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EFFECT OF OMEGA-3 SUPPLEMENTATION ON OXIDATIVE STRESS, INFLAMMATION AND GUT–BRAIN AXIS DISRUPTION IN PROPIONIC ACID-INDUCED AUTISM IN YOUNG RATS

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Abstract

This study examined the omega-3 supplementation effects on oxidative stress, inflammatory biomarkers, gut permeability-related markers, serum neurotransmitters, and short-chain fatty acids concentration in the propionic acid (PPA) (250 mg/kg/day) induced autism rat model. The PPA Group showed significantly increased lipid peroxide compared to other groups and reduced glutathione levels compared with Omega-3 and PPA. IL-6 was significantly higher in the PPA than in the Omega-3 Group. Serotonin levels were significantly lower in the PPA than in the Control and Omega-3 Group. The PPA + Omega-3 Group had significantly lower lipid peroxide and higher glutathione levels than the PPA Group. Histological assessment showed reduced neuronal and villous degeneration in the PPA + Omega-3 Group. Correlation and regression analyses showed associations among selected oxidative stress, gut permeability-related markers, SCFA, inflammatory, and serum neurotransmitter markers, but these findings should be interpreted as exploratory. Overall, in PPA-exposed rats, omega-3 supplementation primarily improved oxidative stress markers, but exhibited limited effects on gut permeability, short-chain fatty acids (SCFAs), and neurotransmitters.

Rezumat

Studiul a investigat efectele suplimentării cu acizi grași Omega-3 asupra stresului oxidativ, a biomarkerilor proinflamatori, a markerilor asociați permeabilității intestinale, a neurotransmițătorilor serici și asupra concentrației de acizi grași cu lanț scurt într-un model de autism indus cu acid propionic (PPA, 250 mg/kg/zi) la șobolani. Grupul PPA a prezentat niveluri semnificativ crescute de peroxizi lipidici comparativ cu celelalte grupuri și niveluri reduse de glutation comparativ cu grupurile tratate cu Omega-3 și PPA + Omega-3. Concentrațiile de IL-6 au fost semnificativ mai mari în grupul PPA decât în grupul Omega-3. Nivelurile de serotonină au fost semnificativ mai scăzute în grupul PPA comparativ cu grupurile control și Omega-3. Grupul PPA + Omega-3 a prezentat niveluri semnificativ mai reduse de peroxizi lipidici și niveluri mai crescute de glutation comparativ cu grupul PPA. Evaluarea histologică a evidențiat o reducere a degenerării neuronale și a vilozităților intestinale în grupul PPA + Omega-3. Rezultatele experimentale au arătat că la șobolanii expuși la PPA, suplimentarea cu Omega-3 a îmbunătățit în principal markerii stresului oxidativ, dar a avut efecte limitate asupra permeabilității intestinale și a nivelurilor neurotransmițătorilor.

Keywords: omega-3 fatty acids, autism spectrum disorder, propionic acid, intestinal permeability, neuroinflammation, oxidative stress, neurotransmitters

Introduction

The microbiota-gut-brain axis describes bidirectional communication between the gastrointestinal tract and the central nervous system through neural, immune, endocrine, and metabolic pathways (1). This interaction is mediated partly through vagal and enteric signalling, hypothalamic-pituitary-adrenal axis activity, immune mediators, and microbial metabolites that can influence neuroimmune function and neurotransmission (2, 3). Experimental studies in rodents have shown that alterations in gut microbiota during development can affect stress responses, social behaviour, and synaptic signalling, supporting the relevance of this axis in models of neurodevelopmental disturbance (4, 5).

Autism spectrum disorder (ASD) is a diverse neurodevelopmental disorder characterized by impaired social communication and restricted or repetitive behaviour.

Individuals with ASD frequently report gastrointestinal symptoms, and some studies have associated these symptoms with behavioural severity (6). Potential contributors to autism-related phenotypes have been explored, including changes in the gut microbiota, microbial metabolites, intestinal barrier dysfunction, immune activation, oxidative stress, and neurotransmitter imbalance, but the mechanisms are not completely defined (7, 8).

Propionic acid (PPA) is a short-chain fatty acid produced by gut bacteria that has been experimentally used to induce autism-like biochemical and behavioural changes in rodents (9). Models based on PPA have been linked to oxidative stress, inflammatory changes, altered monoamine neurotransmission, and changes in gut-related markers (10, 11). Even though these models do not replicate the full complexity of ASD, they provide a useful framework to examine selected biological pathways that may link gut-derived metabolic disturbance with neurochemical and inflammatory changes.

Omega-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid and docosahexaenoic acid, are involved in neuronal membrane composition, lipid mediator production, and regulation of inflammatory signalling (12). Former investigations have demonstrated that omega-3 fatty acids may influence oxidative stress, cytokine responses, gut barrier-related markers, and microbial composition (13). Clinical studies in ASD have reported variable findings, and the biological pathways through which Omega-3 supplementation may affect gut–brain axis–related outcomes remain unclear (14).

Despite growing interest in nutritional approaches in neurodevelopmental models, limited evidence is available on whether omega-3 supplementation affects oxidative stress, inflammatory biomarkers, neurotransmitter levels, gut permeability-related markers, and short-chain fatty acid concentrations within the same PPA-induced model. Therefore, the present study evaluated the effects of omega-3 supplementation on selected oxidative stress, inflammatory, neurotransmitter, gut permeability-related, and microbial metabolite markers in young rats exposed to PPA.

Materials and Methods

Animals and housing

A total of 24 male weaning Wistar Albino rats, weighing 50 - 100 g, were obtained from the Experimental Animal Care Centre, King Saud University, Riyadh, Saudi Arabia, and acclimatized for one week before experimentation. Rats were housed in polypropylene cages under standardized environmental conditions: temperature, 24 - 25°C; relative humidity, 45 - 55%; and a 12-h light/dark cycle. Food and tap water were provided *ad libitum*. All experimental procedures were conducted in accordance with the relevant ethical guidelines for the care and use of laboratory animals (KSU-SE-25-6).

Diet

Throughout the adaptation and experimental periods, rats were fed a standard pellet diet prepared according to the Saudi Grains Organization (SAGO) formulation (F-1005). The composition included crude protein (20%), crude fat (4%), crude fibre (3.5%), calcium (1.0%), phosphorus (0.06%), ash (6.0%), salt (0.50%), vitamin A (20 IU/g), vitamin D (2.20 IU/g), vitamin E (70 IU/kg), and trace minerals (cobalt, copper, iodine, iron, manganese, selenium, zinc). The metabolizable energy content was 2850 kcal/kg.

