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Gender-Related Disparities in induced biochemical autistic features using propionic acid rat model

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ABSTRACT

The dramatical increase in autism spectrum disorder (ASD) prevalence as a neurodevelopmental disorder highlighted the urgent need for early detection and intervention as its symptoms vary across individuals. The question of whether females and males show ASD differently is still up for debate. Research examining the relationship between sex and symptoms of autism and other psychiatric issues typically lacks a general population comparison group, making it difficult to determine whether observed differences are unique to autism or reflect general development patterns. Methods: The present study aimed to understanding sex-related differences in vulnerability to develop autistic features in propionic acid (PPA) induced autism in the rodent model. Thirty-two Wistar albino juvenile male and female rats were divided equally into four groups. Group 1 received saline and served as a control. Group 2 received buffered PPA at 250 mg/kg b.w/day for three consecutive days. While sixteen female Wistar albino juvenile rats were divided into two groups (8 rats/group) as follows: Group 3 received saline and served as a control. While, Group 4 received buffered PPA at 250 mg/kg b.w/day for three consecutive days. Serotonin, dopamine (DA), gamma-aminobutyric acid (GABA) and glutamate were measured. Brain-derived neurotrophic factor (BDNF), caspase 9 and NF-κB were evaluated in all groups. Moreover, Expression Analysis of neurodevelopmental regulator genes (FMR1 and FOXP1) and inflammatory gene (COX-1) was detected. Results**:** PPA administration significantly reduced dopamine, GABA, and FMR1 gene with significant elevation in serotonin, glutamate, BDNF, caspase 9, NF-κB and Expression of FOXP1 and COX-1 genes. Conclusion: Our research highlighted different biochemical parameters that can be promising biomarkers for ASD. the study also discussed sex differences found in animal models of ASD, to provide a possible explanation of the neurological mechanisms underpinning the different presentation of autistic symptoms in males and females.

Keywords: *Autism, Sex differences, Propionic acid (PPA), Neurodevelopmental regulator genes, Neurotransmitters.*

Introduction:

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that alters attitude, connecting with others, and social interaction [1]. When seen from a behavioral point of view, ASD is also identified by restricted and repetitive activities along with social-communication difficulties. The core defining features that characterize individuals with ASD are represented by these two behavioral dimensions in the most recent conceptualization of the disorder. ASD's diversity can be illustrated by using related factors like cognitive and language abilities [2]. The term "spectrum" refers to the distribution of the frequency of a particular difficulty in social communication and interaction, which fluctuates in severity of manifestation and throughout time. This indicates that the autism spectrum encompasses individuals with diverse clinical traits. Every person with autism is distinct, as there are various combinations of this syndrome, which appears structurally similar but differs in symptom intensity, quality, and quantity [3]. In individuals with ASD, imaging and post-mortem examinations have revealed that the cerebellum is one of the primary brain regions affected, exhibiting a reduction in volume and a decrease in the number of Purkinje cells (PCs) [4].

Over the years, reports have indicated an increase in the prevalence of ASD, with significant variations in prevalence observed among different countries [1]. The ASD prevalence increased dramatically to reach 1:36 in 2020 [5], with male-to-female ratio 4.5:1 [6] The rise in ASD prevalence could be attributed to many factors, such as increased awareness, a rise in associated risk factors, the widespread use of new screening tools, and the young age at which children are first evaluated by health professionals [7].

However, research has consistently shown that males are more likely than females to have attention deficit/hyperactivity disorder and other developmental disorders, so a male preponderance is not exclusive to ASD [8]. Compared to males, females with autism are believed to exhibit a greater capacity for forming traditional friendships, relatively higher social motivation, fewer externalizing behaviors (hyperactivity/impulsivity and conduct problems), and fewer repetitive and stereotyped behaviors. They are also thought to experience more internalizing problems, including anxiety, depression, and eating disorders [9].In addition, it is suggested that females are more likely than males to conceal their autistic traits by employing compensatory strategies and masking, also known as "camouflaging," in order to navigate social environments [10]. As a result, autistic females are more likely to be misdiagnosed primarily with internalizing or personality disorders or diagnosed later compared to autistic males. This is noticed in females at the milder end of ASD [11], where the autistic symptoms are difficult to distinguish from other forms of psychopathology or extremes of typical behavior [12]. On the other hand, there is growing awareness of the 'female protective effect' (FPE) resulting in presence of two X chromosomes that provide genetic redundancy, [13], which suggests that females may have high genetic resistance to developing ASD and may require more etiologic load to diagnose the behavioral impairment due to genetic and hormonal factors [14].

