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Neuroprotective Role of Formononetin in a Scopolamine-Induced Alzheimer's Disease Mouse Model

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Abstract: The progressive neurodegenerative illness known as Alzheimer's disease (AD) is typified by memory loss, cognitive decline, and neural malfunction. A common tool for creating experimental models of cognitive impairments resembling AD is scopolamine, a muscarinic receptor antagonist. Because of flavonoids' anti-inflammatory, antioxidant, and neuroprotective qualities, the hunt for natural neuroprotective drugs has accelerated in recent years. This study investigated the neuroprotective effects of formononetin (10, 20, and 30 mg/kg; p.o) in a scopolamine-induced AD model. It examined its impact on oxidative stress indicators and cognitive performance. Scopolamine (1 mg/kg; i.p), administered for 3 days, significantly impaired memory and locomotor activity. Memory and learning capacities were assessed using behavioral tests such as the Open Field Test and the Y-maze Test. The findings revealed that pretreatment with formononetin, at a dosage of 30 mg/kg, significantly mitigated the behavioral changes induced by scopolamine. Formononetin improved memory, as evidenced by a higher percentage of spontaneous alterations in the Y-maze test. Additionally, it enhanced locomotor activity, as indicated by a higher square-crossing count in the OFT. Biochemical analysis showed that scopolamine therapy elevated TBARS levels and reduced GSH levels in the brain. In contrast, formononetin restored approximately 95% of GSH levels and reduced TBARS levels by 95%. The present study aims to investigate the neuroprotective efficacy of formononetin against scopolamine-induced cognitive and biochemical impairments that mimic AD pathology.

Keywords: Formononetin; Memory; Alzheimer's disease; Oxidative stress; GSH, TBARS.

1. Introduction

A chronic neurological illness that affects millions of people worldwide, Alzheimer's disease (AD) is characterized by increasing memory loss, cognitive impairment, and behavioral abnormalities. About 60-70% of dementia cases arise globally, and as populations age, its incidence is anticipated to increase substantially. The underlying pathophysiology of AD is complex and includes oxidative stress as a major contributor, as well as amyloid-beta (Aβ) plaque formation, neurofibrillary tangles, cholinergic dysfunction, and neuroinflammation [1]. It is a major public health concern, especially in older populations, and is the most frequent cause of dementia [2]. This has stimulated research into new therapeutic agents, including natural substances with neuroprotective qualities, as possible AD therapies [3]. Oxidative stress

has a major role in the onset and progression of AD. It results from an imbalance between the production of reactive oxygen species (ROS) and the brain's antioxidant defense mechanisms. Overproduction of ROS can cause DNA damage, lipid peroxidation, protein misfolding, and cellular damage, all of which can contribute to synaptic dysfunction and neuronal death in AD [4]. The potential neuroprotective benefits of natural substances, especially flavonoids and isoflavones, in reducing the pathophysiology of neurodegenerative illnesses, such as AD, have come to light in recent years [5]. Widely found in fruits, vegetables, and medicinal plants, flavonoids are polyphenolic chemicals with anti-inflammatory, neuroprotective, and antioxidant qualities [6]. Owing to their chemical resemblance to estrogen, isoflavones may have neuroprotective properties that might lessen the neurodegenerative processes associated with AD [7]. Red clover (*Trifolium pratense*) and other leguminous plants are the main sources of formononetin, a naturally occurring isoflavone that has attracted interest due to its wide range of pharmacological actions, including estrogenic, anti-inflammatory, and antioxidant properties [8]. The importance of estrogenic substances like formononetin in AD research is highlighted by the fact that estrogen shortage is known to increase the risk of developing AD, especially in postmenopausal women [9]. Formononetin has been shown in recent research to reduce oxidative stress, suppress neuroinflammation, and alter apoptotic pathways, which are strongly linked to the pathogenesis of AD [10, 11]. Formononetin has demonstrated potential in preclinical research to improve memory impairments and restore cognitive abilities in animal models of dementia. We explored the formononetin's dose-dependent efficacy in reversing scopolamine-induced behavioral and oxidative impairments in an AD model. Unlike prior flavonoid studies, we highlight its dual impact on memory and redox balance [12]. Moreover, formononetin has been shown to improve neuronal survival, reduce pro-inflammatory cytokine production, and attenuate Aβ-induced neurotoxicity in vitro [13]. Furthermore, formononetin's promise as a treatment option for AD is supported by its favorable safety profile and ability to cross the blood-brain barrier (BBB) [14]. In order to replicate several characteristics of AD, such as memory loss and oxidative stress, scopolamine, a muscarinic receptor antagonist, is frequently employed to cause cognitive impairment in animal models [15]. Therefore, this study aims to explore the effects of formononetin on memory and locomotion using the scopolamine model, while accounting for the intricate roles of oxidative stress in Alzheimer's disease and the potential of isoflavones to mitigate this stress.