Experimental design and treatments

Following adaptation (7 days), rats were randomly divided into two groups, namely Group 1 (Healthy/ Normal rats) and Group 2 (Autistic rats). The ASD-like features in group 2 were induced by administering a neurotoxic dose of PPA (250 mg/kg body weight/ day) orally for three consecutive days (15). Studies (16-18) have shown that oral administration of PPA at 250 mg/kg body weight induces autism-like behavioural phenotypes in rats, including impaired social interaction, repetitive behaviours, anxiety-like behaviour, hyper locomotion, and reduced exploratory activity. On day 4, the respective dietary and supplementation regimens commenced, and groups were as follows: (i) Control (n = 6): Healthy/Normal rats + Standard diet only; (ii) Omega-3 (n = 6): Healthy/Normal rats + Standard diet + Omega-3 fatty acids (200 mg/kg/day); (iii) PPA (n = 6): Autistic rats + Standard diet; and (iv) PPA + Omega-3 (n = 6): Autistic rats + Standard diet + Co-treated with omega-3 fatty acids (200 mg/kg/day),

Omega-3 fatty acids were administered orally at 200 mg/kg body weight/day for four weeks (19). Figure 1 shows the experimental design showing the four rat groups and their respective treatments.

Tissue and blood collection

Blood samples were obtained from rats at the end of the experimental period. Animals were allowed to fast overnight before collection to minimize variability. The rats were euthanized by ketamine-xylazine reagent (20), in compliance with ethical guidelines for animal experimentation, before brain and intestinal tissue collection. The brain and small intestine were carefully excised and preserved in formaldehyde. Blood was withdrawn from a cardiac puncture using sterile equipment and collected into plain tubes for serum biochemistry. After collection, samples were centrifuged at 3000 rpm for 15 minutes at 4°C, and then the serum samples were stored at -80°C until analysis.

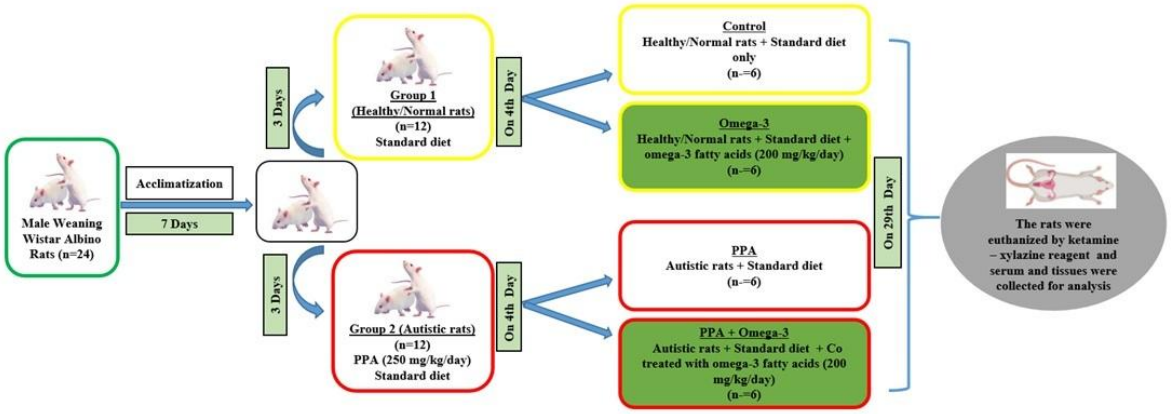

Figure 1.
Experimental design showing the experimental groups and their respective treatments

Biochemical and tissue measurements

Intestinal barrier function. Intestinal barrier function was evaluated by quantifying the expression of claudin-2 (CLDN2; Cat: ELK9666) and tight junction protein-1 (TJP1; ELK7196) in serum using enzyme-linked immunosorbent assay (ELISA) kits (ELK Biotechnology Co., LTD, Wuhan, Hubei, China) following manufacturer instructions. TJP1 and CLDN2 were selected as tight junction-related markers of epithelial integrity. The microtiter wells were pre-coated with antibodies specific to either TJP1 or CLDN2. The microtiter well plates were incubated with 100 µL of standards or appropriately diluted serum samples at 37°C for 80 min. Then the wells were washed thrice with buffer to remove unbound proteins. Afterwards, 100 µL of biotin-conjugated detection antibody was added to each well and incubated at 37°C for 50 min, followed by three additional washes. After washing, 100 µL of streptavidin-horseradish peroxidase (HRP) was added, and then incubation was performed at 37°C for 50 minutes, followed by five washes to remove unbound enzyme conjugate. Colour development (blue) was initiated by adding 90 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to each well and incubating in the dark at 37°C for 20 minutes. Finally, the reaction was stopped with 50 µL of stop solution, and the liquid turned yellow. Optical density was measured immediately at 450 nm, and protein concentrations were determined by comparison with a standard curve.

Inflammatory biomarkers. Neuroinflammation was assessed by measuring circulating concentrations of the pro-inflammatory cytokines tumour necrosis factor (TNF-α) (Cat: ELK139) and IL-6 (Cat: ELK1158) through an ELISA kit supplied by ELK Biotechnology Co., LTD (Wuhan, Hubei, China) following manufacturer instructions. The microtiter well plates provided in these kits were pre-coated with antibodies specific to each cytokine. In brief, 100 µL of either standards or samples was added to microtiter wells pre-coated with cytokine-specific antibody and incubated at 37°C for 80 minutes. The wells were then washed three times with wash buffer (200 µL) to remove any unbound material. A biotinylated detection antibody (100 µL) was subsequently added and incubated at 37°C for 50 minutes, followed by another wash and incubation with streptavidin-HRP (100 µL) for 50 minutes. After a final washing step, the TMB substrate (90 µL) was added and incubated in the dark for 20 minutes. The reaction was terminated by the addition of acid stop solution (50 µL), and the liquid turned yellow from blue, and finally, the absorbance was measured at 450 nm. Cytokine concentrations were determined by comparison to standard curves generated from known concentrations of recombinant rat IL-6 and TNF-α.

Short-chain fatty acids (SCFA). Rat short-chain fatty acid (SCFA) concentrations were quantified using a commercially available ELISA kit (MyBioSource, Cat. No. MBS2615590), following the manufacturer's instructions. The assay is based on a double-antibody sandwich principle using a pre-coated anti-rat SCFA monoclonal capture antibody and a biotinylated polyclonal detection antibody. Firstly, 100 µL of standards or samples were added to a microtiter plate, which was pre-coated with anti SCFA monoclonal antibodies. Then the microtiter plate was incubated at 37°C for 90 min, followed by two washes to remove unbound material. After washing, biotinylated anti-SCFA anti-body was added, and again incubation was performed at 37°C for 60 min, followed by additional washes. Afterwards, horseradish peroxidase (HRP)–avidin conjugate was added and incubated at 37°C for 30 min. After extensive washing, TMB substrate was added for colour development (blue) in the dark. At the end, the reaction was terminated using the stop solution, turning the yellow colour. Absorbance was measured at 450 nm using a microplate reader within 10 minutes. SCFA concentrations were calculated from the standard curve, and sample values were adjusted for dilution factors

where applicable. All samples and standards were assayed in duplicate

Neurotransmitter analysis. Both 5-hydroxytryptamine (5-HT) and Rat dopamine (DA) ELISA assays were performed using competitive inhibition enzyme immunoassay kits (ELK Biotechnology, China) according to the manufacturer's protocols. In each assay, microtiter wells pre-coated with the respective antigen (5-HT or DA) were incubated for an hour at 37°C with either standard solution (50 µL), followed by a biotinylated-conjugated antibody (50 µL) specific to the target analyte. After incubation and washing for 3 times to remove unbound reagents, streptavidin-horseradish peroxidase (HRP, 100 µL) was added to form a detectable complex and was covered with a plate cover and incubated for an hour at 37°C, and then aspiration was repeated, and washing was performed 5 times. Subsequent addition of 3,3′,5,5′-tetramethylbenzidine (TMB, 90 µL) substrate followed by incubation at 37°C for 20 minutes, produced a colorimetric change proportional to analyte concentration. The reaction was stopped with stop solution (50 µL), and the liquid turned yellow from blue, and absorbance was measured at 450 nm using a microplate reader. The concentration of 5-HT and DA was calculated by comparing the sample optical densities with standard calibration curves.

