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Therapeutic Potential of Pomegranate Juice in Wistar Rats against Aluminium Induced Neurotoxicity

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Abstract

Neurodegenerative disorders are the major challenges faced by the global health care system in recent years. It is a well-known fact that a number of environmental as well as lifestyle factors are responsible for neurodegenerative diseases and the contribution by aluminium (Al) plays a major role in this. A diet containing considerably high traces of Al may cause neurotoxicity leading to memory loss, impaired coordination as well as a cognitive disturbance. The present study is designed to evaluate the neuroprotective role of Pomegranate Juice (PJ) since it is rich in polyphenols that have antioxidant properties. We evaluated neurodegenerative changes induced by $AlCl_3$ in the hippocampus of Wistar rats as well as cognitive changes displayed by them. Then compared it with that of PJ treated rats which were previously administered with $AlCl_3$. A significantly decreased ($p<0.001$) number of viable neurons were seen, in different regions of the hippocampus in the $AlCl_3$ administered rats in comparison to the animals of the control group. However, the number of viable neurons significantly boosted ($p<0.001$) in PJ treated rat hippocampus. This suggests that neurotoxic changes induced by the aluminum can be ameliorated by treatment with PJ.

Keywords: Aluminium Neurotoxicity, Hippocampus, Neurodegeneration, Pomegranate

1. Introduction

As the percentage of the aging population is considerably increasing in recent years, concurrent progression of neurodegenerative disorders has become a major challenge faced by the global health care system. It is a well-known fact that a number of environmental as well as lifestyle factors are responsible for neurodegenerative diseases, among which contribution by aluminum (Al) plays a major role[1]. It is the third extensive element on the earth's crust and is widely distributed in the environment[2], A diet containing considerably high traces of Al may lead to increased neurotoxic effects on the human CNS such as, memory loss, impaired coordination, partial paralysis and cognitive disturbances[3]. Association between neurodegenerative changes in different areas of the brain and Al-induced oxidative stress has been reported[4]. Aluminum can cross the blood-brain barrier and accumulate in brain tissues[5]. It mainly accumulates in the hippocampus, the region of the brain that is concerned with learning and memory and in AD this region of the brain is specifically affected. Though there are number of drugs to combat such neurological deficits, it is not managed sufficiently by them[6]. Recently, derivatives of plant products in the form of herbal

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medicine are gaining popularity because of their abundant polyphenols and considerably low side effects caused by them[7].Among such alternative therapeutic sources, pomegranate is receiving much attention because of its rich polyphenols. It has revealed its neuroprotective potential in different experimental models[8–10].However, the effect of PJ on Al-induced neurotoxicity has not been documented sufficiently. Therefore, the present study is carried to investigate the effect of PJ against neurotoxicity caused by AlCl3 in rat brain by evaluating neuromorphological as well as behavioral parameters.

2. Material and Methods

2.1 Animal Groups

The study was conducted on Wistar strain albino rats of 180-200g body weight. The animals were housed under the controlled condition of temperature and light anddark cycle (12:12hr). The study was reviewed and approved by the Institutional Animal Ethics Committee (MC/MNG/IAEC/07-2019 dated 15.02.2019).

The rats were divided into 4 groups with 6 rats in each group.

Group I - normal control,

Group II - AlCl3 treated rats wherein AlCl3powderwas dissolved in distilled water

Group III - Experimental group that received 70mg/kg body weight of AlCl3 in the morning + 40% pomegranate juice (p.o.) in the evening, for 30 days

Group IV - PJ control where animals received 40% pomegranate juice alone for 30 days (p.o).

2.2 Pomegranate Juice Preparation

For PJ dosage selection, initially, the rats were observed for the volume of water consumed by them on a daily basis for 5 days and a dosage of 40% PJ (oral) was selected according to the suggestion from the previous studies[9,12].

Pomegranate juice was made freshly each day for administration. The seeds of fresh pomegranate fruit were removed, blended in a mixer and filtered with the help of an ordinary sieve to obtain the juice. After that, the juice was concentrated to 40% by adding in drinking water and was served to the animals in their drinking bottle.

2.3 Experimental Plan

AlCl3 powder was dissolved in distilled water and administered intraperitoneally (i.p.), at a dose of 70mg/kg/body weight[11], for 30 days, to all rats except to those of the control group.

The rats consumed about 5ml of water daily and accordingly the animals received 2ml (40%) of PJ per day.

