

Toraseamide Improves the Propionic Acid-Induced Autism in Rats: A Histopathological and Imaging Study

ABSTRACT

Objective: Autism spectrum disorder is a neurodevelopmental disease in which impaired social behaviors, impaired sociality, and restricted and repetitive behaviors are seen. Bumetanide is a loop diuretic that inhibits $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter 1 and it is currently used in clinical phase studies in patients with autism spectrum disorder. In present research, it is purposed to demonstrate the beneficial effects of toraseamide which is another $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter 1 inhibitor on an experimental autism model induced with propionic acid by providing imaging and brain tissue investigations.

Methods: Male Wistar rats were used in the present study ($n=30$). Propionic acid of 250 mg/kg/day was administrated intraperitoneally in rats to induce autism for 5 days. Three groups were created for present study as follows: group 1, normal control ($n=10$); group 2, propionic acid and saline given group ($n=10$); group 3, propionic acid + toraseamide-administrated group ($n=10$).

Results: Toraseamide group scored higher on behavioral tests compared to saline group. The brain levels of malondialdehyde, tumor necrosis factor-alpha, interleukin-2, interleukin-17, and Nuclear Factor kappa B (NF- κ B), Glial fibrillary acidic protein (GFAP) were remarkably higher in propionic acid + saline group. In histopathology assessments, toraseamide group had higher neuronal count of Cornu Ammonis 1, neuronal count of Cornu Ammonis 2 in hippocampus, and Purkinje cells in cerebellum. GFAP immunostaining index (Cornu Ammonis 1) and cerebellum were lower in toraseamide group. Magnetic resonance spectroscopy revealed that mean lactate value was higher in propionic acid + saline group compared to toraseamide group.

Conclusion: Our experimental results showed that toraseamide might enhance gamma-aminobutyric acid activity. Toraseamide can be considered another promising $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter 1 inhibitor in the treatment of autism with a longer half-life and less side effects after further studies.

Keywords: Autism, diuretic, toraseamide, treatment

Introduction

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder that is characterized by abnormal social behaviors, impaired interaction, difficulties in interests and activities, and repetitive behaviors.¹ Language development and motor abnormalities are commonly disturbed in ASD.² The ASD is seen as common as 1 in 150 children globally.³ The gender difference is important in ASD and men are affected 3 times more than women.⁴

Although ADS is considered a common disorder, the exact etiology cannot be defined yet.⁴ Moreover, the current therapeutic approaches are so restricted based on insufficient knowledge of etiology as well as associations between the environment and behaviors. Because obtaining brain tissue from living humans is nearly impossible in daily practice and researches, animal models are important for identifying the etiology of ASD and for creating treatment strategies. There have been several environmental agents which were used for creating animal model of ASD.⁵ Propionic acid (PPA) is a potential candidate which causes

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several behavioral and neuro-inflammatory changes in the rat model of ASD. Actually, PPA is a metabolic outcome of enteric bacteria and it has ability to cross the gut–blood and gut–brain barriers. After transmission, PPA accumulates in the cells and leads intracellular acidification^{6,7}; thus, it can affect the normal functions of numbers of neurotransmitters.⁷ Currently, PPA is considered as a well-established agent for the development of experimental model of ASD.⁸⁻¹⁴

The imbalance between neurotransmitters which have excitatory and inhibitor properties is known to be an etiological factor for ASD.¹⁵ Gamma-aminobutyric acid (GABA) is a pivotal inhibitory neurotransmitter in the central nervous system (CNS). The inhibitor effect of GABAergic neurotransmission is highly important for brain development as well as neurotransmission regulation. Thus, GABA has been taken great interest in the etiologies of numerous neurological and mental diseases including schizophrenia, depression, and anxiety.^{16,17} The experimental studies have also demonstrated that dysfunction in GABAergic signaling pathway was significantly associated with core symptoms of ASD.¹⁸ The high percentage of comorbidity of epilepsy with ASD also confirms this suggestion.¹⁹

The accumulating evidence demonstrated that the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1 (NKCC1) and K^+/Cl^- cotransporter 2 (KCC2) were significantly associated with GABAergic signaling.²⁰ The chloride importer NKCC1 and the chloride exporter KCC2 play important roles in GABA receptors.²¹ Bumetanide is an NKCC1 inhibitor, and clinical phase studies showed that use of oral solution form of bumetanide had improved the symptoms including social reciprocity and behavioral symptoms in children and adolescents with ASD.²²⁻²⁵ Torasemide is a non-acid loop diuretic which also inhibits NKCC1, similar to bumetanide.²⁶ However, there is neither any clinical nor an experimental study that investigated the effects of torasemide on ASD symptoms. There have been 2 studies which investigated the effects of torasemide on experimental-induced seizures. Hampel et al²¹, in his first study, investigated the pharmacokinetic-pharmacodynamic properties of torasemide, azosemide, and bumetanide in mouse brain. They demonstrated that all 3 agents hardly pass the blood–brain barrier (BBB); however, free brain concentrations of bumetanide and torasemide were found to be within the range of NKCC1 inhibitory activity. The later study, which was also reported by Hampel et al²⁷, again demonstrated that azosemide, torasemide, and bumetanide had similar effects in experimental-induced epilepsy model.

