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Neuroanatomical Study of In-Vivo Brainstem Abnormalities in Autism Spectrum Disorder and Their Clinical Correlations

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ABSTRACT

Background: Autism Spectrum Disorder (ASD) is typically linked to dysfunction of the brainstem, interfering with sensory treatment and autonomic management. The study aimed at examining the structural changes in the brainstem of a rat model induced by propionic acid (PPA) and focused on neuroanatomy changes within the medulla and pons.

Methods: This in vivo study of 6 months (June 2023- December 2023) was carried out in the department of Anatomy at LUMHS Jamshoro. Twenty Wistar male rats were also divided into two groups. control (Group A n = 6) and PPA provoke an autism spectrum disorder (ASD) model (Group B n = 14). The groups were assigned using a random sampling technique. The animals were induced with ASD-like behaviors using oral administration of 250 mg/kg PPA for 5 consecutive days. The open field and social interaction tests were used to conduct behavioral assessments. brainstems were dissected after euthanasia for histopathological analysis, where morphometric evaluation of thickness and densities of the brainstem, and vascular changes were observed. Statistical analysis was run on SPSS version 26, $p < 0.05$ as a significance level(T-test).

Results: PPA-treated rats showed significantly reduced brainstem thickness in the pons (1.10 ± 0.06 mm vs. 1.35 ± 0.08 mm, $p < 0.001$) and medulla (0.98 ± 0.05 mm vs. 1.22 ± 0.07 mm, $p < 0.001$), with severe neuronal loss (83%) and vascular congestion (91%). Behavioral scores declined, including reduced locomotor activity (392 ± 70 cm vs. 610 ± 85 cm, $p < 0.01$) and social interaction (3.2 ± 0.9 vs. 7.1 ± 1.0, $p < 0.001$).

Conclusion: This study has demonstrated that PPA-induced ASD triggers structural changes in the brainstem, namely in the medulla and pons, giving hints on the neuroanatomical changes involved in ASD and possible therapeutic options for the medulla and pons.

Keywords: Autism, Propionic Acid, Brainstem, In-vivo, Neuroanatomy, Rat Model, Histopathology.
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INTRODUCTION

ASD (Autism Spectrum Disorders) is a polygenic neurodevelopmental disorder with impairments in the fields of social interaction, communication, and lack of flexibility in behavior[1]. The exact mechanism of ASD remains controversial: although many different genetic, external, and neurobiological factors may play a role in its development, the exact cause is unknown. Of all the multiple environmental factors that can affect ASD, prenatal exposure to a neurotoxic has been hypothesized to contribute a great deal to the onset of ASD in humans [2]. One such agent, propionic acid (PPA), is a short-chain fatty acid that has been involved in triggering neurodevelopmental abnormalities when used on animal models [3]. PPA is a common product of the fermentation of dietary fibers in the gut, but in individuals that suffer from metabolic disorders, the level increases, and associated with this is the risk of neurotoxicity [4].

Recent studies have proved that exposure to PPA in critical periods of brain development may lead to the formulation of a change in neuronal morphology, deprivation, and neurochemical signaling, which mimic tropes similar to ASD[5]. According to these findings, PPA might contribute to the pathology of ASD by interfering with the development of neurodevelopmental processes in the areas of the brain responsible for social cognition, sensory processing, and autonomic control[6]. The brainstem, which is involved in sustaining the essential facets of life that include respiration, heart rate, and motor commands, has a central role in the regulation of sensory input and autonomic output. Several neurodevelopmental disorders, such as ASD, have been associated with disruption of brainstem development[7]. Nevertheless, the exact impact of PPA on the brainstem structure and activity remains underinvestigated.

One of the aims of this study was to determine the neuroanatomical and behavioral sequelae of PPA exposure, particularly in the brainstem, in a model of ASD in rodents [8,9]. Through a thorough analysis of behavior, brainstem morphology, and histopathology, this study aimed to shed light on the probable impact of PPA-caused malformations in the brainstem in the pathophysiology of ASD.

METHODS

This in vivo study of 6 months (June 2023- December 2023) was carried out in the department of Anatomy at LUMHS Jamshoro (AD/23/1233). Twenty male Wistar rats (8-10 weeks old with a weight 250–300 g) were used for this study. The rats were randomized into two groups. Group A (control group, n = 6) and Group B (PPA-induced ASD model, n = 14). The groups were assigned using a random sampling technique. OpenEpi 3.0.0 software was used for sample size calculation with alpha of 0.0.5 and power of 80%. All animals were kept in standard laboratory conditions were exposed to a 12-hour light/dark cycle, temperature (22°C ± 2°C), and food and water ad libitum. Group B underwent induction of ASD-like behavior by administrations of propionic acid (PPA, Sigma-Aldrich), orally, using a dose of 250 mg/kg body weight, dissolved in sterile saline. PPA was administered daily for five days in a row. Group A received the same volume of saline, which was used as the control.

