

Volume: 78
Issue No. 3
Year: 2026



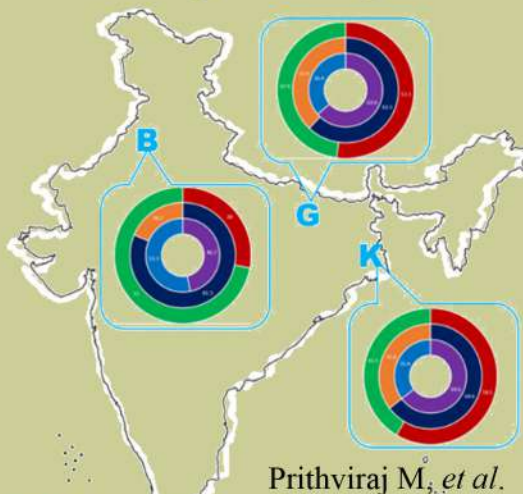
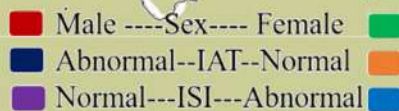
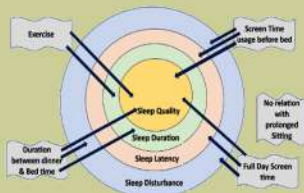
Indian Journal of Physiology and Allied Sciences

An official peer-reviewed journal of

The Physiological Society of India

IJPAS

Vol. 78; No. 3; Year 2026

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Dose- and time-dependent polystyrene microplastic exposure alters brain metabolic markers and behavior in male Wistar rats

Sumana Mondal, Susmita Chakraborty, Jyotirmoy Saha, Sudipta Pal* 

ABSTRACT

Background: As plastic production increased, researchers have continued to focus on the health effects of microplastics (MPs). Energy depletion and bioenergetics alterations caused by MPs could negatively affect cognition and lead to neurodegenerative diseases. The current study aims to assess metabolic parameters in the cerebral cortex and cerebellum across various doses and time points spanning sub-acute to sub-chronic periods. **Materials and Methods:** Rat brain metabolism was evaluated after oral exposure to polystyrene microplastics (PS-MPs) at three doses (0.5, 5, and 50 mg/L) for 28, 56, and 84 days. **Results:** Blood glucose was significantly decreased in the treated groups at all time points. Enhanced lactate dehydrogenase (LDH) activity in the cerebral cortex with shorter durations was reversed with longer exposure. Pyruvate depletion was observed in the PS-MPs-treated cerebral cortex, whereas enhanced pyruvate levels were found in the cerebellum. Decreased cerebral free amino nitrogen (FAN) was observed at the lowest dose after 28 days, whereas FAN increased in other doses and durations. The cerebral GOT activity was enhanced at the middle and high doses after 28 days, but decreased after longer exposures. However, cerebral GPT activity was stimulated after 8 and 12 weeks of treatment. The FAN content was elevated among different doses and durations in the cerebellum. Cerebellar transaminase functions were reduced after PS-MPs treatment. **Discussion:** The study indicates that PS-MPs induce hypoglycemia. In the cerebral cortex, reduced glycolysis was associated with increased glycolysis in the cerebellum. Additionally, behavioral studies indicate that rats treated with PS-MPs exhibited greater curiosity and exploratory behavior at 56 and 84 days after exposure to middle and high doses. The study suggests that the effects of PS-MPs on brain metabolic markers and resulting behavioral changes depend on treatment duration and dose, acting in a dose-dependent manner and leading to differential alterations in cerebral and cerebellar metabolism.

Keywords: Polystyrene microplastics; Cerebral cortex; Cerebellum; Lactate Dehydrogenase; GOT; GPT; Free Amino Nitrogen; Open Field Test; Light-Dark Box Test.

Indian Journal of Physiology and Allied Sciences (2026);

DOI: 10.55184/ijpas.v78i03.609

ISSN: 0367-8350 (Print)

INTRODUCTION

Plastic products are used extensively in daily life worldwide. By 2025, it was anticipated that 11 billion tons of plastic would be dispersed globally.¹ Microplastics (MPs) are well-defined as plastic elements having a diameter less than 5 mm, and are considered as evolving environmental contaminants. Because of their small size and widespread production, MPs have been found in a variety of environmental media, including soil, water, and air, leading to unavoidable human exposure through ingestion, inhalation, and skin contact.²⁻⁹ It increases the health risk as MPs circulate and aggregate in various organs via the circulatory system.¹⁰⁻¹² Numerous *in vivo* investigations in mice have demonstrated that PS-MPs can accumulate and distribute in the testes, livers, kidneys, and gastrointestinal tracts.¹³⁻¹⁵ There is growing evidence that MPs have neurotoxic effects as they build up in the brain.¹⁶⁻¹⁸

One of the most prevalent types of MPs in the environment is polystyrene (PS) microplastic.¹⁹⁻²⁰ PS-MP is distributed in human blood samples and the placenta, raising questions about its possible effects on organ and embryonic development. It has been revealed that exposure to nanomaterials and micro particles in fish brains can cross the blood-brain barrier.^{23,24} It is evident that PS-MPs also suppress acetylcholinesterase activity in the fish brain.²⁵ Moreover, in male mice, MPs treatment can reduce the number of synaptic proteins and also disrupt hippocampal-dependent learning

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How to cite this article: Mondal S, Chakraborty S, Saha J, Pal S. Dose- and time-dependent polystyrene microplastic exposure alters brain metabolic markers and behavior in male Wistar rats. *Indian J Physiol Allied Sci* 2026;78(3):22-34.

Conflict of interest: None

Submitted: 08/08/2026 **Accepted:** 27/08/2026 **Published:** 30/09/2026

and memory.²⁶ On the other hand, MP causes neurotoxicity in cortical spheroids and reduces cell viability in pluripotent stem cell-derived forebrain cortical spheroids.²⁷ According to current research on zebrafish, PS-MPs induced depression like behaviour by reducing exploration, locomotor activity, and rehabilitating social interactions.²⁸ In addition, PS-MPs caused circadian rhythm disruption, resulting in altered expression of important clock genes (*per1b*, *per2*, *per3*) and cryptochrome genes (*cry1a*, *cry2*), along with elevated pro-inflammatory cytokines (IL-6, IL-1 β).²⁹ It was further stated that the exposure to PS-MPs reduced the count of Nissl bodies in the prefrontal cortex.³⁰ While most of the evidence supports

neurological dysfunctions, neurodegenerative disorders, and region-specific neuronal changes by microplastic exposure, scant information is available so far regarding the metabolic effect of that pollutant on different brain regions at differential doses and durations. The research question thus arises how polystyrene microplastics affect differential brain metabolic biomarkers, especially in relation to energy metabolism. Unlike earlier studies that use molecular and genetic markers to establish polystyrene-mediated neuronal changes, the present study primarily targets energy metabolic pathways to evaluate how different brain regions, such as the cerebral cortex and cerebellum, respond to polystyrene microplastics by altering their metabolic profiles. Energy depletion and altered bioenergetics induced by microplastics are implicated in cognitive dysfunction and the pathogenesis of neurodegenerative disorders. This study bridges ecotoxicology and human health by identifying metabolic alterations in certain mammalian species that inform how persistently this synthetic polymer bioaccumulates and alters human metabolic homeostasis. The present study aims to assess metabolic parameters in the cerebral cortex and cerebellum across varying doses over subacute to subchronic durations.