2.4 Histomorphological Study

On the final day of treatment, animals were anesthetized by sodium pentobarbital and then the animals for histological evaluation were perfused and fixed with 10% formaldehyde. The cranial cavity of these animals was dissected and the brain was removed and processed further for histology. Coronal sections of the brain with the hippocampal region of (6-7-µm thickness) was taken. Twenty-fifth and 26th sections from each animal was studied to maintain uniformity. Cresyl violet staining was used to study the quality of neurons. From every section of cornuaamonis areas (CA1, CA2 CA3, and CA4) and Dentate Gyrus (DG) of 250µm length was selected and a number of viable neurons were counted. The slides were screened using a light microscope at 40X magnification (Magnus, Olympus, Pvt, Ltd), quantitative analysis was done. Nikon trinocular microscope (H600L) was used for screening. Imaging software NIS Elements Br version 4.30 will be used to quantify the neurons[13].The cell count was expressed as the number of cells per unit length of the cell field (cell/250µm length).

2.5 Behavioral Study

2.5.1 Open Field Test

This test is the most commonly used method for assessing the exploratory and motor activities of rodents[13]. This is performed within a rectangular wooden chamber (100x100x40cm) with 25 equal squares (5x5cm) in the floor. Sixty centimeters above the center of the floor 100Watts bulb is fixed for illumination. The rats were placed at one corner of the chamber at the commencement of the test. The number of crossings (peripheral and central) by the rats in 10min. the duration was documented. The motor activities like rearing (elevation of limbs) and grooming (cleaning various parts of the body using tongue & forelimb) was documented.

2.5.2 Active Avoidance Test

It is performed within a wooden shuttle box with shutters in front[13]. Its floor has grids and a median grid separates it into two parts. Each part hasa separate electrical circuit. A buzzer is provided within the box. Individual rats were given 5min of time to explore the test box. After 10sec it was given a discriminate stimulus through the buzzer wherein, the rattries to escape the shock by dashing to the other part. It is termed 'Shock Avoidance' (SA). The test includes 30 trials/day and it will be repeatedly performed for 5 successive days. Under normal circumstances, SA

values usually increase from day 1 to day 5. The average the number of SA during 5 days of testing is calculated as the mean score. Any decrease in this score indicates learning impairment. The retention of memory was assessed by repeating the test after a week. Retention Score (RTS) was calculated by comparing both the performances and used for analysis.

$$RTS = \frac{\text{Mean of retest score} \times \text{Mean scoring during 5 days of testing}}{\text{Mean score during day 5 of the test}}$$

The retention score is presented in percentage. Any decrease in the retest score and retention score is suggestive of memory impairment.

2.5.3 Passive Avoidance Test

A passive avoidance test was used to assess the memory retention test. This test is to determine the ability of rat to remember foot shock delivered 24 hr prior to the memory retention test[13]. Passive avoidance apparatus consists of a wooden box with a larger, bright compartment and a smaller, dark compartment with a grid floor, which is attached to a shock source. The connection between the two compartments can be closed with a sliding door.

On the first day of the test, rat was allowed to explore the center of the illuminated large compartment facing away from the entrance to the dark, small compartment. The door between the two champers remained open at this time. Both chambers were explored by the rat for five minutes, this is called the exploration test method. This was followed by three test trials of five minutes by each rat. At the end of each trial animal was kept back in their home cage where it remained during an inter-interval of 5 minutes. In each trial, fraction of time spent in each compartment was noted. At the end of the third test trial, rat was placed in the dark compartment, the connection door was closed and a foot shock was delivered through the grid floor (50 Hz,1.5mAfor 1 second). The rats were kept there for additional 10 seconds, to allow the animal to form an association between the properties of the chamber and foot shock. Then the rat was returned to the home cage. This trial is known as 'passive avoidance acquisition.

After 24 hours of foot shock, the memory retention test was done. The rat was placed in the bright compartment and the time taken for the rat to enter the dark compartment for first time was recorded using a stopwatch. A maximum of 5 minutes was given for the rat to explore. This is known as the 'memory retention test'. Apparatus was cleaned with spirit in between each test. Normal rats avoid entering the dark chamber, where they received a shock on the previous day, suppressing their normal behavior of exploring the dark compartment. Decreased latency to enter the dark compartment and more time spent in the dark compartment will suggest poor memory retention.

