ORIGINAL ARTICLE

To Study the Histomorphological Changes in Cerebellar Purkinje Cells after Exposure to Fine Particulate Matter in C57BL/6J Mice

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ABSTRACT

Objective: To study the histomorphological changes in cerebellar Purkinje cells after exposure to fine particulate matter in C57BL/6J mice.

Study Design: Laboratory based experimental study.

Place and Duration of Study: The study was conducted in the Anatomy department of the Army Medical College, Rawalpindi, from 15 June to 15 September 2020, in coordination with the Military Hospital, Rawalpindi, and the National Institute of Health (NIH), Islamabad.

Materials and Methods: Thirty male and female C57BL/6 mice, 8 weeks of age, weighing 37± 2 gm were obtained from NIH, Islamabad. The animals were divided in two groups, 15 mice in each group (8 male and 7 female) Group A were marked as control, received regular diet and water ad libitum. Group B (experimental group) received dynamic inhalation of 3mg/m³ fine particles (soot) through air circulation for 6h/d for 12 weeks, in plastic cabin measuring 2x2x2 feet fitted with two small fans for evenly distribution of Particulate Matter. After exposure period, the animals were sacrificed. After sectioning the tissue and staining, the microscopic analysis was carried out. Purkinje cell margins were evaluated. Number of Purkinje cells and changes in Purkinje cell size were noted. Data was collected, analyzed with the statistical package for social sciences version 23. A p value ≤ 0.05 was considered significant.

Result: The Purkinje cell margins were observed to become irregular and corrugated in the experimental groups B when compared with control group A. The number and size of Purkinje cells also showed difference when compared to the control group A.

Conclusion: The present study concluded that fine particulate matter induces changes in histomorphological features of mice cerebellar tissue including Purkinje cells.

Key Words: *Air Pollution, Cerebellum, Fine Particulate Matter, Purkinje Cells.*

Introduction

Air pollution is a major public concern that has adverse health and economic effects. Fine particulate matter containing many chemical components within these gradients, which can differ dynamically with time and space and with an aerodynamic diameter of < 10µm, is often associated with air pollution in large cities. The presence of particulate matter is responsible for more damage to human health as compared to ground level ozone.[3,4] Aerodynamic diameter is usually known as particulate matter (PM), ranging from coarse (between 2.5 and 10µm PM10) to fine (<2.5µm PM2.5) to ultrafine (<100nm or 0.1µm; UFP). Ambient UFPs, which result mainly from ignition processes, include the burning of fossil fuels, has primary source in the form of automobile emissions.[5,6] In several parts of the world, atmospheric air quality remains a most important concern, despite attempts to regulate atmospheric pollutant pollution. Total percentage of world's population exposed to pollutant concentrations above the recommended level of the World Health Organization (WHO) is more than eighty percent and from household related sources it accounts for about 4 million and about 3.6 million deaths can be due to environmental air pollution. In addition, air pollution can alter habitats, destroy constructions and

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memorials, as well as affect the energy balance of the planet and thus causes change in climate.[7] PM can be released directly into the atmosphere, called primary PM or it can be produced from gaseous precursors known as secondary PM. The scale of PM ranges from groups of molecules with a diameter of a few nanometers up to abrasion products of a micrometer. In the varying composition and characteristics of PM measured at source and receptor sites, this large dimensional range is expressed. Particulate Matter species are important atmospheric components and play a role in the climate system of the Earth. Some components of PM species absorb visible light and make the atmosphere warm i.e. black carbon, while other species, i.e. organic substances and sulphates reflect sunlight back into space and cool the environment.[8]

Air pollution is a multidimensional environmental toxin which through various pathways can attack the CNS. Inflammation and oxidative stress have been recognized as the common and essential mechanisms by which air pollution causes harm, including adverse effects in the CNS. It is known that black carbon can cause inflammation or that exposure to particulate matter in mice induces the development of pro-inflammatory cytokines (IL1-b, TNFa, and IFNg) in olfactory bulbs.[9,10] When neuroinflammation has started, it causes neuron damage and loss, stimulates microglia, leading to cytokine and ROS production, and causes damage and dysfunction of the blood brain barrier. Both of these events contribute to lipid peroxidation, astrogliosis, and damage to DNA and CNS diseases.[11] Epidemiological studies have linked air pollution with cognitive impairments reduced mental development index and IQ scores, attention-related disorders, anxiety/depression , nonverbal reasoning deficits, autism spectrum disorder (ASD) and delayed psychomotor development. Programmed cell death, or apoptosis, is an evolutionarily conserved process triggered by a variety of stimuli.[12] Exposure to PM has been associated with neuronal shrinkage, micro abscesses, and cerebellar edema.[1]

Studies have focused the histological effect of fine particulate matter on multiple organs for example lungs, heart, nasal mucosa, maternal exposure, and some components of brain. Despite considerable literature there is still a need for studies, which should investigate the histomorphological changes in brain because of rapidly increasing rate of dispersion of air pollutants, especially in the developing country like Pakistan. So, a research was planned to study the histomorphological changes in cerebellar Purkinje cells after exposure to fine particulate matter in C57BL/6J mice.