MPs are available in various forms, such as fibers, fragments, beads, films, and foams. Primary MPs are manufactured at specific sizes to which humans may be exposed through ingestion, inhalation, and dermal contact. MPs are ingested mainly through drinking bottled water, consuming packaged foods, using polystyrene plastic utensils, eating seafood, and consuming table salt; hence, this is the most widely studied route. In the present study, polystyrene microplastics are administered to rats by gavage, the most common route of microplastic administration in humans.

MATERIALS AND METHODS

Materials

Polystyrene microplastics (1µm diameter) were obtained from Sigma-Aldrich Co., USA, Source BCCJ0518 (Cat no.89904-5ML-F). Other chemicals were procured from SRL (India). Glucose assessment kits were obtained from Transasia Bio-Medicals Ltd, Mumbai, India. Millipore ultrapure water was used to make all mixtures to avoid metal pollution.

Animal Maintenance

Male Wistar rats were purchased from Chakraborty Enterprise, Kolkata, India, a CCSEA-registered breeder. The rats were maintained with an adequate protein diet and drinking water *ad libitum*. All animals were housed in polypropylene cages in a well-ventilated room at 25°C under a 12-hour light/12-hour dark cycle.

Treatment schedule

In this time- and dose-dependent investigation, 72 Wistar albino rats were divided into four groups for 28-, 56-, and 84-day treatment schedules; each group consisted of 24

animals. The sample size is calculated considering the effect size (d, usually the difference between two groups), the population standard deviation (σ), the desired power of the experiment to detect the postulated effect (80% power = 0.84), the significance level (Zα 0.05, 1.96), and the ratio of sample size (r). A pilot study has already been conducted to obtain the r(1), d(148), and σ(128) values for a defined set of experiments and to determine the sample size for two means. The total sample size for two means was approximately 11.73 or 12. Considering four groups or four means (n = 6/group), the sample size for each set of experiments will be 24 (4x6). For each time period, four groups of rats were included: one control (n = 6), and three polystyrene-exposed groups: treated 1 (n = 6), treated 2 (n = 6), and treated 3 (n = 6) groups with equal average body weight (130-135 g).

For the duration of the study, PS-MP was administered orally at doses of 0.5 mg/L (low dose; T1), 5 mg/L (medium dose; T2), and 50 mg/L (high dose; T3), which were selected from the literature.^{30,31} The stock microplastic solution (10%) was mixed with a definite volume of Millipore-purified water to make a 50 mg/L solution. It was mixed thoroughly by hand first, then sonicated for ten minutes (frequency 40kHz) before administration. This solution was diluted 10 times to obtain a concentration of 5 mg/L, and the same mixing procedure was followed. The low dose was prepared by diluting a 5 mg/L solution 10-fold and mixing it with a sonicator before use. Finally, 0.5 ml of each of these solutions was administered orally using the curved feeding needle at 100 gm body weight per rat per day for three different time periods: 28 days, 56 days, and 84 days.

Ethical Clearance

Experiments were conducted after getting ethical clearance from the Institutional Animal Ethics Committee (IAEC) [Approval No. TU/IAEC/2023/I/2-1 dated 11.12.2023].

Animal Sacrifice and Tissue Collection

All the animals were sacrificed after a specified time interval, in accordance with the ethical guidelines of the CCSEA (Committee for Control and Supervision of Experimental Animals). The whole brain was carefully collected, washed in cold saline, dried, and dissected to isolate the cerebral cortex and cerebellum. Isolated brain regions were preserved at -20°C for biochemical studies.

Body Weight and Organo-somatic Index

Body weight was measured routinely throughout the treatment period until sacrifice. Furthermore, the organ weight (whole brain) was documented after sacrifice. The brain-somatic index was determined using the following formula, as described elsewhere.³²

$$\text{Organosomatic index} = \frac{\text{weight (g) of organ}}{\text{final body weight at the time of sacrifice (g)}} \times 100$$

Food intake pattern

Food intake for each animal was measured daily during the

treatment period by recording the amount of food left and the amount given.

Biochemical Parameters

Blood glucose

The blood glucose level in the tissue under study was determined using the GOD-POD kit.³³ Before being sacrificed, the animals were fasted for the entire night. Blood was drawn from the hepatic vein, and the Erba glucose kit was used to measure blood glucose using the glucose oxidase–peroxidase method (Transasia Biomedicals Ltd., Mumbai, India). The color intensity of quinoneimine derivatives is directly proportional to the glucose concentration in the solution.

Pyruvate content

Tissue pyruvate content was estimated by the method of Segal *et al.*³⁴ To enable protein precipitation, 4 ml of 5% TCA was added to 1 ml of 2% tissue homogenate (in 0.1 M phosphate buffer, pH 7.4). For ten minutes, it was centrifuged at 3000 RPM. A test tube with twice the volume of distilled water and an equivalent volume of 0.1% 2,4-DNPH was filled with the supernatant (1 mL). After properly mixing it and letting it sit in the dark for 20 minutes, 5 mL of toluene was added, and the mixture was shaken by hand for 3 minutes. After a period of waiting, the lower layer that had separated was removed from the mixture and placed in another test tube containing 4 mL of 10% Na₂CO₃ solution. The mixture was then agitated for another 3 minutes. The sample was taken from the top layer after the liquid phase had separated. After mixing 3 mL of the sample with NaOH solution (1.5 mL), the mixture was left for 5 minutes. Using a photoelectric colorimeter with the blank set to “0” density at 420 nm, the absorbance of the color solution was measured.

Free amino nitrogen content

To remove the protein-free filtrate, the tissue homogenate was centrifuged for 10 minutes at 3000 rpm. According to Rosen, the filtrate was combined with ninhydrin and cyanide acetate buffer, then heated to 100°C.³⁵ As the color began to appear, isopropyl alcohol was added, and absorbance at 570 nm was measured after cooling. The value for free amino nitrogen concentration was leucine in g per 100 g of tissue. Leucine was measured in grams per 100 grams of tissue.

Lactate dehydrogenase (LDH) activity

LDH activity was assessed according to Bergmeyer's protocol.³⁶ To estimate this enzyme activity, a 5% tissue homogenate in 0.05 M phosphate buffer, pH 7.5, was made, and the supernatant was obtained by centrifuging it at 10,000 RPM at 25°C. In a 3 mL cuvette, 2.92 mL of phosphate pyruvate solution was added, followed by 0.05 mL of NADH solution and 0.03 mL of the sample (supernatant). At 340 nm, absorbance was measured every 30 seconds for 5 minutes. The enzyme activity was determined using the molar

extinction coefficient of NADH. The unit of measurement for the enzyme activity was $\mu\text{moles/min/mg}$ of protein.