2.6 Statistical Analysis

The data areexpressed as mean ±SE and the significance of differences between the groups was analyzed using ANOVA followed by Bonferroni's multiple comparison post hoc test. The P-value <0.05 considered as significant.

3. Result

3.1 Behavioral Studies

3.1.1 Open Field Test

As shown in Figure 1,$AlCl_3$ administered rats demonstrated reduced locomotor activity which was evident in the form of significantly decreased (p<0.001) no. of crossings when compared to that of control rats. Whereas, $AlCl_3$ administered rats, when treated with PJ, displayed a significant increase (p<0.001) in no. of crossings when compared to that of rats intoxicated with $AlCl_3$. The rats that received only PJ also demonstrated a significant increase (p<0.001) in total no. of crossing when compared to both $AlCl_3$ and $AlCl_3$ +PJ groups.

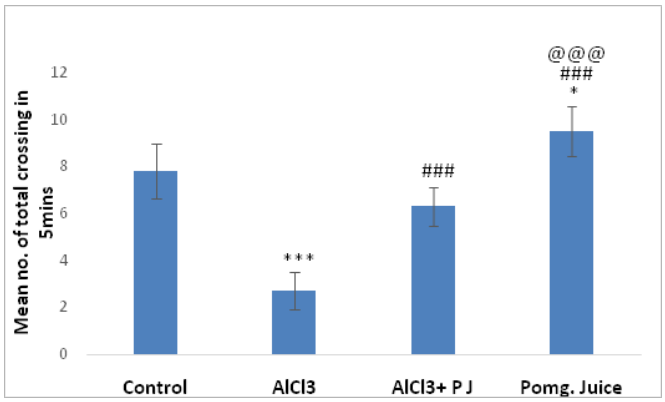


Figure 1. Comparison of total crossings in open field test, by rats of different groups.

Values are expressed as mean ± SD, n=6. Control vs AlCl3 *** p<0.001, Control vs PJ * p<0.05, $AlCl_3$ vs $AlCl_3$+PJ ### p<0.001, $AlCl_3$ vs PJ ### p<0.001, $AlCl_3$+PJ vs PJ @@@ p<0.001.

3.1.2 Active Avoidance Test

The result of this test is expressed as the mean no. of avoidance score, retest score, retention score in

percentage(Figures 2 and 3). $AlCl_3$ administered rats displayed a significantly reduced number of avoidance score ($p<0.001$), memory retention test score ($p<0.001$) and % retention score ($p<0.001$). The PJ-treated $AlCl_3$ administered rats demonstrated significantly increased mean no. of avoidance scores ($p<0.001$), memory retention test scores ($p<0.001$) and % retention score ($p<0.001$). The summary of the active avoidance test is that $AlCl_3$ induced chronic toxicity has adversely affected the learning abilities and memory retention in rats. The pomegranate treatment has reversed this adverse effect in $AlCl_3$ administered rats and also interestingly it has enhanced the learning abilities and ability to retain the memory of normal control rats.

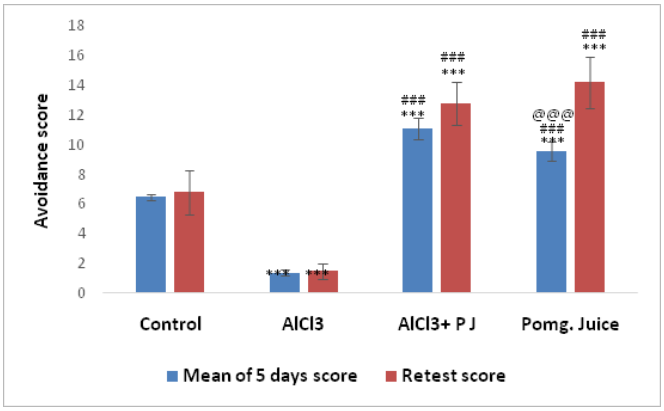


Figure 2. Comparison of no. of shock avoidance in active avoidance test, by rats of different groups. Values are expressed as mean ±SD, n=6.

3.1.3 Mean of 5 Days Score

Control vs. $AlCl_3$*** $p<0.001$, Control vs. $AlCl_3$+PJ*** $p<0.001$, Control vs. PJ*** $p<0.001$, $AlCl_3$ vs. $AlCl_3$+PJ ### $p<0.001$, $AlCl_3$ vs. PJ ### $p<0.001$, $AlCl_3$+PJ vs. PJ @@@ $p<0.001$.