Oxidative stress and antioxidant status. Serum levels of lipid peroxides and glutathione (GSH) were measured to evaluate oxidative stress. Lipid oxidation was measured by thiobarbituric acid reactive substances (TBARS) according to the method of Ruiz-Larrea *et al*. (21). Glutathione levels were determined by the method of Beutler *et al*. (22). This assay is based on the reaction of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) with sulfhydryl groups, producing a stable, yellow-coloured compound. Lipid peroxide levels were determined as an index of membrane lipid oxidation, and glutathione was determined as an endogenous antioxidant.

Histological analysis. The small intestine was carefully excised from the abdomen, and approximately 0.5 cm^3 of the tissue was fixed in 4% formaldehyde (equivalent to 10% buffered formalin), using a volume 5 - 10 times greater than the tissue volume. Tissues were fixed at room temperature for 24 h, trimmed into smaller pieces, and dehydrated through graded ethanol concentrations of 30%, 70%, 80%, and 95% for 1 h each, followed by three changes of absolute ethanol for 1 h each. The samples were then cleared in three changes of xylene and infiltrated with molten paraffin wax at 58 - 60°C for two cycles of 2 h each. Paraffin blocks were prepared and then sectioned at 5 µm using a rotary microtome (Model: SRM 200 CW), as described by Cai *et al*. (23). The paraffin ribbons were floated on a 40 - 45°C water bath, mounted on standard glass slides, air-dried for 30 min, placed on a hot plate, and baked overnight at 38 - 43°C. Sections were stained with haematoxylin and eosin (H&E), following Tellez *et al*. (24). Brain tissues, including the hippocampus and cortex, were processed using the same protocol and examined for histopathological changes.

Histopathological analysis of rat brain and intestinal tissue sections stained with H&E was done under a light microscope. Degenerated neurons and intestinal villi were quantified by counting the number of affected neurons and villi in approximately 10 microscopic fields *per* specimen. Coded images were used for histological evaluation in a blinded manner to minimize observer bias. The counts obtained were averaged and expressed as mean ± SD for statistical analysis.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, USA), R version 4.4.3, and IBM SPSS Statistics (version 26, IBM Corp.). Normality of data distribution was evaluated using the Shapiro–Wilk test, and homogeneity of variances was assessed using Levene's test. For comparison of group means, one-way analysis of variance (ANOVA) was used to assess overall group effects among Control, Omega-3, PPA, and PPA + Omega-3 treatment groups. When significant main effects were identified, Tukey's post hoc test was used for pairwise multiple comparisons. Before Pearson correlation analysis, variable normality was assessed, and scatterplots were inspected to evaluate linearity. Pearson correlation coefficients were then calculated to examine associations between outcome variables. Pairwise group differences were further examined using regression-based contrasts to estimate treatment effects between experimental groups. Statistical significance was set at $p < 0.05$.

Results and Discussion

Histopathological observations

Representative H&E-stained sections of rat brain and small intestine tissues are shown in Figure 2 and in Figure 3. In brain sections (Figure 2), the Control and Omega-3 only Groups showed preserved neuronal morphology, with visible neuronal cell bodies and nuclei. In contrast, the PPA Group showed apparent neuronal alterations, including cells with pyknotic nuclei, irregular morphology, and fewer morphologically normal neurons. The PPA + Omega-3 Group appeared to show fewer visibly degenerated neuronal cells compared with the PPA Group.

Small intestine sections from the Control and Omega-3 only groups showed preserved villous architecture with intact apical epithelium (Figure 3). In the PPA Group, the intestinal sections showed visible villous shortening and degeneration, epithelial detachment, and inflammatory cell infiltration in the lamina propria. The PPA + Omega-3 Group appeared to show less

pronounced villous alteration and fewer inflammatory cells compared with the PPA Group.

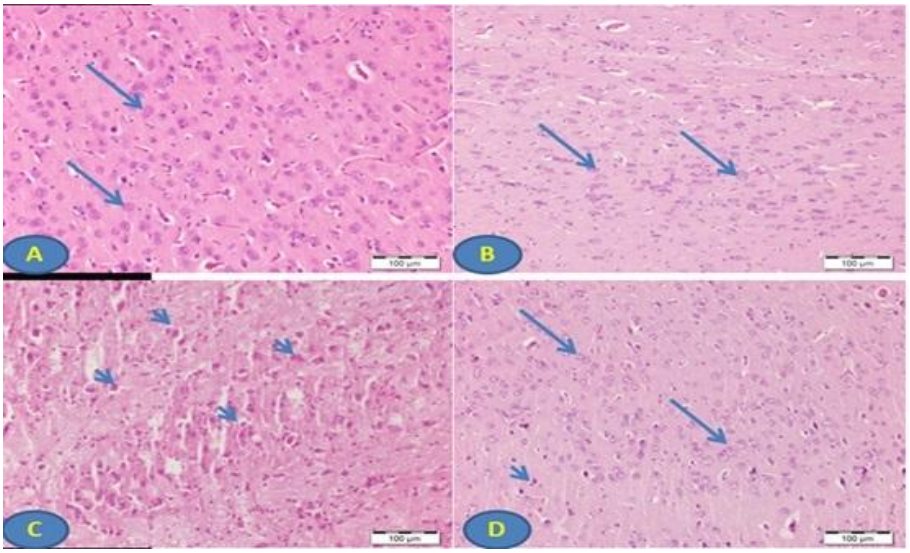

Figure 2.

H&E-stained rat brain tissue; scale bar: 100 μm

(A) Brain tissue from a Normal / Control rat shows normal neurons (arrows). (B) Brain tissue from a rat that received Omega-3, showing normal neuronal cells (arrows). (C) Brain tissue from a rat that received PPA shows many degenerated neuronal cells (arrowheads) and a few normal cells (arrow). (D) Brain tissue from a rat that received both PPA and Omega-3 shows a marked decrease in degenerated neuronal cells (arrowhead) with many normal cells (arrows).