2. Materials and Methods

2.1. Drugs and chemicals.

All of the drug solutions had been prepared freshly before their utilization. Scopolamine received from the Sigma company, Delhi. Donepezil was received from the Sun Pharma company, Chandigarh. Formononetin (CAS no 485-72-3) was provided by the BLD pharma company. All the other chemicals/reagents used in the study were obtained from the central store of Desh Bhagat University.

2.1.1. Experimental animals.

Mice (*Swiss albino* species), weighing between 20 and 30 g and of female sex, were utilized for this study. They were acclimatized within the central animal house of Desh Bhagat University. Mice were supplied with a standard laboratory pellet chow diet and an unrestricted supply of water. They were subjected to a 12-hour light and 12-hour dark cycle. The

experiments were carried out within the hours of 09:30 to 17:30 in a semi-soundproof laboratory setting. The experimental protocol received proper approval from the Institutional Animal Ethics Committee (IAEC) 2136/PO/Re/S/21/CPCSEA/1. The experiments were conducted following the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of Environment and Forest, Government of India.

2.2. Experimental design.

Thirty-six animals were divided into six groups using a random allocation process. Scopolamine (1 mg/kg; i.p) will be administered for 5, 6, 7th day to induce Alzheimer's. Formononetin (10, 20, and 30 mg/kg, p.o) and standard donepezil were administered for 7 consecutive days as shown in Table 1. The behavior of the animals, such as motor coordination, locomotor activity, catalepsy, and exploratory behavior, was assessed one hour after the respective treatment on day 7.

On day 7, after behavioral analysis, the animals were anesthetized. Thereafter, the animals were sacrificed, and brain tissue was harvested for biochemical analysis (oxidative stress) as shown in Figure 1.

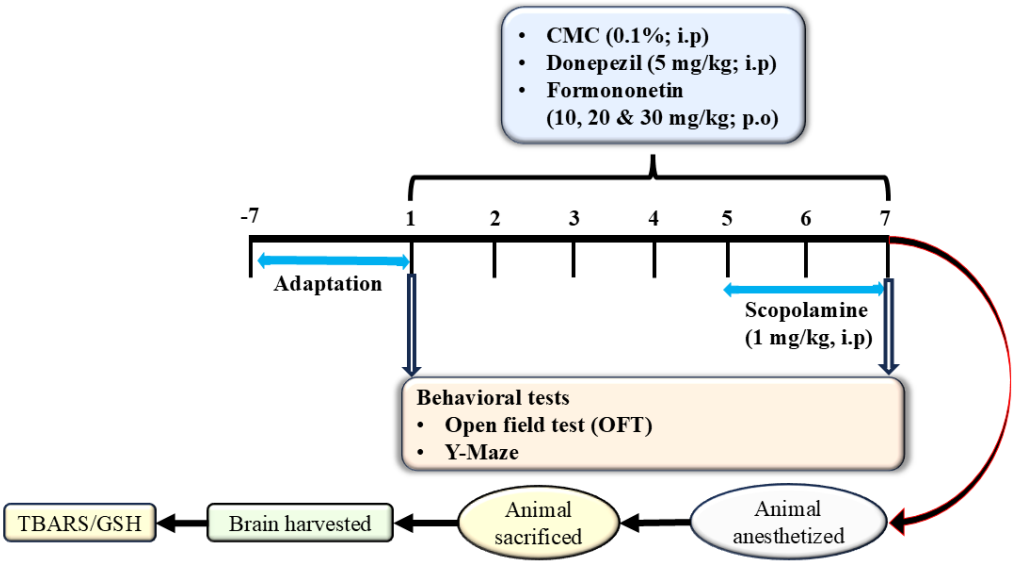

Figure 1. A detailed schematic diagram depicting the experimental protocol.