3.1.4 Retest Score

Control vs.$AlCl_3$*** $p<0.001$, Control vs. $AlCl_3$+PJ *** $p<0.001$, Control vs. PJ*** $p<0.001$, $AlCl_3$ vs. $AlCl_3$+PJ ### $p<0.001$, $AlCl_3$ vs. PJ ### $p<0.001$.

3.1.5 Retention score

Control vs. $AlCl_3$*** $p<0.001$, Control vs. $AlCl_3$+PJ*** $p<0.001$, Control vs. PJ*** $p<0.001$, $AlCl_3$ vs. $AlCl_3$+PJ ### $p<0.001$, $AlCl_3$ vs. PJ ### $p<0.001$.

3.1.6 Passive AvoidanceTest

Rats administered with $AlCl_3$ displayed significantly decreased latency ($p<0.001$) to enter the dark compartment when compared to control rats indicating their poor learning capacity. After PJ treatment the $AlCl_3$ administered rats demonstrated significantly increased ($p<0.001$) latency to enter the dark compartment indicating their improved ability for learning and memory **(Fig.4)**.

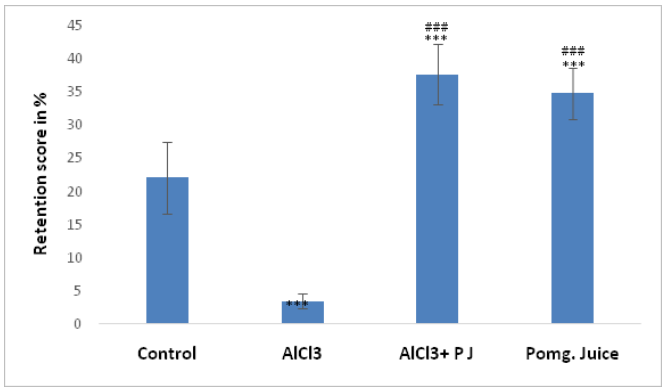


Figure 3. Comparison of retention score in active avoidance test, by rats of different groups. Values are expressed as mean ± SD, n=6.

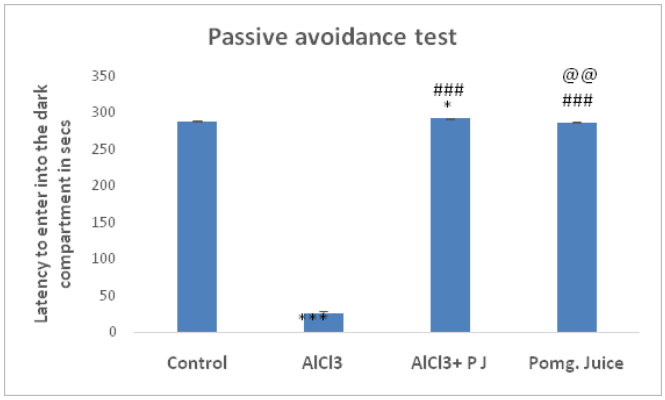


Figure 4. Comparison of latency in passive avoidance test, by rats of different groups. Values are expressed as mean ± SD, n=6.

Latency to enter into the dark compartment: Control vs $AlCl_3$ *** $p<0.001$, Control vs $AlCl_3$+PJ* $p<0.05$, $AlCl_3$ vs $AlCl_3$+PJ ### $p<0.001$, $AlCl_3$ vs PJ ### $p<0.001$, $AlCl_3$+PJ vs PJ@@ $p<0.01$.

3.2 Histomorphological Study

3.2.1 Neuronal Assay

The neurons showing irregular cell membranes with pyknotic and hyperchromatic nuclei are considered as degenerated neurons in the present stud (Figure 5). Those neurons were not considered for counting where the

border between the nucleus and cytoplasm was not clear. Accordingly, the expression of the number of viable neurons in DG& CA4 region has reduced significantly ($p<0.001$) in AlCl3 administered rats when compared to control. But there was a significantly increased ($p<0.001$) number of viable neurons in that of PJ treated Al intoxicated rats when compared to $AlCl_3$ alone administered rats. In CA1, CA2, CA3 region of the hippocampus the expression of the number of normal neurons did not show any statistically significant difference ($p>0.05$), except for a marginal difference between rats of $AlCl_3$+PJ group and $AlCl_3$ group. Pomegranate alone has not altered much of the neuronal expression when compared to control (Table 1).