Gamma-aminobutyric acid (GABA) and glutamate are essential neurotransmitters that preserve the excitatory-inhibitory balance in the brain. ASD is one of the neurological conditions that may arise from an imbalance between these systems [15]. On the other hand, Dopamine (DA) is responsible for social behavior, social communication and movement control [16], while Serotonin plays a critical role in cortical proliferation, cell division, cortical plasticity, and synaptogenesis. Abnormal dopamine and serotonin levels have been reported in ASD

[15,16]. brain-derived neurotrophic factor (BDNF) participates in different neurophysiological processes and is found in nearly all brain regions. The most critical functions of BDNF is involved in the regulation of glycogenesis, neurogenesis, and synaptogenesis, as well as neuroprotection and control of short- and long-duration synaptic interactions that affect memory and cognition [17]. While caspase-9 was not much studied in ASD, high level of caspase 9 was reported to impairs normal brain maturation and induces autistic phenotype and may be associated with remarkable mitochondrial dysfunction as an etiopathological mechanism of ASD [18]. ASD pathophysiology is influenced by inflammatory responses in the neocortical anterior regions. Furthermore, astrocytes and microglia activation are correlated with cognitive activities has led to neuroinflammatory responses in these patients [19]. It has been demonstrated that nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) controls the production of pro-inflammatory cytokines and the response to external stress. Patients with ASD have abnormally high levels of NF-κB, particularly in highly activated microglia, and this protein plays a part in the molecular cascade that leads to neuroinflammation, especially in inhibitant immune cells in brain regions associated with behavioral abnormalities in ASD [20].

FMR1 gene is located on chromosome X and it is associated with Fragile X syndrome (FXS) which is the most common single-gene disorder associated with ASD. This support the reason of severe manifestations in ASD males than females [21]. While, FOXP1 gene is found extensively in the hippocampus, neocortex and striatum during development and maturation. FOXP1 mutations have been noticed in patients with intellectual disabilities and ASD, as well as language deficiency [22]. COX enzymes are implicated in the regulation of hippocampus cell proliferation, neurogenesis, synaptic plasticity and the maintenance of the blood-brain barrier. moreover, they play critical roles in modulation of inflammation and immune response. Polymorphisms in COX genes have also been linked to ASD [23]

Animal studies have shown that administering propionic acid (PPA) through brain infusion or oral dosing in rat pups can mimic many biochemical features observed in individuals with autism. [15,24]. In addition, behavioral characteristics have been recorded, including hyperactivity, poor social interaction, decreased exploratory activity, and increased repetitive behaviors, along with histopathological alterations like neuronal loss, astrogliosis, and hyaline bodies [15,24]. PPA accumulation within cells results in intracellular acidification. which inhibits gap junctions, alters neurotransmitter release, and promotes intracellular calcium release. These changes can impair neuronal communication and behavioral disorders [25]**.** High PPA levels are also accompanied by oxidative stress, developmental delays, and immune or metabolic disturbances similar to those of autism [26]. Therefore, our study aimed to evaluate the variation in different biochemical parameters among male and female autistic rats, through determination of Serotonin, dopamine, gamma-aminobutyric acid (GABA) and glutamate .Also, BDNF, caspase 9 and NF-κB were evaluated in all groups along with expression Analysis of neurodevelopmental regulator genes (FMR1 and FOXP1) and inflammatory gene (COX-1).