Glutamate-pyruvate transaminase activity (GPT) and Glutamate-oxaloacetate transaminase activity (GOT)

Tissue transaminase levels were estimated using the Reitman and Frankel protocol (assay kit provided by Coral Clinical Systems, Goa, India).³⁷ In GOT, aspartate reacted with α -ketoglutarate to form pyruvate, whereas in GPT, the color was produced by the chemical reaction of α -ketoglutarate. Each tube was incubated at 37 °C for 30 minutes after adding the sample, and the tissue homogenates were then combined with their corresponding substrates. The optical density at 505 nm was determined after all procedures were finished. The activities were expressed as $\mu\text{g/min/100 mg}$ of tissue. The GOT activity was expressed as $\mu\text{g/min/100 mg}$ of tissue protein, and GPT activity was expressed as $\mu\text{g/min/100 mg}$ of tissue.

Tissue protein content

The protein content was measured by the Lowry *et al.* method using bovine serum albumin as a standard.³⁸ Protein content was expressed in grams per 100 grams of tissue.

Behavioural Studies

Behavioral studies were conducted just before the day of sacrifice at various time points.

Open field test

After 28, 56, and 84 days of oral exposure to the PS-MPs, the open field test was used to evaluate the locomotor functions and anxiety-like behaviors of the animals before euthanasia. Each rat was placed in an open box with elevated boundaries on four sides. The box measures 100 cm $\times$ 100 cm $\times$ 40 cm. The box was divided into sixteen small squares. The central four squares were designated the inner zone, and the remaining squares were designated the outer zone.³⁹ Rats were allowed to roam the field for 10 min. The time spent in each zone, the rearing, and the number of times they entered the inner zone were recorded by an overhead camera.

Light-Dark Box Test

After 28, 56, and 84 days of PS-MP exposure, the light-dark box test was performed on living animals. The box was made of black-and-white-painted plexiglass sheets. It contained two identical chambers connected by a central entrance, allowing the rats to move freely between them. The brightness of the light chamber was approximately 100 lux (15 lm/watt40), whereas the dark chamber was completely dark. An individual animal was placed in the light chamber, facing the dark chamber entrance, and allowed to move freely for 5 min. The duration of stay in each chamber was recorded using an overhead mobile camera.

Statistical Analyses

The ‘Kruskal-Wallis test’ was used to determine whether there was a statistically significant difference among

the experimental groups. Moreover, a 'Mann-Whitney U test' was performed to assess the significance of the difference between two specific group means using PAST (PAleontological STatistics) software. $P < 0.05$ was considered to be statistically significant.

RESULTS

Polystyrene microplastics caused a decrease in body weight of rats following sub-acute to sub-chronic exposure

Four weeks' exposure decreased the body weight of the exposed animals by 16.5% ($p = 0.005$), 28.97% ($p = 0.005$), and 38% ($p = 0.005$) in the T1, T2, and T3 groups, respectively (Table 1). Eight weeks' treatment caused a significant decline in body weight by 17.3% ($p = 0.005$), 28.9% ($p = 0.005$), and 37.5% ($p = 0.005$) in the low-dose, medium-dose, and high-dose treated animals. Similarly, body weight decline after twelve weeks' PS-MP exposure was also found in the treated rats; the proportion variations were calculated to be 12.6% ($p = 0.03$), 22.3% ($p = 0.02$), and 27.9% ($p = 0.005$) in the T1, T2, and T3 groups, respectively.

A decrease in daily average food consumption was noted in PS-MP-exposed animals in both time and dose-dependent ways

Animals exposed to polystyrene microplastics for 84 days exhibited significant changes in their food consumption patterns (Table 1). There was a clear decline in regular food intake in the treated rats after 28 days of exposure, followed by a gradual decline during the subsequent exposures at 56 and 84 days.

Absolute brain weight and brain somatic index (BSI) following varied doses and durations of polystyrene exposure

The absolute organ weight of the treated rats was decreased in group 2 (10.6%, $p = 0.005$) and group 3 (21.74%, $p = 0.01$)

after 28 days of exposure. On the contrary, after 56 days and 84 days of exposure, there was no change in the absolute brain weight of the treated rats (Table 1). Furthermore, the brain somatic index (BSI) gradually increased in the treated rats (Table 1). There was a 17% ($p = 0.005$), 25% ($p = 0.005$), and 29% ($p = 0.005$) increase in the BSI in the T1, T2, and T3 groups after 28 days' exposure. Following 56 days' treatment, the BSI was found to be elevated by 25.6% ($p = 0.005$), 38.5% ($p = 0.005$), and 52.6% ($p = 0.005$) in the T1, T2, and T3 groups, respectively. Similarly, 84 days' exposure to PS-MP also caused a significant increase in the BSI by 15.5% ($p = 0.005$) and 35.7% ($p = 0.005$) in the T1, T2, and T3 groups, respectively.

PS-MP reduced blood glucose in experimental rats both time and dose dependently

A substantial decrease in blood glucose levels was observed across all treated groups at various time points, except in the T1 group (dose 0.5 mg/L) after 28 days of exposure (Figure 1). After 56 days of exposure, the changes were recorded as 22.56% ($p = 0.005$), 31.41% ($p = 0.005$), and 48.85% ($p = 0.005$) in the T1, T2, and T3 groups, respectively. In the 84-day treatment, polystyrene reduced the blood glucose level by 22.5% ($p = 0.005$), 38% ($p = 0.005$), and 50.33% ($p = 0.005$) in the low-, medium-, and high-dose exposure groups, respectively.

Differential metabolic changes in cerebral cortex and cerebellum following varied doses and durations of PS-MP exposure

The LDH function in the cerebral cortex was increased in medium- and high-dose-exposed groups after 28 days' exposure. After 56 days of exposure, the LDH function was also stimulated by 50.64% ($p = 0.005$), 71.21% ($p = 0.005$) and 72.52% ($p = 0.004$) in low-, medium- and high-dose exposed groups. On the contrary, after 84 days of treatment, there was a significant decline in that enzyme function by 10.21% ($p = 0.02$) in treated group 3 (Figure 2A).