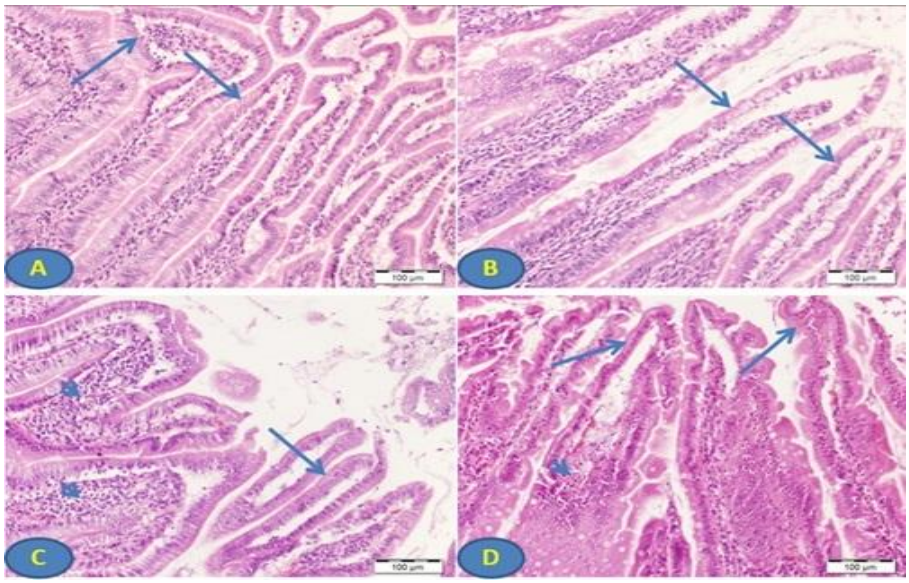

Figure 3.

H&E-stained rat small intestine; scale bar: 100 μm

(A) Intestinal tissue from Normal/Control rat shows normal villi (arrows). (B) Intestinal tissue from a rat that received Omega-3, showing normal villi (arrows). (C) Intestinal tissue from a rat that received PPA shows many degenerated villi with detached apical epithelium that are degenerated (arrows) and severe inflammatory cell infiltration in the lamina propria of the core of villi (arrow heads). (D) Intestinal tissue from a rat that received both PPA and Omega-3 shows a marked decrease in degenerated villi (arrows) with few inflammatory cells (arrowhead).

The quantitative histopathological analysis (Table I) of H&E-stained brain rat tissue revealed the absence of any degenerated neurons in the Control and Omega-3 Groups. The PPA Group (autistic) showed a substantial rise in the number of degenerated neurons (mean = 6.3). However, the treatment with Omega-3 showed a partial histological improvement and a decrease in the number of degenerated neurons in the PPA + Omega-3 Group (mean = 3.2). Similarly, the quantitative histopathological evaluation of intestinal tissue revealed a low number of degenerated intestinal villi in the Control (mean = 1.2) and Omega-3 Group

(mean = 1.1), while the PPA Group exhibited a marked increase in degenerated villi (7.9). Omega-3 treatment reduced the degeneration in the PPA + Omega-3 Group (mean = 3.9).

Table I

Quantitative histopathological assessment of degenerative neurons and intestinal villi changes among experimental groups across representative microscopic fields

Field Number	Number of Degenerative Neurons	Number of Degenerative Intestinal villi	Number of Degenerative Neurons	Number of Degenerative Intestinal villi	Number of Degenerative Neurons	Number of Degenerative Intestinal villi	Number of Degenerative Neurons	Number of Degenerative Intestinal villi
	Control	Control	Omega-3	Omega-3	PPA	PPA	PPA + Omega-3	PPA + Omega-3
1	0	1	0	1	6	9	4	3
2	0	0	0	1	8	7	3	4
3	0	1	0	0	5	8	3	5
4	0	1	0	1	5	6	5	4
5	0	0	0	0	6	6	2	3
6	0	2	0	1	9	7	2	2
7	0	1	0	1	4	10	5	3
8	0	3	0	2	5	9	4	4
9	0	1	0	1	6	8	1	5
10	0	2	0	3	9	9	3	6
Mean ± SD	0	1.2 ± 0.919	0	1.1 ± 0.876	6.3 ± 1.767	7.9 ± 1.370	3.2 ± 1.317	3.9 ± 1.197

Result has been reported as Mean ± SD

Effect of omega-3 on inflammatory biomarkers

TNF-α levels were numerically higher in the PPA group than in the Control Group; however, this difference was not statistically significant (Figure 4A). TNF-α levels were also numerically lower in the PPA + Omega-3 Group than in the PPA Group, but this difference also did not reach statistical significance. IL-6 levels showed a statistically significant difference between the PPA and Omega-3 only Groups. IL-6 was significantly higher in the PPA group than in the Omega-3 only Group (p = 0.0441) (Figure 4B). IL-6 levels were numerically lower in the PPA + Omega-3 Group than in the PPA Group; however, this difference was not statistically significant.

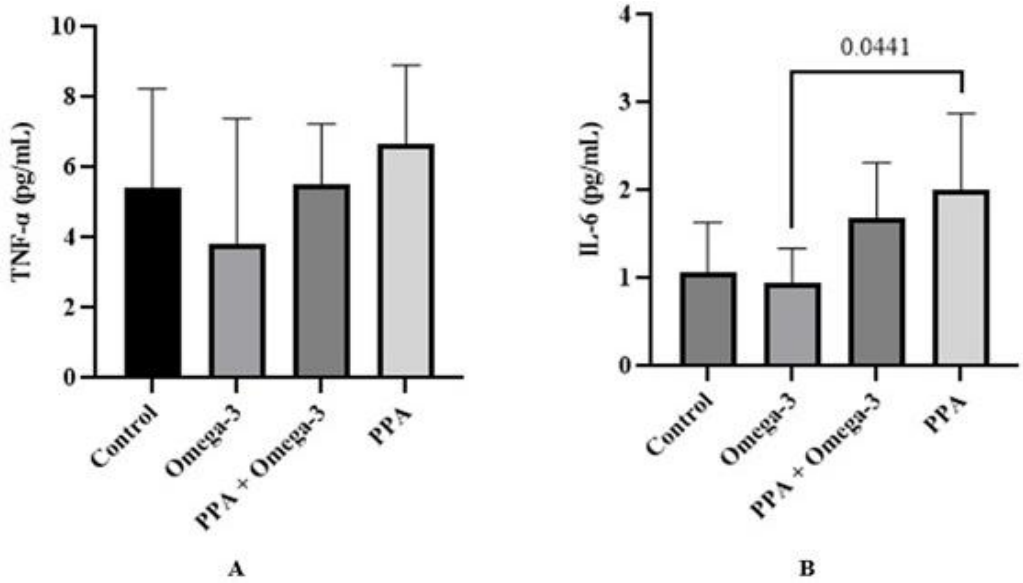

Figure 4.

Effects of omega-3 on inflammatory biomarkers (A) TNF-α (B) IL-6 levels across groups

Effects of omega-3 on gut permeability biomarkers

CLDN2 levels differed significantly between selected groups (Figure 5A). The PPA Group showed the lowest mean CLDN2 expression. The Omega-3 only Group showed significantly higher CLDN2 expression than the Control Group (p = 0.0316). CLDN2 expression was also significantly lower in the PPA Group than in the Omega-3 only Group (p = 0.0186). Although CLDN2 levels were numerically higher in the PPA + Omega-3 Group than in the PPA group, this difference was not statistically significant. TJP1 levels showed a similar numerical pattern across groups; however, the differences did not reach statistical significance (Figure 5B).