1. Material and methods:

1.1. Animals:

Total of thirty-two (sixteen male and sixteen female) Wistar albino juvenile rats (3-4 weeks old, 31-45 g) were obtained from the animal house of the National Research Center, Dokki, Giza, Egypt. They were fed a standard diet supplied by El-Nasr Pharmaceutical Company, Egypt. All national, international, and institutional guidelines for animal care and

use were followed. Animals were individually housed at a controlled temperature (21±1°C) with ad libitum access to food (Prolab rat chow) and tap water.

1.2. Ethical approval:

The protocol was approved by the Medical Research Ethics Committee (MREC), National Research Centre, Dokki, Giza, Egypt, (Approval No. 1244052021). The National Institutes of Health's Guidance for the Care and Use of Laboratory Animals was followed during the procedures (NIH Publication No. 85-23, revised 1996). The experiment's layout complied with the ARRIVE 2.0 recommendations.

1.3.Experimental design:

Autism was induced by administering buffered PPA (250 mg/kg b.w/day) for 3 days, according to Alfawaz et al. [27]. The experiment continued for 30 days. Sixteen male Wistar albino juvenile rats were divided into two groups (8 rats/group) as follows: Group 1 received saline and served as a control. Group 2 received buffered PPA at 250 mg/kg b.w/day for three consecutive days. While Sixteen female Wistar albino juvenile rats were divided into two groups (8 rats/group) as follows: Group 3 received saline and served as a control. While, Group 4 received buffered PPA at 250 mg/kg b.w/day for three consecutive days.

1.4. Preparation of blood sampling:

At the end of the 30-day experiment, the animals were fasted for 12-14 hours. Blood samples were taken in clean, dry from orbital plexus of eyes, sterilized centrifuge tubes. The samples were centrifuged for 10 minutes at 4 °C at 3000 rpm to obtain serum, which was stored at -80 °C for biochemical analysis.

1.2. Brain tissue sampling:

After blood collection, rats were sacrificed by decapitation. The brain tissue was rapidly removed, perfused with ice-cold isotonic saline to remove blood clots, blotted on filter paper and weighed for biochemical analysis. The tissue was immediately stored at -80 °C for biochemical analyses.

2.6. Determination of Brain neurochemistry and neurotransmitters:

2.6.1. Determination of Dopamine and Serotonin levels in brain tissue:

Dopamine, and serotonin levels were measured in brain tissue homogenate samples using an Agilent Technologies 1100 series (Santa Clara, California, USA) HPLC system with a quaternary pump (G131A model).

2.6.2. Determination of GABA and Glutamate (GLU) levels:

As directed by the manufacturer, an ELISA assay kit (My BioSource, USA) (Cat No. MBS269152) was used to detect the amount of gamma-aminobutyric acid (GABA) in serum. Glutamate (GLU) levels were also measured in serum using an ELISA test kit (Cat. No. MBS756400) provided by MyBioSource (USA) according to the manufacturer's instructions.

2.7. Measurement of Serum BDNF Levels: Serum brain-derived neurotrophic factor (BDNF) levels were quantified using a commercial ELISA kit (Cat. No. E1302Hu; Biosensis Mature BDNF ELISA Kit, Thebarton, Australia) according to the manufacturer's instructions. All samples, reagents, equipment, and standard dilutions were prepared as recommended. The optical density (OD) was measured within 10 min using a microplate reader. Each sample was assayed in duplicate, and the mean value was used for statistical analysis

2.8. Assessment of caspase-9

The concentration of caspase-9 was quantified using a commercial ELISA kit (CUSABIO®, Germany) in serum according to the manufacturer's instructions. This assay is

based on a competitive enzyme immunoassay principle and the absorbance was measured at 450 nm using a microplate reader. Caspase-9 levels in the samples were calculated by comparing the optical density (OD) values with the standard curve provided in the kit.

2.9. Determination of NF-κB level

Serum NF-κB levels were quantified using a commercial ELISA kit (Cat. No. SEA616Ra; Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm within 30 minutes using a Kinetic Microplate Reader (Molecular Devices). The mean optical density (OD) values of duplicate wells were calculated and plotted against the standard concentrations to determine NF-κB levels in the tested samples.

