuropathological mechanism regarding its development is still poorly understood. Various hypotheses have been proposed including a disturbed balance between dopaminergic and cholinergic system, dysfunction in GABA neurons [12], excitotoxicity via glutamate receptors and oxidative stress [5–7]. Neither of them individually explains the pathogenesis of tardive dyskinesia but over the time oxidative stress and excitotoxicity theory has gained ample support from the literature. Overproduction of free radicals derived from the metabolism of dopamine or from the enhancement of glutamatergic transmission caused by the blockade of presynaptic dopamine receptors, seems to participate in the genesis of TD [4, 28, 30, 40].

In the striatum, dopamine D2 receptors are reported to interact with high affinity adenosine subtype 2 (A2) receptors. Adenosine receptors, especially A2A receptors are involved in several drug induced motor behaviors [27, 29] as well as other related behaviors such as benzodiazepine-induced withdrawal symptoms [22]. Chronic intraperitoneal treatment with haloperidol was reported to increase the density of striatal A2A receptors by 33% whereas there was no change in the striatal D1 and A1 receptors [32]. Administration of clozapine and sulpiride did not affect the receptor density in the striatum [32]. Based on reciprocal antagonistic interaction between adenosine A2A receptors and dopaminergic D2 receptors, A2A receptors could be expected to be a target in TD. Antagonists of A2A receptors are also known to provide protection against the neuronal damage caused by toxins as well as they can also protect the cell against damage inflicted by reactive oxygen species.

In the present study, we have investigated the effect of non-selective adenosine receptor antagonist, theophylline in haloperidol-induced orofacial dyskinesia in rats, a potential animal model of tardive dyskinesia.

### Materials and Methods

#### Animals

Male Wistar rats (180–220 g) bred in the Central Animal House facility of Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a 12:12 h light-dark cycle with free access of food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used only once in the experiment. All the experiments were carried out between 9.00 and 15.00 h. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the guidelines of Indian National Science Academy for the use and care of experimental animals.

#### Drugs and treatment schedule

The following drugs were used in the present study: haloperidol (Serenece, Searle India, India) diluted with distilled water and theophylline dissolved in slightly warm water. The first group received vehicle, second group received haloperidol (1 mg/kg) plus vehicle, third group received haloperidol (1 mg/kg) plus theophylline (25 mg/kg), fourth group received haloperidol (1 mg/kg) plus theophylline (50 mg/kg) and fifth group received only theophylline (50 mg/kg). Haloperidol and theophylline were administered intraperitoneally in a constant volume of 0.5 ml per 100 g of body weight of rat.

#### Induction of orofacial dyskinesia

Haloperidol (1 mg/kg ip) was administered chronically to rats for a period of 21 days to induce oral dyskinesia. All the behavioral assessments were carried out every week and last behavioral quantification was done 24 h after the last dose of haloperidol [31].

#### Behavioral assessment of orofacial dyskinesia

On the test day, rats were placed individually in a small (30 × 20 × 30 cm) Plexiglas cage for the assessment of oral dyskinesia. Animals were allowed 10 min to get used to the observation cage before behavioral assessments. To quantify the occurrence of oral dyskinesia, hand-operated counters were em-
ployed to score tongue protrusion and vacuous chewing frequencies. In the present study, VCMs are referred to as single mouth openings in the vertical plane not directed toward physical material. If tongue protrusion or VCMs occurred during a period of grooming, they were not taken into account. Counting was stopped whenever the rat began grooming, and restarted when grooming stopped. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral dyskinesia when the animal was faced away from the observer. The behavioral parameters of oral dyskinesia were measured continuously for a period of 5 min. In all the experiments, the scorer was unaware of the treatment given to the animals [31].