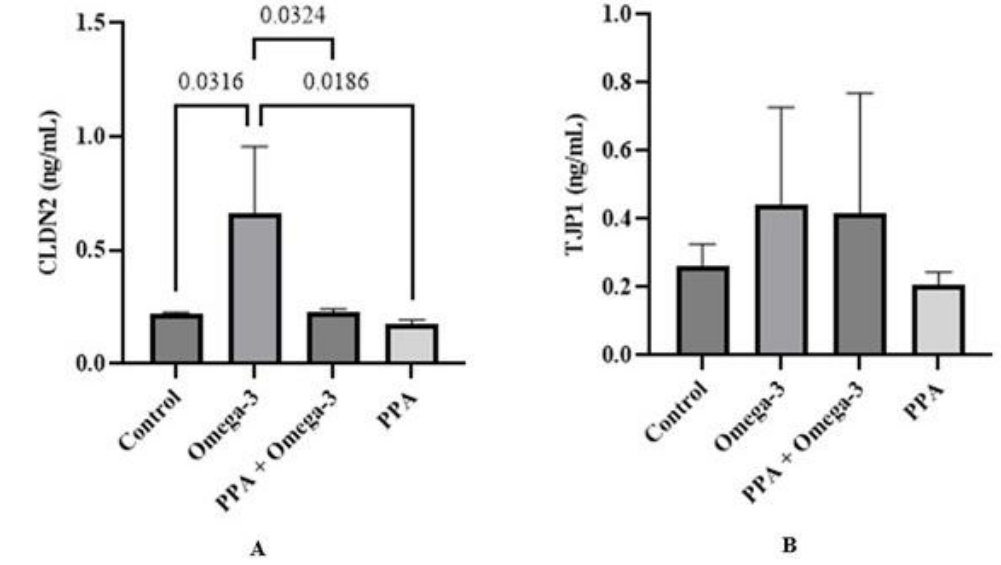

Figure 5.
Effects of omega-3 on gut permeability biomarkers (A) CLDN2 (B) TJP1 levels across groups

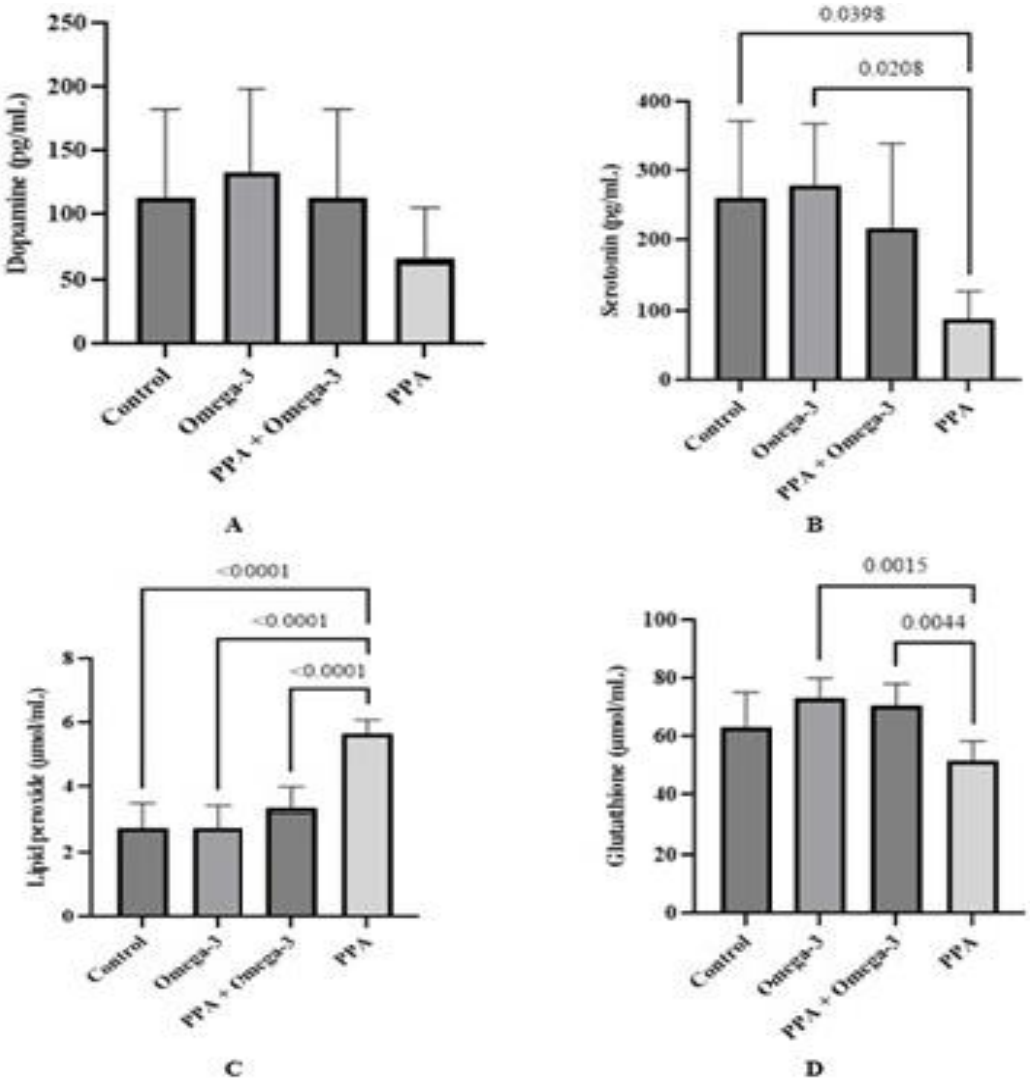

Figure 6.
Effect of Omega-3 on Neurotransmitter Biomarkers (A) Dopamine (B) Serotonin and oxidative stress (C) Lipid peroxide (D) Glutathione

Effects of omega-3 on neurotransmitter and oxidative stress biomarkers

Dopamine levels were numerically lower in the PPA Group than in the other groups; however, the differences did not reach statistical significance (Figure 6A). The PPA + Omega-3 and Omega-3-only Groups showed numerically higher mean dopamine levels than the PPA Group, but these differences were not statistically significant. Serotonin levels were significantly lower in the PPA Group than in the Control Group ($p = 0.0398$) and Omega-3-only Group ($p = 0.0208$) (Figure 6B). The PPA + Omega-3 Group showed a numerically higher mean serotonin level than the PPA Group; however, this difference was not statistically significant. Lipid peroxide levels were significantly higher in the PPA Group than in all other groups ($p < 0.0001$) (Figure 6C). The PPA + Omega-3 Group showed significantly lower lipid peroxide levels than the PPA group ($p < 0.0001$). Glutathione levels were significantly lower in the PPA Group than in the Omega-3 only group ($p = 0.0015$) and the PPA + Omega-3 Group ($p = 0.0044$) (Figure 6D). No further interpretation was made beyond these group differences.