This research aimed to demonstrate the beneficial effects of torasemide on the PPA-induced autism model by providing imaging and brain tissue investigations.

MAIN POINTS

- Torasemide was found to have beneficial effects on behavioral tests.
- Torasemide group had favorable results compared to saline group in terms of brain neurochemical findings and histopathological comparisons.
- Magnetic resonance spectroscopy assessment supported the superiority of torasemide to saline group.
- Torasemide can be considered a promising agent in the treatment of autism.

Methods

Animals

This study used 30 male albino Wistar rats weighing 150-200g and 10-12 weeks of age. This study was certified by Local Animal Ethics Committee (Demiroğlu Bilim University, number: 19210702). The rats which were used in present study were from Experimental Animal laboratory of Demiroğlu Bilim University.

Experimental Stages

For this study, 30 male Wistar rats were taken. Propionic acid of 250 mg/kg/day was given intraperitoneally to 20 rats to induce autism for 5 days. Ten rats were selected as control. The PPA-exposed rats were grouped as follows: group 2 (PPA + saline, n = 10): PPA via oral gavage 1 mL/kg/day 0.9% NaCl saline group; group 3 (PPA + torasemide, n = 10) PPA via oral gavage 1 mg/kg/day torasemide (Sutril tablets 5 mg, Adeka). The rats in study groups were treated for 15 days. By the fifteenth day, the behavioral tests were tested in all groups. Animals were assessed by magnetic resonance (MR) spectroscopy procedure under ketamine anesthesia (50 mg/kg).

Behavioral Tests

Three-Chamber Sociability Test: This test was performed on rats as previously described with minor modifications.^{28,29}

Open-Field: The open field test was performed in present study as previously described.^{28,29}

Passive Avoidance Learning: This behavioral test was performed according to previous data.³⁰

Magnetic Resonance Imaging Protocol and Magnetic Resonance Spectroscopy

Conventional Magnetic Resonance Imaging: A 3.0 T MRI/magnetic resonance spectroscopy (MRS) scanner (Magnetom, Siemens Healthcare, Germany) was used for evaluation. Conventional MR sequences were performed as previously described.³¹

Magnetic Resonance Spectroscopy: Magnetic resonance spectroscopy was administrated as previously described³¹ and Magnetom software was used to assess the raw data which were stored in a workstation.³¹

Hippocampus and Cerebellum Histopathology

The Cornu Ammonis (CA) 1 and CA 3 regions of hippocampus were selected for the investigations of hippocampus. The brains of rats were removed and stored for 3 days in 10% formaldehyde in 0.1 M phosphate-buffer saline. The histopathological examinations were performed according to previous descriptions.³² GFAP immunohistochemistry was performed by primary antibodies against GFAP (Abcam, Inc., Mass, USA; 1/1000) for 24 hours. Olympus BX51 microscope was used for obtaining the histopathological images. GFAP immunostaining index was calculated as follows: GFAP-positive cells were counted at 40× magnification in randomized sections (3-4) for each rat. An imaging system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) was used for the assessments.^{8,33}

Brain Biochemical Analysis

The brain levels of nerve growth factor (NGF), TNF- α , interleukin (IL)-2, IL-17, and lactate levels in the brain were assessed by enzyme-linked

immunosorbent assay kits. Confirmation was made for all investigations. A microplate reader was used for the measurement of the Absorbances (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, USA).³³

Measurement of Brain Lipid Peroxidation

Lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA). Malondialdehyde levels were analyzed with a standard calibrator using tetraethoxypropane.³³

Measurement of Brain Protein Levels

Bradford's method was used for all measurements of proteins.

Statistical Analysis

The obtained data were assessed by Statistical Package for Social Sciences version 15.0 for Windows (SPSS Inc.; Chicago, IL, USA). For defining normality and homogeneity of variance, Shapiro-Wilk's and Levene's tests were used. Analysis of variance test was used to compare the numerical data between the groups. Tukey test was used for post hoc analysis. The results are presented as mean $\pm$ standard deviation. The value of $P < .05$ was accepted as statistically significant.

Results

Behavioral Tests

Sociability test (time spent with stranger rat percent) mean point was higher in PPA+torasemide and control groups compared to PPA+saline group ($P = .0006$, $P = .008$, and $P < .007$, respectively). The control group had higher scores on sociability test compared to PPA+torasemide group ($P = .048$). The mean score of open field test (number of ambulation) was found to be higher in control and PPA+torasemide groups compared to PPA+saline group ($P = .001$, $P = .0009$, and $P = .04$, respectively) and it was similar between control and PPA+torasemide groups ($P = .051$). The mean scores of passive avoidance learning (PAL) latency were also higher in control group and PPA+torasemide group compared to PPA+saline group ($P = .001$, $P = .007$, and $P = .041$, respectively). The control group had higher scores on PAL test compared to PPA+torasemide group ($P = .044$) (Table 1).

