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Beneficial effect of omega-3 fatty acids supplementation on leaky gut, inflammation and oxidative stress in propionic acid-induced autism in aged rats

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ABSTRACT

This study examined the effects of omega-3 fatty acids supplementation on gut barrier integrity, systemic inflammation, neurotransmission and oxidative stress, in an aged rat model of propionic acid (PPA)-induced neurotoxicity. Twenty-four aged male rats were divided into four groups: control, omega-3, PPA and PPA + omega-3. Serum cytokines, tight-junction proteins (TJP1), dopamine, serotonin, short-chain fatty acids (SCFAs), oxidative stress markers, and histopathology of the brain and small intestine were evaluated. PPA exposure significantly increased tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and reduced TJP1 expression, confirming gut barrier disruption and systemic inflammation. Omega-3 fatty acids supplementation selectively reduced IL-6 but did not reverse PPA-induced TNF-α elevation or oxidative stress. CLDN2 expression increased in PPA + omega-3 rats, suggesting a compensatory but incomplete barrier response. Dopamine, serotonin, and SCFA levels showed upward trends with supplementation but were not statistically significant. Histological analysis demonstrated partial preservation of neuronal and intestinal structure in the PPA + omega-3 group. Overall, omega-3 fatty acids exerted modest anti-inflammatory effects but failed to fully restore oxidative balance or barrier integrity in aged rats, suggesting that omega-3 fatty acids may be more effective as a preventive rather than restorative intervention in ageing-related gut-brain axis disruption.

Keywords: omega-3 fatty acids, propionic acid, aged rats, gut-brain axis, neuroinflammation, oxidative stress, neurotransmitters

INTRODUCTION

Ageing is a complex biological process that affects multiple systems in the body, particularly the gut and brain (1). As we age, the integrity of the intestinal barrier weakens, leading to a condition often referred to as "leaky gut," where the gut lining becomes more permeable, allowing harmful substances to enter the bloodstream. This phenomenon is accompanied by chronic low-grade inflammation, also known as inflammaging, which

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has been shown to contribute to a range of age-related diseases, including neurodegenerative disorders (2). Alongside increased permeability, ageing is characterised by oxidative stress, which further damages cellular structures and exacerbates inflammation. These changes in the gut and systemic inflammation can negatively impact the brain, contributing to cognitive decline and an increased risk of diseases such as Alzheimer's (3). Disruption of the gut-brain axis, through the communication between the gut and the central nervous system, plays a central role in these processes. It has been demonstrated that microbial imbalance and gut inflammation can impact brain function by activating microglia and altering neurotransmitter levels (4).

Propionic acid (PPA), a short-chain fatty acid produced by gut microbiota, has been widely used to model gut-brain dysfunction, particularly in relation to neurodevelopmental and neurodegenerative diseases. While PPA is usually present in small amounts as a byproduct of fermentation, excessive concentrations are associated with inflammatory responses in the gut and brain (5). PPA-induced neurotoxicity has been shown to result in increased intestinal permeability, leading to systemic inflammation and oxidative stress, which further exacerbates cognitive and behavioural deficits (6). In the context of ageing, PPA treatment in rats mimics several aspects of age-related neurodegenerative processes, such as neuroinflammation, gut barrier disruption and neurotransmitter imbalances, making it a useful model to investigate the effects of dietary interventions, including omega-3 fatty acids supplementation. Furthermore, PPA has been shown to impair brain function by altering neurotransmitter metabolism, particularly dopamine and serotonin, both of which are critical for mood regulation, cognition, and overall brain health (7).

Omega-3 polyunsaturated fatty acids (PUFAs) are essential nutrients with well-documented anti-inflammatory, antioxidant and neuroprotective properties. These fatty acids have been shown to modulate multiple pathways involved in inflammation, oxidative stress and brain health. Omega-3 fatty acids exert anti-inflammatory effects by incorporating into cell membranes and producing specialised pro-resolving mediators such as resolvins and protectins, which help resolve inflammation and prevent the escalation of immune responses (8). In the context of ageing, omega-3 fatty acids supplementation has been associated with reduced levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), as well as improvements in cognitive function and mood regulation (9). Omega-3 fatty acids have been shown to support the integrity of the intestinal barrier by modulating tight junction proteins, thus potentially protecting against leaky gut and its systemic consequences (10). Despite these promising effects, studies investigating the efficacy of omega-3 fatty acids in aged models with induced neurotoxicity, such as PPA, remain limited. While omega-3 fatty acids supplementation has shown benefits in younger populations or models of acute disease, its role in restoring gut barrier function and mitigating neuroinflammation in aged rats exposed to PPA remains unclear.

Despite promising evidence that omega-3 fatty acids supplementation can benefit inflammation, gut barrier integrity and cognitive function, a clear gap exists in the literature regarding its effects in aged models exposed to PPA-induced neurotoxicity. The current research primarily focuses on younger populations or acute disease models, leaving an important void in understanding how omega-3 fatty acids influence the gut-brain axis in ageing, especially under conditions of chronic inflammation and oxidative stress. Additionally, while many studies have explored individual biomarkers of gut and brain

health, few have provided a comprehensive, multi-level assessment of omega-3 fatty acids' effects on inflammation, gut barrier function, neurotransmitter regulation, microbiome metabolites and oxidative stress simultaneously. This study aims to fill this gap by investigating the effects of omega-3 fatty acids supplementation on gut permeability, neuroinflammation, oxidative stress, and neurotransmitter function in aged rats exposed to PPA.