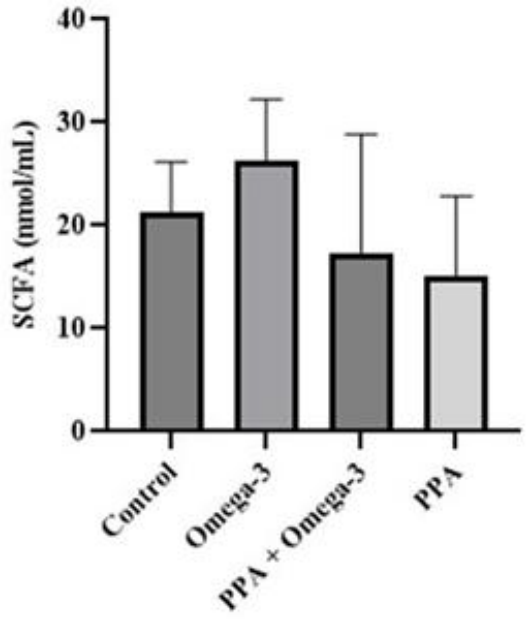

Figure 7.
SCFA levels across groups

Effects of omega-3 on SCFA concentration

SCFA concentrations did not differ significantly among the experimental groups (Figure 7). The PPA Group showed the lowest mean SCFA concentration, while the Omega-3 only Group showed the highest mean SCFA concentration. The PPA + Omega-3 Group showed a numerically higher mean SCFA concentration than the PPA group; however, this difference was not statistically significant.

Correlation analysis between biomarkers

Due to the exploratory nature of the study and the small sample size (n = 6 rats/group), correlation and regression analyses were conducted as preliminary analyses and interpreted cautiously. Non-significant numerical trends were not considered definitive evidence. Pearson correlation analysis was performed to examine associations among the measured biomarkers (Figure 8). IL-6 was negatively correlated with serotonin ($r = -0.55$) and dopamine ($r = -0.28$). TNF-α showed a weak negative correlation with serotonin ($r = -0.19$). CLDN2 was positively correlated with TJP1 ($r = 0.58$), SCFA ($r = 0.46$), and glutathione ($r = 0.52$), and negatively correlated with lipid peroxide ($r = -0.45$). TJP1 was positively correlated with SCFA ($r = 0.47$) and glutathione ($r = 0.48$) and negatively correlated with lipid peroxide ($r = -0.31$). Serotonin was positively correlated with dopamine ($r = 0.50$) and negatively correlated with lipid peroxide ($r = -0.54$), IL-6 ($r = -0.55$), and TNF-α ($r = -0.19$). Glutathione was negatively correlated with lipid peroxide ($r = -0.53$). These results describe correlations only and do not indicate causal relationships.

Regression Analysis between Biomarkers

Variable-specific Regression

Regression analyses were conducted using selected biomarkers as dependent variables. When IL-6 was treated as the dependent variable, no predictors reached statistical significance. Similarly, when dopamine was treated as the dependent variable, no significant predictors were identified. However, when TJP1 was treated as the dependent variable, SCFA reached the threshold for statistical significance ($p = 0.050$) (Table II). Glutathione did not reach statistical significance in this model ($p = 0.060$). When lipid peroxide was treated as the dependent variable, glutathione was statistically significant ($p = 0.047$) (Table II). Other predictors in the lipid peroxide model did not reach statistical significance.

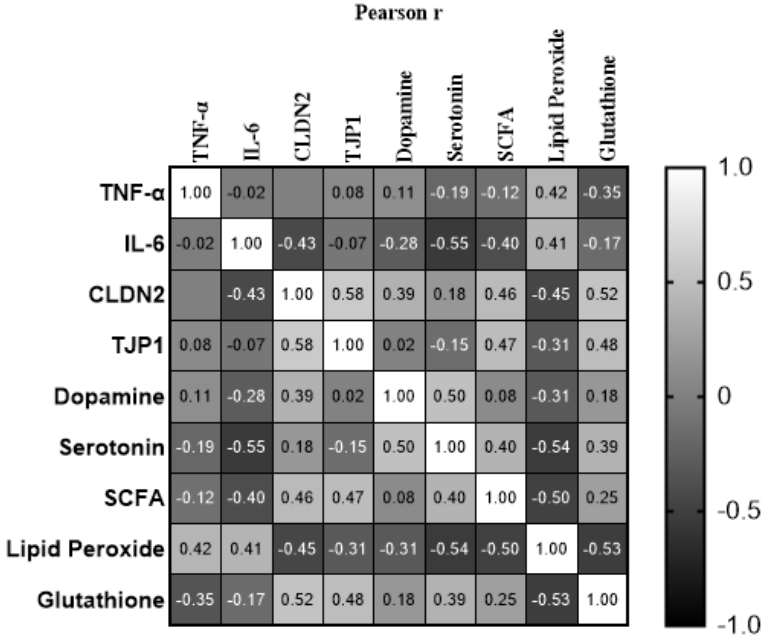

Figure 8.
Pearson correlation matrix of measured outcomes

Table II
Regression coefficients for TJP1 and lipid peroxide as dependent variables

Coefficient [a, b]								
Model		**Unstandardized B**	**Coefficients Std. Error**	**Standardized Coefficients β**	***t***	***p***	**Collinearity Statistics**	
							Tolerance	**VIF**
[a]**TJP1**	**(Constant)**	-0.609	0.457		-1.331	0.200		
	IL-6	0.045	0.068	0.142	0.664	0.515	0.746	1.341
	Dopamine	0.000	0.001	-0.034	-0.172	0.866	0.859	1.165
	SCFA	0.013	0.006	0.465	2.102	0.050*	0.694	1.441
	LP	0.015	0.045	0.084	0.329	0.746	0.518	1.930
	Glutathione	0.009	0.004	0.436	2.007	0.060	0.719	1.390
[b]**LP**	**(Constant)**	7.739	1.697		4.561	< 0.001		
	IL-6	0.279	0.353	0.154	0.790	0.440	0.753	1.328
	TJP1	0.402	1.221	0.071	0.329	0.746	0.614	1.627
	Dopamine	-0.003	0.004	-.160	-0.896	0.382	0.896	1.117
	SCFA	-0.055	0.033	-.351	0.643	0.114	0.643	1.555
	Glutathione	-0.049	0.023	-.420	0.736	0.047*	0.736	1.359

[a]Dependent Variable: TJP1; [b]Dependent Variable: LP; SCFA: short chain fatty acids; IL-6: interleukin- 6; TJP1: tight junction protein-1; LP: lipid peroxide; *t*: test statistics; *p*: statistical significance (*p < 0.05)

Pairwise group comparisons

Pairwise group comparisons were conducted across the measured biomarkers (Table III). For inflammatory biomarkers, IL-6 levels were significantly lower in the Omega-3 only Group than in the PPA Group (p = 0.044). The Control Group showed numerically lower IL-6 levels than the PPA group, but this comparison was not statistically significant (p = 0.084). No significant group differences were observed for TNF-α. For gut permeability biomarkers, CLDN2 expression was significantly higher in the Omega-3 only Group than in the Control Group (p = 0.032), PPA Group (p = 0.019), and PPA + Omega-3 Group (p = 0.032). The difference between the PPA Group and the PPA + Omega-3 Group was not statistically significant. TJP1 levels did not show statistically significant group differences. For neurotransmitter biomarkers, serotonin levels were significantly lower in the PPA group than in the Control Group (p = 0.040) and Omega-3 only Group (p = 0.021). The difference between the PPA and PPA + Omega-3 Groups was not statistically significant. Dopamine levels did not show statistically significant group differences. SCFA levels did not show statistically significant differences between groups. The Omega-3 only Group showed numerically higher SCFA levels than the PPA Group, but this comparison did not reach statistical significance (p = 0.096). For oxidative stress biomarkers, lipid peroxide levels were significantly higher in the PPA Group than in the Control,

Omega-3 only, and PPA + Omega-3 Groups ($p < 0.001$). Glutathione levels were significantly higher in the Omega-3-only Group ($p = 0.001$) and PPA + Omega-3 Group ($p = 0.004$) than in the PPA group.

