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Research Article

Influences of Exogenous Pro- and Anti-oxidants on Aluminum-induced Behavioral Alterations in Elevated Plus Maze and Passive Avoidance Activity of Rats

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Abstract: Exposure to aluminum is always associated with oxidative stress, while, oxidant imbalance is a common observation for degenerative neuropathologies. Recently, oxidative stress and cognitive impairment were associated together for the neurodegenerative processes and age-related behavioral pathologies were linked with neuroglial oxidant homeostasis. This study is aimed to explore the implication of external oxidant interventions in the aluminum induced neurobehavioral toxicity to evaluate the role of oxidant balance in functional neurodegenerative changes. Male NIN-Wistar rats were exposed to aluminum (Al_+) or vehicle (Al_0) for 4 weeks. During the period of aluminum exposure, the animals were also exposed to ethanol (0.2-0.6 g/Kg bw) and α-tocopherol (5 IU/day). After the completion of treatment protocol, their behaviors were evaluated with the help of elevated plus maze (EPM) and passive avoidance (PA) test. Time spent at different places, acquisition time (transfer latency), retention of memory for 24hrs and 48hrs were evaluated for the behavioral tests. All data were processed through two-way ANOVA with replication to find out the impact of aluminum treatment and oxidant imbalance. The differences between the groups were evaluated through Tukey's honestly significant difference (HSD) test. Significant contribution of the interactions between aluminum and pro-oxidant exposure had been observed in terms of choosing closed arm and central area of EPM having prominent effects on 3rd day especially in higher group of pro-oxidant exposures, whereas, ethanol exposure alone could influence the time spent in open arm on day 3 without significant difference between Al_0 and Al_+ animals. Supplementation with antioxidant could prevent the observed impacts of highest dose of ethanol exposure. However, similar protection by antioxidant is not observed in PA. Significant contribution of aluminum was observed on the day 1 and interaction with pro-oxidant exposure was significant for step down latency for all the three trials. The behavioral alterations caused by aluminum in EPM study was found to be influenced by pro-oxidant exposures and could also be ameliorated by antioxidant supplementation. However, lone aluminum did not cause alterations in PA parameters and the observed alterations could only corrected partly by the used antioxidant supplementation. Therefore, the aluminum-induced behavioral alterations are depending on oxidant status and exogenous supplementation of α-tocopherol can prevent the neurodegenerative changes, at least partially.
Keywords: Aluminum, Oxidative stress, transfer latency, step down latency (SdT) and step through latency (StL).

INTRODUCTION

Being the most used metal in regular domestic life and most available in earth's crust, exposure to aluminum is unavoidable. Because of unique physicochemical properties, it does not possess any utility value in the biological system. Thus, its presence in body is not warranted; unfortunately, existence and deleterious effects of aluminum were reported in many tissues[1]. The concept of being 'biological inert' promoted extensive use of aluminum in all spheres of life, however, study of toxic effects of aluminum started with the report of its neurotoxicity and probable link with age-related neurodegenerative disorders.

Increased use of aluminum nanoparticles in the medicine and cosmetics enhanced the iatrogenic exposure to aluminum. Nevertheless, the unregulated use of aluminum remained the major source of aluminum from environmental, dietary and occupational exposures[2]. Although, restricted solubility and high reactivity of aluminum allows only minute amount of it to be assimilated, non-renewability and susceptibility made neuronal cells specifically vulnerable to aluminum insult. The neurotoxicity of aluminum is well accepted for more than a century; still the mechanism(s) of neurotoxicity is/are not clear. Many hypotheses have been put forward to encompass the array of toxic impacts seen in experimental animals

and associated neurodegenerative disorders. Interestingly, most of these hypotheses include oxidative stress directly or indirectly, while the trivalent aluminum ion does not possess any redox activity in physiological solutions.