EXPERIMENTAL

Chemicals and reagents

Propionic acid was purchased from Merck KGaA (Germany), ketamine was obtained from Alfasan WOERDEN (The Netherlands) while xylazine from Bioveta, a.s. (Czech Republic). Sodium chloride and trisodium citrate were purchased from Loba Chemie Pvt Ltd. (India), EDTA from Techno Pharmachem (India), disodium hydrogen phosphate dihydrate from VWR BDH Chemicals (Belgium), potassium dihydrogen orthophosphate from Fischer Scientific UK Ltd. (UK), metaphosphoric acid from Acros Organics (USA), thiobarbituric acid and trichloroacetic acid from Merck. ELK Biotechnology Co., Ltd. (China) supplied the kits for inflammatory biomarkers, intestinal barrier function and neurotransmitters, while My Biosource (USA) supplied the kit for short chain fatty acids.

Holista Omega-3 syrup (1500 mg in 5 mL) was supplied from Holista Health (Canada). As per manufacturer, each 5 mL contains 436 mg of docosahexaenoic acid and 700 mg of eicosapentaenoic acid, derived from purified marine fish oil (anchovy, mackerel and sardine).

Animals and housing

A total of twenty-four aged (18–24 months) male Wistar albino rats (250–300 g) used in this study were obtained from the Experimental Animal Care Centre (King Saud University, Riyadh, Saudi Arabia). Male rats were selected because autism spectrum disorder (ASD) shows a strong male predominance in humans and also avoids the potential confounding effects of hormonal fluctuations associated with the oestrous cycle in females, which may influence neuro-inflammatory, metabolic and behavioural parameters (11). Additionally, the use of male animals ensures greater homogeneity and reduces biological variability, thereby improving the reliability and consistency of the experimental outcomes. The animals were housed in polypropylene cages under controlled environmental conditions (24–25 °C, 45–55 % relative humidity, and a 12 h light/dark cycle). Food and water were provided *ad libitum*. All rats were acclimatized for one week prior to experimental procedures on a standard pelleted diet (Saudi Grains Organisation, SAGO, Saudi Arabia). All experimental protocols of this study were approved by the Animal Ethical Committee of King Saud University (Riyadh, Saudi Arabia).

Experimental design and treatments

Following acclimatization (7 days), rats were randomly divided into two groups, namely, control and autistic group, and each group consisted of 12 rats. Both groups were provided with a standard diet and water *ad libitum*. In autistic group, 250 mg kg^{-1} per day

of PPA was administered orally for three consecutive days to induce neurotoxicity (5). On day 4, dietary and supplementation protocols commenced, and the groups were assigned as: control (aged control + standard diet); omega-3: (aged control + standard diet + omega-3 fatty acids (orally, 200 mg kg^{-1}); PPA (autistic rats + standard diet); PPA + omega-3 (autistic rats + standard diet + omega-3 fatty acids, 200 mg^{-1} kg^{-1}).

Omega-3 fatty acids were administered orally at 200 mg kg^{-1} body mass daily for four weeks (12). The omega-3 fatty acids were freshly prepared in distilled water and administered orally once daily *via* gastric gavage at a volume adjusted weekly according to body mass to ensure precise dosing, whereas the control group received an equivalent volume of distilled water as the vehicle.

Tissue and blood collection

Blood samples were obtained from rats at the end of the experimental period. Animals were allowed to fast overnight prior to collection to minimize variability. The rats were euthanized using a ketamine-xylazine reagent (13). The brains and small intestines 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 min at 4 °C and then the serum samples were stored at –80 °C until analysis.

Serum analyses

Inflammatory biomarkers. – Serum levels of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were quantified using enzyme-linked immunosorbent assay (ELISA) commercial kits according to the manufacturer's protocols.

Intestinal barrier function. – Gut barrier integrity was assessed by quantifying the tight junction proteins claudin-2 (CLDN2) and tight junction protein-1 (TJP1) in serum using ELISA kits.

Neurotransmitter analysis. – Serum dopamine and serotonin concentrations were determined using ELISA kits to evaluate the modulatory effects of omega-3 fatty acids supplementation on neurotransmission.

Short-chain fatty acids (SCFAs). – Serum concentrations of SCFAs were measured using ELISA kits according to the manufacturer's protocols.

Oxidative stress and antioxidant status. – In this study, lipid oxidation was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS), following the method described by Ruiz-Larrea *et al*. (14). Glutathione levels were determined using the method of Beutler *et al*. (15) which involves the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with sulfhydryl groups to produce a stable yellow-coloured compound.

Histological analysis

The small intestine was removed carefully from the abdomen and about 0.5 cm^3 of the tissue was immersed in 10 % neutral buffered formalin (containing 4 % formaldehyde) at

a fixative to tissue volume ratio of 5–10:1 and kept at room temperature for 24 hours. Fixed tissues were trimmed, then dehydrated sequentially in 30, 70, 80 and 95 % ethanol for one hour each, followed by three one-hour washes in 100 % ethanol. Clearing was done with xylene in three changes. The tissues were then immersed in melted paraffin wax at 58–60 °C for two two-hour cycles. After preparation of paraffin blocks, they were cut into 5 μm thick slices with a rotary microtome (16). The paraffin ribbons were then immersed in a hot water bath at around 40–45 °C. The sections were mounted on ordinary glass slides, air dried for 30 minutes, and then placed on a hot plate before being heated overnight at 38–43 °C for staining with haematoxylin and eosin (H&E). Haematoxylin stains the nuclei with deep blue colour while eosin stains cytoplasm with pink colour (17). Brain sections (hippocampus and cortex) were also examined similarly for evidence of neuronal damage and inflammatory changes.