2.10. Expression Analysis of neurodevelopmental regulator genes (FMR1 and FOXP1) and inflammatory gene (COX-1):

Total RNA was isolated from rat's brain tissues by the standard TRIzol® Reagent extraction method (Invitrogen, Germany). Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis [28]. Aliquots were used immediately for reverse transcription (RT), otherwise stored at -80 °C.

Reverse transcription (RT) reaction

RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) was used to obtain the cDNA copy of rat tissue samples. The complete Poly(A)$^+$ RNA isolated from tissue samples was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany).

The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C [29]. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through Real Time polymerase chain reaction (RT-PCR).

Real Time-Polymerase Chain Reaction (RT-PCR)

StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the tissue samples of rat's copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template [30,31]. The sequences of specific primer of the genes used were designed and listed in Table 1. The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method [32].

Table [1]: Primers sequence used for qRT-PCR

Gene	Primer sequence	NCBI Reference
FMR1	F: cct ggg gtc act gct att ga	NM_001431612.1
	R: tca ccc tca caa ctc ctg ac	
FOXP1	F: tta ctt ccg acg caa tgc ag	NM_001034131.2
	R: tca gcc atg gaa gcc tgt aa	
COX-1	F: acc cac ctt ccg tag aac ag	S67721.1
	R: ttc agc aag tca cac aca cg	

GAPDH	F: aac gac ccc ttc att gac ct	XM_063285518.1
	R: ccc cat ttg atg tta gcg gg	

FMR1: fragile X messenger ribonucleoprotein 1; FOXP1: forkhead box P1; COX-1: cyclooxygenase isoform; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

2.13. Statistical Analysis

Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 20 software package (USA). Results were expressed as mean ± S.D of 8 rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), combined with the Costat software computer program, where unshared letters are significant at P value ≤ 0.05.

3. Results:

3.1. Brain neurochemistry and neurotransmitters:

3.1.1. Dopamine and Serotonin levels in brain tissue:

Our study revealed a significant decrease in brain dopamine levels compared to the control groups (NC♂ and NC♀). In addition, normal male rats (NC♂) exhibited high ($p<0.05$) brain dopamine levels, as compared to normal control female rats (NC ♀), while PPA female rats showed the higher improvement as illustrated in table (2).

Regarding brain serotonin significant elevation ($p<0.01$) was recorded in the different experimental groups, compared to normal control groups (NC♂ and NC♀). In addition, normal control male rats (NC♂) showed significant lower level of serotonin compared to normal control female rats (NC ♀).

3.2.2. GABA and Glutamate levels:

Data in Table (2) illustrate significant elevation ($p<0.001$) in serum glutamate levels in PPA induced groups, compared to normal control groups (NC♂ and NC♀). Unlikely, serum gamma amino butyric acid (GABA) levels were decreased autistic groups, compared to normal control (NC♂ and NC ♀). In addition, normal control male rats (NC♂) showed significant elevation ($p<0.05$) in serum GABA levels compared to normal control female rats (NC ♀).

3.3. Serum BDNF, caspase-9 and NF-κB levels:

Our results showed that levels of BDNF, Caspase-9 and NF-κB were increased significantly (6.31±0.30, 3.65±0.28 and 74.15±3.66, respectively) in serum samples of PPA male rats versus control male rats (1.54±0.17, 1.84±0.24 and 27.79±2.60, respectively).

In the same trend, the levels of BDNF, Caspase-9 and NF-κB were increased significantly (5.47±0.35, 2.58±0.24 and 58.93±4.21, respectively) in serum samples of PPA female rats versus control female rats (1.40±0.15, 1.72±0.22 and 25.86±3.10, respectively) (Figs. 1-3).

3.4. Expression Analysis of FMR1 gene:

The expression levels of the FMR1 gene in brain of male PPA rats were lower (0.52±0.044) than those in male control rats (1.00±0.033). The expression levels of the FMR1 gene brain of male PPA rats were down-regulated by 52% as compared to brain control rats (Figure 4).