**Elevated plus maze test**

The elevated plus maze was used to evaluate spatial long-term memory, following the procedure as described earlier [33]. Briefly, the apparatus consisted of two open arms and two closed arms. The arms extended from a central platform, and the maze was elevated to a height of 50 cm from the floor. On the first day, each animal was placed at the end of an open arm. Transfer latency (TL), that is the time taken by the rat to move into one of the closed arm, was recorded on the first day. If the animal did not enter a closed arm within 90 s it was gently pushed into one of closed arms and the TL latency was assigned as 90 s. The rat was allowed to explore the maze for 20 s and then was returned to the home cage. The rat was again placed in the maze on the next day (24 h later) and TL was recorded [16]. Percent retention was calculated by the formula:

\[
\text{Transfer Latency (Day 1)} - \text{Transfer Latency (Day 2)} / \text{Transfer Latency (Day 2)} \times 100.
\]

**Locomotor activity**

The locomotor activity was monitored using an activity meter (IMCORP, India). Before subjecting the animal to cognitive task, they were individually placed in the activity meter and the total activity count was registered for 5 min. The locomotor activity was expressed in terms of total photo beam interruption counts/5 min per animal [33].

**Stereotypic rearing assessment**

Stereotypic rearing was measured in different groups of animals. Each animal was individually placed in a 1000 ml beaker and rearing was scored for the time period of 5 min. Stereotypic rearing was scored as ++++ (14–16) = very high, +++ (10–12) = high, ++ (8–10) = moderate, + (4–6) = low [20].

**Dissection and homogenization**

On day 22, after behavioral quantification, animals were randomized into two groups on the basis of their behavioral characteristics. The animals were sacrificed by decapitation. In one group, the brains were removed, forebrain was dissected out and cerebellum was discarded. Brains were put on ice and the cortex, striatum and subcortical regions were separated and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post-nuclear fractions for catalase assay were obtained by centrifugation of the homogenate at 1000 × g for 20 min, at 4°C and for other enzyme assays, it was centrifuged at 12,000 × g for 60 min at 4°C. The subcortical region of brain comprised all the remaining parts of the forebrain which included the hippocampus, thalamus, hypothalamus, amygdala and other subthalamic structures. Whole brains (excluding cerebellum) of one set of animals from each group were separately stored at −80°C for HPLC studies. On the day of experiment, forebrain was dissected into two parts: cortical and subcortical (including the striatum).

**Oxidative stress parameters**

Several oxidative stress parameters such as lipid peroxidation, reduced glutathione and antioxidant enzyme activities (catalase and superoxide dismutase) were analyzed. The quantitative measurement of lipid peroxidation in the forebrain was performed according to the method of Wills [41]. Reduced glutathione was estimated according to the method of Ellman [11]. Superoxide dismutase activity was assayed according to the method of Kono [18] whereas catalase activity was assayed by the method of Luck [26]. The protein content was measured according to the method of Lowry [25] using bovine serum albumin as standard [31].
**Estimation of neurotransmitter levels**

Biogenic amines (dopamine) were estimated by HPLC with electrochemical detector by the method of Church [9]. Waters standard system consisting of a high pressure isocratic pump, a 20 μl sample injector valve, C18 reverse phase column and electrochemical detector were used. Data were recorded and analyzed with the help of Empower software. Mobile phase consisted of 2% citric acid, 2% KHPO₄, 1 mM EDTA, 1.2% MeOH, and 70 mg/ml of sodium octyl sulfate. pH of the mobile phase was adjusted to 3 with the help of HCl (6M). Electrochemical conditions for the experiment were +0.800 V, sensitivity ranges from 5–50 nA. Separation was carried out at a flow rate of 1 ml/min. Samples (20 μl) were injected manually. On the day of experiment, frozen forebrain samples were taken out and thawed. They were homogenized in ho-

![Graph A](image1)