Statistical analysis

All statistical analyses were performed using SPSS (IBM, USA), R software (version 4.4.3; R Foundation for Statistical Computing, Austria), and GraphPad Prism version 10 (GraphPad Software, USA). Data were expressed as mean ± standard deviation (SD). Group differences in outcome variables were assessed using one-way analysis of variance (ANOVA) followed by appropriate *post hoc* tests for pairwise comparisons.

Pearson correlation analysis was conducted to determine the relation among the measured biomarkers. Multiple linear regression models were applied in two formats: (*i*) outcome-specific models with each biomarker as the dependent variable, and (*ii*) group-wise regression models estimating adjusted pairwise differences between experimental groups. Effect sizes, including partial eta squared (η^2) for ANOVA models and coefficient of determination (R^2) for regression models, were calculated to quantify the magnitude of observed effects. Statistical significance was set at $p < 0.05$ for all analyses.

RESULTS AND DISCUSSION

Comparison of the different measured biomarkers among different groups

Fig. 1a demonstrates the significant increase in TNF-α levels in PPA-treated rats as a rodent model of autism compared to the omega group ($p = 0.0254$), indicating a pro-inflammatory effect of PPA. Co-administration of omega-3 fatty acids in PPA-induced rats lowered TNF-α concentrations; however, the reduction did not reach statistical significance. Similarly, IL-6 concentration was also found to be highest in the PPA group but significantly reduced in the PPA + omega group ($p = 0.0107$) (Fig. 1b). As shown in Fig. 1c, claudin-2 protein, a tight junction protein (CLDN2), concentrations in the control, omega, and PPA groups remained low and did not differ significantly from one another, with the least value observed in the PPA-rodent model of ASD. The result suggests that omega-3 fatty acids supplementation enhanced CLDN2 expression when administered in PPA treated rats, as a significant increase in CLDN2 in PPA + omega-3 rats has been observed when compared with control ($p = 0.0134$), omega ($p = 0.0314$) and PPA ($p = 0.0220$) (Fig. 1c). Tight junction protein 1 (TJP1, also known as Zonula occludens-1; ZO-1) was significantly ($p = 0.0373$) reduced in the PPA-rodent model of ASD, with insignificant increase in the treatment

group (Fig. 1d). These results suggest that PPA exposure was associated with reduced expression of the tight junction protein TJP1, while omega-3 fatty acids supplementation in PPA rats did not restore TJP1 levels. Figs. 1e and 1f show changes in dopamine and serotonin levels after PPA treatment and omega-3 fatty acids supplementation. PPA reduced dopamine and serotonin levels, while omega-3 fatty acids increased them toward control values. However, none of these changes was statistically significant. These results suggest that PPA may lower dopaminergic and serotonergic activity, and omega-3 fatty acids tend to normalize these neurotransmitters, though not significantly. SCFA concentrations were lowest in the PPA group, with higher levels in the control and omega-3 groups. Omega-3 fatty acids supplementation in PPA rats (PPA + omega-3) increased SCFA levels compared to PPA alone, nearing control values. However, these differences were not statistically significant (Fig. 1g). This suggests that PPA reduces SCFA production, while omega-3 fatty acids may help in restoring SCFA levels, potentially enhancing gut microbial activity. The levels of lipid peroxides and GSH in all groups studied are shown in Figs. 1h and 1i. Lipid peroxides recorded a significantly elevated level in the PPA-treated group ($p = 0.001$) and a lower level only in omega rats with insignificant difference between PPA--treated group and PPA + omega-3 group, while glutathione concentrations were similar across all four groups, with no statistically significant differences detected.

Supplementation with omega-3 fatty acids in aged autistic rats produced a selective benefit along the gut-brain axis. In the PPA neurotoxicity model, omega-3 fatty acids reduced systemic inflammation by lowering IL-6 in PPA + omega-3 *vs.* PPA, whereas TNF-α remained elevated relative to healthy omega-supplemented rats and was not reversed by supplementation (PPA *vs*. omega-3). Indices of intestinal permeability revealed a disrupted barrier in PPA rats, as indicated by lower TJP1 levels, while CLDN2 exhibited a divergent pattern across analyses, suggesting complex or context-dependent tight-junction responses rather than uniform restoration. Dopamine, serotonin and SCFAs trended upward with omega-3 fatty acids in PPA rats but did not reach statistical significance. Oxidative stress persisted despite treatment, with lipid peroxides being markedly elevated in PPA and PPA + omega-3, while glutathione levels remained unchanged. The anti-inflammatory effect of omega-3 fatty acids supplementation observed in this study, particularly the reduction of IL-6 in PPA-treated aged rats, is consistent with previous reports that long-chain omega-3 PUFAs modulate cytokine activity through incorporation into cellular membranes and the production of specialised pro-resolving mediators such as resolvins and protectins (8, 18). Interestingly, TNF-α remained elevated despite supplementation, suggesting that once this cytokine cascade is strongly induced, omega-3 fatty acids alone may be insufficient to reverse it. This finding aligns with earlier studies indicating that omega-3 fatty acids exert stronger effects on IL-6 driven pathways than on TNF-α signalling, particularly in the context of ageing and chronic inflammation (9, 19). Alterations in intestinal barrier proteins paralleled these inflammatory changes. As expected, TJP1 was reduced in the PPA group, confirming barrier disruption as described in inflammatory gut disorders (20). However, CLDN2 was unexpectedly elevated in the PPA + omega-3 group, a finding that mirrors report of CLDN2 upregulation under inflammatory stress, where it forms pore-like channels that increase paracellular permeability (21). This suggests that while omega-3 fatty acids supplementation mitigated some aspects of cytokine signalling, its capacity to restore epithelial integrity may be limited under conditions of established damage, a conclusion consistent with prior studies reporting mixed outcomes for omega-3 fatty acids and tight junction repair (10, 22).