Materials and Methods

This study was conducted in Rawalpindi, Department of Anatomy, Army Medical College, in collaboration with the National Institute of Health (NIH), Islamabad, from June 2020 to September 2020, after getting approval by the Army Medical College, Rawalpindi, and the National University of Medical Sciences, Islamabad, Ethical Review Committee (ERC/ ID/ 10). Laboratory based experimental study was carried out and non-probability convenience sampling technique was used. Thirty male and female C57BL/6 mice, 8 weeks of age, weighing 37± 2 gram were procured from NIH, Islamabad. They were kept in separate cages in animal house of NIH under standard laboratory conditions with temperature 22 ± 2° C and 12-hour light/dark cycle. The animals were fed on standard laboratory rat chow and water *ad libitum.* Mice with any obvious injury and disease were excluded. The animals were divided in two groups, 15 mice in each group (8 male and 7 female).

Group A served as control. They received standard diet and water *ad Libitum.*

Group B (experimental group). Mice in experimental group B received dynamic inhalation of 3mg/m³ fine particles in the form of carbon soot through air circulation for 6h/d for 12 weeks.[13,14] 15 mice (8 males and 7 females) in two separate cages were placed in a plastic cabin measuring 2x2x2 feet fitted with two small fans opposite each other in NIH Islamabad. Air circulation was maintained by fan running at moderate speed. The particulate matter was evenly dispersed in the cabin through fan.[15, 16] The dose of particulate matter was calculated in mg/number of breaths in one minute. The dose was weighed by using digital precision balance. Fine particulate matter was purchased from Amazon.com. The particle name was Carbon powder with normal sensitivity and refractive index of 2.4. The size of the particle ranged from 0.020 to 2000.000µm with specific surface area of 0.555m^2/g. At the end of 03 months, after 18hrs of last exposure the animals

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were euthanized by placing the animals in the jar with cotton soaked in ether.[17] The animals were positioned on a dissecting board and decapitated by cutting the neck close to the head with the help of a sharp blade. Skin of the skull was removed by giving a longitudinal incision in the midline of the head. With the help of a bone cutter the cranium was opened along the sagittal suture and the bones were cracked open by using a forceps in the center. Brain was exposed and carefully lifted at its anterior end. Optic nerves were identified, and brain was separated from them and removed from the cranium by detaching from the spinal cord. The brain was placed on the dissection board and the cerebellum was identified. The cerebellum was separated from the cerebrum by locating and cutting through the layer of duramater (tentorium cerebelli) between the two. The middle cerebellar peduncle was identified, and the cerebellum was separated from the brain stem by cutting through this peduncle.

After washing the specimens with normal saline, the entire cerebellum was put into 10% formalin enough to cover all the tissue (about 3times the volume of tissue). From each cerebellum, splitting it into upper and lower halves, three transverse sections were obtained. All parts have been stored in tissue tek cassettes and processed in the automated tissue processor LEICA TP 1020. Sections were processed and cleared in xylene through the rising concentration of alcohol from 70 percent to 100 percent. For penetration and embedding, paraffin wax was used with a melting point of 58oC. LEICA EG 1160 Paraffin Embedding Center was used for this purpose. The blocks were allowed on a cold plate to solidify.[18] The rotary microtome (Leica rm 255) was used to cut 5µm thick cross sections. At 45˚C, pieces were floated in a hot water bath and then placed on glass slides. For 30 minutes, the slides were held in a slanting position to remove excess water and sections were dried at 65°C in a hot air oven for 60 minutes. Identification details were inscribed using the diamond tip pencil at the extreme corner of the glass slides. With the Leica auto-Stainer X, the sections were stained. Hematoxylin and Eosin (H&E) stains have been used to study cerebellum histology. Purkinje cell margins were observed in complete Purkinje cell layer in one slide per specimen at 40X magnification. The cell margins were recorded as regular (having normal pyriform somata with pale nuclei and prominent nucleoli) or irregular (having shrunken Purkinje cell bodies with irregular outline, deeply stained cytoplasm and hardly identified nuclei).[19] Number of cells was counted at high power field in complete Purkinje cell layer in one slide per specimen.[20] Size of Purkinje cells was measured by taking ten consecutive high-power fields from left to right and three cells per field were considered. Both length and width of Purkinje cell was calculated with the help of micrometry and mean was taken. It was compared with the control.[21] Micrometry is a technique to measure microscopic organisms and parameters using calibrated scale i.e., ocular micrometer and stage micrometer. The linear unit of measurement in micrometry is micron. One micron is equal to 1/1000mm. With the help of the stage micrometer, the eyepiece scale was calibrated, and the former was then used for measurements. By comparing the ocular micrometer scale with a calibrated stage micrometer, the eyepiece micrometer was calibrated. Once calibrated the ocular micrometer was used for measurement. 0.25 micrometer calibration factor was obtained for 40X and was valid for the optical combination. The stage micrometer was removed and on the microscope stage, slides of the cerebellum were placed. For the Purkinje cell size, the number of eyepiece divisions was counted and multiplied by 0.25. Using the eyepiece of the Olympus DP22 light microscope, the Sony digital camera (16 megapixels) was used. The zoom of camera was 3X. The images were corrected and modified by Photo Scape software. The magnification was calculated according to the formula below:

Magnification Power of eye piece (10) x Power of objective (10&40) x Camera zoom (3x)

Statistical package for data analysis SPSS version 23 was used to analyze data. Intergroup comparison for quantitative variables was done by independent sample t-test. A p value ≤ 0.05 was considered significant. Results were represented as mean ± standard deviation (mean ± SD). Qualitative variables were presented by frequency and percentage. Chi-square test was applied for comparison of qualitative variables

Results

Between the periods of 15 June 2020 to 15 September 2020, 15 out of 30 mice with average weight of 37±2 gram were given dynamic inhalation of 3mg/m³ fine particles through air circulation for 6h/d for 12 weeks. The mice were placed in a plastic cabin measuring 2x2x2 feet fitted with two small fans opposite each other in NIH Islamabad. Air circulation was maintained by fans running at moderate speed. The particulate matter was evenly dispersed in the cabin through fans. Till the end of the experiment, all the animals remained alive. The purkinje cells in control group A showed regular margins i.e., irregularity ratio was 0%.Whereas in experimental group B 73% of purkinje cells had irregular and distorted margins and 27% had regular margins (Fig l). The intergroup comparison between A and B was done by Fisher's exact test which showed significant results with p-value 0.000 (Table I).

Mean ± SD number of Purkinje cell number in control group A was 392 ± 62 whereas in experimental group B the mean ± SD of Purkinje cell number was 328 ± 67 (Fig. ll). The intergroup comparison between group A and B was done by independent sample t-test which yielded significant result with p-value 0.012 (Table ll). Mean ± SD size of Purkinje cells in control group A was 6.0 ± 0.4 µm whereas in experimental group B the mean ± SD size of Purkinje cells was 3.2 ± 0.36 µm (fig III). The intergroup comparison between group A and B was done by independent sample t-test which showed significant result with p-value 0.000 (Table ll).

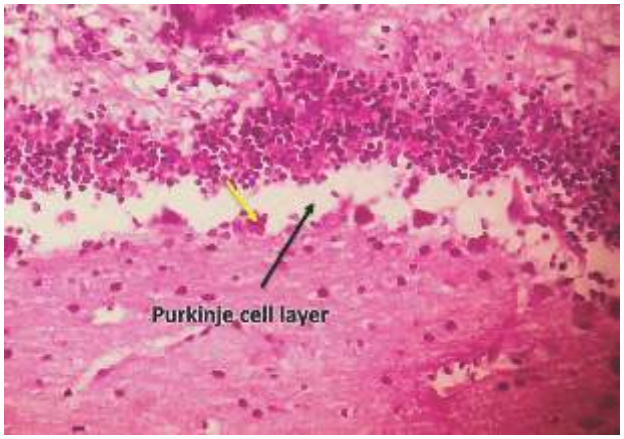

Fig. 1: Photomicrograph of Histological Section of Cerebellum in B2F Showing Purkinje Cell Layer (black arrowhead). Purkinje Cell Margin is Shown by Yellow Arrowhead. H&E 1200X

Table l Frequency of Purkinje Cell Margin Among the Control Group A and Experimental Groups B.