**Fig. 1.** (A) Vacuous chewing movements (VCMs), (B) Tongue protrusions, (C) Number of facial jerking episodes recorded on day 7, 14, 22 (test day) in rats chronically treated with: vehicle, haloperidol (1 mg/kg, p 21 days), theophylline (50), theophylline (25) + haloperidol (1), theophylline (50) + haloperidol (1). Data are expressed as the mean ± SEM. a p ≤ 0.05 as compared to control group (on the day of behavioral assessment), b p ≤ 0.05 as compared to haloperidol treated group (on the day of behavioral assessment), c p ≤ 0.05 as compared to theophylline (25) + haloperidol (1)
mogenizing solution containing 0.1 M perchloric acid. After that samples were centrifuged at 12000 \( \times \) g for 5 min. The supernatant was further filtered through 0.25 \( \mu \)m nylon filters before injecting in the HPLC injection pump. Data were recorded and analyzed with the help of Empower software [9].

**Statistical analysis**

One specific group of rats was assigned to one specific drug treatment condition and each group comprised six rats (n = 6). All the values are expressed as the means \( \pm \) SEM. The data were analyzed by using analysis of variance (ANOVA) followed by Dunnett’s test. In all tests, the criterion for statistical significance was \( p < 0.05 \).

**Results**

**Behavioral assessment**

**Assessment of orofacial dyskinesia**

Haloperidol (1 mg/kg, \( ip \)) treatment resulted in time-dependent increase in VCMs, tongue protrusion and facial jerking. Co-administration of theophylline (25 and 50 mg/kg) dose dependently inhibited the time-dependent increase in haloperidol-induced VCMs, tongue protrusions and facial jerking. Theophylline (50 mg/kg) *per se* did not produce any significant change in VCMs, tongue protrusions and facial jerking as compared to control (Fig. 1 A, B, C).

**Stereotypic rearing assessment**

Haloperidol (1 mg/kg, \( ip \)) treatment resulted in a decrease in stereotypic rearing up to 7th day which was thereafter increased up to the last behavioral quantification (day 22). Co-administration of theophylline (25 and 50 mg/kg) prevented the increase in stereotypic rearing. Theophylline (50 mg/kg) *per se* did not produce any significant change in stereotypic rearing behavior as compared to control (Fig. 2).

**Locomotor activity**

Haloperidol (1 mg/kg, \( ip \)) treatment resulted in a decrease in total locomotor activity (ambulatory and rearing) up to 14th day which was thereafter increased until the last behavioral quantification (day 22). Co-administration of theophylline (25 and 50 mg/kg) prevented this increase in locomotor activity. Theophylline (50 mg/kg) *per se* did not produce any significant change in total locomotor activity (ambulatory and rearing) as compared to control (Fig. 3).

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**Fig. 2.** Stereotypic rearing behavior recorded before drug administration (baseline), day 7, 14, 22 (test day) in rats chronically treated with (a) vehicle, haloperidol (1 mg/kg, \( ip \) 21 days), theophylline (50), theophylline (25) + haloperidol (1), theophylline (50) + haloperidol (1). Data are expressed as the mean \( \pm \) SEM. "p \( \leq \) 0.05 as compared to control group.
Elevated plus maze test

Haloperidol (1 mg/kg, *ip*) treatment resulted in a significant decrease in % retention as compared to control group on day 22. Co-administration of theophylline at 50 mg/kg, prevented the decrease in % retention whereas co-administration of theophylline at 25 mg/kg did not show any effect. Theophylline (50 mg/kg) *per se* did not produce any significant change in % retention as compared to control (Fig. 4).

![Graph of total locomotor activity](image1)

**Fig. 3.** Total locomotor activity recorded before drug administration (baseline), day 7, 14, 22 (test day) in rats chronically treated with: vehicle, haloperidol (1 mg/kg, *ip* 21 days), theophylline (50), theophylline (25) + haloperidol (1), theophylline (50) + haloperidol (1). Data are expressed as the mean ± SEM. *p* ≤ 0.05 as compared to control group, *p* ≤ 0.05 as compared to haloperidol-treated group.