Finally, oxidative stress markers highlighted one of the most apparent limitations of omega-3 fatty acids supplementation in this model. Lipid peroxides remained markedly elevated in PPA-treated rats despite supplementation, while glutathione levels were unchanged. These findings contrast with studies that report the antioxidant benefits of omega-3 fatty acids, but align with evidence that, under a high oxidative load, polyunsaturated fatty acids themselves are prone to peroxidation unless coupled with protective antioxidants, such as vitamin E (23). These findings suggest that omega-3 fatty acids may

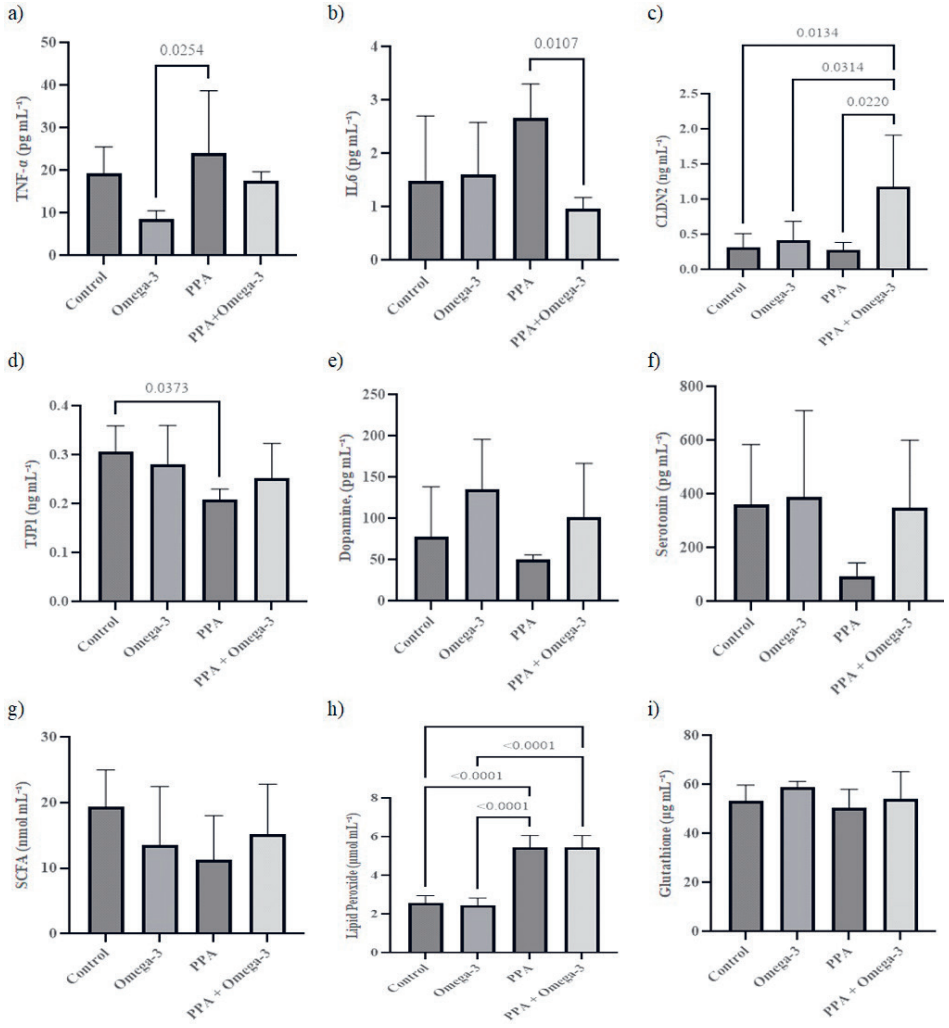

Fig. 1. Comparison of measured biomarkers between control (n = 6), omega-3 (n = 6), PPA (n = 6) and PPA + omega-3 (n = 6) group. a) TNF-α; b) IL-6; c) CLDN2; d) TJP1; e) dopamine; f) serotonin; g) SCFAs; h) lipid peroxides; i) glutathione. Data represent mean ± SD. CLDN2 – claudin-2, IL-6 – interleukin-6, TJP1 – tight junction protein-1, SCFAs – short-chain fatty acids, TNF-α – tumour necrosis factor-alpha.

be more effective in preventing oxidative stress than reversing it once established, a conclusion that resonates with the context-dependent outcomes reported across ageing and neurotoxicity models.

Correlation analysis and regression analysis

Pearson correlation analysis among the nine measured outcomes is presented in Fig. 2. Lipid peroxide showed a moderate positive correlation with TNF-α (R = 0.350) and CLDN2 (R = 0.333), and a moderate negative correlation with TJP1 (R = –0.448) and glutathione (R = –0.309). Glutathione was negatively correlated with TNF-α (R = –0.442) and positively correlated with dopamine (R = 0.462). TNF-α was strongly negatively correlated with dopamine (R = –0.614) and moderately negatively correlated with TJP1 (R = –0.411). No strong associations were observed between SCFA and other measured outcomes, although small negative correlations were noted with lipid peroxide (R = –0.186) and CLDN2 (R = –0.143). Dopamine and serotonin were positively correlated (R = 0.318). These relationships suggest coordinated alterations linking oxidative stress, inflammation, gut barrier integrity, and neurotransmitter levels, particularly the inverse association between inflammatory markers and dopamine.