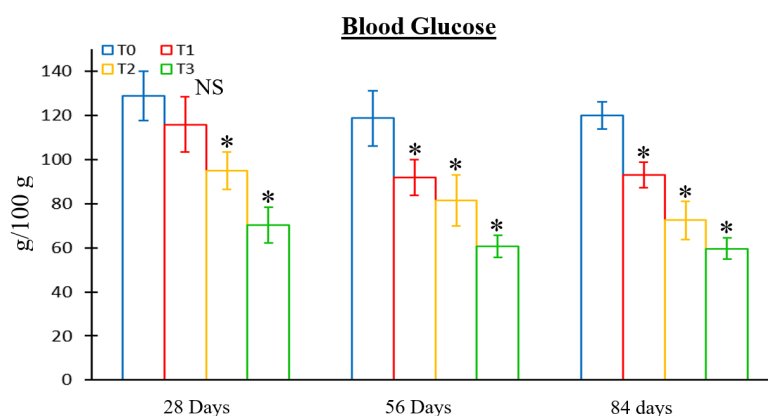


Figure 1: Effect of sub-acute, sub-chronic, and chronic exposures of graded doses of PS-MP on blood glucose level in rats. [T0 rats received only sterile water, T1 received 0.5 mg/L, T2 received 5 mg/L, and T3 received 50 mg/L of PS-MP; Values are Means $\pm$ SD. * $P < 0.05$ was considered statistically significant, NS = not significant]

Table 1: Time-dependent effect of respective doses of PS-MP exposure

Group of animals	28 Days			
	T0	T1	T2	T3
Brain somatic index	1.00 ± 0.06	1.17 ± 0.05*	1.25 ± 0.09*	1.29 ± 0.19*
Absolute brain weight (g)	1.61 ± 0.07	1.57 ± 0.11	1.44 ± 0.15*	1.26 ± 0.29*
Body weight (g)	161.67 ± 7.84	135.00 ± 11.35*	114.83 ± 9.72*	100.17 ± 12.81*
Average food consumption/day/rat (g)	24.37 ± 4.98	12.32 ± 3.90*	11.01 ± 3.90*	8.98 ± 1.48*
Group of animals	56 Days			
	T0	T1	T2	T3
Brain somatic index	0.78 ± 0.14	0.98 ± 0.02*	1.08 ± 0.07*	1.19 ± 0.09*
Absolute brain weight (g)	1.52 ± 0.12	1.60 ± 0.05	1.52 ± 0.19	1.46 ± 0.17
Body weight (g)	198.00 ± 20.44	163.83 ± 8.18*	140.83 ± 17.50*	123.67 ± 15.83*
Average food consumption/day/rat (g)	21.55 ± 3.90	16.08 ± 3.81*	13.41 ± 3.43*	12.81 ± 6.03*
Group of animals	84 Days			
	T0	T1	T2	T3
Brain somatic index	0.84 ± 0.10	0.97 ± 0.04*	1.04 ± 0.19	1.14 ± 0.13*
Absolute brain weight (g)	1.57 ± 0.03	1.60 ± 0.06	1.61 ± 0.06	1.53 ± 0.18
Body weight (g)	189.00 ± 20.93	165.17 ± 9.70*	146.83 ± 15.68*	136.17 ± 24.76*
Average food consumption/day/rat (g)	20.78 ± 3.45	16.95 ± 3.38*	14.45 ± 3.21*	13.20 ± 4.86*

Values are Means ± SD. * indicates $P < 0.05$ between the control and the respective treated group means.

The GOT function in the cerebral cortex remained almost unchanged at the lowest dose of polystyrene exposure, though a slight rise was found in group 2 (19.72%) and group 3 (19.59%) after 28 days' exposure, whereas after 56 days' treatment, a remarkable decline in the GOT function was observed as 40.53% ($p = 0.005$), 45.62% ($p = 0.005$), 50.64% ($p = 0.005$) in all the treated groups. Additionally, after 84 days of exposure, the GOT function was inhibited by 21.30% ($p = 0.02$), 17.62% ($p = 0.03$), and 37.27% ($p = 0.005$) at the low, middle, and high exposure doses, respectively (Figure 2B). On the other hand, the function of cerebral GPT declined in the T1 group, whereas it increased in the T2 and T3 groups after four weeks of exposure. After 56 days of exposure, the cerebral GPT activity was increased in the T2 and T3 groups by 12.24% ($p = 0.006$) and 30.38% ($p = 0.005$), respectively. After 84 days of exposure, the GPT function steadily deteriorated in the PS-MP-treated group; the percentage changes were 15.94% ($p = 0.03$) and 40.26% ($p = 0.006$) in the T2 and T3 groups (Figure 2C).

It was further revealed that the LDH function in the rat cerebellum was increased in all three durations of PS-MP treatment (Figure 3A). In the case of subacute exposure, the increase was 22.81% ($p = 0.01$) and 24.76% ($p = 0.01$) in medium-, and high-dose-exposed animals. On the other hand, the increase in LDH activity was noted in all the cerebellum tissue exposed to PS-MP for 56 days. In the 84-day treatment groups, the percentage changes were 54.64% ($p = 0.005$), 60.68% ($p = 0.005$), and 67.93% ($p = 0.005$) in T1, T2, and T3, respectively.

In the cerebellum PS-MP also caused a significant decline in the GOT function at different time periods; the percentage changes were 11.30% ($p = 0.005$), 17.43% ($p = 0.005$), and

20.39% ($p = 0.005$) in PS-MP T1, T2, and T3 groups after 28 days; the percentage decrease was recorded 12.68% ($p = 0.05$), 17.91% ($p = 0.005$), and 31.19% ($p = 0.005$) in the treated groups following 56 days of treatment. Moreover, PS-MP treatment for 84 days caused a significant decline in the GOT activity by 16.41% ($p = 0.005$), 29.32% ($p = 0.005$), and 36.18% ($p = 0.005$) in treated groups T1, T2, and T3, respectively (Figure 3B).

It was further observed that after four weeks of treatment, GPT enzyme activity in the rat cerebellum decreased by 10% ($p = 0.004$), 12% ($p = 0.004$), and 36.18% ($p = 0.004$) across all treated groups. After 56 days of exposure, the GPT function decreased by 19.20% ($p = 0.005$), 24.18% ($p = 0.005$), and 40.96% ($p = 0.005$) in treated groups of animals 1, 2 and 3, respectively. On the other hand, after 84 days of exposure, the enzyme activity declined by 26.99% ($p = 0.005$), 44.73% ($p = 0.005$), and 41.32% ($p = 0.005$) in treated groups T1, T2, and T3, respectively (Figure 3C).

Moreover, the pyruvic acid content decreased in PS-MP-exposed cerebral tissue after 28 days' treatment in a dose-responsive fashion (Figure 4A). With eight weeks of exposure, the parameter also declined in that brain region in a dose-responsive fashion. Similarly, after twelve weeks of treatment, PS-MP caused a significant decline in the cerebral cortex pyruvic acid level by 8.46% ($p = 0.01$), 13.56% ($p = 0.04$), and 24.10% ($p = 0.005$) in the treated groups T1, T2, and T3, respectively.