Results of Brain Tissue Investigations

Brain MDA level was found to be higher in PPA+saline group compared to control group and PPA+torasemide group ($P = .0001$, $P = .0006$, and $P = .031$, respectively). It was also higher in PPA+torasemide group compared to control group ($P = .001$). Brain TNF- α level

was higher in PPA+saline group compared to control group and PPA+torasemide group ($F = 8.99$; $P = .0001$, $P = .0007$, and $P = .043$, respectively) and it was higher in PPA+torasemide group compared to control group ($P = .045$). Brain IL-2 level was found to be higher in PPA+saline group than in control and PPA+torasemide groups ($P = .0008$, $P = .0009$, and $P = .021$, respectively). Brain IL-2 level was higher in PPA+torasemide group compared to control group ($P = .001$). Brain IL-17 level (pg/mg protein) was found to be higher in PPA+saline group compared to control group and PPA+torasemide group ($P = .0005$, $P = .0007$, and $P = .030$, respectively). Brain IL-17 level was also higher in PPA+torasemide group than in control group ($P = .034$). Brain NF-KB level was higher in PPA+saline group than control and PPA+torasemide groups ($P = .0007$, $P = .0008$, and $P = .010$, respectively) and it was found to be higher in PPA+torasemide group compared to control group ($P = .004$). Brain lactate level was higher in both PPA+saline and PPA+torasemide groups compared to control group ($P = .0004$; $P = .0005$, and $P = .0006$, respectively) and it was higher in PPA+saline group compared to PPA+torasemide group ($P = .001$). Brain NGF level was higher in control group and PPA+torasemide group compared to PPA+saline group ($P = .0001$, $P = .0001$, and $P = .0002$, respectively). It was also found to be higher in PPA+torasemide group compared to control group ($P = .0009$). The results are presented in Table 2.

Hippocampus and Cerebellum Histopathology

Neuronal count CA1 was higher in control and PPA+torasemide groups compared to PPA+saline group ($P = .0004$, $P = .0006$, and $P = .013$, respectively). It was also higher in control group compared to PPA+torasemide group ($P = .003$). Neuronal count CA3 was higher in control and PPA+torasemide groups compared to PPA+saline group ($P = .0001$, $P = .0004$, and $P = .0003$, respectively). Neuronal count CA3 was found to be similar between control and PPA+torasemide groups ($P = .621$). GFAP (CA1) immunostaining index was found to be lower in control and PPA+torasemide groups compared to PPA+saline group ($P = .0003$, $P = .0004$, and $P = .0005$, respectively). It was found to be similar between control and PPA+torasemide groups ($P = .699$). GFAP immunostaining index (CA3) was also lower in control group and PPA+torasemide group than PPA+saline group ($P = .007$, $P = .008$, and $P = .031$, respectively). GFAP immunostaining index (CA3) was also higher in PPA+torasemide group compared to control group ($P = .037$). Purkinje count cerebellum was found to be higher in control group and PPA+torasemide group ($P = .006$, $P = .008$, and $P = .030$, respectively). Purkinje count cerebellum was found to be similar between PPA+torasemide group and control

Table 1. Comparison of the Results of Behavioral Test Between Groups

	Normal Control (n = 10)	PPA and Saline (n = 10)	PPA and Torasemide (n = 10)	Post Hoc Tukey
Sociability test: time spent with stranger rat percent (%)	61.8 (9.5)	30.7 (5.1)	51.9 (6.6)	$P = .008^a$ $P = .007^b$ $P = .048^c$
Open field test: number of ambulation	13.5 (2.3)	6.9 (1.2)	10.1 (2.5)	$P = .0009^a$ $P = .040^b$ $P = .051^c$
Passive avoidance learning (PAL) latency (Sec.)	285.2 (23.5)	101.5 (56.8)	211.2 (42.4)	$P = .007^a$ $P = .041^b$ $P = .044^c$

^aNormal control and PPA and saline; ^bPPA and torasemide and PPA and saline; ^cNormal control and PPA and torasemide.

Significant values are presented as bold characters.

PPA, propionic acid.