Table III

Pairwise group comparisons for each biomarker outcome using regression analysis

Outcomes	Group Comparison	SE	*t*	*p*	R^2
Lipid Peroxide	**Control - Omega-3**	0.349	0.040	p = 1	0.823
	Control - PPA	0.349	-8.249	p < 0.001*	0.823
	Control - PPA + Omega-3	0.349	-1.627	p = 0.387	0.823
	Omega-3 - PPA	0.349	-8.289	p < 0.001*	0.823
	Omega-3 – PPA + Omega-3	0.349	-1.667	p = 0.366	0.823
	PPA - PPA + Omega-3	0.349	6.622	p < 0.001*	0.823
SCFA	**Control - Omega-3**	4.592	-1.110	p = 0.688	0.259
	Control - PPA	4.592	1.360	p = 0.537	0.259
	Control - PPA + Omega-3	4.592	0.847	p = 0.832	0.259
	Omega -3- PPA	4.592	2.467	p = 0.096	0.259
	Omega-3- PPA + Omega-3	4.592	1.956	p = 0.237	0.259
	PPA - PPA + Omega-3	4.592	-0.513	p = 0.955	0.259
Serotonin	**Control - Omega-3**	55.198	-0.337	p = 0.986	0.428
	Control - PPA	57.892	2.959	p = 0.040*	0.428
	Control - PPA + Omega-3	61.713	0.712	p = 0.891	0.428
	Omega-3 - PPA	57.892	3.280	p = 0.021*	0.428
	Omega-3 - PPA + Omega-3	61.712	1.013	p = 0.744	0.428
	PPA - PPA + Omega-3	64.133	-1.986	p = 0.232	0.428
Dopamine	**Control - Omega-3**	35.627	-5.60E-01	p = 0.943	0.164
	Control - PPA	35.627	1.34E+00	p = 0.547	0.164
	Control - PPA + Omega-3	35.627	2.30E-15	p = 1	0.164
	Omega-3 - PPA	35.627	1.90E+00	p = 0.258	0.164
	Omega-3 - PPA + Omega-3	35.627	5.60E-01	p = 0.943	0.164
	PPA - PPA + Omega-3	35.627	-1.34E+00	p = 0.547	0.164
CLDN2	**Control - Omega-3**	0.129	-3.428	p = 0.032*	0.694
	Control - PPA	0.138	0.333	p = 0.986	0.694
	Control - PPA + Omega-3	0.138	-0.016	p = 1	0.694
	Omega-3 - PPA	0.129	3.783	p = 0.019*	0.694
	Omega-3 - PPA + Omega-3	0.129	3.410	p = 0.032*	0.694
	PPA - PPA + Omega-3	0.138	-0.349	p = 0.984	0.694
IL-6	**Control - Omega-3**	0.367	0.328	p = 0.987	0.359
	Control - PPA	0.367	-2.538	p = 0.084	0.359
	Control - PPA + Omega-3	0.367	-1.692	p = 0.353	0.359
	Omega-3 - PPA	0.367	-2.865	p = 0.044*	0.359
	Omega-3 - PPA + Omega-3	0.367	-2.0203	p = 0.214	0.359
	PPA - PPA + Omega-3	0.367	0.845	p = 0.832	0.359
TNF-α	**Control - Omega**	1.728	0.923	p = 0.793	0.154
	Control - PPA	1.728	-0.717	p = 0.889	0.154
	Control - PPA + Omega-3	1.833	-0.064	p = 1	0.154
	Omega-3 - PPA	1.728	-1.640	p = 0.387	0.154
	Omega-3 - PPA + Omega-3	1.833	-0.935	p = 0.787	0.154
	PPA - PPA + Omega-3	1.833	0.612	p = 0.927	0.154
TJP1	**Control - Omega-3**	0.132	-1.371	p = 0.531	0.188
	Control - PPA	0.132	0.436	p = 0.972	0.188
	Control - PPA + Omega-3	0.132	-1.157	p = 0.66	0.188
	Omega-3 - PPA	0.132	1.807	p = 0.299	0.188
	Omega-3 - PPA + Omega-3	0.132	0.214	p = 0.996	0.188
	PPA - PPA + Omega-3	0.132	-1.593	p = 0.405	0.188
Glutathione	**Control - Omega-3**	4.850	-2.062	p = 0.2	0.541
	Control - PPA	4.850	2.337	p = 0.123	0.541
	Control - PPA + Omega-3	4.850	-1.580	p = 0.412	0.541
	Omega-3 - PPA	4.850	4.399	p = 0.001*	0.541
	Omega-3 - PPA + Omega-3	4.850	0.482	p = 0.962	0.541
	PPA - PPA + Omega-3	4.850	-3.917	p = 0.004*	0.541

SCFA: short chain fatty acids; IL-6: interleukin- 6; TJP1: tight junction protein-1; CLDN2: claudin-2; SE: standard error; *t*: test statistics; *p*: statistical significance; R^2: coefficient of determination indicating the proportion of variance in the outcome explained by regression model

Dietary and nutritional factors have received increasing attention in autism spectrum disorder (ASD) research, particularly because current management approaches are largely supportive and focus on behavioural, educational, and symptom-directed interventions (25). In our study, although the behavioural tests were not included, the PPA model used here has been previously reported to induce ASD-like behavioural abnormalities in rodents at similar dosing regimens (16-18). Therefore, the present study focused on biochemical, inflammatory, oxidative stress, gut permeability-related, serum neurotransmitter, and histopathological changes within an established PPA-induced experimental model.

PPA exposure was associated with increased lipid peroxide levels and reduced glutathione levels, whereas the autistic rats treated with omega-3 showed significantly lower lipid peroxide and higher glutathione levels. These findings are consistent with previous PPA-based studies reporting lipid peroxidation, reduced antioxidant defence, and redox imbalance in experimental ASD models (11, 26). Experimental evidence has shown that natural compounds and probiotics can reduce oxidative stress in disease models (27-28). This supports their therapeutic potential. Studies have found that omega-3 fatty acids can influence oxidative balance through lipid mediator production, membrane-related mechanisms, and antioxidant pathways (13, 29). However, the interpretation should remain specific to the measured oxidative stress markers. Our findings support an omega-3-associated improvement in lipid peroxidation and glutathione status, but they do not establish broad neuroprotection or complete reversal of PPA-induced pathology.

The histopathological findings were broadly consistent with the oxidative stress results. Autistic rats showed increased degeneration counts in brain and intestinal tissues, while the autistic rats treated with omega-3 showed lower numbers of degenerated neurons and villi. Similar tissue-level alterations have been reported in PPA-induced models, where oxidative stress and inflammatory changes are often accompanied by neuronal and intestinal structural abnormalities (30-32). The lower degeneration counts in the PPA + Omega-3 group suggest that omega-3 supplementation was associated with less apparent tissue injury under the present experimental conditions. Nevertheless, these findings should be interpreted as histological observations rather than evidence of functional recovery. More detailed morphometric assessment and behavioural testing would be needed to strengthen the tissue-level interpretation.