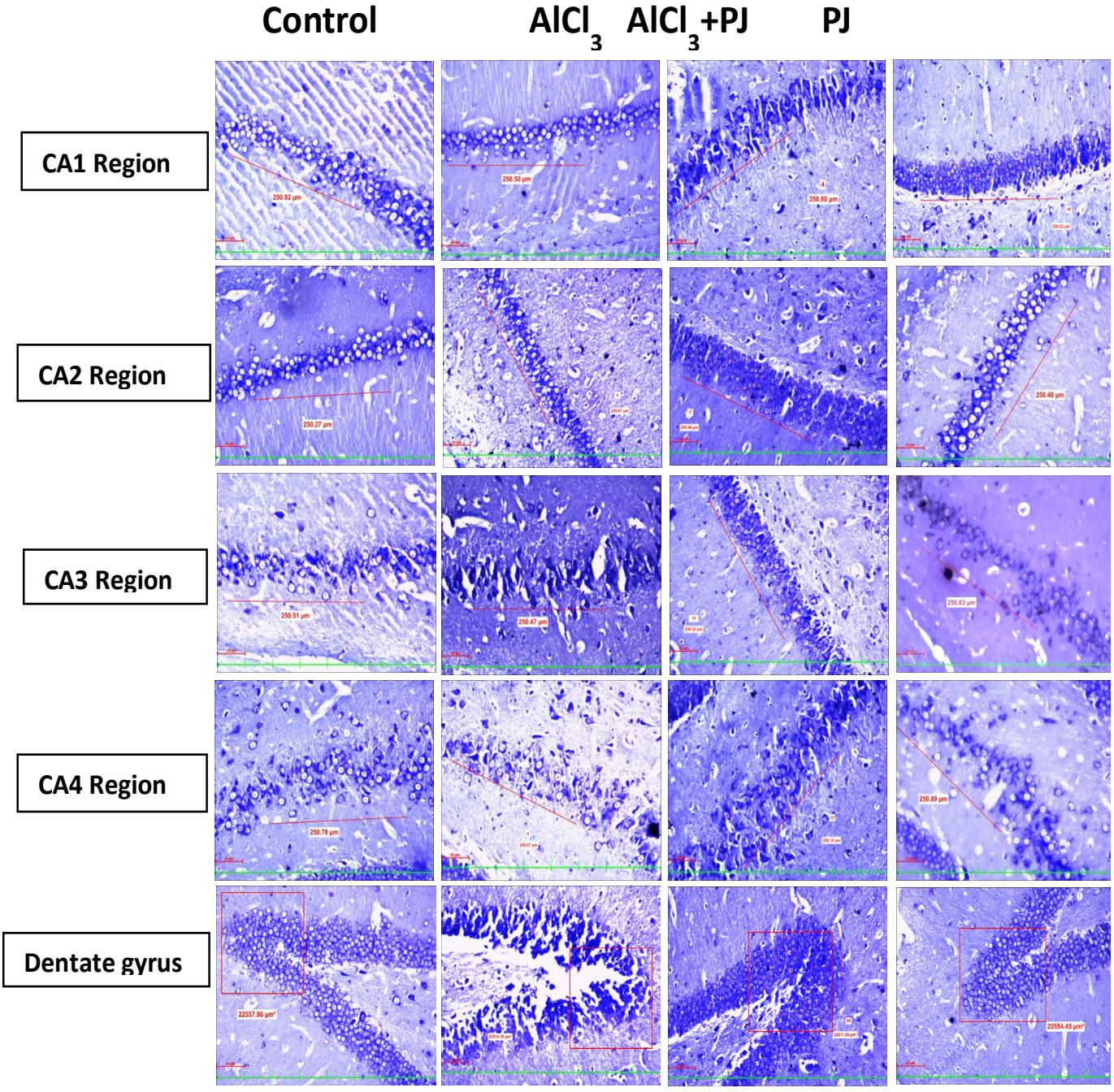


Figure 5. Photomicrographs of the hippocampal subregions in different groups of rats stained with cresyl violet stain. Length of 250μm area was selected in each region of the hippocampus. CA – Cornu Ammonis; DG – Dentate gyrus

Table 1. Number of viable neurons in Hippocampus of different groups; Values expressed as Mean ± SD

