



## EFFECT OF TOCOTRIENOL SUPPLEMENTATION ON NEUROBEHAVIORAL PARAMETERS OF RAT

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Article Received on 15/11/2016

Article Revised on 25/11/2016

Article Accepted on 05/12/2016

### ABSTRACT

**Background:** Role of free radicals is suggested in neurodegenerative diseases. Hence, antioxidants may have putative benefits against neuropathological changes. Unsaturated isoprenoid side chain provided Tocotrienol (TT), a constituent of vitamin E, better antioxidant activity. Several *in vitro* studies have demonstrated strong neuroprotective nature of TT and recent *in vivo* studies also support so. **Objective:** Current study is aimed to evaluate the effect of tocotrienol supplementation on the neurobehavioral parameters of rat. **Materials and Method:** Male albino Wistar rats were treated with tocotrienol (10 mg/day) for two weeks and maintained for four weeks (TT<sub>+</sub>) along with rats those are not exposed to TT (TT<sub>0</sub>). Spontaneous motor activity (SMA), Rota-rod performances (RRP) and open-field activity (OFA) are evaluated fortnightly starting from pretreatment phase. Performances in Morris water maze (MWM) are recorded once at the end of experiment. Data collected from both the groups are statistically processed with Kruskal-Wallis test and Mann-Whitney pairwise comparisons. **Results:** Significantly improved SMA score was recorded by TT<sub>+</sub> group in 4<sup>th</sup> week. Improved RRP were also noticed throughout the study in case of TT<sub>+</sub> group. However, no significant difference between the TT<sub>+</sub> and TT<sub>0</sub> group was noted in terms of OFA and MWM experiments. **Conclusion:** From the results it can be suggested that the dose and duration of tocotrienol supplementation has improved some of the tested neurobehavioral performances and the impact continues at least for 4 weeks after the supplementation. With the modification of dose and duration of TT supplementation it can be used against specific neuropathologies to prevent the behavioral declension.

**KEYWORDS:** Tocotrienol, Brain, Spontaneous motor activity (SMA), Rota-rod performance (RRP), Open-field activity (OFA), Morris water maze (MWM).

### INTRODUCTION

Four types ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) of tocotrienols (TT) and tocopherols are there in the vitamin E family, which are well known for their antioxidant activity. Sometimes back, the neuroprotective property of vitamin E has come into the limelight; however, studies with tocopherol are not so encouraging. Independent of its potent antioxidative activity, TT has recently been recognized for its cholesterol lowering effect, anti-angiogenesis effect, anti-cancer effect, anti-inflammatory effect and neuroprotective effect.<sup>[1]</sup>

As the brain is highly susceptible to oxidative damage<sup>[2]</sup>, it is likely that antioxidants will provide protection against the oxidative damages. However, neurodegeneration is not limited to oxidative stress only. In this regard, the TT is the preferable candidate as it provides protection to the neurons by both oxidative and non-oxidative means. It has been suggested that the antioxidative effects of TT are due to its capacity to induce specific antioxidant enzymes like superoxide

dismutase (SOD), NADPH:Quinone oxidoreductase (NQO) and glutathione peroxidase (GPx); and thereby mediate the quenching of free radicals<sup>[3]</sup> and removal of lipoperoxyl radicals.<sup>[4]</sup> On the other hand, TT attenuates the cytosolic phospholipase A<sub>2</sub> to restrict the glutamate-mediated neurotoxicity, suppresses the arachidonic acid metabolism by interacting with 12-lipoxygenase, blocks the protooncogene Src tyrosine kinase, upregulates the multidrug resistance protein 1, induces arteriogenic tissue inhibitor of metalloprotease 1 while weakening the effects of multidrug resistance-associated protein 1 and thus provides protection to the neural tissues.<sup>[5]</sup> There are studies with TT to provide neuroprotection against a variety of neurodegenerative threats like diabetic neuropathy, alcoholic neuropathy, in cognitive deterioration induced by chronic alcohol exposure and streptozotocin and glutamate-induced neurodegeneration.<sup>[3]</sup> However, neuroprotective effects of TT should also be effective against the natural wear and tear process when there is no overt threat of neural damage or the neurotrophic impacts of TT are likely to

be demonstrable by improvement of cognitive and psychomotor functions by TT in normal individuals.