Cognitive deterioration has been proven many times as impact of aluminum exposure[3] and neurobehavioral alterations has been suggested as early indicator of aluminum toxicity[4] and proposed that it can be detected even before the neurochemical or neuroanatomcial alterations. Promotion of inadvertent plaques formation in specific brain regions have been documented as an impact of aluminum intoxication[5] proposed to be the reason of aluminum-induced neurobehavioral alteration[6]. While products of redox imbalance *e.g.* increased lipid peroxidiation, increased formation of protein carbonyls and decreased reduced glutathione, are commonly seen on those tissues[7]. To explain the mechanism of oxidative stress, often indirect influence of β-amyloid has been considered[8-9], while β-amyloid plaque and neurofibrillary tangles were not implicated in the proposed mechanism of aluminum-induced neurobehavioral toxicity[10]. However, irrespective of the mechanism, both oxidative stress and cognitive impairments were considered as the toxic impacts of oral aluminum exposure.

Notwithstanding the controversies related to the link between aluminum exposure and Alzheimer's disease, there is conformity that both of these bear common consequences[11]. Neurochemical and molecular approaches are being used to make strategy against the neurodegenerative disorders, however, importance of early diagnosis of neurodegenerative disorders have been recently emphasized[12]. Discerning the role of aluminum in neurobehavioral alterations as it has been implicated in many age-related neurodegenerative disorders may help to understand the course of these neuropathologies.

Previous studies suggest that exposure to aluminum induction will increase dementia and behavioral changes in human and experimental animals[13]. In view of our earlier observations of aluminum-induced alteration of oxidant handling capacity[14], the current study was aimed to evaluate the behavioral alterations in aluminum-exposed animals which are concomitantly exposed to conditions of pro-oxidant dominance, antioxidant dominance or both.

MATERIALS AND METHODS

Animal maintenance and treatments

The experimental protocol was approved by the Institutional Animal Ethics Committee. The animals were obtained, maintained and treated in the Registered Animal House of NRI Medical College & General Hospital and the procedures were performed according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, India).

Male adult NIN-Wistar rats were used in the study. The animals were maintained with standard conditions[14]. After one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into groups (Table 1). Ethanol, aluminum and tocopherol were force fed (daily for 4 weeks) through orogastric tube. Ethanol or distilled water was given in the forenoon session while aluminum or vehicle and tocopherol were given in the afternoon session daily. Because of inconclusive toxicokinetic interactions of ethanol and aluminum, different treatment sessions were maintained[15]. Morning sessions were preferred for ethanol exposures to avoid impact of ethanol on food intake.

Behavioral Study

At the end of the treatment protocol, all groups of animals were subjected to behavioral study in Elevated Plus Maze (EPM) and Passive Avoidance (PA) Activity. The behavioral recordings were carried out in three consecutive days.

Elevated Plus maze: The maze consists of two closed and two open arms of size 50cm length, 30cm Elevated from the base height. The rat was dropped gently at the open arm facing towards the open end of the open arm[16-17]. Time required to enter any of the closed arms with its four legs inside the closed arm area was noted as transfer latency. Then the animal was allowed to explore the EPM freely for 5 minutes. The time spent in center stage, closed and open arms were noted by digital counters.

Passive avoidance activity: The unit consists of two chambers, one is a bigger chamber of 30×30×30cm, inside this chamber there was another small dark cabinet of 15×12×10cm with an entry-hole. The floor inside this cabinet was a grill of metallic tubes of diameter 0.5cm. The rat was placed gently on the small cabinet and then the time requires to step down from the stage with all four legs on the floor (step down latency; SdL), time to enter into the dark chamber with all four legs inside the dark chamber (step through latency; StL) were recorded for a span of 5 minutes. First day the animals were given electric pulses whenever it entered the dark chamber. Second and third day SdL and StL were noted as passive avoidance parameters.

Statistical analysis

Six individual data were collected from each group and were processed for statistical analysis using two-way ANOVA with replication to get the F value. The differences between individual means were analyzed by Tukey's HSD test. Statistical significance for two-way ANOVA with replication and Tukey's HSD test were

collected from the tables accepting probability (p) ≤ 0.05.