![Graph of percentage retention](image2)

**Fig. 4.** Percentage retention recorded on day 22 (test day) in rats chronically treated with: vehicle, haloperidol (1 mg/kg, *ip* 21 days), theophylline (50), theophylline (25) + haloperidol (1), theophylline (50) + haloperidol (1). Data are expressed as the mean ± SEM. *p* ≤ 0.05 as compared to control group, *p* ≤ 0.05 as compared to haloperidol-treated group.
Theophylline, adenosine receptor antagonist in tardive dyskinesia

Theophylline (50) + Haloperidol (1)

Cortex Striatum Subcortical

Unit SOD activity/mg protein (% of control)

Unit CAT activity/mg protein (% of control)

Unit CAT activity/mg protein

Unit CAT activity/mg protein

Unit SOD activity/mg protein

Unit SOD activity/mg protein

Fig. 5. Estimation of (A) Lipid peroxidation, (B) Reduced glutathione levels, (C) Catalase activity, (D) Superoxide dismutase activity in different brain regions (cortex, subcortical regions, striatum) in rats chronically treated with: vehicle, haloperidol (1 mg/kg, ip 21 days), theophylline (50), theophylline (25) + haloperidol (1), theophylline (50) + haloperidol (1). Data are expressed as the mean ± SEM. a, p ≤ 0.05 as compared to control group. b, p ≤ 0.05 as compared to haloperidol-treated group. c, p ≤ 0.05 as compared to theophylline (25) + haloperidol (1).
Biochemical assessment

Oxidative stress parameters

Chronic haloperidol treatment (1 mg/kg, ip) resulted in a significant increase in lipid peroxidation, decrease in reduced glutathione and antioxidant enzyme (catalase and superoxide dismutase) levels in all the regions (cortex, subcortical, striatum) of the brain as compared to control animals. Co-administration of theophylline (25 and 50 mg/kg) dose-dependently prevented the increase in lipid peroxidation, decrease in reduced glutathione and antioxidant enzyme (catalase and superoxide dismutase) levels in the cortex and striatum but not in the subcortical region. Theophylline (50 mg/kg) per se did not produce any significant change in lipid peroxidation as compared to control (Fig. 5 A, B, C, D).

Neurochemical assessment

Neurotransmitter estimation

Chronic administration of haloperidol decreased level of dopamine in extracellular space in cortical and subcortical regions (including the striatum) which was prevented dose-dependently by co-administration of theophylline (25 and 50 mg/kg). Theophylline (50 mg/kg) per se did not produce any significant change in dopamine levels as compared to control (Tab. 1).

Discussion

In the present study, theophylline, an adenosine receptor antagonist attenuated chronic haloperidol-induced orofacial dyskinesia and associated behavioral, biochemical and neurochemical parameters. Traditionally, purine receptor ligands provide protection against ischemia and stroke [34, 36] but their interactions with other receptors such as dopamine, GABA and excitatory neurotransmitter glutamate suggest a possible role of these ligands in neurodegenerative diseases [42]. As suggested by recent reports, the role of glutamate receptors and oxidative damage in the pathophysiology of tardive dyskinesia and its increased prevalence with aging, made it a possible neurodegenerative disease [5].

Preventive effects of theophylline in haloperidol-induced orofacial dyskinesia and associated parameters are possible because of its ability to block adenosine receptors. Theophylline is a non-selective antagonist of adenosine receptors. Recent reports suggest that adenosine, an agonist of A1 receptors, and antagonists of A2 receptors are protective against haloperidol-induced orofacial dyskinesia [2]. These drugs also showed protective effects in different ROS-generated pathologies, suggesting A2A receptor antagonists as potential neuroprotective agents [37]. It is quite possible that preventive effect of theophylline is due to its ability to block A2A receptors. Previously, it has been reported that theophylline has some neuroprotective actions. Chronic administration of theophylline showed moderate or marked subjective improvement in parkinsonian patients and was suggested as a useful adjunct to the routine therapy [19, 28]. It is also involved in excitatory transmission caused by the hypoxia [10].