Accordingly, the present work is aimed to evaluate the neuroprotective effects of orally supplemented TT on selected neurobehavioral parameters pertaining to explorative learning, spatial learning and motor skill learning. Repeated evaluations of spontaneous motor activity, Rota-rod performances, open-field activity and one time evaluation of Morris water maze performances have been carried out in rats supplemented orally with TT.

## MATERIALS AND METHODS

**Materials** – Oryza tocotrienol©-90 was kindly donated by the Oryza Oil & Fat Chemical Co. Ltd, Japan.

**Animal maintenance and Treatment** – The experimental protocol was approved by the Institutional Animal Ethics Committee. Male albino Wistar rats weighing 120-140 g were obtained, maintained and treated in the Central Animal House of NRI Medical College & General Hospital and the procedures were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). After one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into two groups - TT<sub>+</sub> [exposed to Oryza-tocotrienol©-90 (10mg/day) orally for two weeks] and TT<sub>0</sub> [exposed to sham treatment for two weeks].

### **Behavioral evaluation**

**Spontaneous motor activity (SMA):** Spontaneous horizontal locomotor activity of rats has been measured by using digital photoactometer. The infrared photocells of the instrument record the interruptions caused by the horizontal locomotor activities of the animal. During activity testing, only one animal has been placed in the chamber and the activity is measured for 5 minutes without prior acclimatization to the test chamber. The digital counter indicates the number of interruptions. This has been repeated three times in three days and mean of three counts has been taken as an individual weekly score.

**Rota-rod performance (RRP):** Motor coordination is tested using a Rota-rod apparatus designed by Dunham and Miya.<sup>[6]</sup> The apparatus consists of a horizontal iron rod 21cm perimeter and 61.5cm long with roughened surface, moving on its axis at different speeds. The animals have been placed on the moving rod facing the direction of rotation and the digital time counter is switched on. The rationale of this study is that the animals whose motor coordination has been deteriorated fall from the rod into a tray 10 cm below and the counter is switched off. Thus, the counter shows the time for which animal could maintain themselves on the rotating rod. The animals have been trained for two days (two sessions each) prior to initiation of the experiment. On

the day of the test, the abilities of the animals to stay on the rod for a test period upto 180 seconds have been observed. The endurance time (from placing on the rotating rod to first fall) for each animal has been noted. Rats naturally try to stay on the rotating cylinder, or Rota-Rod and avoid falling to the ground. The length of time that a given animal stays on this rotating rod is a measure of their balance, coordination, physical condition, and motor-learning.

**Open-field activity (OFA):** The open-field test is a generally accepted paradigm for measurement of explorative and locomotor behaviours in animals. It has been extensively exploited in neuroscience studies of behavior and brain functions in rats.<sup>[7]</sup> In spite of high frequency of studies, controversies are there about the representativeness of animal behavior at the levels of general locomotor activity or neurobiology.<sup>[8]</sup> The test allows evaluation of the responses towards new and unfamiliar environments as well as habituation to the environment. Therefore, in addition to being used for the estimation of novelty seeking behavior, it can be used for the study of the reward system and the pharmacological actions of addictive substances. Additionally, it has been considered to provide measures of discrimination and recognition memory, and it has been used in drug and lesion studies and in screening for genes and other molecular targets responsible for learning and memory processes.<sup>[7]</sup> Anxiety plays an important role in this type of study, as anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal's breeding or natural environment).<sup>[9]</sup>