Parameter	Finding	Group A n=15	Group B n=15	P-value A v B
Margins of Purkinje cell	Regular	100%	4(27%)	0.000**
	Irregular	0%	11(73%)	

p-value ≤0.05Significant*
p-value <0.001Highly significant**

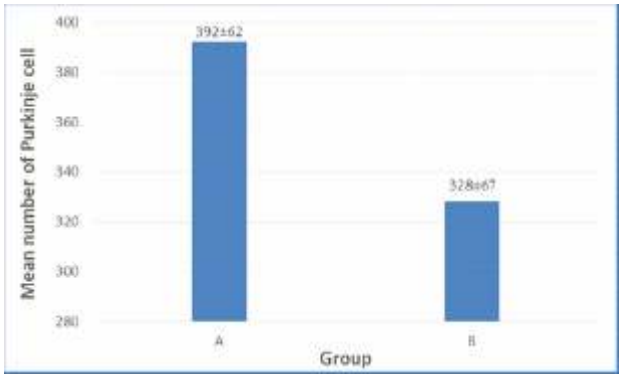

Fig. 2 : Bar Chart Showing Intergroup Comparison of Mean Number of Purkinje Cells among Control Group A and Experimental Group B (P- Value 0.012)

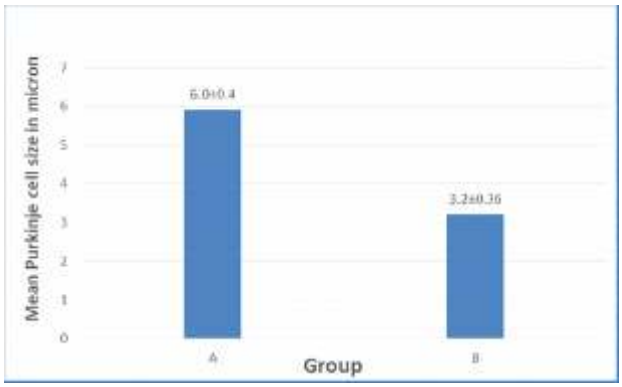

Fig. 3 : Bar Chart Showing Intergroup Comparison of Mean Size of Purkinje Cells Among Control Group A And Experimental Groups B (P-Value 0.000)

Table ll: Mean Cell Count and Cell Size of the Control Group A and Experimental Groups B.

Parameter	Group A n=15	Group B n=15	P- value
Number of Purkinje cell	392 ± 62	328 ± 67	0.012*
Size of Purkinje cell	6.0 ± 0.4µm	3.2 ± 0.36µm	0.000**

P value < 0.05 significant*
P value < 0.01 highly significant**

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Discussion

The aim of this study was to assess the effects of fine particulate matter exposure on the histomorphology of mice cerebellar Purkinje cells for which thirty C57BL/6 mice of 8 weeks of age were selected. All animals remained alive during the experimental period. Regarding the histomorphological examination the Purkinje cell margins were found to be smooth and regular in control group A, whereas in experimental groups B they were found to be highly irregular and corrugated, shrunken with deeply stained cytoplasm ($p<0.05$). These results were similar to a study conducted by El-Dien[19-22] in which the purkinje cell margins were irregular after exposure to fluoride which has same mechanism of action as particulate matter i.e. oxidative stress leading to neurodegenerative diseases. There was decreased number of Purkinje cells in experimental group B as compared to control group A with P-value 0.012. The decrease in number of Purkinje cells is most probably due to degenerative changes and stress imposed by exposure to FPM and also due to process of apoptosis stimulated by exposure to fine particulate matter as documented by Xiaozheng Zhu[23]. Also, there is an assumption that due to presence of spongiosis the Purkinje cells might have displaced from their normal place creating empty spaces in Purkinje cell layer. These results are in correlation with a study conducted Wallauer[20] in which there is decreased number of Purkinje cells after exposure to tobacco smoke. In another study done by Kivrak[21-24] in which there was reduced number of Purkinje cells after exposure to radiation which has similar mode of action as particulate matter. The size of Purkinje cells was decreased in experimental group B as compared to control group A with P-value 0.000. This is most probably due to degenerative changes in cells and stress imposed by exposure to particulate matter and due to development of spongiosis which compresses the cells. The results are similar to a study in which there is decrease in size of cells after exposure to radiation[21] with P-value <0.01 and also to a study conducted to find out quantitative analysis of purkinje cells in nude mice[25].

Conclusion

The present study concluded that fine particulate matter in the form of carbon soot induces changes in histomorphological features of mice cerebellar tissue. The major effects of exposure included both neurodegenerative and neuroinflammatory changes along with decreased size and number of Purkinje cells, which were presented by irregular and distorted margins.

Ethics statement

The rules and regulations regarding the handling and care of animal set forth by the ethics committee of the Army Medical College / National University of Medical Sciences were followed for the research (Dated: 2nd Feb 2020, NO: ERC/ID/10).

Author contribution

SH designed the project, SM & SMK helped in experimental procedure, ABJ analyzed the data. All authors approved the final version of manuscript.

Conflict of interest

Author has no conflict of interest to declare

Disclosure

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CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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Authors have declared no specific grant for this research from any funding agency in public, commercial or nonprofit sector.

DATA SHARING STATMENT

The data that support the findings of this study are available from the corresponding author upon request.