Multiple linear regression models were fitted for each outcome variable, with the remaining measured parameters entered as predictors. For lipid peroxide, IL-6, SCFA, glutathione, TNF-α, CLDN2, TJP1, dopamine and serotonin, no predictors reached statistical significance after adjustment for multiple comparisons. The closest associations to significance were observed when glutathione was the dependent variable, with serotonin as a positive predictor (p = 0.093) (Table I). Conversely, when serotonin was the dependent variable, glutathione was a positive predictor (p = 0.093). Although these findings did not

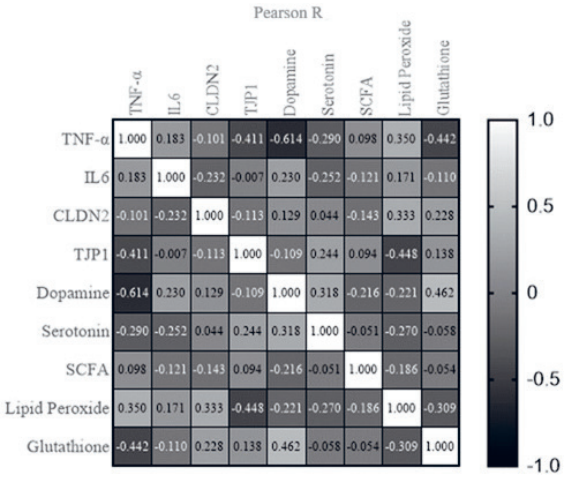

Fig. 2. Correlation matrix showing relationships among inflammatory markers, tight-junction proteins, neurotransmitters, SCFAs and oxidative stress indices. CLDN2 – claudin-2, IL-6 – interleukin-6, TJP1 – tight junction protein-1, SCFAs – short-chain fatty acids, TNF-α – tumour necrosis factor-alpha.

Table I. Multiple linear regression coefficients for predictors of glutathione and serotonin

	Coefficient				
Model	B	SE	β	*t*-value	*p*-value
Dependent variable: glutathione					
Constant	104.150	8.764		11.884	0.053
Lipid peroxide	–0.778	0.471	–0.130	–1.651	0.347
IL-6	–3.074	0.614	–0.130	–5.010	0.125
SCFA	–1.029	0.301	–0.910	–3.412	0.182
TNF	0.019	0.176	0.015	0.110	0.930
CLDN	–2.832	4.003	–0.203	–0.707	0.608
TJP1	–70.915	13.053	–0.557	–5.433	0.116
Dopamine	0.065	0.024	0.448	2.717	0.225
Serotonin	–0.019	0.003	–0.512	–6.825	0.093
Dependent variable: serotonin					
Constant	5263.236	1038.792		5.067	0.124
Lipid peroxide	–39.254	25.249	–0.246	–1.555	0.364
IL-6	–155.747	40.471	–0.589	–3.848	0.162
SCFA	–51.786	20.025	–1.707	–2.559	0.237
Glutathione	–50.786	7.441	–1.913	–6.825	0.093
TNF	0.895	9.019	0.027	0.099	0.937
CLDN	–129.372	215.897	–0.349	–0.599	0.656
TJP1	–3570.953	972.860	–1.056	–3.671	0.169
Dopamine	3.320	1.425	0.847	2.302	0.261

B – unstandardized regression coefficient, SE – standard error of B, β – standardized regression coefficient, t – t-test statistics, p – two tailed significance level ($p \leq 0.05$).

meet statistical significance, they suggest a possible relationship between antioxidant status and serotonergic activity that warrants further investigation.

Separate regression models were fitted for each biomarker to assess differences between experimental groups while accounting for within-group variability. Statistically significant findings emerged primarily in inflammatory markers, tight junction proteins, and measures of oxidative stress (Table II). TNF-α concentrations were significantly lower in the omega group compared with PPA ($p = 0.0254$, $R^2 = 0.439$), indicating that the inflammatory elevation observed in PPA rats was absent in omega-supplemented healthy rats. Comparisons between PPA and PPA + omega-3 showed no significant difference, suggesting

that omega-3 fatty acids supplementation did not fully reverse TNF-α elevation once induced. IL-6 levels were significantly reduced in PPA + omega-3 rats compared with PPA ($p = 0.0107$, $R^2 = 0.394$), demonstrating a measurable anti-inflammatory effect of omega-3 fatty acids supplementation in the PPA model. Other pairwise comparisons were not statistically significant. TJP1 levels were higher in control rats compared with PPA ($p = 0.0371$, $R^2 = 0.347$), confirming impaired tight junction integrity in the PPA group. Omega-3 fatty acids supplementation did not significantly alter TJP1 levels in PPA rats. CLDN2 concentrations in PPA + omega-3 rats were significantly higher than in control ($p = 0.0135$), omega-3 ($p = 0.0315$) and PPA ($p = 0.022$), with a model $R^2 = 0.479$. No statistically significant group differences were detected for dopamine ($R^2 = 0.269$) or serotonin ($R^2 = 0.255$). SCFA concentrations did not differ significantly between groups in the adjusted model ($R^2 = 0.165$), indicating that the modest increases observed in descriptive comparisons were not statistically supported in the regression framework. Lipid peroxide levels were markedly elevated in both PPA and PPA + omega-3 groups compared with control ($p < 0.001$) and omega-3 ($p < 0.001$),