A circular (diameter: 60cm), natural colored and equally illuminated steel field has been used for the study.<sup>[10]</sup> Each animal has been left in the arena (placed in the central zone) for 10 minutes and their movements and activities have been recorded with video.<sup>[11]</sup> The recorded videos have been converted into JPEG (Joint Photographic Experts Group) photographs with a snap at every 200ms with the help of Free Video to JPG Converter 1.8.7. Later, these photographs have been processed with modified TLD<sup>®</sup> software (Kindly provided by Prof. Zdenek Kalal with GNU General Public License) to identify Time stationary (Seconds), Ambulatory time (Seconds), Total distance covered (Meters), Average speed of movement (Centimeters / Second), Time spent at the centre (Seconds), Number of right angle turns (Counts), Number of wall climbing efforts (Counts), Time spent at First approach Quadrant (Seconds), Time spent at concentric quadrants (Seconds). The spatial distribution of exploration has been used to calculate the thigmotaxic behaviors, while bursts of movements against time have been used to calculate the slope of temporal bursts activity. Other behaviors like Grooming and defecation have also been counted manually by watching the video.

Morris Water Maze: The test is performed following the protocol described by Thippeswamy *et al.*<sup>[12]</sup> Rats have been trained to swim to a visible platform in a circular pool (180 cm diameter and 60 cm in height) located in an experimental room. Rats can escape from swimming by climbing onto the platform and over time the rats apparently learn the spatial location of the platform from any starting position at the circumference of the pool. The pool has been divided into four equal quadrants and filled with water to a height so that rats are not able to touch the bottom of the pool. During the acquisition phase, a square platform (9cm) has been placed in one of the quadrants of the pool approximately 2cm below the water. The water has been made opaque by adding a non toxic dye (titanium dioxide) and four equidistant locations have been identified around the edge of the pool (A, B, C and D) and have been used as starting points. Animals have received a training session consisting of four trials with a gap of 5 minutes between the two trials on day 40. Four different starting positions have been used for the successive training sessions in random orders. Rats have been released into the maze facing towards the wall of the pool and latencies to find the platform have been recorded for a maximum period of 90 seconds. If the rat did not find the platform within 90 seconds, it has been guided to the platform and has been allowed to remain there for 20 seconds. The time required for the rat to find the platform has been considered as the acquisition time. The time taken to find out the platform (retention time) has been assessed on day 41(24 hrs later) and day 42 (48hrs later). On the day 43, the platform has been removed from the pool and rats are placed into the pool and allowed to explore the pool for 5 minutes. The time spent in the target quadrant where the platform placed has been noted, the change in time spent by the rats at the target quadrant has been used as an indicator for spatial memory.

**Statistical analyses** – Variance and difference between the groups are analyzed by Kruskal-Wallis (KW) test and Mann-Whitney pairwise comparisons (MW), respectively, accepting the probability of 5% or less as significant using PAST statistical software (ver. 3.12; Copyright: Ø. Hammer 1999-2016).<sup>[13]</sup>

## RESULTS

As mentioned in the methodology section, the basal values for the repeatedly evaluable behavioral parameters *e.g.* SMA, RRP, OFA, are recorded before beginning of the treatment protocol on acclimated animals. These basal values were regarded as Wk 0 value. The animals are then maintained for two weeks with (TT<sub>+</sub>) or without (TT<sub>0</sub>) oral supplementation of TT and those parameters are evaluated at the end of two weeks, Wk 2 values. Then the animals are maintained for another 4 weeks with evaluation of those behavioral parameters twice, Wk 4 and Wk 6. The repeatedly measured parameters have been expressed as percentage alterations of basal (Wk 0) value of respective animal.