RESULTS

The transfer latencies in EPM for three consecutive days are presented in Table 2. The transfer latency for first day for each animal was considered as acquisition time to learn the presence of closed arms. For next two days the transfer latencies were used for evaluation of retention of learned preferred area in the EPM. The retention time at day 3 of Al_+ animals of P-II and P-III groups were significantly higher than that of Al_0 animals of the respective groups. In fact, the retention time of Al_+P-III animals on day 3 was found to significantly higher to all the groups of animals except Al_0P-II animals. Accordingly, the contribution of aluminum alone (F = 24.538) and its interaction with ethanol (F = 5.24) were found to be statistically significant by two-way ANOVA with replication. In other days there was no statistically significant difference was found in day wise transfer latency values for all tested groups, except, Al_0P-III vs Al_0P-II on the day of acquisition.

The time spent by Al_0 and Al_+ animals of each pro-oxidant group in the different areas of EPM during three days post-treatment were presented in figure 1. During first day of trial, the Al_+P-0 animals found spending most time in closed arms. With increase in pro-oxidant doses, Al_+ animals tend to spend less time in the closed arms but not reaching out to open arms. On the other hand, Al_0P-0 animals were found to come out of the closed arms and spending time in center area. All the pro-oxidant exposed Al_0 animals were showing comparable behavior in terms of their choices to spend time mostly in closed arm. Two-way ANOVA with replication evinced significant contribution of interactions (F = 4.83) of aluminum and pro-oxidant exposures on the time spent by the animals at the closed arm of EPM. Statistically significant differences between Al_0 and Al_+ animals were observed P-0 and P-III groups in case of closed arm preference. Consequently, appreciable differences were observed between Al_0 and Al_+ animals in terms of time spent in central area, however, the difference was statistically significant for only P-0 group (Day 1; Figure 1). Two-way ANOVA with replication reported significant contribution of ethanol as pro-oxidant alone (F = 3.04) and in interaction with aluminum (F = 7.64). After 24 hours (Day 2; Figure 1), Al_0P-0 animals maintained their tendency of spending more time in central area. However, Al_0 animals of P-III group demonstrated reduction in preferring closed arms while spending more time in central area. Statistically, only difference between Al_0 and Al_+ animals of P-0 group was found to be significant in case of closed arm opting and two-way ANOVA with replication found interaction between aluminum and pro-oxidant to be significant (F = 4.39). On day 3, the time spent at open arms were found to be significantly influenced by the pro-oxidant exposure (two-way ANOVA with replication, F = 9.33) demonstrating significant differences between the pro-oxidant groups (Al_0P-I vs Al_0P-II; Al_+P-0/I/III vs Al_+P-II; Figure 1). In case of time spent in closed arms, two-way ANOVA with replication calculated significant contribution of pro-oxidant exposure (F = 6.45) and interactions of exposure to aluminum and pro-oxidant (F = 6.88). Accordingly, Al_0P-0 animals spent significantly less time in the closed arms than their Al_+ counterpart, while Al_+ animals spent significantly less time in that are in comparison to their counterparts of P-II and P-III groups. Corroborating these observations, Al_+ animals of those groups spent significantly more time in the central area. Two-way ANOVA with replication demonstrated significant contributions of exposure to aluminum (F = 6.61), pro-oxidant (F = 10.60), as well as their interactions (F = 6.04).

Temporal pattern of positioning themselves in the area of preferences were noted for the antioxidant set of animals for three consecutive days and presented in figure 2. Statistically, the Al_0 and Al_+ animals were not differing in their time spent in different areas in either of the groups or of the days.