Table II. Regression estimates of pairwise group differences in biomarkers

Outcome	Inter-group comparison	Estimate	SE	*t*-value	*p*-value	R^2
TNF-α	Control – omega	10.617	4.502	2.358	$p = 0.129$	0.439
	Control – PPA	–4.919	4.987	–0.986	$p = 0.759$	0.439
	Control – (PPA + omega)	1.533	4.987	0.307	$p = 0.990$	0.439
	Omega – PPA	–15.536	4.799	–3.237	$p = 0.0254$*	0.439
	Omega – (PPA + omega)	–9.084	4.799	–1.893	$p = 0.272$	0.439
	PPA – (PPA + omega)	6.452	5.257	1.227	$p = 0.620$	0.439
IL6	Control – omega	–0.119	0.487	–0.245	$p = 0.995$	0.394
	Control – PPA	–1.180	0.487	–2.425	$p = 0.104$	0.394
	Control – (PPA + omega)	0.532	0.487	1.092	$p = 0.698$	0.394
	Omega – PPA	–1.061	0.487	–2.180	$p = 0.163$	0.394
	Omega – (PPA + omega)	0.651	0.487	1.337	$p = 0.551$	0.394
	PPA – (PPA + omega)	1.712	0.487	3.518	$p = 0.011$*	0.394
TJP1	Control – omega	0.0262	0.035	0.750	$p = 0.876$	0.347
	Control – PPA	0.099	0.033	2.975	$p = 0.037$*	0.347
	Control – (PPA + omega)	0.054	0.035	1.550	$p = 0.43$	0.347
	Omega – PPA	0.073	0.035	2.087	$p = 0.195$	0.347
	Omega – (PPA + omega)	0.028	0.037	0.766	$p = 0.868$	0.347
	PPA – (PPA + omega)	–0.045	0.035	–1.286	$p = 0.583$	0.347
CLDN2	Control – omega	–0.101	0.248	–0.407	$p = 0.977$	0.479
	Control – PPA	0.036	0.277	0.131	$p = 0.999$	0.479
	Control – (PPA + omega)	–0.859	0.248	–3.462	$p = 0.014$*	0.479
	Omega – PPA	0.137	0.277	0.495	$p = 0.959$	0.479
	Omega – (PPA + omega)	–0.758	0.248	–3.0556	$p = 0.032$*	0.479
	PPA – (PPA + omega)	–0.895	0.277	–3.228	$p = 0.022$*	0.479

Serotonin	Control – omega	–28.951	144.847	–0.200	$p = 0.997$	0.255
	Control – PPA	264.944	138.680	1.910	$p = 0.259$	0.255
	Control – (PPA + omega)	10.135	138.680	0.073	$p = 1.000$	0.255
	Omega – PPA	293.895	138.680	2.119	$p = 0.185$	0.255
	Omega – (PPA + omega)	39.086	138.680	0.282	$p = 0.992$	0.255
	PPA – (PPA + omega)	–254.809	132.226	–1.927	$p = 0.252$	0.255
Dopamine	Control – omega	–57.618	36.115	–1.595	$p = 0.414$	0.269
	Control – PPA	26.632	41.702	0.639	$p = 0.918$	0.269
	Control – (PPA + omega)	–24.764	38.306	–0.646	$p = 0.915$	0.269
	Omega – PPA	84.250	41.702	2.020	$p = 0.23$	0.269
	Omega – (PPA + omega)	32.854	38.305	0.858	$p = 0.826$	0.269
	PPA – (PPA + omega)	–51.396	43.613	–1.178	$p = 0.650$	0.269
SCFA	Control – omega	5.869	4.239	1.385	$p = 0.523$	0.165
	Control – PPA	8.128	4.239	1.917	$p = 0.253$	0.165
	Control – (PPA + omega)	4.165	4.239	0.983	$p = 0.761$	0.165
	Omega – PPA	2.256	4.239	0.532	$p = 0.950$	0.165
	Omega – (PPA + omega)	–1.704	4.239	–0.402	$p = 0.977$	0.165
	PPA – (PPA + omega)	–3.960	4.239	–0.934	$p = 0.787$	0.165
Glutathione	Control – omega	–5.667	4.395	–1.289	$p = 0.58$	0.164
	Control – PPA	2.889	4.395	0.657	$p = 0.912$	0.164
	Control – (PPA + omega)	–0.778	4.395	–0.177	$p = 0.998$	0.164
	Omega – PPA	8.555	4.395	1.947	$p = 0.241$	0.164
	Omega – (PPA + omega)	4.889	4.395	1.112	$p = 0.686$	0.164
	PPA – (PPA + omega)	–3.667	4.395	–0.834	$p = 0.838$	0.164
Lipid peroxide	Control – omega	9.40E-02	0.296	3.18E-01	$p = 0.989$	0.907
	Control – PPA	–2.88E+00	0.296	–9.73E+00	$p < 0.001$*	0.907
	Control – (PPA + omega)	–2.88E+00	0.296	–9.73E+00	$p < 0.001$*	0.907
	Omega – PPA	–2.97E+00	0.296	–1.00E+01	$p < 0.001$*	0.907
	Omega – (PPA + omega)	–2.97E+00	0.296	–1.00E+01	$p < 0.001$*	0.907
	PPA – (PPA + omega)	4.44E-16	0.296	1.50E-15	$p = 1.000$	0.907

Estimate – regression coefficient representing the mean difference between two groups (first group minus second group), SE – standard error, t – t-test statistics, R^2 – coefficient of determination indicating the proportion of variance in the outcome explained by regression model. * Denotes statistical significance ($p < 0.05$).

with a very high explanatory power ($R^2 = 0.907$). No significant difference was observed between PPA and PPA + omega-3, indicating that omega-3 fatty acids supplementation did not attenuate lipid peroxidation once induced. Glutathione levels showed no statistically significant differences between groups ($R^2 = 0.164$), consistent with the absence of group effects in the descriptive analysis. The group-wise regression identified clear omega-3 fatty acids-associated improvements in IL-6 and CLDN2, but no significant impact on

TNF-α, TJP1, neurotransmitters, SCFA or glutathione. Persistent lipid peroxide elevation in both PPA and PPA + omega groups highlight that oxidative stress was not alleviated by supplementation in this model.