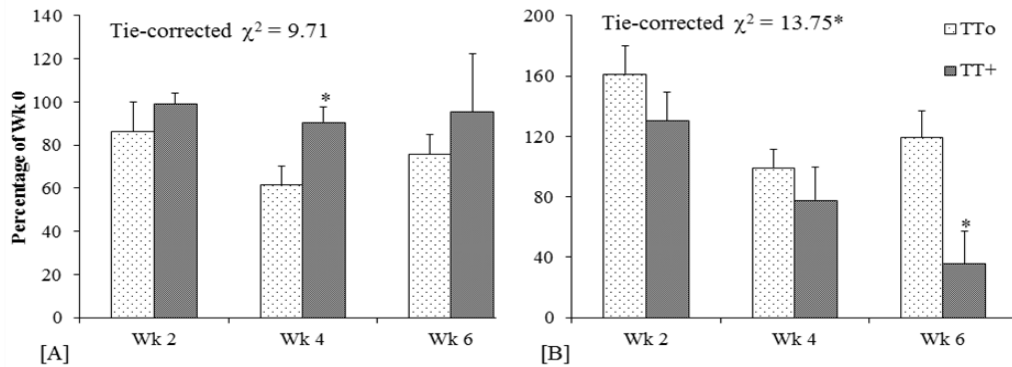
Comparison of percentage alterations of spontaneous motor activities of TT<sub>0</sub> and TT<sub>+</sub> animals during Wk 2, Wk 4 and Wk 6 are presented in figure 1A. Higher values of TT<sub>+</sub> animals are noted during all the three measurements, however, the difference with TT<sub>0</sub> animals is statistically significant only during Wk 4. On the other hand, lower number of fecal boli is dropped during the experimental session by the TT<sub>+</sub> animals for all the three measurements (Figure 1B). The difference between the percentage alterations of fecal boli from TT<sub>+</sub> and TT<sub>0</sub> animals is statistically significant during Wk 6 only.

Repeated evaluations of RRP include highest distance travelled by each animal at one stretch (*i.e.* without falling), numbers of falls during the session of three minutes and number of fecal boli dropped during the session. Significantly higher endurance and distance travelling is seen in TT<sub>+</sub> animals during all the Wk 2, Wk 4 and Wk 6 evaluations (Figure 2A). Even though, TT<sub>+</sub> animals have also demonstrated higher number of falls (Figure 2B) and a higher number of fecal boli (Figure 2C) during all the fortnightly evaluations, none of the differences are found to be statistically significant.

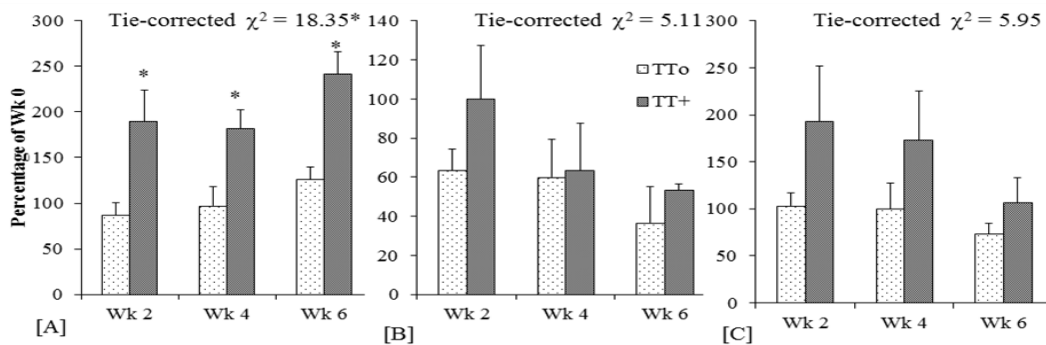
Compared to the TT<sub>0</sub> animals, lower total stationary times (Figure 3A) are noticed in TT<sub>+</sub> animals during all the three repeat fortnightly evaluations of OFA, however, the differences between the percentage alterations of scores of TT<sub>0</sub> and TT<sub>+</sub> animals are not statistically significant. On the other hand, decrements in average speed in relation to the Wk 0 values are found to be 6.6%, 30% and 27% in TT<sub>+</sub> animals while 40%, 57% and 43% in TT<sub>0</sub> animals in Wk 2, Wk 4 and Wk 6, respectively (Figure 3B). More ambulatory period with higher average speed in TT<sub>+</sub> animals allow them to explore through a greater distance within the study field, however, the differences between the averages of percentage change in travelled distances of animal groups are not statistically significant (data not shown). Throughout the study period, both TT<sub>0</sub> and TT<sub>+</sub> groups maintain similar numbers of right angle turns as that was during the Wk 0 (Figure 3C). Abrupt decrease in slope of temporal burst activities of TT<sub>+</sub> is observed (Figure 3D) during Wk 2 itself and this is maintained for Wk 4 and Wk 6 also, while there is gradual decrease in slope of temporal burst activities of TT<sub>0</sub> (70% vs 50% in Wk 2, 42% vs 33% in Wk 3 and 5% vs 28% in Wk 6). Figure 3E compares the thigmotaxic behaviors of TT<sub>0</sub> and TT<sub>+</sub> groups of rats. Increased thigmotaxis is observed in TT<sub>+</sub> animals compared to their counterparts, nevertheless, the difference is statistically insignificant. Maintained numbers of wall climbing efforts are observed in TT<sub>+</sub> animals, while the efforts are found to be gradually increased in TT<sub>0</sub> animals (Figure 3F).