TD is a hyperkinetic movement disorder developing due to multiple reasons. One of the important reasons among them is dopamine receptor supersensitivity [2, 31]. The increase in dopamine receptor sensitivity on the day of the last behavioral quantification was correlated with the increase in stereotypic rearing and total locomotor activity (ambulatory and rearing activity). Co-administration of theophylline prevented this development of dopamine receptor supersensitivity. The increase in motor hyperactivity with the simultaneous increase in stereotypy in haloperidol-treated groups and reversal of this in theophylline-
treated groups suggested the potential interrelationship between the two [2].

It is often observed that A2A receptor stimulation results in an increased transmitter release. A2A receptor activation shows an excitatory action on the transmitter release including glutamate, an effect probably produced by increasing presynaptic calcium influx [10, 21, 35, 38]. Consistent with this finding is that A2A receptor antagonist CGS15943 could depress glutamate release. Presynaptic dopamine D2 receptor inhibits the release of glutamate from excitatory cortical striatal projections [40]. Chronic neuroleptic blockade of these receptors increases the synaptic release of aspartate and glutamate in different brain regions, which results in neuronal degeneration. In addition, oxygen radicals generated by chronic administration of neuroleptics can inhibit presynaptic glutamate uptake, inactivate enzymatic defense against cellular damages, disrupt mitochondrial electron transport chain and stimulate nitric oxide synthase by the influx of calcium mediated by NMDA receptors, generation of highly reactive peroxynitrite radicals which resulted in generation of redox active species and extra neuronal excitatory amino acids [40]. Orofacial dyskinesia developed by chronic administration of haloperidol and acute administration of reserpine is associated with the increase in glutamate in the extracellular space followed by calcium influx [5]. In our study, theophylline dose-dependently prevented various measures of oxidative damage such as lipid peroxidation, decrease in reduced glutathione and antioxidant enzyme defense system. The effect is most prominent in the striatum region which is most affected in tardive dyskinesia. Involvement of striatum region points towards the larger involvement of A2A receptors, further suggesting that A2A blockade by theophylline is responsible for its protective action. Different reports emphasize that A2A receptor antagonists may have potentially wide use as neuroprotective agents against cell injuries in many situations where damage is produced by either glutamate receptor accumulation, increase in oxygen free radicals or combination of the two [37, 38]. Theophylline might have resulted in limiting the glutamate excitotoxicity indirectly by inhibiting A2A receptors.

Chronic administration of the dopamine receptor antagonist resulted in depletion of the extracellular levels of dopamine in the cortical as well as in subcortical (including striatum) regions. Chronic administration of haloperidol resulted in increased density of dopamine receptors [1, 2, 13]. The increased receptor density results in development of supersensitivity and increase in the number of dormant receptors. Besides, this decreased level of dopamine also suggests its increased metabolism which may lead to production of free radicals [8, 39]. Theophylline prevented this decrease in dopamine in the cortical as well as subcortical areas (including striatum). This suggests that theophylline prevents both development of supersensitivity of dopamine receptors and generation of free radicals.

Chronic administration of haloperidol resulted in decreased retention in the plus maze task which was dose-dependently prevented by theophylline. Neurodegeneration might be a possible reason of the decrease in % retention and prevention of this by theophylline might be attributed to neuroprotective action of theophylline or possibly due to its ability to antagonize adenosine receptors in the hippocampus and cortex, the brain areas involved in cognition. Besides this positive action of theophylline on information processing and performance might be attributed to improvement of behavioral routines, arousal enhancement and sensorimotor gating [2, 15].

In conclusion, the finding that theophylline inhibited the development of haloperidol-induced orofacial dyskinesia further supports the involvement of adenosinergic receptor system in this debilitating movement disorder. Theophylline possibly prevents the development of dopamine supersensitivity and decreases the oxidative damage caused by either increased glutamate in extracellular space or increased metabolism of dopamine. Taken together, the present findings substantiate the involvement of adenosinergic receptor system in development of tardive dyskinesia and suggest potential therapeutic option in theophylline.

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