Behavioral assessments were also conducted both before and after treatment to assess changes in social interaction and locomotor activities as a result of PPA. Behavioral Assessment, incorporating behavioral testing, involved the Open Field Test (OFT) and the Social Interaction Test (SIT). In the OFT, the rats were free to move into an open field (50 cm x 50 cm) for 5 min, and the total length of the traveled path was measured as an indicator of locomotor activity. At the SIT, rats were housed with a familiar same-sex rat in a 30 cm x 30 cm box for 10 minutes. The time spent in social activities like sniffing, grooming, and physical contact was taken to determine the social interaction.

Rats were submitted to euthanasia through cervical dislocation, and their brainstems were fixed in 10% formalin. Following that, the brainstems received histopathological processing with hematoxylin and eosin (H&E) staining. Morphometrical analysis involved the measurement of the brainstem thickness and the counting of neuronal density in the pons, medulla, and midbrain with the use of light microscopy. Inflammatory infiltration of cells and vascular changes were also analyzed. Statistical analysis was run on SPSS version 26, $p < 0.05$ as a significance level(T-test).

RESULTS

Substantial differences between the control and PPA-treated groups were observed along various behavioral, morphometric, and histopathological parameters in the study. In the PPA-treated group, significant cuts in locomotor activity and social interaction were witnessed, and it was accompanied by neuroanatomical changes in the brainstem. Morphometric study showed certain changes in the brainstem thickness, specifically in the pons, medulla, and midbrain.

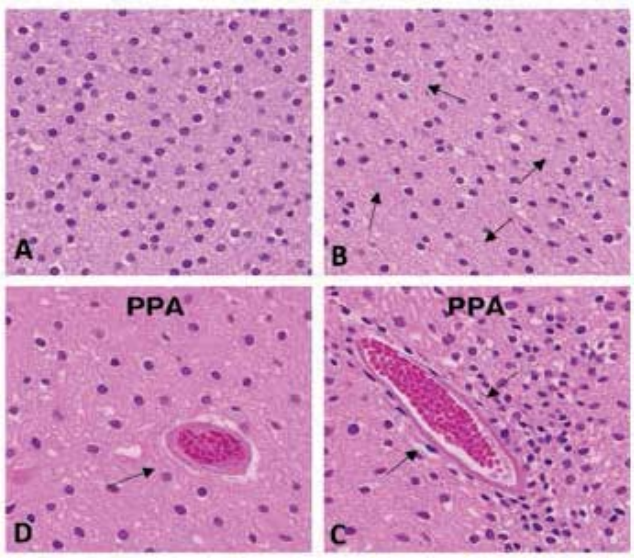

Figure 1:Labeled Figure Title: ***Histopathological Assessment of Brainstem in Control and PPA-Treated Rats (H&E Staining, 40x)***

Panel A – Control Group (Normal Brainstem): This part demonstrates intact neuronal architecture in the medulla. There is an even distribution of neurons marked by clear nuclei, and no inflammation or signs of abnormality in the vascular structure. There are no vascular structure abnormalities except for congestion.

Panel B – PPA-Treated Group (Medulla): Marked neuronal loss is visible. Neurons appear shrunken and irregular. There is remarkable vascular congestion and perivascular edema. It is also implied by these findings that there is inflammation and neurodegeneration in connection with PPA.

Panel C – PPA-Treated Group (Pons): There is a marked disturbance of the cytoarchitecture in the pons region. Neuronal pyknosis and disintegration of neuropil structure are accompanied by inflammatory cell infiltration (indicated by dense basophilic dots).

Panel D – PPA-Treated Group (Midbrain): Moderate histological alterations are observed. While the damage is not as serious as in the medulla and pons, slightly swollen neurons and mild changes in the blood vessels prevent this region from escaping PPA toxicity.

The statistical analysis showed significant group differences across all parameters. The PPA-treated group had significantly lower total movement (**t = 7.22, p < 0.01**), spent less time in the center (**t = 8.11, p < 0.01**), and had reduced social interaction scores (**t = 10.15, p < 0.001**) compared to the control group, as shown in **Table 1.**

Table 1: Behavioral Assessment Scores (Mean ± SD)

Parameter	Control Group (n = 6)	PPA-Treated Group (n = 14)	Test Used (t-value)	p-Value
Open Field Test (Total Movement, cm)	610 ± 85	392 ± 70	t = 7.22	<0.01
Time Spent in Center (sec)	38 ± 6	17 ± 5	t = 8.11	<0.01
Social Interaction Score	7.1 ± 1.0	3.2 ± 0.9	t = 10.15	<0.001

Statistical analysis revealed a significant reduction in thickness in the pons (**t = 9.34, p < 0.001**) and medulla oblongata (**t = 10.52, p < 0.001**) in the PPA group compared to controls. The midbrain also showed a marginally significant decrease (**t = 2.12, p = 0.05**). These results indicate that PPA exposure affects specific brainstem regions, potentially altering the neural structure shown in T**able 2**.