In the same trend, the expression levels of the FMR1 gene in brain of female PPA rats were lower (0.74±0.049) than those in female control rats (1.00±0.046). The expression levels of the FMR1 gene brain of female PPA rats were down-regulated by 74% as compared to brain control rats.

3.5. Expression levels of the *FOXP1* and *COX-1* genes:

The expression levels of the *FOXP1* and *COX-1* genes in brain of male PPA rats were higher (5.27±0.219, 3.88±0.185, respectively) than those in male control rats (1.00±0.102, 1.00±0.074, respectively).In the same trend, the expression levels of the *FOXP1* and *COX-1* genes in brain of female PPA rats were higher (4.34±0.184, 3.06±0.153, respectively) than those in female control rats (1.00±0.089, 1.00±0.086, respectively) (Figs. 5&6).

2.3.1. Discussion:

This study helps to explain the sex difference in ASD through highlighting the susceptibility of male to develop glutamate excitotoxicity, impaired dopamine signaling, increase in BDNF, caspase 9, NF-κB levels along with elevation in expression of FOXP1 and COX-1 genes than females.

In the current study, oral administration of male and female rats with propionic acid (PPA♂ and PPA♀) induced biochemical autistic features compared to control group (NC♂ and NC♀). This result is in agreement with Akbulut et al. [33] who reported that the using of PPA for induction of ASD represent one of the most approved and widely used in experimental model as it induced autistic features similar to that of human [33,34].

Data shown in table (2) showed a significant elevation in serotonin level along with depletion in dopamine level in both male and female groups after administration with PPA compared to normal untreated control rats. This observed elevation in serotonin is an indicative to their respond to chemical stimulation (PPA) and the increased signals from gut microbiota to release serotonin as a response to PPA administration [35]. This result is in agreement with the previous studies of Ali et al. [36], who reported the depletion in dopamine level in PPA intoxicated rats compared to normal rats [37]. The decreased level reflects its role in behavior flexibility and cognitive control. The reduction of DA level is associated with attention-deficit hyperactivity disorder which is obvious in ASD children. This is in line with Bhat et al. [38], who noticed decrease in dopamine level in rodent model of autistic rats. also, we recorded a significant increase in DA level in male groups than female groups. This result is in parallel with Munro et al., [39] who documented that men had markedly greater dopamine release than women in the striatal regions this may be resulting from the elevation of striatal dopamine receptors in males than females [40]. The significant elevation in serotonin level in female groups compared to female groups is in the same line with Weiss et al., which may be due to serotonin-metabolizing monoamine oxidases (MAO-A and MAO-B) are encoded by genes on the X chromosome and are good functional candidates to influence serotonin level [41].

Data in the present study (table 2) demonstrated significant elevation in glutamate with decline in GABA levels in PPA induced biochemical autistic features in rats compared to normal untreated male and female groups. Similar effect was found by Paudel et al. [42], who found PPA treated rats showed significant decreased in GABA and increased glutamate concentration in brain as compared to control group, reflecting the role of excessive glutamate in activation excitotoxicity damage neurons and reduce synaptic plasticity[42]. The elevation of reactive oxygen species (ROS) resulting from PPA administration has been shown to decrease the activity of glutamate transporter, weakening synaptic clearance of glutamate associated with the increase in the levels of extracellular glutamate [42,43]. Our results noticed higher glutamate level with depletion in GABA level in female groups compared to male groups. This may be due to the effect of progesterone in increases the expression of transporters GLT-1 and EAAT3 which are responsible for glutamate uptake [44]. While males were found to have

significantly higher levels of messenger RNA levels of glutamate decarboxylase, the rate-limiting enzyme in GABA synthesis [45]. In parallel with heffield et al., [46]. A novel finding showing concentration of GABA in women is actually less than that of men, explaining discrepancy seen between mood disorders, which are more prevalent in females and appear to correlate with low cortical GABA. Our results are not in accordance with Al-Suwailem et al. [47] who reported that female rats exhibited significantly lower levels of Glu that males.