Table 2. Comparison of the Results of Brain Tissue Analysis Between Groups

	Normal Control (n = 10)	PPA and Saline (n = 10)	PPA and Torasemide (n = 10)	Post Hoc Tukey
Brain MDA level (nmol/g protein)	54.8 (9.2)	179.5 (11.7)	115.5 (9.5)	$P = .0006^a$ $P = .031^b$ $P = .001^c$
Brain TNF-alpha level (pg/mg protein)	14.1 (2.6)	113.1 (15.5)	68.7 (8.2)	$P = .0007^a$ $P = .043^b$ $P = .045^c$
Brain IL-2 level (pg/g protein)	2.1 (0.2)	265.3 (19.5)	166.1 (17.8)	$P = P = 0.0009^a$ $P = .021^b$ $P = .001^c$
Brain IL-17 level (pg/g protein)	225.8 (12.3)	558.1 (16.6)	367.7 (21.1)	$P = .0007^a$ $P = .030^b$ $P = .034^c$
Brain NF-KB level (pg/g protein)	17.5 (1.8)	232.4 (21.3)	104.1 (6.2)	$P = .0008^a$ $P = .011^b$ $P = .004^c$
Brain lactate level (mmol/100 g wet weight)	1.12 (0.01)	3.36 (0.2)	2.62 (0.4)	$P = .0006^a$ $P = .0005^b$ $P = .001^c$
NGF level (pg/mg protein)	82.5 (5.7)	41.9 (4.5)	50.1 (7.7)	$P = .0001^a$ $P = .002^b$ $P = .0009^c$

^aNormal control and PPA and saline; ^bPPA and torasemide and PPA and saline; ^cNormal control and PPA and torasemide.

IL, interleukin; MDA, malondialdehyde; NGF, nerve growth factor; TNF, tumor necrosis factor alpha. Significant values are presented as bold characters.

group ($P = .521$). GFAP immunostaining index (cerebellum) was found to be lower in control group and PPA + torasemide group ($P = .008$, $P = .009$, and $P = .023$, respectively) compared to PPA + saline group. GFAP immunostaining index (cerebellum) was similar between the PPA + torasemide and control groups ($P = .519$). Figures 1, 2, 3, and 4 demonstrate the histopathology examinations (Figure 1-4) (Table 3).

Magnetic Resonance Spectroscopy

Mean lactate value (percentage of normal control) was higher in PPA + saline group compared to control group and PPA + torasemide group ($P = .0003$, $P = .0007$, and $P = .0004$, respectively) and it was higher in PPA + torasemide group compared to control group ($P = .019$) (Figure 5) (Table 3).

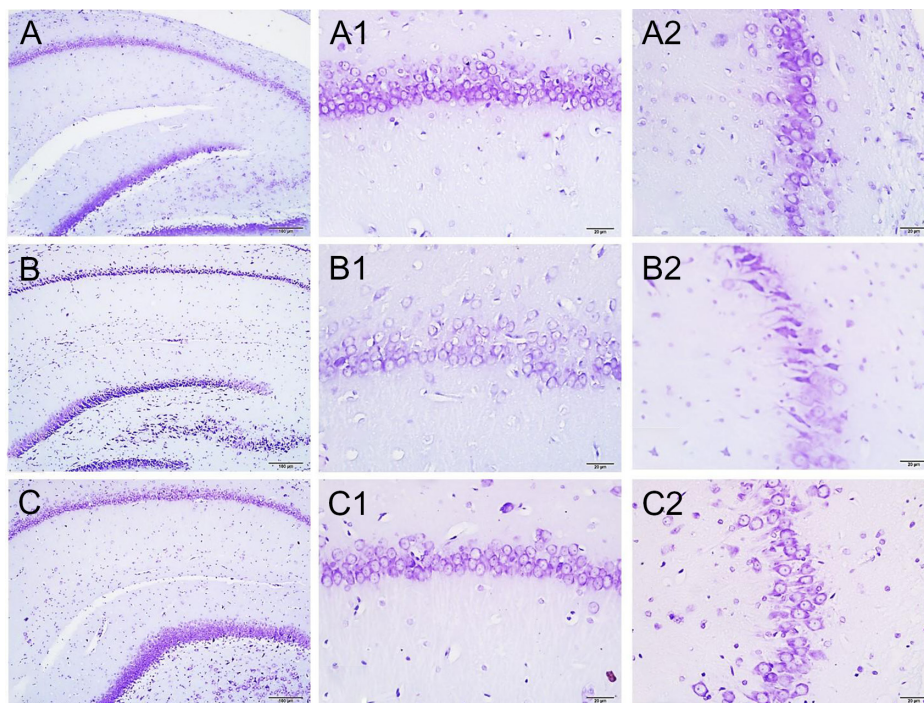


Figure 1. CA3 and CA1 regions of hippocampus in Crystal violet stain 4 at 40× magnification. A-A1-A2: normal control group male rats (CA3 and CA1). Normal pyramidal neuron. B-B1-B2: PPA and saline group male rats have neural body degeneration and decreased neural count and dysmorphological changes (CA3 and CA1). C-C1-C2: PPA and torasemide group male rats have increased neural count and improved neural morphology changes (CA3 and CA1). PPA, propionic acid.