	Control	AlCl3	AlCl3+PJ	PJ
Dentate gyrus	33.3 ± 2.94	4.2 ± 0.75	14.3 ± 3.98	31.7 ± 3.55
CA4 region	21.7 ± 2.80	3 ± 0.89	11 ± 1.78	19 ± 1.41
CA3 region	21.5 ± 1.87	3.7 ± 1.21	11.3 ± 1.50	21 ± 2.89
CA2 region	16.5 ± 1.37	2.8 ± 0.75	12.3 ± 1.75	19.2 ± 1.47
CA1 region	21.8 ± 2.63	3.2 ± 0.75	15.2 ± 2.48	17.8 ± 3.92

4. Discussion

Diet containing considerably high traces of Al causes neurotoxicity that leads to memory loss, impaired coordination as well as cognitive disturbances[11]. Cognitive impairment resulted after chronic administration of aluminum chloride, was in the form of poor memory and spatial learning. Reduced locomotor activity was seen when the rats were administered with a sublethal dosage of Aluminium lactate for 25 days[14]. These rats experienced oxidative damage, muscular incoordination, cognitive impairment, locomotor and exploratory disability. The present study agrees with the above reports because $AlCl_3$ administered rats in our study have shown a significantly decreased number of total crossings in the open field test (Figure 1) suggesting locomotor inability. Whereas, PJ administered rats displayed improved behavioral changes that were previously impaired by aluminum chloride administration (Figure 1) Further, an improvement in avoidance response was also observed in PJ-treated animals (Figures 2, 3 and 4). This is in agreement with the previous studies wherein PJ has improved behavioral patterns in an AD model of mouse[12]. It is a normal behavior of the rats to enter into any dark corner or chamber whenever they are allowed to explore. The decreased latency to enter the dark chamber where foot shock is given in the passive avoidance test indicates poor memory of rats. In our study, $AlCl_3$ administered rats showed decreased latency to enter the dark chamber and this supports Al-induced cognitive impairment. The transgenic mice model of AD has shown apositive effect of PJ on cognitive and behavioral deficits[15]. In another study on mice the neuroprotective effect of PJ against Al-induced neurotoxicity is reported in the form of enhanced memory after ingestion of PJ[16]. Roskams and Connor opine that Al crosses Blood-brain Barrier (BBB) through certain receptors and induces neurodegeneration[17]. Brain inflammation induced by Al can cause loss of memory. Morphological indication of Al-induced neurodegeneration was demonstrated after 12 weeks of $AlCl_3$ administration, in the form of clumpy neuronal and glial cells[18].In the present study extent of neuronal damage was evident in the form of swollen cell, disrupted cell membrane, and pyknotic nuclei (Figure 5).

$AlCl_3$ induced pathology is thought to spread progressively to DG, CA3, CA1, CA2 and CA4 regions of the hippocampus[19]. We agree with the above reports because we also observed significantly decreased neuronal count in all the above-mentioned regions of the hippocampus in $AlCl_3$ administered rats when compared to that of control rats (Table 1). In dementia, the hippocampus and frontal cortex are the regions of the brain mainly involved and a correlation between cognitive impairment and cholinergic pathway has been reported in AD patients. Neuronal loss observed in the present study after administration of $AlCl_3$ agrees with the above description (Figure 5).

PJ has augmented memory in older and middle aged adults, as reported in the literature[20]. Pre-administration of Pomegranate offered a neuroprotective benefit against brain injury that is caused by cerebral ischemia[21]. It decreased DNA damage through its antioxidant and anti-apoptotic properties. A fairly similar result was observed in the present study wherein the number of viable neurons are significantly ($p<0.001$)increased in the DG and CA1 to CA4 regions of the hippocampus of animals treated with PJ when compared with that of $AlCl_3$ administered rats(Table 1 and Figure 5). On the contrary, in the Lewis rat model of Parkinson's disease, PJ did not show any neuroprotective role, instead, it exacerbated the rotenone-induced nigrostriatal degeneration[22]. Therefore, the present study leaves avenues for future research to substantiate the neuroprotective and nutraceutical potential of PJ in neurodegenerative diseases in larger samples.

5. Conclusion

In the present study, the neurodegeneration and cognitive impairment induced by $AlCl_3$ is ameliorated by treatment with PJ. This provides a promising neuroprotective potential of pomegranate juice on Al-induced neurotoxicity and an

added proof for nutraceutical value of Pomegranate to take it as supplementary diet though further studies on large samples are needed to firmly ascertain this neuroprotective effect.

6. Conflict of Interest

The authors declare that they have no conflict of interest.

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