The interplay between inflammation and neurotransmission was also evident in our results. Although dopamine and serotonin levels did not differ significantly between groups, the upward trends observed with omega-3 fatty acids supplementation in PPA rats align with the literature, which links DHA and EPA to improved monoamine signalling, synaptic plasticity and neurogenesis (24, 25). Our correlation data further support this link, as TNF-α was strongly inversely related to dopamine, highlighting the vulnerability of dopaminergic pathways to inflammatory burden. Thus, the modest neurotransmitter recovery we observed may reflect a secondary effect of reduced inflammation rather than a direct pharmacological action of omega-3 fatty acids. A similar pattern emerged for gut microbial metabolites. Although SCFA concentrations did not significantly improve with omega-3 fatty acids supplementation, their upward trajectory is consistent with reports that omega-3 fatty acids promote enrichment of SCFA-producing bacteria such as *Bifidobacterium* and *Lactobacillus*, which in turn reinforce barrier function and exert neuroprotective effects (26–28). The lack of statistical significance in our study could be related to the short duration of intervention in an aged host, where microbial communities are less plastic than in younger models.

The correlation and regression analyses provided additional mechanistic insight into the interplay of inflammation, oxidative stress and neurotransmission. The strong negative correlation between TNF-α and dopamine underscores the established link between inflammatory burden and suppression of dopaminergic activity. In contrast, the positive association between glutathione and dopamine highlights the contribution of antioxidant capacity to neurotransmitter stability. This suggests that the modest improvements observed in neurotransmitter levels with omega-3 fatty acids may have been mediated indirectly through partial attenuation of inflammatory and oxidative pathways. However, the persistence of elevated lipid peroxides and the incomplete normalisation of TNF-α reinforce the interpretation that omega-3 fatty acids supplementation is more preventive than curative in the context of established neuroinflammatory and oxidative stress states. Correlation patterns linked higher lipid peroxides to higher TNF-α and CLDN2, and to lower TJP1 and glutathione, and inversely tied TNF-α to dopamine, underscoring an inflammation-oxidative stress-neurotransmitter coupling. Taken together, the data indicate that in aged hosts, omega-3 fatty acids confer a measurable anti-inflammatory effect (particularly on IL-6) but is insufficient to reverse established oxidative injury or fully normalise gut barrier signatures under PPA challenge.

Histological findings

H&E-stained rat brain tissue (Fig. 3) from the control group (a) showed normal neurons with preserved morphology (arrows). Omega-3 supplemented (b) controls also displayed normal neuronal architecture. In contrast, PPA-treated rats (c) exhibited numerous degenerated neuronal cells (arrowheads) with fewer normal cells (arrows). In the PPA + omega-3 group (d), there was a marked reduction in degenerated neurons (arrowhead) and a higher proportion of normal cells (arrows), indicating partial preservation of neuronal integrity with omega-3 fatty acids supplementation. This observation is consistent with

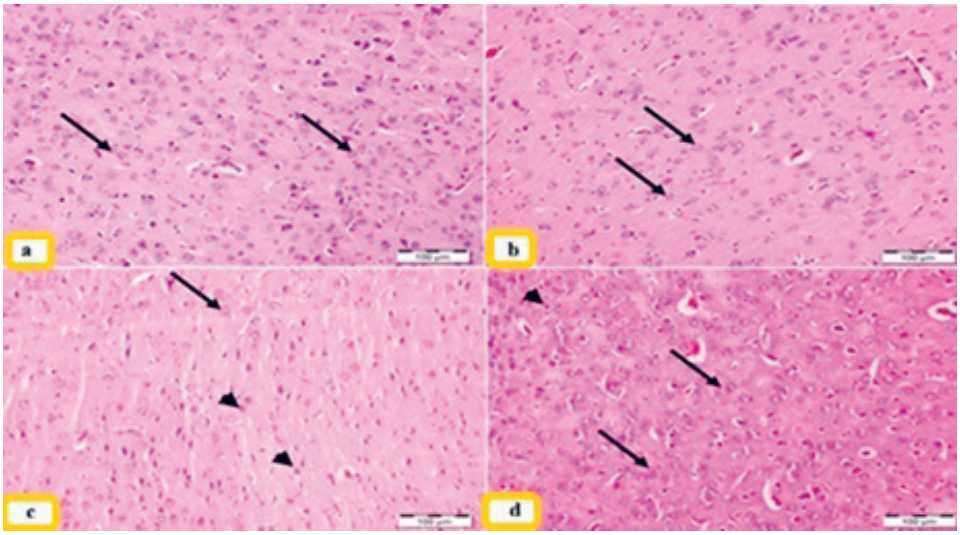

Fig. 3. H&E-stained rat brain sections (bar 100 μm): a) control: normal neuronal morphology; b) omega-3: preserved neuronal architecture; c) PPA: numerous degenerated neuron (arrow heads) with reduced normal cells; d) PPA + omega-3: decreased neuronal degeneration (arrow head) and improved structural preservation.

previous findings demonstrating a significant increase in Purkinje neuronal cell size and density in PPA-induced rodent models fed three types of fish, an effect that may be attributable to the high omega-3 fatty acid content of these fish (29). H&E-stained rat small intestine from control group (a) displayed intact villi with normal epithelial lining (arrows) (Fig. 4). Omega-3 fatty acids supplemented (b) controls showed similar villous morphology. PPA-treated (c) rats exhibited multiple degenerated villi with detached apical epithelium (arrows) and severe inflammatory cell infiltration within the lamina propria (arrow heads). In the PPA + omega-3 group (d), the proportion of degenerated villi was reduced (arrows), with only mild inflammatory cell infiltration (arrowhead), suggesting that omega-3 fatty acids supplementation partially ameliorated PPA-induced intestinal damage. In a previous report, H&E staining revealed an increase in inflammatory cells and disruption of crypt architecture in the colonic tissues of the valproic acid (VPA) group, while PUFA supplementation in VPA-treated rats attenuated these changes and significantly increased the expression of colonic junction proteins (30).