Table 1. Description and grouping of animals

Groups	Treatment	No. of animals
I	0.1% CMC (p.o)	6
II	Scopolamine (1 mg/kg; i.p)	6
III	Donepezil (5 mg/kg; p.o) + Scopolamine (1 mg/kg; i.p)	6
IV	Formononetin (10 mg/kg p.o) + Scopolamine (1 mg/kg; i.p)	6
V	Formononetin (20 mg/kg p.o) + Scopolamine (1 mg/kg; i.p)	6
VI	Formononetin (30 mg/kg p.o.) + Scopolamine (1 mg/kg; i.p)	6
	Total	36

2.3. Behavioral model.

2.3.1. Y-maze test.

The three similar painted wooden arms (40 cm long, 35 cm high, and 12 cm broad) positioned at equal angles and designated A, B, and C in the Y-labyrinth form the Y-maze. Each animal was positioned in the middle of the three arms at the beginning of the trial, and

they were given five minutes to explore the maze. If the mice's rear paws were entirely inside the designated arm, the entrance was considered the length of the arm. Among the three arms, animals constantly evaluate their different arms. Multiple inputs into each of the three arms on successive options (i.e., ABC, CBA, DBA, and BCA) are referred to as spontaneous alternation [16-19]. The number of arm entries and alternations was recorded, and the spontaneous alternation percentage (SAP) was calculated using the following equation:

$$Spontaneous\ alteration\ (\%) = \frac{Alterations}{Total\ number\ of\ possible\ alterations} \times 100$$

2.3.2. Open field test (OFT)

A wooden box (32 cm × 32 cm × 32 cm) with a floor separated into 16-square grids served as the basis for the open field apparatus. Prior to the test, each animal was given ten minutes to acclimatize. Following their separate acclimation, each animal was brought into the central square section of the apparatus and allowed to roam about freely for five minutes. For a measurement of the mice's locomotor activity, the number of squares they crossed in five minutes was counted. The correct square crossing was considered when all four paws of the mice left the previous square. Before each trial, the instrument was cleaned with an alcohol solution to remove any olfactory-related bias [20].

2.4. Biochemical estimations.

2.4.1. Collection of samples.

The animals were sacrificed by cervical dislocation under anesthesia at the conclusion of the Behavioural analysis. Brain samples were isolated, and homogenates were prepared in phosphate buffer (pH 7.4). One hundred milliliters of phosphate buffer (pH 7.4) was used to homogenize one hundred milligrams of minced brain. After that, the homogenates were centrifuged at 3000 rpm for fifteen minutes. Thiobarbituric acid reactive species (TBARS) and reduced glutathione (GSH) were biochemically analyzed using the produced supernatants.

2.4.2. Estimation of TBARS level in the brain samples.

In this test, the production of pink color resulting from the reaction of TBA with MDA and other aldehydes serves as an indicator of lipid peroxidation. In this procedure, 0.1 ml of brain homogenate supernatant was added to a 2 ml mixture (1:1:1 ratio) of thiobarbituric acid, trichloroacetic acid, and hydrochloric acid (TBA-TCA-HCl). After that, the mixture was heated to 100°C for fifteen minutes in a water bath. The mixture was centrifuged for 10 minutes at 1000 rpm after cooling. After centrifugation, the absorbance of the clear supernatant was measured at 535 nm. 1,1,3,3-tetramethoxypropane (TMP) was used as the standard in order to create the standard curve. Tissue (µM/mg) was used to express the results [21].

2.4.3. Estimation of GSH level in the brain samples.

To estimate the GSH level, this method was applied with minimal modification. Yellow color was produced in the test as a result of DTNB's interaction with the sulfhydryl group of GSH. During this process, 0.25 ml of newly made 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.5 ml of supernatant, and 2 ml of 0.3 M disodium hydrogen phosphate buffer (pH 8.4) were

added. A UV spectrophotometer was used to measure the absorbance of the resulting yellow solution at 412 nm. Reduced GSH was used as a benchmark to create the standard curve. Tissue (µM/mg) was used to express the results [21].