The cerebral free amino nitrogen declined in the lowest dose-exposed group, whereas a significant increase was detected in the highest dose after four weeks' treatment. On the other hand, there was a significant dose-dependent increase in the free amino nitrogen among the treated group

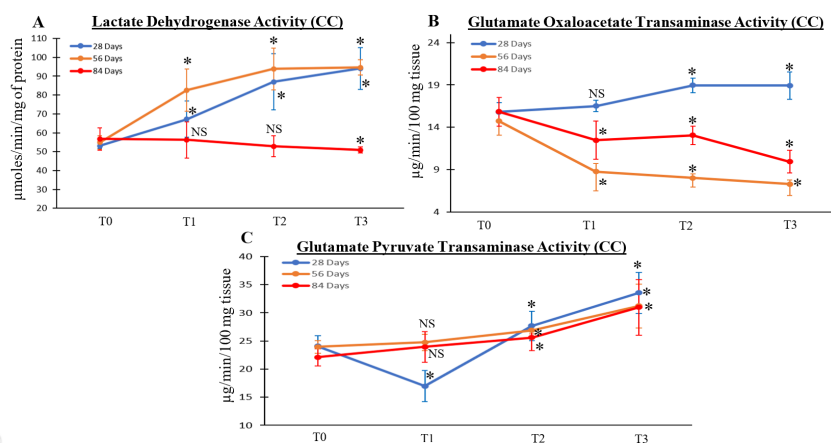


Figure 2: Time-dependent alteration in the cerebral cortex of polystyrene-treated rats. A. Lactate dehydrogenase activity of cerebral cortex, B. GOT activity of cerebral cortex, C. GPT activity of cerebral cortex. [T0 rats received only sterile water, T1 received 0.5 mg/L, T2 received 5 mg/L, and T3 received 50 mg/L of PS-MP; Values are Means $\pm$ SD. * $P < 0.05$ was considered statistically significant, NS = not significant]

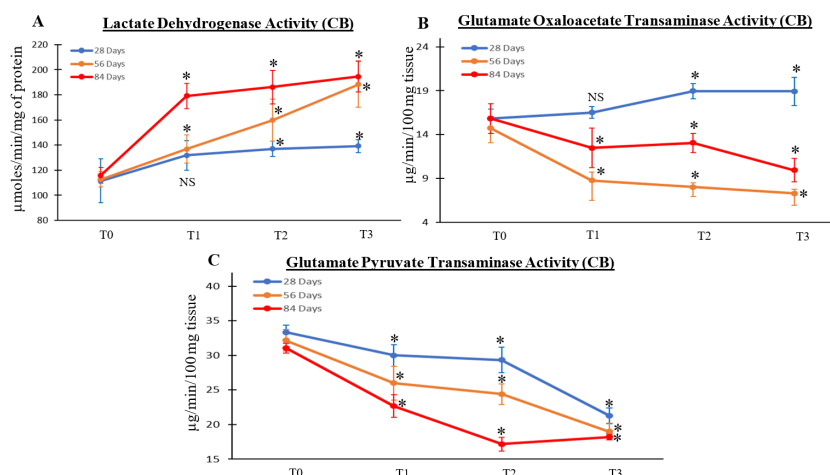


Figure 3: Time-dependent alteration in the cerebellum of polystyrene-treated rats. A. Lactate dehydrogenase activity of cerebellum, B. GOT activity of cerebellum, C. GPT activity of cerebellum. [T0 rats received only sterile water, T1 received 0.5 mg/L, T2 received 5 mg/L, and T3 received 50 mg/L of PS-MP; Values are Means $\pm$ SD. * $P < 0.05$ was considered statistically significant, ns = not significant]

of animals after 56 days' treatment. Similarly, after 12 weeks of exposure, a significant increase in free amino nitrogen was observed in the rat cerebral cortex compared with the untreated group (Figure 4B). Furthermore, it was observed that the total protein content of the cerebral cortex remained nearly unchanged after 4, 8, and 12 weeks of exposure at varying doses (Figure 4C).

Pyruvic acid content was increased in the low, medium, and high dose-exposed animals' cerebellum after 28 days of treatment. After eight weeks of exposure, cerebellar pyruvic acid increased in the treated groups T2 and T3; the percentage changes were 96.19% ($p = 0.005$) and 107.62% ($p = 0.005$), respectively, compared with the control. In case of twelve weeks' exposure, the cerebellar pyruvate content was elevated by 260.59% ($p = 0.005$), 270.21% ($p = 0.005$), and 375.29% ($p = 0.005$) in the treated groups, respectively (Figure 5A).

In the cerebellum, after 28 days of exposure, the free amino nitrogen levels were elevated by 50.00% ($p = 0.005$), 297.57% ($p = 0.005$), and 285.30% ($p = 0.005$) in the respective treated groups. After 56 days' PS-MPs exposure, there was a 11.58% ($p = 0.005$), 78.42% ($p = 0.004$), and 108.07% ($p = 0.005$) increase in the level of free amino nitrogen in the T1, T2, and T3 groups, respectively. Similarly, after 84 days, the free amino nitrogen in the cerebellum increased in all PS-MP-treated groups of rats (Figure 5B). Moreover, the total protein content in the cerebellum remained unaltered after subacute and subchronic exposures to polystyrene (Figure 5C).

PS-MP significantly altered the behavioral activities of the treated animals in a time-dependent fashion

In the light-dark box test, the treated animals of eight weeks and twelve weeks of exposure spent more time in the light chamber than in the dark (Table 2). After 28 days of exposure

Table 2: Time-dependent effect of graded doses of PS-MP exposure on Light-Dark Box Test

Test parameter	Light- Dark Box Test			
	28 DAYS			
	T0	T1	T2	T3
Dark (Sec)	333.67 ± 11.79	341.00 ± 3.29	342.83 ± 4.22	341.67 ± 10.33
Light (Sec)	26.33 ± 11.79	19.00 ± 3.29	17.17 ± 4.22	18.33 ± 10.33
Rearing	1.33 ± 0.82	1.50 ± 0.55	5.17 ± 1.17*	9.50 ± 1.38*
Transition	1.67 ± 0.52	1.50 ± 0.55	1.66 ± 0.52	1.50 ± 0.55
56 DAYS				
Dark (Sec)	337.17 ± 6.68	297.63 ± 43.19*	312.67 ± 5.39*	298.17 ± 25.15*
Light (Sec)	19.67 ± 3.14	40.83 ± 12.02*	47.33 ± 5.39*	61.50 ± 24.70*
Rearing	2.20 ± 1.17	1.16 ± 0.75	6.66 ± 2.25*	9.40 ± 3.73*
Transition	2.00 ± 0.89	3.33 ± 1.75	1.17 ± 0.41	1.83 ± 0.75
84 DAYS				
Dark (Sec)	349.33 ± 6.80	328.00 ± 19.86*	318.50 ± 10.31*	307.00 ± 25.06*
Light (Sec)	17.20 ± 1.83	32.20 ± 5.15*	44.00 ± 7.97*	56.00 ± 20.91*
Rearing	1.80 ± 1.17	1.00 ± 0.63	5.20 ± 2.93*	4.83 ± 1.72*
Transition	1.60 ± 0.49	3.20 ± 1.33*	3.00 ± 0.89*	5.17 ± 1.47*

Values are Means ± SD. * indicates $P < 0.05$ between the control and the respective treated group means.