The Morris water maze experiment is carried out at the end of experiment once only. No difference in transfer latency (TL), retention time after 24 hours (RT24) and retention time after 48 hours (RT48) is seen between the groups of rats (Figure 4A). Slight higher time is spent by

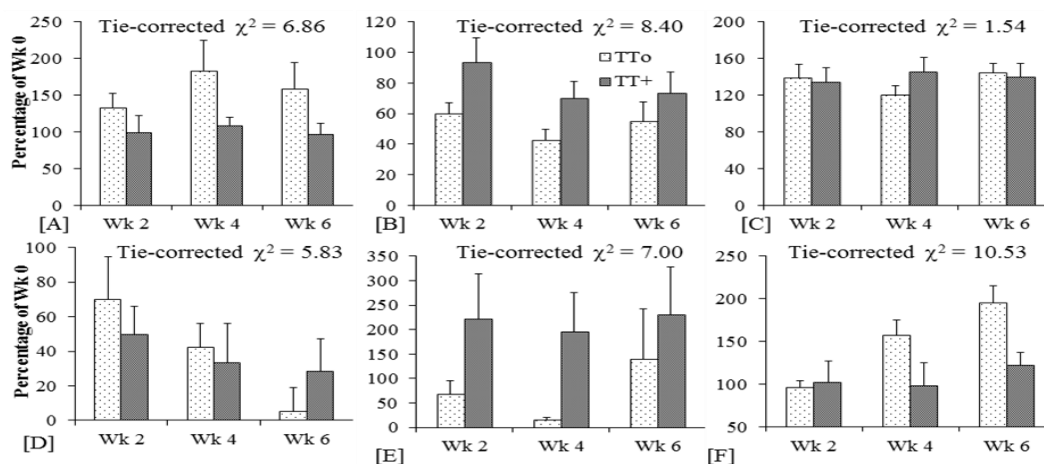
TT<sub>+</sub> rats in the target quadrant during the seeking behavior test (Figure 4B), however, the difference with the TT<sub>0</sub> group is not statistically significant.