Cytokines influence the central nervous system (CNS) through several pathways, including passage through vulnerable regions of the blood–brain barrier (BBB), active transport *via* cytokine-specific carriers, signalling along cranial nerves, and release from peripherally activated monocytes, which may then influence neurodevelopment and synaptic plasticity. The inflammatory markers varied in our study. In our study, TNF-α did not differ significantly among the experimental groups, which limits conclusions regarding TNF-α-mediated inflammation in this model, and IL-6, even though increased in the PPA group, but the difference was significant only with the Omega-3 group. This contrasts with some previous studies that reported wider cytokine reductions following omega-3 supplementation (33-35). Differences in animal age, omega-3 formulation, dose, duration, inflammatory challenge, tissue compartment, or sample size may partly explain these discrepancies. In the current study, inflammatory modulation appears limited and marker-specific rather than robust across cytokines.

We found that CLDN2 was not significantly restored in the autistic rats treated with Omega-3 compared with the PPA Group, although its expression differed significantly between selected groups, with the Omega-3 group showing significantly higher expression than all other groups, demonstrating a group-specific rather than uniform treatment effect. CLDN2 is a pore-forming tight junction protein allied with changes in permeability between intestinal cells, so CLDN2 changes should be interpreted with caution. We did not observe a significant change in TJP1, which indicates that CLDN2 modulation reflects molecular tight junction remodelling rather than a clear modification in overall intestinal barrier functions that requires further investigation. Previous studies have linked omega-3 fatty acids with regulation of tight junction proteins and modulation of intestinal barrier function, which was mediated through anti-inflammatory signalling pathways and host–microbiota interactions (36-38).

Short-chain fatty acids (SCFAs) are vital microbial metabolites found to be involved in intestinal epithelial metabolism and microbiota-host signalling, and altered SCFA profiles have been reported in ASD-related gut-brain axis studies (7). Gut microbiota imbalance has been linked to increased oxidative stress and inflammation, supporting its role as a therapeutic target (39). In the present study, the limited effect of omega-3 on SCFAs suggests that its main action may be downstream in the regulation of oxidative stress rather than directly changing the microbial metabolic output. Moreover, the present study measured SCFAs with an ELISA-based assay rather than GC-MS or LC-MS, which may provide more sensitive and comprehensive metabolomic characterization.

Serotonin was significantly lower in the PPA group than in the Control and Omega-3 only groups, whereas the PPA + Omega-3 group did not differ significantly from the PPA group, and dopamine did not show significant group differences. So, serum neurotransmitter findings should also be interpreted with caution. Previous experimental studies have linked PPA exposure with altered monoamine-related markers and neurochemical changes in ASD-related

models (40, 41). However, in the present study, serotonin and dopamine were measured only in serum, not in brain tissue. The observed decrease in serotonin in PPA rats suggests peripheral neurochemical disturbance consistent with the pathophysiology of ASD, they do not directly reflect the brain neurotransmitter concentration, while the ability of omega-3 to maintain normal levels in healthy rats suggests its potential for prevention. Blood levels, however, do not directly reflect concentrations of neurotransmitters in the brain. Serum monoamines may be influenced by peripheral sources, including the gastrointestinal tract, platelets, and systemic metabolism, and may not accurately reflect brain-region-specific neurotransmitter activity. These peripheral measures are commonly used as supportive evidence in neurodevelopmental research, though brain tissue analysis is needed for direct confirmation.

The exploratory correlation and regression analyses provided additional context but should not be overinterpreted. The negative association between glutathione and lipid peroxide is biologically plausible given their opposing roles in redox balance (42, 43). Similarly, the connotation between SCFA and TJP1 may recommend a possible relationship between microbial metabolites and tight junction-related markers. However, the small sample size limits the stability of these estimates.

The findings of our study show that omega-3 supplementation exerts selective biological effects in the PPA-induced autism model. We observed that omega-3 supplementation primarily acts as a highly targeted antioxidant intervention in PPA-exposed rats, rather than a broad-spectrum cure. In this study, although the omega-3 supplementation exerted a limited effect on gut permeability, short-chain fatty acids, and neurotransmitter levels, the findings demonstrate that its therapeutic benefits in this model are more likely attributable to its antioxidant properties than to modulation of gut-brain axis components. This pattern may help explain why clinical studies of omega-3 supplementation in ASD have reported mixed outcomes, with some studies showing modest behavioural or symptom-related changes and others reporting minimal effects (14). The variability may reflect differences in dose, duration, developmental stage, baseline fatty acid status, outcome selection, and the heterogeneity of ASD. These outcomes are pharmaceutically vital as they suggest that Omega-3 should not be viewed as a standalone treatment for all neurodevelopmental symptoms, but rather as a specific supporting therapy aimed at managing oxidative damage. This study's strength lies in its integrated analysis of the microbiota–gut–brain axis. Assessing cytokines, gut permeability, neurotransmitters, SCFAs, and oxidative stress, it offers a broad view of omega-3's impact in a PPA-induced autism model. The inclusion of correlation and regression analyses adds robustness by not only identifying group differences but also exploring mechanistic links and predictive relationships. But this study also has several limitations. In this study, the major limitation is the small sample size (n = 6 rats/group), which may have reduced statistical power and increased the risk of unstable estimates, particularly in the correlation and regression analyses. Therefore, these analyses should be considered exploratory in nature. The small sample size may also have limited the detection of significant differences in outcomes such as dopamine, SCFAs, TJP1, TNF-α, and CLDN2 changes in the PPA + Omega-3 Group. Behavioural assessments were not included, which is a major limitation for an autism-related experimental model. Therefore, the current findings should be interpreted as biochemical and histopathological observations within an established PPA-induced model, rather than as evidence of behavioural improvement or recovery. Only male rats were used, which has limited the generalizability across sex. In addition, serotonin and dopamine were measured only in serum and not in brain tissue; therefore, these biomarkers cannot be used to infer central neurotransmitter activity directly. The study also did not include microbiome sequencing, which limits the interpretation of SCFA-related changes. Finally, although correlation and regression analyses provided useful preliminary information, they do not establish causality and require validation in larger studies.

Conclusions

Omega-3 supplementation in the PPA-induced autism rat model showed the strongest association with oxidative stress-related outcomes, while evidence for effects on inflammation, intestinal barrier-related markers, SCFA concentration, and peripheral neurotransmitter markers was limited. However, the histological analysis of the brain revealed neuronal alterations, while that of small intestine histology showed shortening and degeneration in the PPA Group (autistic), which improved with omega -3 supplementation. Further studies using larger sample sizes, behavioural assessments, brain tissue analysis, microbiome profiling, and both sexes are needed to clarify the role of omega-3 supplementation in PPA-induced models.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the NIH. The animal study protocol was approved by the Research Ethics Committee at King Saud University (IRB # KSU-SE-25-6), Riyadh, Saudi Arabia. Informed Consent was not applicable in this study.

AI use disclosure

The authors used Quillbot to improve readability and language. The authors reviewed all outputs from these tools and took full responsibility for the content of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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