BDNF level in PPA rats compared to normal control groups along with non-significant change between male and female groups. In the same line, Barbosa et al., [17] documented. The average BDNF serum concentration level was statistically higher for children with ASD ($P < 0.000$) compared to the control group. This may be contributed to the role of BDNF in the pathophysiology of ASD development and evolution [17]. Moreover, the enhanced BDNF response in some individual with autism is thought to represent an immune cell response that is dysregulated in ASD. Elevated BDNF levels may therefore contribute to neuronal synaptic dysfunction [48].

A significant increase in caspase-9 level in PPA intoxicated rats compared to normal control groups with significant increase in PPA male group compared to female PPA rats was observed in our study. Also, El-Ansary et al., [18] reported the same result along with depletion in GABA level. Caspase 9 as pro-apoptotic proteins can be related to neuronal excitation, and imbalance of the inhibition system, which can be used as reliable predictive biomarkers for ASD [18]. The significant increase in PPA male compared to female PPA rats may be related to the female protective effect in ASD.

A significant elevation in NF-κB level was noticed after administration of PPA in both male and female groups. Naik et al. [49] documented significant increase in NF-kB DNA binding activity in peripheral blood samples of children with autisms. This may be corelated to the production of ROS as it plays a role in activation of NF-κB [49]. Elevated amounts of NF-kB in children with autism can strengthen the conceptual frameworks of the role of innate immunity and ROS in the etiopathology of this condition in agree with Doğan et al. [50].

A noticed significant depletion in FMR1 expression in PPA rats compared to normal male and female groups with a significant decrease in male PPA rats compared to the females. Also, Fyke & Velinov [21] reported that Multiple pathways regulated by FMRP are found to be dysfunctional in ASD patients who do not have fragile X syndrome (FXS) [21]. This may be because FMR1 is associated with FXS which is the most common single-gene disorder associated with ASD. The loss of this protein disrupts synaptic functioning, leading intellectual disability, social anxiety, speech and language delays, and repetitive behaviors. The significant lower expression in autistic male rats than females resulting from having a single "X" chromosome while, Females with FXS may experience a milder phenotype due to the presence of a second, typically functioning X chromosome [51].

FOXP1 is abundant in the neocortex, hippocampus and striatum during development and maturation. *FOXP1* mutations have been seen in a small number of human patients like intellectual disabilities and ASD, as well as language deficiency [22]. Chien et al. [52] reported significant elevation of the mRNA level of *FOXP1* in patients with ASD compared to that in the control group [52]. Our finding suggests that increased *FOXP1* expression may be involved in the pathogenesis of ASD.

COX enzymes are essential for the production of prostaglandins, which are significant regulators of inflammation and neuronal activity in the brain. Changes in the

expression or function of the COX gene could interfere with the development of the nervous system, leading to the development of ASD [23]. Our results showed an increase in COX gene level in autistic rats compared to normal groups which reflects its role in neuroinflammation. In the same line Choi et al., [53] administered the COX-1 inhibitor to Alzheimer's disease mice, which progressively develop extracellular amyloid plaques, intracellular neurofibrillary tangles, and cognitive impairment leads to significantly reduces amyloid deposits, tau hyperphosphorylation, and neuroinflammation, and ameliorates cognitive deficits [53].

The significant elevation in Caspase-9 and NF-κB levels with *FOXP1 and COX1* gene expression in male autistic rats compared to female autistic rats support the female protective effect among people with ASD. Our findings suggested that females are less susceptible to develop ASD.