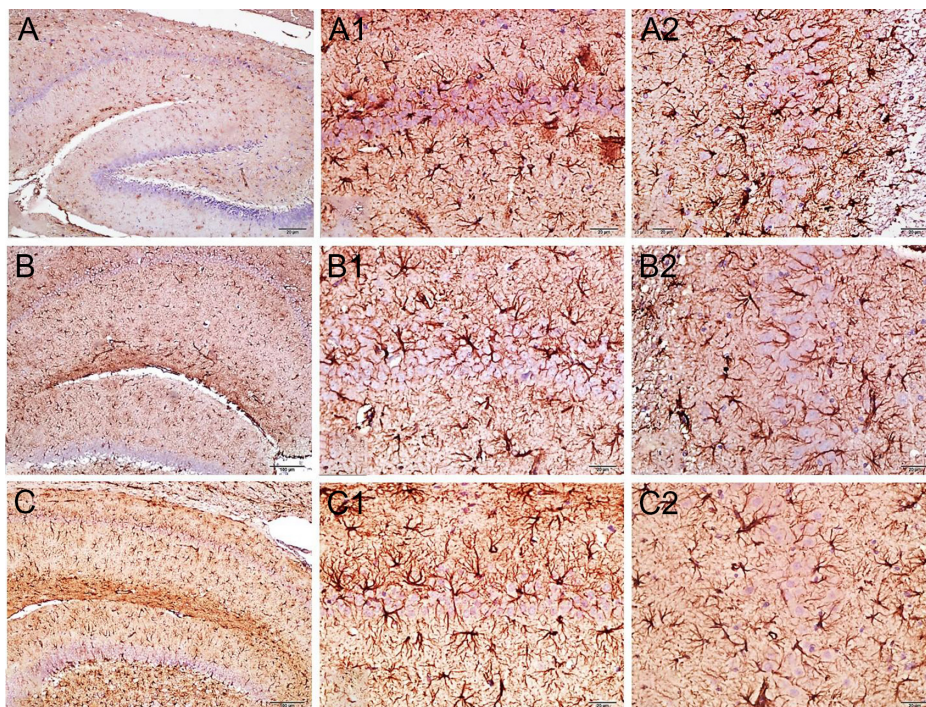


Figure 2. CA3 and CA1 of hippocampus at 40× magnification. Astroglial activity was characterized by GFAP immunostaining (Brown staining). A-A1-A2: normal control group male rats (CA3 and CA1), B-B1-B2: PPA and saline group male rats have increased glial activity (CA3 and CA1). C-C1-C2: PPA and torasemide group male rats have decreased glial activity (CA3 and CA1). PPA, propionic acid.

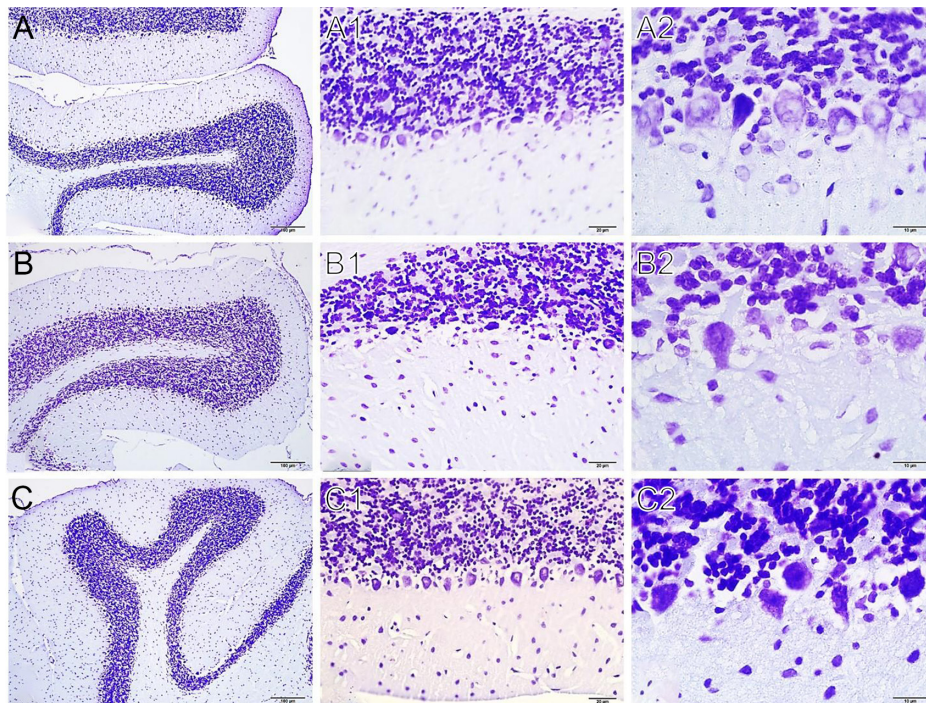


Figure 3. Cerebellum at 4×, 40×, 100× magnifications in crystal violet stain. A-A2-A3: normal control group male rats' cerebellum and normal Purkinje neuron. B-B1-B2: PPA and saline group male rats have decreased count and dysmorphic Purkinje neuron. C-C1-C2: PPA and torasemide group male rats have increased count and improved neural morphological changes Purkinje neuron. PPA, propionic acid.