The step down (SdT) and step through (StL) latencies for the passive avoidance activity of pro-oxidant groups of animals are presented in figure 3. Of all the tested groups, the SdT of Al_0P-III animals was found to be highest during the day 1 trial and it was significantly differing from that of Al_+ animals of same group (Figure 3). For day 1 trials, two-way ANOVA with replication found significant contribution of both aluminum (F = 5.891), ethanol exposures (F = 3.959), as well as their interactions (F = 5.908). Similarly, StL on the same day was found to be influenced by exposure to aluminum (F = 11.082), ethanol (F = 25.96) and their interactions (F =24.797) as per two-way ANOVA with replication. In the same line, Al_0 animals were significantly different from Al_+ animals for all the ethanol-exposed groups in terms of their StL. However, the StL value was lower for Al_+ animals in P-III group, while it was higher in other ethanol-exposed groups (Figure 3; Day 1). The StL of Al_+ animals of any group was not differing significantly from that of their respective Al_0 animals and accordingly treatment was found to be ineffective in day 2 as per two-way ANOVA with replication. On the other hand, two-way ANOVA with replication found significant contribution of ethanol exposure (F = 7.321) and its interaction with aluminum exposure (F = 17.049) on SdL of day 2 with alternating differences between Al_0 and Al_+ animals of P-II and P-III groups (Figure 3; Day 2). On the final day of testing, significant contributions of aluminum (F = 4.87) and its interaction with ethanol exposure (F = 3.31) on the SdL were evinced by two-way ANOVA with replication, even though the differences between Al_0 and Al_+ animals were not significant in either of the pro-oxidant groups (Figure 3; Day 3). On the contrary, Al_+ animals recorded significantly lower StL in

comparison to their Al_0 counterparts in all the ethanol-exposed groups (P-I/II/III) but the StLs of Al_+P-0 and Al_0P-0 animals was noted to be equally high. Nevertheless, only contribution of aluminum exposure was found to be significant (F = 23.582) as per two-way ANOVA with replication (Figure 3, Day3). In the antioxidant set, significant difference between Al_0 and Al_+ animals were noted only on day 1 for SdL and on day 3 for StL (Figure 4).

Table-1: Groups and their treatment protocol.

Treatment protocol			Groups of animals											
			Control		Pro-Oxidant						Anti-Oxidant			
			P-0		P-I		P-II		P-III		TP-0		TP-III	
Timings	*Treatments*	*Max. Vol.*	Al_0	Al_+	Al_0	Al_+	Al_0	Al_+	Al_0	Al_+	Al_0	Al_+	Al_0	Al_+
9AM	Ethanol (g / Kg bw)	0.2mL	×	×	0.2	0.2	0.4	0.4	0.6	0.6	×	×	0.6	0.6
	Distilled water	0.2mL	✓	✓	×	×	×	×	×	×	✓	✓	×	×
5PM	Aluminum (10 mg / Kg bw)	0.2mL	×	✓	×	✓	×	✓	×	✓	×	✓	×	✓
	Gum Acacia	0.2mL	✓	×	✓	×	✓	×	✓	×	✓	×	✓	×

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.

Table-2: Time taken by the animals to enter the closed arm of EPM with all four limbs inside the closed arm for Day 1 (Acquisition time in seconds) and Days 2 and 3 (Retention time in seconds).

Study sets			**Pro-Oxidant study**				**Anti-Oxidant study**	
Time (sec)			P-0	P-I	P-II	P-III	TP-0	TP-III
Acquisition		Al_0	5.28 ± 0.85	5.35 ± 1.46	9.97 ± 3.36	2.88 ± 0.52	4.25 ± 1.50	2.00 ± 1.25
		Al_+	6.32 ± 1.73	3.76 ± 0.81	8.32 ± 2.98	7.44 ± 1.44	4.25 ± 1.50	3.50 ± 2.25
Retention	Day 1	Al_0	3.76 ± 0.45	5.76 ± 2.11	5.28 ± 3.04	3.28 ± 0.52	3.25 ± 0.75	3.25 ± 1.25
		Al_+	2.72 ± 0.54	2.80 ± 0.61	2.48 ± 0.56	4.00 ± 0.79	2.50 ± 0.90	3.75 ± 1.25
	Day 2	Al_0	1.87 ± 0.49	0.91 ± 0.24	0.78 ± 0.18	0.88 ± 0.07	2.25 ± 0.75	3.25 ± 1.63
		Al_+	1.86 ± 0.28	2.22 ± 0.45	3.81 ±1.13*	5.78 ± 1.26*	2.75 ± 1.63	4.25 ± 1.25