2.5. Statistical analysis.

The mean ± standard error mean (SEM) was used to express the results. The statistical significance was established using Graphpad Prism 10.2.1 software (GraphPad Software Inc., U.S.A.) and two-way analysis of variance (ANOVA) in behavioral and one-way analysis of variance (ANOVA) in biochemical estimates.

3. Results and Discussion

3.1. Effect of formononetin on behavioral parameters.

3.1.1. Effect of formononetin on locomotor activity: OFT.

In the current investigation, on day 7, the scopolamine-treated mice group exhibited a significant reduction in locomotor activity as compared to the vehicle-treated mice group. When comparing the scopolamine-treated group to the control group on day 7, a significant decrease in square crossings was observed. Compared with the scopolamine-treated group, donepezil dramatically increased the frequency of square crossings on day 7. Furthermore, formononetin (10, 20, and 30 mg/kg) treatment significantly increased locomotor activity compared to the scopolamine-treated group on the 7th day. However, formononetin at a dose of 30 mg/kg significantly increased the number of square crossings compared with the scopolamine-treated group and the 10 mg/kg dose of formononetin. Whereas no statistically significant increase was observed compared with the 20 mg/kg dose of formononetin ($p>0.05$) (Figure 2A).

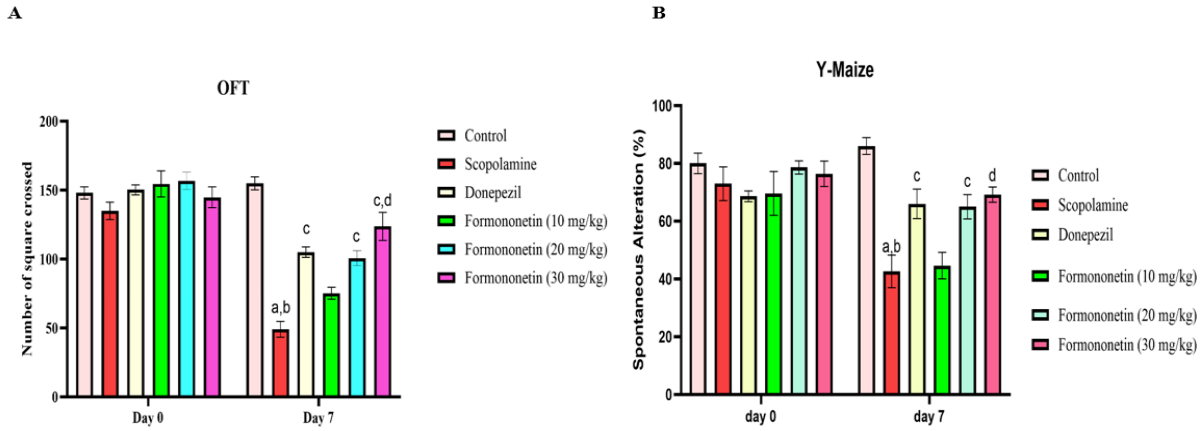

Figure 2. The graph illustrates the effect of formononetin on memory in the **(A)** OFT and **(B)** Y-Maze tests. Statistical significance was determined using a two-way analysis of variance (ANOVA) followed by Tukey's test. [a] $p < 0.05$ vs day 0 scopolamine, [b] $p < 0.05$ vs day 7 control, [c] $p < 0.05$ vs day 7 scopolamine, [d] $p < 0.05$ vs formononetin (10 mg/kg), [e] $p < 0.05$ vs formononetin (20 mg/kg).

3.1.2. Effect of formononetin on memory: Y-maize.

Compared to the vehicle control group, mice treated with scopolamine on day 7 showed a substantial decrease in the percent of spontaneous change. Compared with the scopolamine-treated group, donepezil (5 mg/kg) markedly increased the proportion of spontaneous changes. The proportion of spontaneous alteration was substantially higher in the mice treated with a

higher dose of formononetin (30 mg/kg) than in the mice treated with scopolamine and the mice treated with formononetin (10 mg/kg). Whereas, no statistically significant increase was observed as compared to a 20 mg/kg dose of formononetin ($p>0.05$) (Figure 2B).