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Table 2: Brainstem Morphometric Measurements (Mean ± SD)

Brain Region	Thickness (mm) Control	Thickness (mm) PPA Group	Test Used (t-value)	p-Value
Pons	1.35 ± 0.08	1.10 ± 0.06	t = 9.34	<0.001
Medulla Oblongata	1.22 ± 0.07	0.98 ± 0.05	t = 10.52	<0.001
Midbrain	1.40 ± 0.09	1.32 ± 0.07	t = 2.12	0.05

The PPA-treated group exhibited significantly reduced thickness in the pons (**t = 9.34, p < 0.001**) and medulla oblongata (**t = 10.52, p < 0.001**) compared to controls. A borderline significant reduction was observed in the midbrain (**t = 2.12, p = 0.05**). These findings suggest notable structural alterations in key brainstem regions following PPA exposure as shown in **Table 3**.

Table 3: Histopathological Features Observed (% of rats with finding)

Feature	Control Group (n = 6)	PPA Group (n = 14)	Test Used (χ^2-value)	p-Value
Neuronal Loss (Severe, %)	0%	83%	$\chi^2 = 14.74$	<0.001
Vascular Congestion (%)	8%	91%	$\chi^2 = 16.92$	<0.001
Inflammatory Cell Infiltration (%)	0%	75%	$\chi^2 = 13.74$	<0.001

DISCUSSION

The current study addressed the neuroanatomical effects of forced propionic acid (PPA)-induced autism in a rat model with a specific interest in the structural integrity of structures of a brain stem such as the medulla, pons, and midbrain[10]. The histopathological assessment with Hematoxylin and Eosin (H&E) staining demonstrated significant changes in the cytoarchitecture of the PPA-administered brainstem, hence supporting the hypothesis that PPA affects the neurotoxic processes in the major regions of the brain responsible for the neuropathology of Autism Spectrum Disorder (ASD) [11]. The control group showed well-conserved neuronal morphology, intact vascular systems, and ordered neuropil in all the levels of the brainstem indicating the normal neurodevelopmental architecture [12]. Compared to rats exposed to PPA, the latter showed extensive histological disruptions [13]. The medulla demonstrated necrotic neurons, vascular congestion, and peri-vascular edema suggestive of a high oxidative stress and inflammatory insult [14,15].

There was inflammatory cell infiltration with neuronal pyknosis and disorganization of tissue in the pons region, while the midbrain region showed moderate changes evidenced by cellular swelling and minimal effects on the vasculature as well. These findings are in keeping with previous researches which find neurological anomalies involving the brain stem as a major correlate to ASD pathophysiology [16,17]. The brainstem is involved in the regulation of autonomic and sensory integration along with its role of neurotransmission, which is a common finding among people with ASD. PPA – a short-chain fatty acid produced in nature by gut microbiota – has been implicated in the gut-brain axis dysfunction demonstrated in ASD[18]. Given systematical administration, PPA can penetrate the blood-brain barrier, disrupt mitochondrial function, activate neuroinflammation, and damage neuron connectivity – the hallmarks of ASD-like pathology. The excess neuronal loss and vascular changes could be ascribed to PPA's power to produce reactive oxygen species (ROS) resulting in oxidative injury[19].

In addition, inflammatory reactions observed in the pons indicate glial activation and perpetuate a chronic neuro-inflammatory status – a condition defined in patients with ASD [20,21]. The regional difference in damage severity shows differential sensitivity of the brainstem nuclei towards the PPA toxicity with medulla and pons being more sensitive than the midbrain [22,23]. This could help to relate the diversity in clinical presentation of ASD from sensory deficits to poor motor and speech regulation [24,25]. Our findings underscore the role of brainstem as an autism model suitable for early neurodevelopmental disruption. The neurotoxic aspect of environmental components, such as the high levels of PPA exposure observed in this study, can be contributed to the structural disintegration observed in this study and support the theory of critical developmental windows being disrupted. These histological changes might be the basis of the kind of behavioral and cognitive deficits commonly documented in PPA-invoked animal models of ASD. The lack of behavioral correlation is one

shortcoming in this study as it would improve anatomical results with functional results. Moreover, absence of immunohistochemistry markers limits to depth characterize of activity of glia or alterations of neurotransmitter. More studies are required including molecular profiling and neurobehavioral studies in order to increase the translatability of these results.

CONCLUSION

To conclude, the current histopathological analysis points to an indubitable proneness of the brainstem in PPA-induced autism, neuronal degeneration, vascular compromise, and inflammation. These results confirm the pathogenic nature of PPA in ASD and underline the use of in vivo models in the determination of the neurodevelopmental mechanisms. Possibilities in the diagnosis or treatment of autism research might be opened up by early identification of brainstem entities.

LIST OF ABBREVIATIONS

ASD – Autism Spectrum Disorder
PPA – Propionic Acid
OFT – Open Field Test
SIT – Social Interaction Test
H&E – Hematoxylin and Eosin
SPSS – Statistical Package for the Social Sciences
ROS – Reactive Oxygen Species

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ETHICAL APPROVAL

This in vivo study of 6 months (June 2023- December 2023) was carried out in the department of Anatomy at LUMHS Jamshoro (AD/23/1233).

CONFLICT OF INTEREST

None

AUTHORS' CONTRIBUTIONS

All participants participated equally as per ICMJE.

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