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.
*** = Significant difference with the respective Al_0 group.**

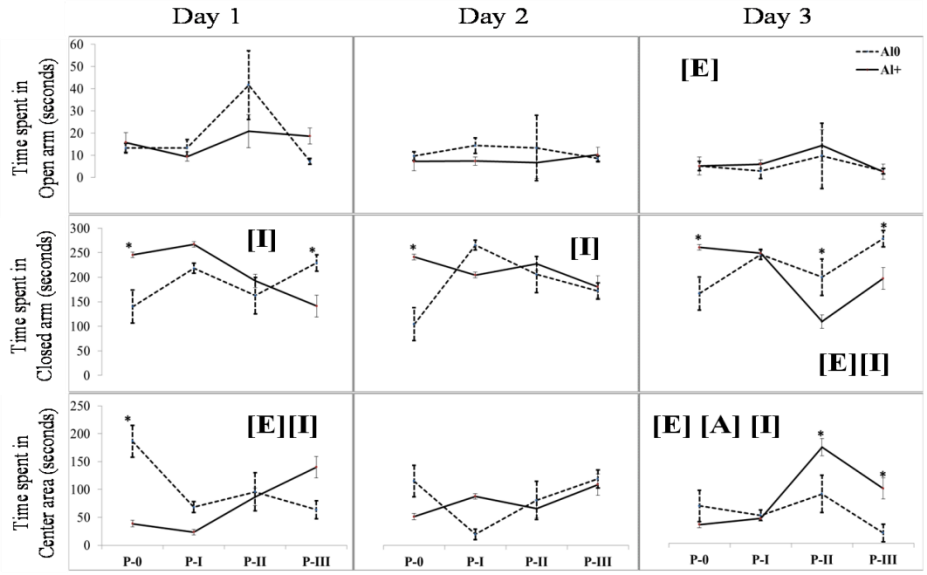

Fig-1: The time spent in closed arm, open arm and central area of EPM by the animals during their three day trials after antioxidant phase of study.

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.
* = Significant difference with the respective Al_0 group. [A], [E] and [I] indicate significant influence of aluminum exposure, ethanol exposure and their interactions, respectively, as per two-way ANOVA with replication.