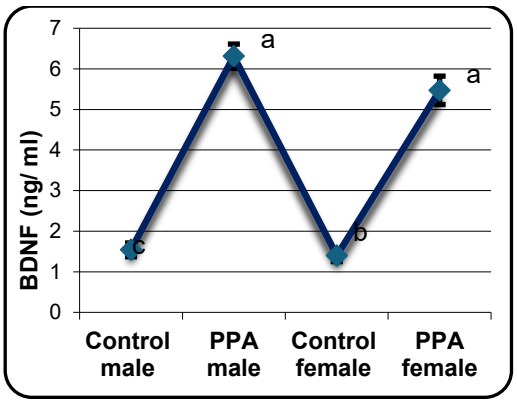
6. Conclusions

This comprehensive scientific and statistical analysis highlighted different biochemical parameters that can be promising biomarkers for ASD. the study also discussed sex differences found in animal models of ASD, to provide a possible explanation of the neurological mechanisms underpinning the different presentation of autistic symptoms in males and females. Our data showed that PPA administration significantly reduced dopamine, GABA, and FMR1 gene with significant elevation in serotonin, glutamate, BDNF, caspase 9, NF-κB and Expression of FOXP1 and COX-1 genes. These data further bring evidence for supporting "female protective effect".

Table 2: Statistical significance of dopamine, serotonin glutamate and GABA levels among the different experimental groups.

Groups	Dopamine (ng/g tissue)	Serotonin (µg/g tissue)	Glutamate (ng/ml)	GABA (pg/ml)	GABA/Glu ratio
NC male	5.91± 0.02[a]	4.56 ± 0.25[d]	37.36 ±0.37[d]	19.53± 0.25[a]	0.522
PPA male	3.58 ± 0.05[c] ↓	7.2± 0.26[b] ↑	119.33 ±0.35[b] ↑	7.17 ± 0.14[c] ↓	0.06
NC female	5.6 ± 0.02[b]	5.29 ± 0.09[c]	42.3 ±0.3[c]	17.6 ±0.26[b]	0.416
PPA female	3.30 ± 0.07[d] ↓	8.44 ± 0.02[a] ↑	133.73 ± 0.25[a] ↑	6.23± 0.15[d] ↓	0.046

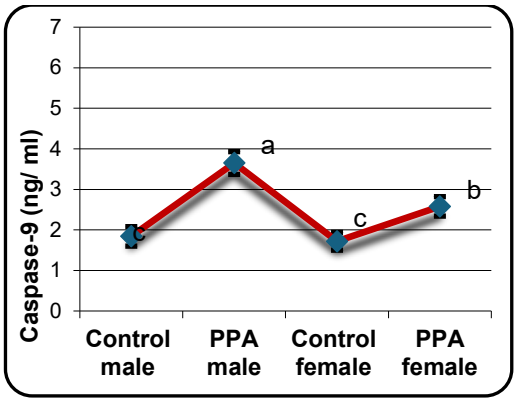
- NC: normal control; PPA: Propionic acid.
- The same superscript letters mean non-significant.
- Least significant difference (LSD) at $p<0.05$



Treatment	Mean	SD	% of control
Control male	1.54	0.30	100.0
PPA male	6.31↑	0.51	409.7↑

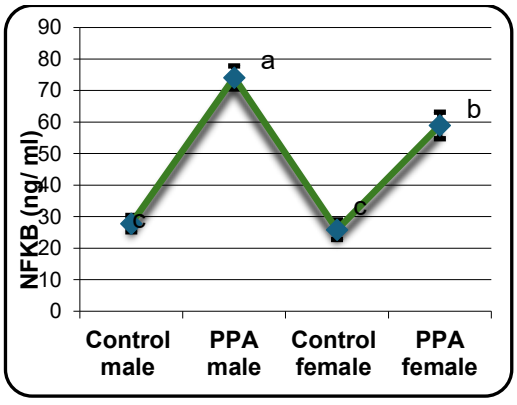
Control female	1.40	0.25	100.0
PPA female	5.47↑	0.61	390.7↑

Fig. 1: BDNF concentrations in serum of control and autism rats (male and female), Data are presented as mean ± SD. Means with different superscripts[(a,b)] between groups are significantly different at $p \leq 0.05$.