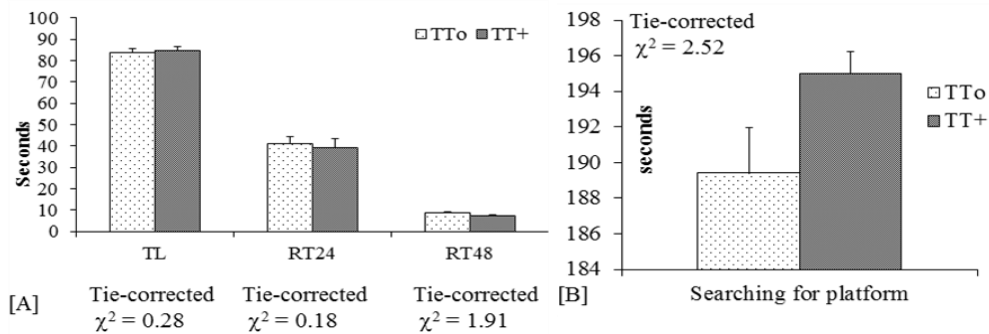
**Figure 1.** Comparison of fortnightly scores of Spontaneous motor activity [A] and number of fecal boli during the activity [B]. Each column represents mean of five observations presented as percentage change of respective Wk 0 values  $\pm$  standard error of mean. \* =  $p < 0.05$ )



**Figure 2.** Comparison of fortnightly scores of Rota-rod performances in terms of distance travelled [A], number of falls during the performances [B] and number of fecal boli during the activity [C]. Each column represents mean of five observations presented as percentage change of respective Wk 0 values  $\pm$  standard error of mean. \* =  $p < 0.05$ )



**Figure 3.** Comparison of fortnightly scores of Open-field activity in terms of total stationary time [A], average speed [B], numbers of right angle turns [C], slope of temporal burst activities [D], thigmotaxic behavior [E] and number of wall climbing [F] during the activity. Each column represents mean of five observations presented as percentage change of respective Wk 0 values  $\pm$  standard error of mean. \* =  $p < 0.05$ )

**Figure 4.**

Comparison of time taken to find out the platform on first exposure (TL) Time taken to find out the platform on 2<sup>nd</sup> day (RT24) and Time taken to find out the platform on 3<sup>rd</sup> day (RT48) in Morris water maze experiment [A], and Time spent on the target quadrant to search the platform [B]. Each column represents mean of five observations  $\pm$  standard error of mean. \* =  $p < 0.05$

## DISCUSSION

Tocotrienols (TT) are the vitamin E congeners having unsaturated isoprenoid side-chains with double bonds in the 3', 7' and 11' positions. Low quantities of esterified forms of TT are available in some monocot seed oils.<sup>[14]</sup> Hagl *et al*<sup>[15]</sup> have demonstrated the beneficial effect of ethanolic extract of rice bran on the basis of their strong mitochondria protective actions. Though, the exact molecule responsible for this effect is not identified, a mixture of tocotrienols are proposed to be the possible candidate for such neuroprotective activity and suggested to be beneficial against neurodegenerative disorders.<sup>[15]</sup> Very recently, the potent neuroprotective function of TT is further emphasized as it is found to help in recovery of cognitive functions from the  $\beta$ -amyloid toxicity and neurodegeneration.<sup>[16]</sup> In this context, the current study is carried out to evaluate the neurobehavioral performances of rats after oral supplementation of *Oryza* tocotrienol© for two weeks when there is no overt neurodegenerative threat.

Significant difference between SMA scores of TT<sub>0</sub> and TT<sub>+</sub> animals during the Wk 4 study but not in Wk 6 study indicate that the effect of oral supplementation could have not been extended beyond that time. On the other hand, the significant reduction in stress during SMA test, as represented by number of fecal boli, suggests that used instrument is no longer novel to the TT<sub>+</sub> animals. Thus, the benefits of oral TT supplementation at a dose of 10 mg /day is seen for a limited time in terms of some parameters while it is extended for longer period in some other parameters. It is also corroborated by the observation of the better performances in RRP till through the 6 weeks. Contrary to these, the stresses during these fortnightly test episodes are found to be higher in TT<sub>+</sub> animals, even though not statistically significant. Similarly, numbers of falls are found to be insignificantly higher during the RRP even immediately after the supplementation is over (Wk 2). In this context, TT-induced elevation of mood cannot be ruled out as a cause of increased number falls, as it can be the result of the escape phenomenon from the

task during RRP tests. Therefore, the specificity of test parameters is crucial factor to identify the impact of TT supplementation as positive neuromotor modulator. As such neurobehavioral tests are always influenced in multifactorial fashion and interpretation of their results is a challenging task.