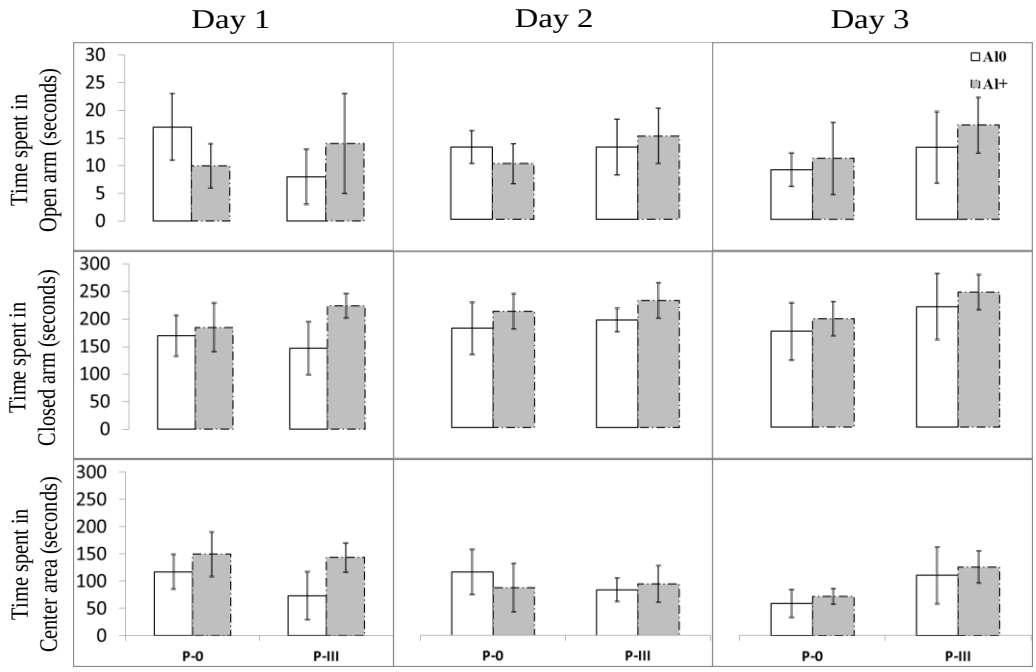

Fig-2: The time spent in closed arm, open arm and central area of EPM by the animals during their three day trials after antioxidant phase of study.

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.

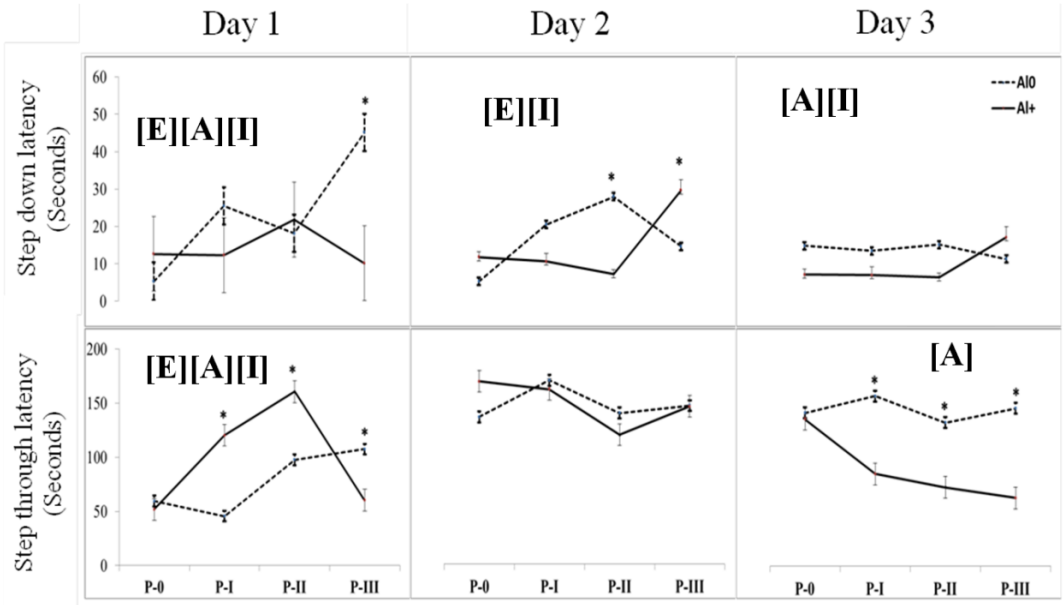

Fig-3: Time taken to step down from the dropping stage (Step down latency) and the time taken to enter into the dark chamber (Step through latency) by the animals during their three day trials after pro-oxidant phase of study.

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.

* = Significant difference with the respective Al_0 group.

[A], [E] and [I] indicate significant influence of aluminum exposure, ethanol exposure and their interactions, respectively, as per two-way ANOVA with replication.