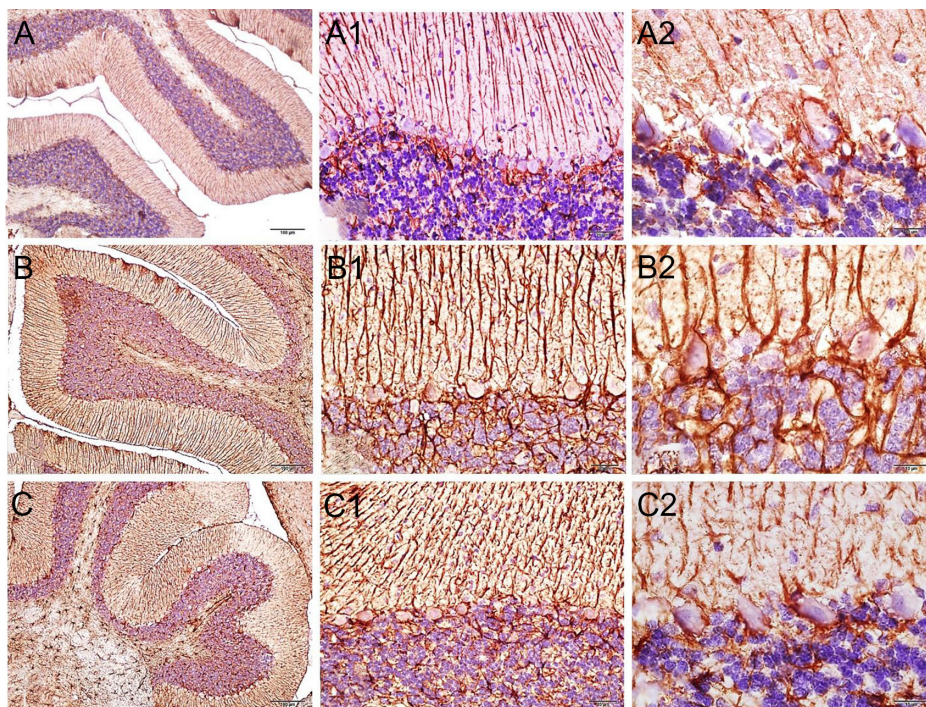


Figure 4. Cerebellum at 4×, 40×, 100× magnifications. Astroglial activity was characterized by GFAP immunostaining (Brown staining). A-A1-A2: normal control group male rats' cerebellum and normal Purkinje neuron. B-B1-B2: PPA and saline group male rats have increased glial activity cerebellum. C-C1-C2: PPA and torasemide group male rats have decreased glial activity cerebellum. PPA, propionic acid.

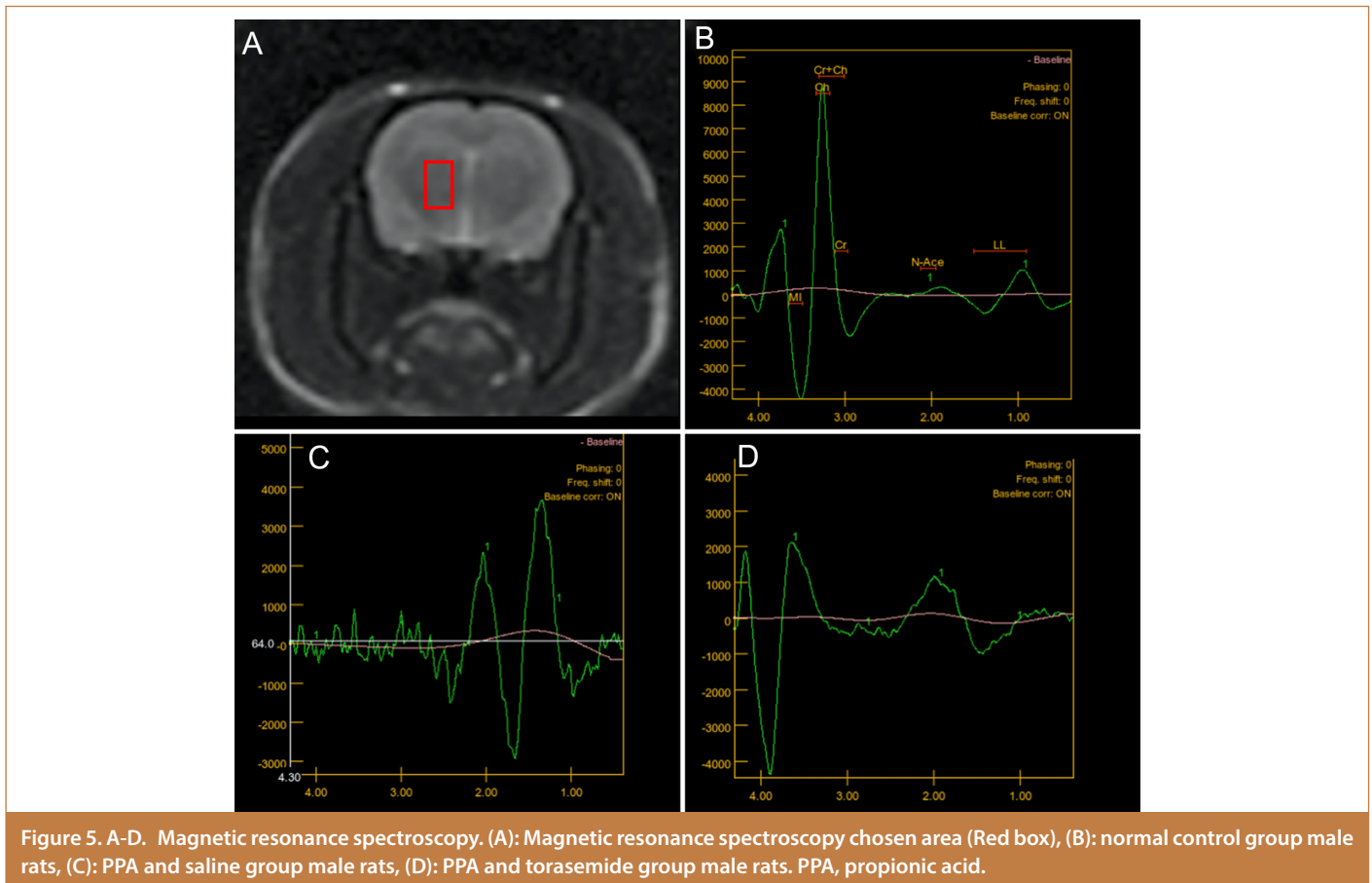
Table 3. Comparison of the Results of Hippocampus and Cerebellum Histopathology and MR Spectroscopy Between Groups