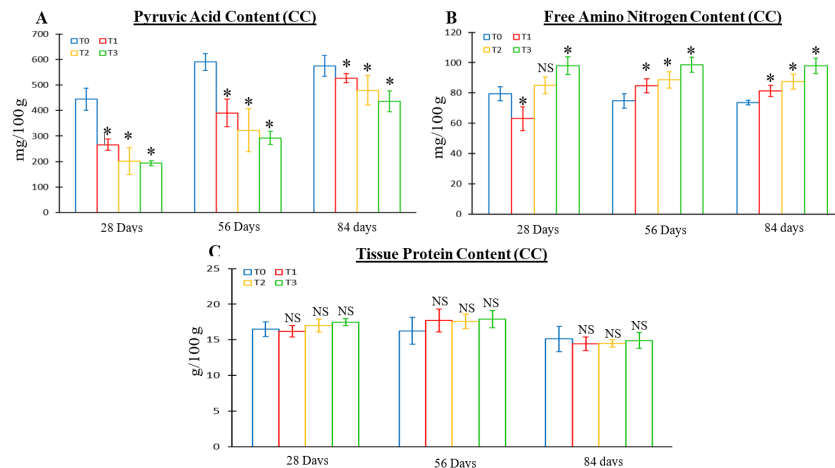


Figure 4: Time-dependent effect of PS-MP of cerebral cortex following graded doses of exposure. A. Pyruvic acid content of cerebral cortex, B. FAN content of cerebral cortex, C. Tissue protein content of cerebral cortex. [T0 rats received only sterile water, T1 received 0.5 mg/L, T2 received 5 mg/L, and T3 received 50 mg/L of PS-MP; Values are Means ± SD. * $P < 0.05$ was considered statistically significant, NS = not significant]

to varying doses of PS-MP, there was no significant difference in exposure time between the light and dark chambers and the control group. On the contrary, after 56 days of exposure, the time spent in the dark chamber gradually decreased compared to the control group. The treated rats preferred to stay in the light chamber longer than in the dark. The percentage change in time reduction in the dark chamber was 11.8% ($p = 0.01$), 7.3% ($p = 0.005$), and 11.6% ($p = 0.006$) in the PS-MP-treated groups T1, T2, and T3, respectively, compared with the control. Similarly, after 84 days of

exposure, the animals in the treated groups spent more time in the light chamber than the control rats. The increase in time spent was calculated to be 87% ($p = 0.005$), 156% ($p = 0.005$), and 225% ($p = 0.005$) in the treated groups T1, T2, and T3, respectively, as compared with the control.

It was further noted that the rearing frequency increased only in the treated 2 and treated 3 groups after 28, 56, and 84 days of PS-MP exposure (Table 2). In case of four weeks of exposure, the average rearing frequency in the T0 group was 1.33; in the T2 group, 5.17 ($p = 0.004$); and in the treated

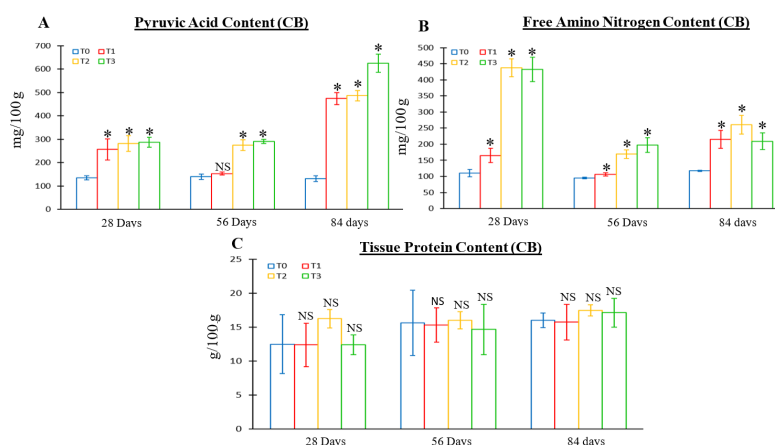


Figure 5: Time-dependent effects of PS-MP of cerebellum following graded doses of exposure A. Pyruvic acid content of cerebellum tissue, B. FAN content of cerebellum, C. Tissue protein content of cerebellum [T0 rats received only sterile water, T1 received 0.5 mg/L, T2 received 5 mg/L, and T3 received 50 mg/L of PS-MP; Values are Means $\pm$ SD. * $P < 0.05$ was considered statistically significant, NS = not significant]

3 group, 9.5 ($p = 0.004$). Similarly, an increase in rearing frequency was observed in the T2 and T3 groups of rats following 56 days of PS-MP exposure. The values were 2.2 in the control group, 1.17 in the T1 group, 6.7 in the T2 group ($p = 0.006$), and 9.4 in the T3 group ($p = 0.006$), respectively. After twelve weeks' PS-MP exposure, the rearing frequency increased in the T2 and T3 groups by 189% ($p = 0.04$) and 168% ($p = 0.008$), respectively, in contrast with the T0 group. Moreover, PS-MP exposure significantly altered the animals' transition frequency only after 12 weeks of exposure (Table 2). The transition frequency was increased by 100% ($p = 0.04$),

87.5% ($p = 0.02$), and 223% ($p = 0.005$) in the treated groups T1, T2, and T3, respectively.

Table 3 represents the behavioral data obtained from the 'Open-Field Test'. Significant changes in rearing frequency were also observed in the medium and highest doses in treated rats at 4, 8, and 12 weeks of exposure. PS-MP treatment for four weeks increased the rearing frequency by 224.7% ($p = 0.005$) and 268.2% ($p = 0.005$) in the T2 and T3 groups, respectively. Similarly, after eight weeks' exposure, the rearing frequency was increased by 100% ($p = 0.03$) and 165.2% ($p = 0.01$) in the T2 and T3 groups of rats. Additionally,

Table 3: Effect of PS-MPs exposure on Open-Field Box Test

Test Parameter	Open-Field Box Test			
	28 DAYS			
	T0	T1	T2	T3
Rearing	2.67 $\pm$ 1.21	7.00 $\pm$ 6.20	8.67 $\pm$ 2.73*	9.83 $\pm$ 3.25*
Time Spent in Inner Zone (Sec)	1.17 $\pm$ 1.47	2.67 $\pm$ 3.44	1.33 $\pm$ 2.07	1.00 $\pm$ 1.55
Time Spent in Outer Zone (Sec)	599.00 $\pm$ 1.55	597.33 $\pm$ 3.44	598.67 $\pm$ 2.07	599.00 $\pm$ 1.55
Number of entries in the central area	0.33 $\pm$ 0.52	0.50 $\pm$ 0.55	0.33 $\pm$ 0.52	0.00 $\pm$ 0.00
56 DAYS				
Rearing	3.33 $\pm$ 1.97	4.00 $\pm$ 1.79	6.67 $\pm$ 1.86*	8.83 $\pm$ 2.23*
Time Spent in Inner Zone (Sec)	1.00 $\pm$ 1.55	0.67 $\pm$ 1.03	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Time Spent in Outer Zone (Sec)	599.00 $\pm$ 1.55	599.33 $\pm$ 1.03	600.00 $\pm$ 0.00	600.00 $\pm$ 0.00
Number of entries in the central area	0.33 $\pm$ 0.52	0.33 $\pm$ 0.52	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
84 DAYS				
Rearing	4.17 $\pm$ 1.94	5.17 $\pm$ 2.32	14.33 $\pm$ 4.13*	19.00 $\pm$ 3.58*
Time Spent in Inner Zone (Sec)	0.67 $\pm$ 1.03	0.00 $\pm$ 0.00	2.00 $\pm$ 3.10	0.00 $\pm$ 0.00
Time Spent in Outer Zone (Sec)	599.33 $\pm$ 1.03	600.00 $\pm$ 0.00	598.00 $\pm$ 3.10	600.00 $\pm$ 0.00
Number of entries in the central area	0.33 $\pm$ 0.52	0.00 $\pm$ 0.00	0.33 $\pm$ 0.52	0.00 $\pm$ 0.00

Values are Means $\pm$ SD. * indicates $P < 0.05$ between the control and the respective treated group means.