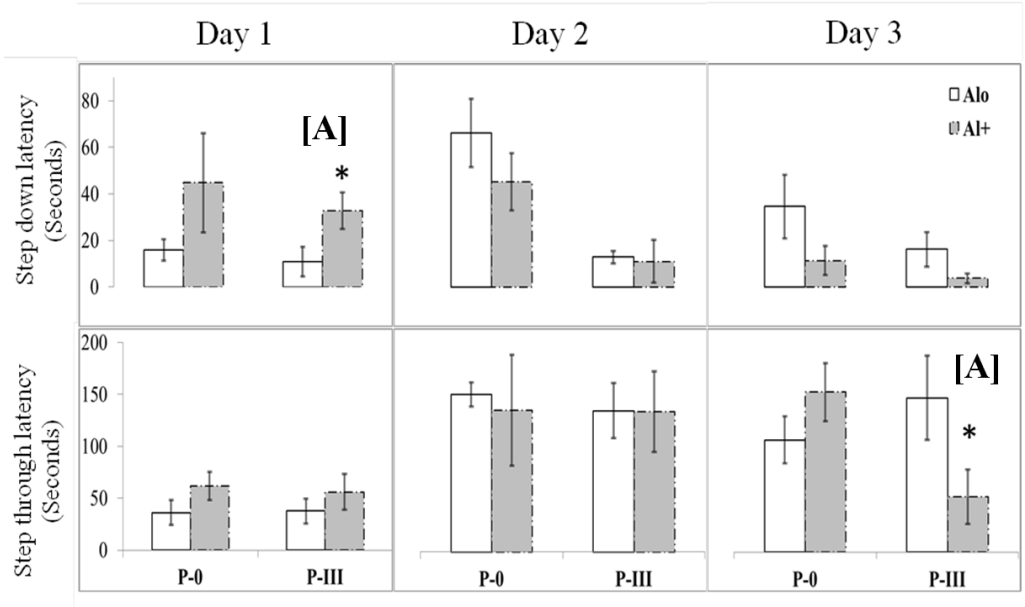

Fig-4: Time taken to step down from the dropping stage (Step down latency) and the time taken to enter into the dark chamber (Step through latency) by the animals during their three day trials after pro-oxidant phase of study.

Al_0 = Group receiving no aluminum exposure; Al_+ = Group receiving aluminum exposure.
* = Significant difference with the respective Al_0 group.
[A] indicate significant influence of aluminum exposure as per two-way ANOVA with replication.

DISCUSSION

As per the current estimate, worldwide millions of people are suffering from neurodegenerative diseases, projecting it as an important societal burden[18]. Unfortunately, even after years of sophisticated researches, problem seems to be unresolved. Multifaceted theories have been put forward to discern the mechanisms of initiation and progression of neurodegenerative changes, and efforts are continuously made to halt or restrict the progress with only limited outcome. As the neurons are highly sensitive to oxidative stress[18], redox imbalance is always having major share in the proposed mechanisms of neurodegneration[19-21]. Oxidative stress in brain is ubiquitously associated with dyshomeostasis of metals, irrespective of their redox status[20], while it is suggested that oxidative stress can be induced by metal toxicity[18]. Proposing multiple mechanisms, induction of oxidative stress and cellular damage have been suggested by presence of aluminum in brain[22] even though it is a redox-inactive metal. On the other hand, it has been suggested that oxidative stress is closely related to cognitive dysfunction and antioxidants may potentially protect impaired cognitive functions[23].

Regardless of the species and age of host, aluminum is a neurotoxin through all routes of administration. However, the extent of neurotoxicity is dose and duration dependent[24]. The implications of aluminum-induced neurotoxicity are more potent in some stage of life and in certain compromised states. There are several reports which suggest exposure to antioxidative measures (vitamins, minerals or herbal products) could possibly ameliorate the toxic impacts of aluminum[25]; while aluminum itself could augment the oxidative stress created by pro-oxidant (ethanol) exposure differentially in cerebrum[26] and cerebellum[2]. Thus, the present study was carried out to evaluate the impact of aluminum on neurobehavioral activities of rats when they are exposed to pro-oxidants in absence and presence of antioxidant supplementation.