Study limitations

Several inherent limitations of our study should be acknowledged. First, the relatively small sample size may have reduced statistical power, particularly for variables such as neurotransmitters and SCFAs, where differences appeared biologically meaningful but did not reach significance. Second, SCFA measurements and microbiome sequencing were conducted only at the endpoint, which limited our ability to track dynamic changes in microbial composition and metabolite production throughout the supplementation period.

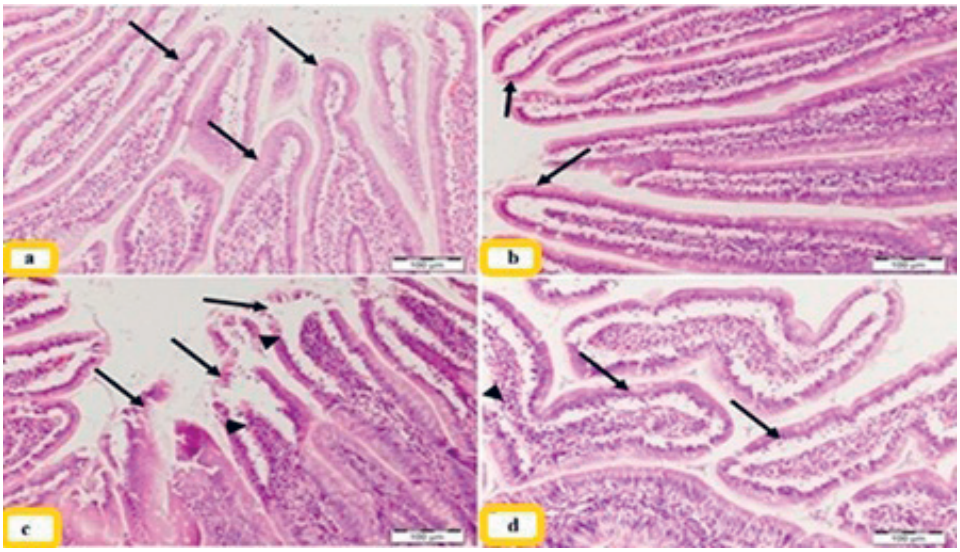

Fig. 4. H&E-stained rat small intestine (bar 100 μm): a) control: normal villous architecture, b) omega-3: intact villi similar to control, c) PPA: degenerated villi, epithelial detachment, and marked inflammatory infiltration (arrow head), d) PPA + omega-3: reduced villous degeneration and mild inflammatory infiltration (arrow head).

Longitudinal sampling would have provided clearer insights into the temporal relationship between omega-3 fatty acids intake, microbial modulation and systemic outcomes. Third, while biochemical and histological analyses were comprehensive, behavioural testing was not included. As a result, we were unable to link molecular and histological improvements to functional outcomes such as cognition, learning or memory, which are critical for translating preclinical findings to ageing-related neurodegeneration in humans. Finally, future studies should investigate multiple doses to establish dose-response relationships and optimal therapeutic ranges. Collectively, these limitations suggest that future studies with larger cohorts, longitudinal microbiome profiling, and behavioural endpoints are needed to validate and extend the current findings.

CONCLUSIONS

Ageing is accompanied by progressive disruption of gut barrier function, heightened systemic inflammation, neurotransmitter imbalances and oxidative stress, all of which converge on the gut-brain axis to accelerate vulnerability to neurodegenerative changes. In this study, omega-3 fatty acids supplementation in aged rats selectively reduced IL-6 and showed modest trends toward improvement in neurotransmitters and microbial metabolites, but failed to reverse oxidative damage or consistently restore tight junction integrity. These findings highlight the potential of omega-3 fatty acids as a supportive strategy to mitigate aspects of age-related neuroinflammation, while underscoring its

limited capacity to repair established oxidative injury. Taken together, our work suggests that omega-3 fatty acids may be most beneficial when employed as a preventive or early intervention, or in combination with complementary therapies, to promote resilience of the gut-brain axis during ageing.

Abbreviations, acronyms, symbols. – ASD – autism spectrum disorder; CLDN2 – Claudin-2; IL-6 – interleukin-6; PPA – propionic acid; SCFAs – short chain fatty acids; TJP1 – tight junction protein 1; TNF- α – tumour necrosis factor- α; ZO-1 – Zonula occludens-1.

Ethical issue. – 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 Research Ethics Committee at King Saud University (IRB # KSU-SE-25-6), Riyadh, Saudi Arabia.

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Conflict of interest. – The authors declare no conflict of interest.

Author's contribution. – Conceptualization, A.A., H.A., and I.A.A.; writing, original draft preparation, A.A., R.M., and H.A.; writing, review and editing, S.A.; supervision, A.A., A.R.K., and H.A.; funding acquisition, H.A. and A.A. All authors have read and agreed to the published version of the manuscript.

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