	Normal Control (n = 10)	PPA and Saline (n=10)	PPA and Torasemide (n=10)	Post Hoc Tukey
Neuronal count (CA1)	81.5 (3.3)	35.2 (4.1)	56.5 (4.4)	<i>P</i> = .0006^a <i>P</i> = .013^b <i>P</i> = .003^c
Neuronal count (CA3)	46.3 (1.8)	30.9 (2.2)	45.8 (3.2)	<i>P</i> = .0004^a , <i>P</i> = .0003^b <i>P</i> = .621^c
GFAP immunostaining index (CA1)	34.5 (2.8)	48.5 (5.3)	35.1 (4.5)	<i>P</i> = .0004^a <i>P</i> = .0005^b <i>P</i> = .699^c
GFAP immunostaining index (CA3)	33.9 (3.9)	53.2 (4.8)	43.3 (2.9)	<i>P</i> = .008^a <i>P</i> = .031^b <i>P</i> = .037^c
Purkinje count cerebellum	17.5 (2.3)	11.1 (1.5)	15.2 (0.9)	<i>P</i> = .008^a <i>P</i> = .030^b <i>P</i> = .521^c
GFAP immunostaining index (cerebellum)	16.1 (2.8)	27.7 (1.8)	19.8 (1.7)	<i>P</i> = .009^a <i>P</i> = .023^b <i>P</i> = .519^c
MR spectroscopy lactate value (% of normal control)	100	265.1 (54.8)	149.8 (33.5)	<i>P</i> = .0007^a <i>P</i> = .0004^b <i>P</i> = .019^c

^aNormal control and PPA and saline; ^bPPA and torasemide and PPA and saline; ^cNormal control and PPA and torasemide.

MR Lac, magnetic resonance spectroscopy lactate value (% of normal Control); NC, neuronal count; Purkinje, Purkinje count cerebellum.

Significant values are presented as bold characters.



Discussion

The pharmacological management of ASD does not exist yet and the current medical approaches in the management of ASD are focused on the improvement of behavioral symptoms and co-existing conditions.³⁴ The patients with ASD are currently attempted to be treated with educational and behavioral interventions.³⁵ The available pharmacological treatments are only approved for the improvement of behavioral symptoms.³⁶ Thus, it can be considered that any proof for the treatment of ASD will be of great interest for both individuals who are suffering from ASD and the researchers.

Bumetanide which is initially used as loop diuretic is currently being considered for the treatment of main symptoms of ASD.²²⁻²⁵ In present study, we demonstrated that scores on open field test, sociability test, and passive avoidance learning were significantly higher in torasemide group compared to saline group. Our results indicate that torasemide improved ASD symptoms in experimental conditions. Gamma-aminobutyric acid signaling is the major inhibitor system in CNS and plays significant roles in cognitive functions and in the development of CNS.¹⁸ The GABA neurons are regulated by the levels of intracellular chloride and they are able to be controlled by NKCC1 and KCC2.³⁷ Thus, an imbalance between excitatory and inhibitory neuronal activity is known to be related to impairments in communication skills, language, and sensory perception.³⁸ Increasing the activity of GABA signaling effects was reported to be associated to enhance the activity of NKCC1.³⁹ Hampel et al²¹ compared the pharmacokinetic-pharmacodynamics properties of torasemide, azosemide,

and bumetanide in mouse brain. All 3 drugs were shown to have free plasma levels which were sufficient to block NKCC1 in CNS. They concluded that azosemide and torasemide would be promising alternatives for CNS disorders with their longer half-life as well as their lower diuretic potency. There is no study that investigated the effects of torasemide on ASD. From this point, torasemide is a promising agent for improving the main symptoms of ASD with a higher half-life and without lower side effects.