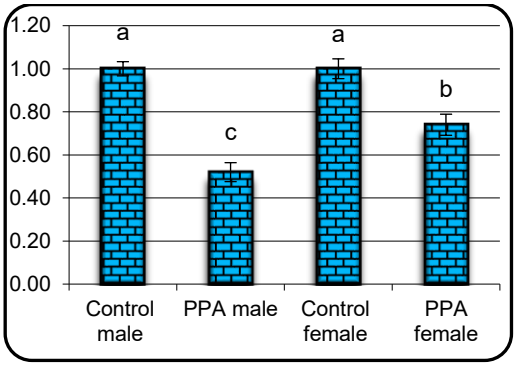
Treatment	Mean	SD	% of control
Control male	1.84	0.41	100.0
PPA male	3.65↑	0.48	198.4↑
Control female	1.72	0.39	100.0
PPA female	2.58↑	0.41	150.0↑

Fig. 2: Caspase-9 levels in serum of control and autism rats (male and female), Data are presented as mean ± SD. Means with different superscripts[(a,b,c)] between groups are significantly different at $p \leq 0.05$.



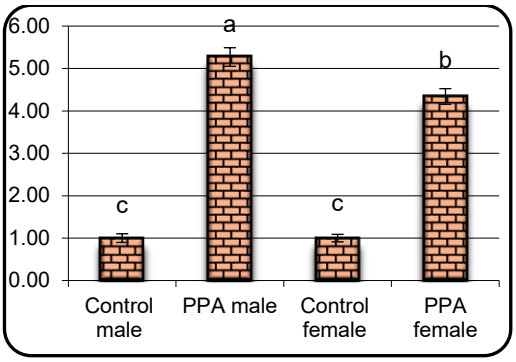
Treatment	Mean	SD	% of contr
			ol
Control male	27.79	4.51	100.0
PPA male	74.15↑	6.33	266.8↑
Control female	25.86	5.36	100.0
PPA female	58.93↑	7.30	227.9↑

Fig. 3: NF-κB levels in serum of control and autism rats (male and female), Data are presented as mean ± SD. Means with different superscripts[(a,b,c)] between groups are significantly different at $p \leq 0.05$.



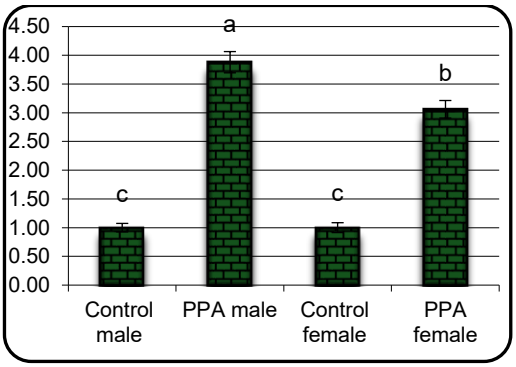
Treatment	Mean	SD	% of control
Control male	1.00	0.06	100.0
PPA male	0.52	0.08	52.0
Control female	1.00	0.08	100.0
PPA female	0.74	0.09	74.0

Figure 4: The expression alterations of *FMR1* gene in brain of control and autism rats (male and female). Data are presented as mean ± SD. [a,b,c:] Mean values within treatment with unlike superscript letters were significantly different ($P<0.05$).



Treatment	Mean	SD	% of control
Control male	1.00	0.18	100.0
PPA male	5.27	0.38	527.0
Control female	1.00	0.15	100.0
PPA female	4.34	0.32	434.0

Figure 5: The expression alterations of *FOXP1* gene in brain of control and autism rats (male and female). Data are presented as mean ± SD. [a,b,c:] Mean values within treatment with unlike superscript letters were significantly different (P<0.05).



Treatment	Mean	SD	% of control
Control male	1.00	0.13	100.0
PPA male	3.88	0.32	388.0
Control female	1.00	0.15	100.0
PPA female	3.06	0.26	306.0

Figure 6: The expression alterations of *COX-1* gene in brain of control and autism rats (male and female). Data are presented as mean ± SD. [a,b,c:] Mean values within treatment with unlike superscript letters were significantly different (P<0.05).

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