3.2. Effect of formononetin on oxidative stress.

3.2.1. Reduced glutathione content.

Compared to the control group, the treatment of scopolamine markedly decreased the brain GSH level. The GSH-lowering impact of scopolamine was considerably reversed by donepezil administration. Pretreatment with formononetin (10 mg/kg) had no significant effect on the GSH-lowering effect of scopolamine. However, the GSH-lowering impact of scopolamine was effectively countered by formononetin (20 and 30 mg/kg). The brain GSH level was dramatically elevated by formononetin (20 and 30 mg/kg) in comparison to the group that received scopolamine treatment. Furthermore, it was found that the effects of the 30 mg/kg formononetin dose were more pronounced than those of the 10 mg/kg dose. (Figure 3A).

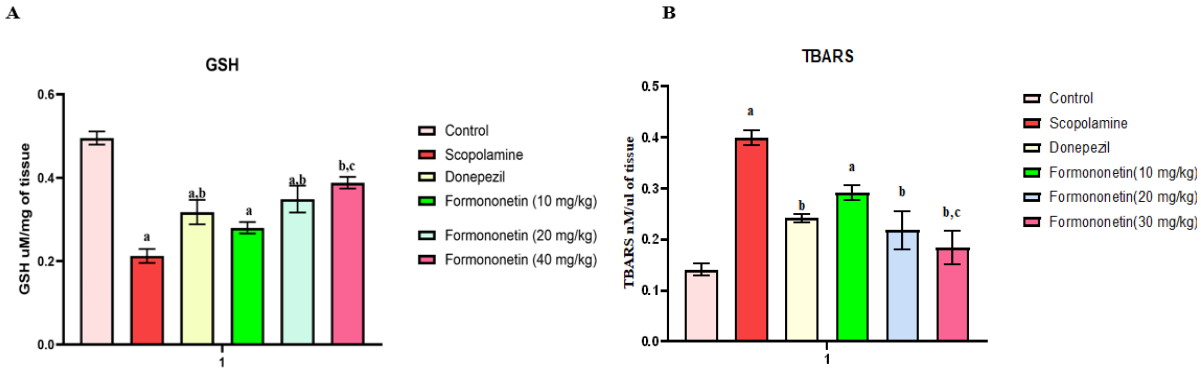

Figure 3. The graph illustrates the effect of formononetin on the brain **(A)** GSH and **(B)** TBARS levels. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's test. a $p < 0.05$ vs control, b $p < 0.05$ vs scopolamine, c $p < 0.05$ vs formononetin (10 mg/kg).

3.2.2. Lipid peroxidation Index.

Scopolamine administration significantly increased the brain TBARS level when compared to the control group. Administration of donepezil significantly reversed the TBAR-suppressing effect of scopolamine. Donepezil significantly downregulated the brain TBARS level as compared to the scopolamine group. Pretreatment with formononetin (10 mg/kg) had no significant effect on the brain TBARS level. However, 20 and 30 mg/kg doses of formononetin significantly reversed the TBARS-increasing effect of scopolamine. A significant reduction in brain TBARS levels was observed with the 20 and 30 mg/kg doses of formononetin compared with the scopolamine-treated group. However, formononetin (30 mg/kg) exhibited a more pronounced effect compared to the 10 mg/kg and 20 mg/kg doses. A significant reduction in brain TBARS levels was observed with a 30 mg/kg dose of formononetin compared with a 10 mg/kg dose (Figure 3B).

3.3. Discussion.

AD advances from an asymptomatic stage to marked cognitive and motor deficits. Early symptoms include short-term memory loss [22], followed by long-term memory deterioration and locomotor decline [23]. Scopolamine replicates key AD features by inducing oxidative stress and neuroinflammation [24], making it a reliable model due to its ability to cross the