The experimental model of ASD which was performed by intracerebroventricular delivery of PPA was shown to increase TNF- α , IL-6, and interferon- γ cytokine levels.^{40,41} In the present study, it was also shown that brain levels of MDA, IL 2, IL 17, TNF- α , and NF-KB which are well-known inflammatory factors were remarkably higher in PPA + saline group compared to other 2 groups. Actually, there is not any evidence that indicates the effects of torasemide on neuro-inflammation. However, several studies reported that torasemide had anti-inflammatory and anti-fibrotic effects on heart tissue.^{42,43} Regarding the decreased levels of MDA, IL 2, IL 17, TNF- α , and NF-KB in torasemide group compared to saline group in the present study, it can be suggested that torasemide can have beneficial effects on the neurobiological errors in the brain tissue of ASD via the prevention of neuro-inflammation. Nerve growth factor is one of the well-known neurotrophic factor which plays a particular role in cell death and/or neurodegeneration.⁴⁴⁻⁴⁶ The data which identified the associations between brain NGF level and ASD are restricted. Lu et al⁴⁷ reported that several NGF single-nucleotide polymorphism could be related to deficits in nonverbal communication. We also demonstrated that

torasemide group had significantly higher NGF level than saline group. These results can also be associated with anti-inflammatory action of torasemide.

Numerous evidences indicated that hippocampus is an important part of the brain in terms of etiology of ASD.⁴⁸⁻⁵⁰ Moreover, cognitive dysfunctions were reported to be significantly associated with hippocampal neurotransmission.⁵¹ The networks in amygdala and hippocampus are considered to be the main region for the “social brain.”⁵² Thus, the abnormalities in hippocampus were reported to be significantly related to core symptoms of ASD.⁵³ Evidences demonstrated that abnormality in glial cells could be significantly related to ASD.^{54,55} Particularly, GFAP was reported to be expressed more in the ASD brain compared to healthy controls.⁵⁶ Furthermore, reduced microbiota complexity was shown to be related to impaired microglial proliferation in an experimental model.⁵⁷ Considering our results, torasemide seems to have beneficial effects on cell count as well as microglial formation in PPA-induced autism model.

The cerebellar dysfunctions were reported to be associated with ASD.⁵⁸ However, the exact etiological model cannot be explained yet. The cerebellar abnormalities in patient with ASD were considered to be related to main symptoms of ASD including social, cognitive, language, and movement symptoms.^{59,60} The clinical studies also confirmed the existence of decreased cerebellar volume in patients with ASD.⁶¹⁻⁶³ Purkinje cells also take interest in terms of etiology of ASD.⁶⁴ The reduce in the amount of Purkinje cells in patients with ASD is reported to be related to alterations in cerebellar proteins which are associated with apoptosis (Bcl2 and p53),⁶⁵ astroglial activation (GFAP),⁶⁶ GABAergic signaling pathway (GAD65/67), and glutamatergic synaptic functions.⁶⁷ In the present study, the cerebellar findings in PPA + saline group support the results of previous studies. Furthermore, Purkinje cell lost and GFAP levels were found to be less in PPA + torasemide group. This novel finding provides the enhancing effects of torasemide on GABAergic neurons.

There have been several in vivo studies that investigated the brain lactate levels which is a biomarker of mitochondrial disease in ASD with magnetic resonance spectroscopy (1H).⁶⁸⁻⁷⁰ The most current one showed increased lactate levels in patients with ASD compared to healthy subjects. In this study, it was concluded that mitochondrial dysfunction would be a neurobiological factor in ASD.⁷¹ In the present study, we succeeded to demonstrate increased lactate levels in PPA + saline group in both tissue investigation and MRS imaging.

The comparison of bumetanide and torasemide would give stronger neurobiological, histopathological, and imaging proofs for torasemide. However, bumetanide is not available in our country; thus, we could not compare the effects of bumetanide and torasemide. This issue is a limitation of this study. However, this limitation will be an interest to further clinical and experimental studies.

Conclusion

The present study is the first to show the benefits of torasemide on the main symptoms of ASD experimentally. Our experimental results showed that torasemide might enhance GABA activity. Torasemide can be considered another promising NKCC1 inhibitor in the treatment of autism with a longer half-life and less side effects after further studies.

Ethics Committee Approval: The experimental procedures employed in the present study were certified by the Animal Ethics Committee (Demiroğlu Bilim University, no: 19210702).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.D.; Design – O.E.; Supervision – M.D.; Materials – M.D.; Data Collection and/or Processing – O.E.; Analysis and/or Interpretation – O.E.; Literature Review – M.D.; Writing Manuscript – Y.A.; Critical Review – Y.A.

Declaration of Interests: The authors have no conflicts of interest to declare.

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