Interestingly, during all the repeat measures, the TT<sub>+</sub> animals are maintaining their scores very close to their Wk 0 values, while the stationary times for TT<sub>0</sub> animals are higher by 30-80% compared to their basic values. These increments in stationary time may be related to the familiarity of the field area. The possibility of familiarity has been ruled out because of only ~50% correlation with the time factor in all the animals, except one (data not shown). In addition, the decrement in average speed is found to be lesser in TT<sub>+</sub> animals compared to their TT<sub>0</sub> counterparts. Even though the differences are not statistically significant, these data suggested that the TT<sub>+</sub> animals are more explorative than the TT<sub>0</sub> animals which may represent the anxiolytic effect of TT. Similar anxiolytic effects of TT have also been noticed in the elevated plus maze study with more number of exits from the closed arms.<sup>[17]</sup>

Slope of temporal burst activities during the session of open-field activity indicates the pattern of reduction in burst activities against time. Naturally, animals abounds the available field in concentric arenas to explore the area with safety pauses. The frequency of these bursts activities is high initially, which reduce gradually with acquaintance of the field. The more negative value of the slope indicates quicker finishing of exploration of the study field. A smaller decrease in the slope of temporal bursts in TT<sub>0</sub> group depicts sluggish learning of the study field, even though animals are being exposed to the same open-field setup fortnightly. Whereas, TT<sub>+</sub> animals remember the study field better as indicated by sharp declining in the slope of temporal bursts. However, maintained burst activities in TT<sub>+</sub> animals may evince the anxiolytic effects of TT or suggestive of its positive psychotropic effects.

Similar numbers of right angle turns in both the groups of animals during Wk 0, Wk 2, Wk 4 and Wk 6 indicates no effect of TT on the explorative behaviors. Hence, the possible anxiolytic effect of TT is not compromising the explorative behaviors of these animals or their self awareness which is critical as a safety measure. Agoraphobia plays an important role in the test of OFA, as the animal is kept alone in the open study field which is quite bigger than its regular housing cage covered with stainless steel mesh. Because of larger and open arena, a feeling of insecurity led the animal to spend more time towards the boundary producing thigmotaxic behaviors in the tested animals. Intensified thigmotaxic behavior along with more distance coverage with higher speed in TT<sub>+</sub> animals may indicate encouraged explorative behavior without breaching security measure.

Oral supplementation with TT has not shown any beneficial effect in terms of spatial learning. Considering the time lag between the evaluation process of MWM experimentation and completion of TT supplementation, the obtained result is not out of expectation. Further studies with immediate evaluation and TT supplementation with higher doses or longer duration may bring in a positive outcome.

Overall, it may be concluded from the current study that this is the first report to support the positive neuroactive impacts of TT in the normal physiological situation. The observed effects are sometimes subtle and not achieving statistical significance, however, the dose and duration used in the current study is also of minimal level. Further studies with higher dose or longer duration or both with more number of animals may achieve the level of significance. Nevertheless, positive effects of TT oral supplementation have been observed in terms of the SMA and RRP tests. These results indicate that TT has an obvious effect in motor learning and performances. Further studies regarding the possible effects of TT on other cognitive functions are warranted.

#### ACKNOWLEDGEMENTS

This work is financially supported (No. ERIP/ER/1204652/M01/1496) by the Directorate of Extramural Research & Intellectual Property Rights (ER & IPR), Defence Research & Development Organization (DRDO), Government of India. Oryza Oil & Fat Chemical Co. Ltd., Japan, has provided the Tocotrienol©-90 sample to carry out this work. The authors wish to thankfully acknowledge the support received from the Management of NRI Academy of Sciences to carry out the work.

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