BBB [25]. As expected, scopolamine impaired spontaneous alternation in the Y-maze and locomotor activity in the OFT, consistent with prior findings [25-29]. This paper underscores the neuroprotective effects of formononetin in reducing scopolamine-induced behavioral and oxidative stress. Scopolamine had profoundly differentiated locomotor activity and memory range, as evidenced by decreased square crossings in the open field test and reduced spontaneous alternation in the Y-maze. Standard therapeutic donepezil compensated both parameters, confirming the model's correctness. The most active pretreatment formononetin dose, 30 mg/kg, showed considerable behavioral recovery. The increased percentage of enhanced square crossings and spontaneous alternation at high doses indicated a dose-dependent improvement in scopolamine-induced deficits. It is interesting to note that a lower concentration of 20 mg/kg elicited minor improvements, and 10 mg/kg did not have any significant effect, indicating that the therapeutic effect is concentration-dependent. Behavioral findings were supported by oxidative stress markers. Brain GSH abundances were reduced drastically, and TBARS abundances were augmented with scopolamine, suggesting redox homeostasis (i.e., impairment). Donepezil was efficient in reversing these trends. 20 mg/kg formononetin and 30 mg/kg formononetin, respectively, replenished the GSH level and lowered TBARS content, indicating antioxidant supplementation. Again, 30 mg/kg proved to be the most successful intervention, and the least effective is 10 mg/kg. All these findings indicate that formononetin has a two-fold therapeutic effect on the neurological system: boosting cholinergic-related behaviors and reducing oxidative stress. The dose-response nature indicates the existence of pharmacodynamic cut-off points, which would surely be through antioxidant mechanisms and cholinergic control. Our study demonstrates that formononetin significantly ameliorates cognitive deficits in scopolamine-induced AD mice, whereas prior investigations in APP/PS1 transgenic models showed that formononetin similarly promoted LRP1-mediated Aβ efflux and preserved hippocampal endothelial integrity. The results provided the premise for further molecular studies, such as its action mechanism, and Nrf2 signal and ROS inhibition, thus being a potential prospect in terms of Alzheimer's therapeutics.

4. Conclusion

AD progresses gradually from a clinically silent phase to severe cognitive impairment and motor dysfunction. The initial manifestations commonly involve deficits in short-term memory, which are subsequently followed by progressive deterioration of long-term memory and impaired locomotor function. Scopolamine-induced neurotoxicity is widely used as an experimental model of AD, as it reproduces several pathological features associated with the disease, including oxidative stress, neuroinflammation, and cognitive deficits. Its ability to readily cross the BBB further enhances its suitability as a reliable pharmacological model. In agreement with previous studies, scopolamine administration resulted in significant impairments in spontaneous alternation behavior in the Y-maze test. It reduced locomotor activity in the open field test (OFT), confirming the induction of AD-like cognitive and behavioral alterations. Donepezil reversed these effects, validating the model. Formononetin (10, 20, and 30 mg/kg) effectively countered scopolamine-induced behavioral impairments. The 30 mg/kg dose offered the most robust restoration of memory and movement, suggesting dose-dependent efficacy.

Additionally, scopolamine-induced oxidative stress, marked by elevated TBARS and diminished GSH, was significantly alleviated by 20 and 30 mg/kg doses of formononetin. These findings support the compound's potential neuroprotective role. Limitations of the

present study include the absence of molecular pathway analysis and the reliance on behavioral and biochemical endpoints. Mechanistic confirmation, such as examining Nrf2 signaling or mitochondrial integrity, was beyond this scope. Implications suggest formononetin may offer therapeutic promise for AD via behavioral and oxidative restoration. However, its exact pharmacodynamic targets remain undefined. Future directions should include molecular docking with oxidative stress-related proteins, exploration of formononetin's role in neuroinflammation, and long-term efficacy in chronic models. Translational studies in aged animals or models with tauopathy would further validate its relevance in AD therapy.

Author Contributions

Conceptualization, A.S. and D.D.; methodology, A.S.; software, A.S.; validation, D.D., P.G., and A.S.; formal analysis, A.S.; investigation, P.G.; resources, D.D.; data curation, A.S.; writing—original draft preparation, A.S.; writing—review and editing, A.S.; visualization, D.D.; supervision, A.S.; project administration, P.G.; funding acquisition, P.G. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Desh Bhagat University(*2136/PO/Re/S/21/CPCSEA/1*.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflict of Interest

Akshay Sahani, Puja Gulati, Diksha Dalal, and Anish Singh declare no conflict of interest.

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