Elevated plus maze is normally used to evaluate the anxiety like behavior in rodents. Recently its use as exteroceptive behavioral tool to evaluate learning and memory in rats has been suggested[27]. By measuring acquisition time and retention time on the following two days the elevated plus maze allow assessment of cognitive function of animals. Both Al_0 and Al_+ animals of P-0 group equally avoided the open arms during all three days of study. Spending more time in the closed arms, Al_+ animals indicated less explorative behaviors supported by relatively more anxious behavior. The Al_+ animals of P-0 group continued to spend more time in the closed arms during the all three days of test and thus corroborated the earlier report of aluminum-induced anxiety-like behaviors[28]. On the other hand, Al_+ animals with concomitant exposure to pro-oxidants with higher doses (P-II/III) spent more time in the central area on third day and suggested either domination of anxiolytic property of ethanol[29-30]. However, significant differences between spatial performances of Al_0 and Al_+ animals of P-II/III groups during third trial may be due to possible state of confusion or reduced

motor activity in Al_+ animals, while, Al_0 animals might had the benefit of better retention memory of pre-exposure experience to EPM maze[31].

Using intragastric aluminum overloading, Zhang et al [32] have demonstrated that rats faced degenerative changes in CA1 region of hippocampus along with oxidative stress and diminished response in passive avoidance learning. In the present study we found that there was no significant difference between the Al_0 and Al_+ animals in terms of step down latency or step thorough latency either during learning (Day 1) or retaining the memory for next two days. Acceptably, this difference in observation is most likely due to difference in dose and duration of treatment. Zhang et al [32] continued the treatment for 12 weeks with 400 mg elemental aluminum / kg body weight for 5 days / week, whereas the current investigation was carried out with only 10 mg elemental aluminum / kg body weight / day for 4 weeks. Therefore, understandably there will be no oxidative stress in brain regions like frontal cortex and temporal cortex[33]. Interestingly, concomitant exposure to pro-oxidants in the form of different doses of ethanol, the same dose of Al_0 and Al_+ animals exhibit significant differences in terms of learning of step through latency and also retaining the memory and performing after 48 hours (Figure 3). Therefore, it can be suggested that the neurobehavioral damage created by aluminum is dependent on the concomitant exposure to other neurotoxicants. On the other hand, the observations of significantly high step down latency of Al_0P-III group on Day 1 may not be related with impact of aluminum or pro-oxidant effect on that, as the response was significantly differing from other ethanol exposure groups (Al_0P-0/I/II). However, impact of aluminum on the same dose cannot be disregarded on the basis of retrieving memory after 24 hours (Figure 3). Aluminum-induced deteriorations of performances in passive avoidance were noted by several authors[34-37] and they have suggested that the observed learning and memory derangements can be prevented by herbal preparations. In the current context, it has been observed that concomitant exposure to conditions of pro-oxidant dominance can also be the cause of aluminum-induced alterations and the noted improvements by the use of herbal preparations[36-37] may be ascribed to the antioxidant activities of those herbal preparations. While evaluated, the antioxidant dominance created by oral administration of α-tocopherol (5μg/day) could prevent the aluminum-induced changes only partially in P-III group of animals (Figure 4). Ahmed et al (2014) demonstrated the amelioration of aluminum-induced loss in learning and memory functions by using antioxidant and anti-inflammatory agents. However, they have demonstrated reduced aluminum content in the frontal cortex and hippocampus in case of coadministration of aluminum with antioxidant and anti-inflammatory agents. The conditional learning was evaluated in passive avoidance as a study of reinforcement which provided the memory and conditional learning with a fear tendency of the rat under study (). However, it has been suggested that the study might not be a true reflection of the exact status of stress and anxiety[38].

The behavioral alterations caused by aluminum in EPM study was found to be influenced by pro-oxidant exposures and could also be ameliorated by antioxidant supplementation. However, lone aluminum did not cause alterations in PA parameters and the observed alterations could only corrected partly by the used antioxidant supplementation. Most likely, higher doses of tocopherol supplementation could have been effective against these behavioral alterations. Therefore, the aluminum-induced behavioral alterations are depending on oxidant status and exogenous supplementation of α-tocopherol can prevent the neurodegenerative changes, at least partially.

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