12 weeks' exposure increased rearing frequency in the exposed rats by 243.6% ($p = 0.004$) in the T2 group and by 355.6% ($p = 0.005$) in the T3 group, respectively. Furthermore, the Open-Field test revealed no significant change in the duration of stay in the outer zone of the box between the control and polystyrene-treated rats. Similarly, PS-MP had no impact on the duration of stay in the inner box compared with the control group.

DISCUSSION

The extensive use of plastic materials has drawn global attention as 'white pollution' due to the continuous release of primary and secondary microplastics (MPs) and nanoplastics (NPs) into the environment, potentially threatening human health.⁴¹⁻⁴³ After subacute exposure to a low dose, there was no significant change in the blood glucose of the treated animals, whereas medium and high doses caused marked reductions in that parameter. On the other hand, after eight and twelve weeks of exposure, a significant reduction in the effect of PS-MP was evident in all treated animals, indicating that, with increasing exposure duration, the low dose also exhibited a hypoglycaemic effect. The reason for this effect might be a change in food intake patterns in PS-MP-treated groups compared with increasing exposure doses and durations in the animals. This assumption is supported by the current observation, which shows that PS-MP exposure significantly decreased average daily food intake across all doses and exposure durations. Environmental pollutants like microplastics may cause stress in exposed animals by inducing stress-related biomarkers, such as elevated blood cortisol levels, as observed in larval zebrafish.⁴⁴ Food absorption and daily food intake may be hampered, causing less improvement in the body weight in the treated animals following sub-acute to sub-chronic exposure. Another suggestive mechanism might be that polystyrene may elevate insulin levels in rats' blood. This statement is supported by a previous report showing that polystyrene elevates serum insulin levels in female rats.⁴⁵ Additionally, PS-MP decreased the absolute brain weight in PS-MP-treated rats administered with 5 mg/l and 50 mg/l of PS-MP for the period of four weeks, whereas no significant change in the absolute brain weight was noticed after 56 days and 84 days of exposure. As body weight decreased, the brain somatic index also decreased in the PS-MP-treated animals throughout the exposure period.

Metabolic changes have been observed in different doses and durations of polystyrene microplastic exposures. Cerebral LDH activity was increased in the medium- and high-dose-exposed groups after 28 and 56 days of exposure. This may lead to increased conversion of pyruvate to lactate, causing less pyruvate accumulation in the cerebral cortex. An increased level of LDH was observed in the human placental explants after time-dependent exposure of PS-MPs.⁴⁶ Stimulation of the LDH function was noted in sub-acute to sub-chronic (56 days of treatment), whereas inhibition of

that enzyme activity was observed after 84 days of exposure. Such a contrasting effect after a long duration (12 weeks) of PS-MP exposure may be due to a lack of a substrate, such as pyruvate, or to suppressed enzyme expression. Pyruvate depletion may lead to significant energy scarcity to feed the TCA cycle. Earlier studies reported that pyruvic acid content decreased after 4 weeks of PS-MP treatment, indicating suppressed glycolytic activity in the cerebral cortex.⁴⁷ PS-MP inhibits glycolysis in mouse liver by changing the expression of the genes encoding glycolytic enzymes, as reported in the hepatic transcriptome studies.^{48,49} Thus, the plausible mechanism of glycolytic inhibition by PS-MP might involve suppression of glycolytic enzyme gene expression, such as glucokinase and pyruvate kinase, as supported by earlier investigations.^{49,50} Accordingly, it is assumed that decreased production of glycolytic enzymes may be responsible for the suppression of glycolysis and the consequent lower pyruvate production. This assumption is supported by a new study that specified that MPs inspire glycolytic inhibition by declining phosphofructokinase function and glucose transporter, and also contributing to nephrogenesis interruption.⁵¹

Additionally, cerebral free amino nitrogen decreased at low PS-MP doses, suggesting that it could serve as an alternative energy-producing gluconeogenic substrate. Conversely, elevated levels of free amino nitrogen in the cerebral cortex at high doses are suggestive of either increased circulation-based mobilization of amino acids resulting from polystyrene-induced animal stress or the conversion of keto acids to amino acids via enhanced transaminase activity. Furthermore, free amino nitrogen was increased at all PS-MP doses after 56 and 84 days of treatment, which might be due to enhanced amino nitrogen production or circulation-based mobilization induced by animal stress caused by polystyrene. The GOT activity was slightly enhanced at the middle and high treatment doses after 28 days of exposure, whereas it decreased in all treated rats after 8 and 12 weeks of exposure, indicating that sub-acute exposure stimulated enzyme activity and sub-chronic exposure inhibited it. Prior research has shown that exposure to PS-MP alters transaminase activities in various tissues, with different microplastic dosages and exposure times showing both stimulation and inhibition of these activities. For example, an earlier report stated that GOT activity increased following low-dose PS-MP administration but decreased after subsequent exposure to high concentrations in crab hepatic tissue.⁵² In another article, increased functions of GOT and GPT were reported in male Wistar rats by pristine polystyrene nanoplastics.⁵³ Protein content in the cerebral cortex remained unaltered following varied doses and durations of PS-MP exposure, indicating that the tissue proteostasis network is not hampered by polystyrene at the current doses and durations. However, the cerebral cortex is primarily involved in higher-level processes such as memory, learning, language, emotion, and other cognitive functions, so changes in metabolic integrity induced by polystyrene

may disrupt functional harmony.

The crucial part of the brain is the cerebellum, which plays a critical role in controlling movement coordination, cognitive function, posture, and balance, and facilitating motor learning. Following PS-MP treatment, pyruvic acid content increased in the cerebellum at subacute and subchronic exposure durations. The elevation of pyruvate might be due to the motivation of glycolysis to supply more pyruvate to the TCA cycle. Another plausible mechanism is the lack of the pyruvate dehydrogenase enzyme, which plays an important role in converting pyruvate to acetyl-CoA for the TCA cycle. Moreover, increased lactate dehydrogenase levels in the rat cerebellum are relevant in the context of elevated pyruvate levels. Furthermore, the production of free amino nitrogen was elevated by PS-MP treatment. The mobilization of free amino nitrogen from other brain tissue to the cerebellum could be the cause to support protein metabolism. Additionally, transaminase activity declined after PS-MP treatment. A decrease in GOT function may sensitize cerebellar tissue to glucose deprivation, as GOT is vital for supplying oxaloacetate under low-glucose conditions, thereby helping preserve redox homeostasis. A decrease in GOT function was evident in juvenile crabs (*E. sinensis*) liver exposed to high doses of polystyrene microplastics (4-40 mg/L) for 21 days.⁵² Additionally, inhibition of GPT by PS-MP might cause glutamate toxicity and overaccumulation of glutamate and pyruvate in that brain tissue. On the other hand, cerebellar protein content shows no significant change at 3 times the duration and across different PS-MP doses, indicating that PS-MPs did not affect the protein synthesis or breakdown machinery in the rat cerebellum at different exposure times.

Additionally, behavioral changes were observed in rats following the PS-MP treatment. Since they are nocturnal animals, they are more active at night and seek cover in dimly lit areas to feel protected from predators. Additionally, darkness provides them with a sense of security, allowing them to explore their surroundings and seek food without worrying about being noticed. After the exposures of PS-MP at three varied doses and durations, it was noticed that the treated animals were more inclined to stay in the light in comparison to the untreated rats following 56 days and 84 days of exposures to PS-MP, but sub-acute (28 days) treatment does not reveal any change in stay duration in the light and dark compartments between the treated and the control animals. The frequency of rearing was increased in the PS-MP-treated rats, especially in the treated 2 (middle dose) and treated 3 (high dose) rats, across all exposure durations. This reveals that polystyrene microplastics at the middle and highest doses prompted the animals to exhibit more curious behavior than the control animals. The transition of the animals between the two chambers (light and dark) was higher in the treated animals than in the controls after 84 days of treatment, indicating that as PS-MP exposure duration increased, the animals became more irritable and

thus exhibited anxiety-like behavior. To further monitor anxiety-related behavior, the open-field test was conducted. The results demonstrated that the animals spent more time in the outer area than in the inner area, indicating anxiety-like behavior in the treated rats. It was demonstrated that more time in the outer arena of the open-field box amplified anxiety-related behavior in the animals.⁵⁴

The light-dark box test relies on the rodent's innate aversion to brightly illuminated areas and its impulsive exploration behavior in response to minor stimuli.^{55,56} Normally, rats preferred to stay in a dark compartment rather than a light chamber.⁵⁷ The current investigation reveals that after 56 and 84 days of PS-MP exposure, the Wistar rats spent more time in the light compartment than in the dark as compared to the control animals. Additionally, PS-MP-treated rats showed increased rearing in the light chamber at medium and high doses for all three durations. These observations indicated that PS-MP-treated rats showed disinhibition of exploratory movements in response to light stimuli compared with controls. According to the investigators, the stressed animals exhibit typical behaviors such as rearing and grooming.⁵⁸ However, additional studies are required to identify the consequences of polystyrene microplastics on neurochemicals to obtain a clear understanding of the stress-mediated changes in the behavior of the treated animals. Compared to smaller NPs, recent reports found that larger MPs have a higher accumulation rate and are therefore more difficult to remove from the body.^{59,60} NPs are rapidly absorbed and cleared from the body due to their smaller size, and they may be less neurotoxic than MPs. Therefore, further study is required to determine the clear relationship between particle size and particle-induced neurotoxicity.⁶⁰ Although the effects of smaller NPs have been extensively evaluated, it remains unknown how unsafe MPs are to the mammalian nervous system.^{17,61}

The open-field test is a widely used paradigm for assessing anxiety-like behavior and locomotion in animals. To determine whether the animals exhibit anxiety-like behavior, parameters such as grooming frequency, rearing frequency, and locomotor activity are assessed.⁵⁸ In the current study, the animals exhibited significantly increased rearing frequency after 28 days, 56 days, and 84 days of PS-MPs exposure, indicative of stereotypical behavior. Stressed animals exhibit stereotypical behaviors, such as rearing and grooming.⁵⁸ By exposure to restraint stress, the male Wistar rats showed such behavioral alteration, which is evidenced by increased latency and decreased ambulation, and also found that age-related changes in anxiety-like behavior became more noticeable.⁶² In another research article, the researchers performed an open-field test under a reversed light-dark cycle and found that the total distance traveled was greater in mice exposed during the light phase than during the dark phase.⁶³ According to an earlier study report, the mice exposed to PS-MPs for a subchronic duration (60 days, low and high doses) exhibited an increase in percentage

freezing duration, which concludes that PS-MPs show anxiety-like behavior.⁶⁴ However, in the current investigation, PS-MP-exposed rats showed increased rearing frequency, indicative of exploratory behavior, in comparison to the control group of rats.

CONCLUSION

Overall, PS-MPs exposure time-dependently induced hypoglycemia at the current doses and durations, which might be due to decreased average daily food consumption. Brain metabolic activity was altered in distinct ways across discrete brain regions. Moreover, behavioral studies observed increased time spent in the light and higher frequencies of rearing and transition. These findings indicate that polystyrene microplastics alter brain metabolic functions and behavioral activities following subacute to subchronic exposures.

This study bridges ecotoxicology and human health by identifying metabolic alterations in certain mammalian species that inform how persistently this synthetic polymer might bioaccumulate and alter human metabolic homeostasis. Energy depletion and altered bioenergetics by microplastics are implicated in cognitive function damage and pathogenesis of neurodegenerative disorders like Alzheimer's disease and dementia. Recent studies detecting PS-MP in human blood, breast milk, liver, and skeletal muscle emphasize the necessity for clinical awareness and routine clinical exposure screening of particulate exposure.

ACKNOWLEDGEMENT

The first author acknowledges the University Grants Commission, New Delhi, for providing a non-NET fellowship to carry out this research. The authors further acknowledge the Co-ordinator of the Advanced State Biotech Hub for supporting the infrastructural facility.

CREDIT AUTHOR CONTRIBUTION STATEMENT

Mondal S: Data curation, formal analysis, draft manuscript writing; Chakraborty S: formal analysis, investigation, reference writing; Saha J: Statistical analyses; Pal S: Conceptualization, project administration, methodology, supervision, validation, review, and editing.

THE DATA AVAILABILITY STATEMENT

On reasonable request, the corresponding author may provide the dataset used in the present study.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.

Gymguard-SCD Clinical Decision Support System

GYM GUARD-SCD: THE FUTURE OF SPORTS CARDIOLOGY AND PREVENTIVE CARDIOLOGY



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SYSTEM AND METHOD FOR PRE-EXERCISE DYNAMIC ELECTRO-AUTONOMIC INSTABILITY DETECTION

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* GymGuard-SCD is an advanced clinical support system designed for multi-parametric sudden cardiac death risk stratification. This innovative device performs pre-exercise electro-autonomic instability detection, representing the future of sports cardiology and supporting the vision of Atmanirbhar Bharat.