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The Independent and Combined Effects of Omega-3 and Vitamin B12 in Ameliorating Propionic Acid Induced Biochemical Features in Juvenile Rats as Rodent Model of Autism

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Abstract

Metabolites of proper fatty acids modulate the inflammatory response and are essential for normal brain development; equally, abnormal fatty acid metabolism plays a critical role in the pathology of autism. Currently, dietary supplements are often used to improve the core symptoms of Autism spectrum disorder (ASD). The present study analyzed the effects of orally supplemented omega-3 (ω-3) and vitamin B12 on ameliorating oxidative stress and impaired lipid metabolism in a propionic acid (PPA)-induced rodent model of autism, together with their effect on the gut microbial composition, where great fluctuations in the bacterial number and strains were observed; interestingly, polyunsaturated fatty acids such as omega-3 induced higher growth of the gram-positive bacterium *Staphylococcus aureus* and decreased the survival rates of *Clostridia sp.* as well as other enteric bacterial strains. Thirty-five young male western albino rats were divided into five equal groups. The first group served as the control; the second group was given an oral neurotoxic dose of PPA (250 mg/kg body weight/day) for 3 days. The third group received an oral dose of ω-3 (200 mg/kg body weight/day) for 30 days after the 3-day PPA treatment. Group four was given an oral dose of vitamin B12 (16.7 mg/kg/day) for 30 days after PPA treatment. Finally, group five was given a combination of both ω-3 and vitamin B12 at the same dose for the same duration after PPA treatment. Biochemical parameters related to oxidative stress and impaired fatty acid metabolism were investigated in the brain homogenates of each group. The effects of the dietary supplements on the gut microbiota were also observed. The PPA-treated autistic model expressed significantly higher levels of lipid peroxides and 5-lipoxygenase (5-LOX) and significantly less glutathione (GSH), glutathione S-transferase (GST), and cyclooxygenase 2 (COX2) than the control group. However, a remarkable amelioration of most of the impaired markers was observed with oral supplementation with ω-3 and vitamin B12, either alone or in combination. Our results concluded that impairment at various steps of the lipid metabolic pathways may contribute to the development of autism; however, supplementation with ω-3 and vitamin B12 can result in a positive therapeutic effect.

Keywords Omega-3 · Vitamin B12 · Oxidative stress · Lipid metabolism · Gut microbiota

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Introduction

Animal models are usually used to test pathological mechanisms of disease and to suggest potential treatments targeting the affected metabolic pathways. Although autism affects humans, animal models of this disorder can help uncover the etiology of autism and test therapeutic agents (Erdogan et al. 2017). MacFabe et al. (2007) and El-Ansary et al. (2012) proposed that brain infusion or oral administration of propionic acid (PPA) to rat pups could induce many of the biochemical traits seen in individuals with autism. Moreover, histopathological changes, such as neuronal loss, hyaline bodies, and astrogliosis, together with several behavioral traits, such as hyperactivity, impaired social interaction, reduced exploratory activity, and increased

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repetitive behaviors, have been recorded (MacFabe et al. (2007); Khalil et al. 2015; Daghestani et al. 2017). Pathogenic overproduction of PPA in autism by Propionibacteria, such as *Clostridia* species, is well documented to contribute to the etiological mechanism of autism (Finegold et al. 2017; Fluegge 2017; Ding et al. 2017), supporting the use of PPA in the creation of an animal model of autism.

Increased oxidative stress has been repeatedly postulated to contribute to the etiology of autism (El-Ansary et al. 2017; Khemakhem et al. 2017; Meguid et al. 2017; Yui et al. 2017). Autistic patients and animal models of autism are reported to exhibit elevated lipid peroxidation and decreased expression of detoxifying agents (e.g., glutathione) and antioxidants involved in the defense system against reactive oxygen species (ROS). Moreover, a positive correlation between reduced levels of antioxidants or elevated ROS and autism severity has been recorded (Chauhan and Chauhan 2006; Khalil et al. 2015; Kałużna-Czaplińska and Jóźwik-Pruska 2016).

In relation to oxidative stress, there is emerging evidence that fatty acid metabolism and homeostasis are impaired in autism, which might be due to dietary insufficiency and abnormalities in fatty acid-metabolizing enzymes (Ming et al. 2005). Abnormal fatty acid metabolism is well documented to affect normal brain function, especially during development. Indeed, a direct relationship between impaired fatty acid metabolism at various sites and the pathophysiology of autism has been repeatedly documented (Chauhan et al. 2004; James et al. 2004). The disturbance of the gut microbial composition due to both impaired fatty acid intake and/or metabolism have been observed (Bakken et al. 2011). The gut consists of millions of microbiota which together with its metabolites might be involved in the pathophysiology of autism. Accumulating evidences showed modulation of the gut microbiota is a potential therapy in treating autism. Several articles have reviewed the influence of the gut microbiota on the animal central nervous system (CNS) and suggested the existence of a microbiota gut-brain axis (Bienenstock et al. 2015; Mayer et al. 2015) which can be greatly affected by dietary intake (Wu et al. 2011). Herstad et al. (2017) reported that higher dietary fat intake greatly influence the gut bacterial composition mainly by increased bile acid secretion. Omega-3 fatty acids, on the other hand, exhibited significant improvements in social behaviors when administered for 12 weeks (Ooi et al. 2015) and most efficient at increasing survival and decreasing bacterial loads (Svahn et al. 2016).

COX-2 has been widely studied as an important enzyme that plays a critical role in polyunsaturated acid (PUFA) metabolism. COX-2 is highly expressed in tissues under inflammatory or neurotoxic stress. ω-3 has been shown to effectively modulate the high expression of COX-2, in addition to its ability to control the ω-6 PUFA level (Boudrault et al. 2010). 5-LOX is an iron-containing dioxygenase that catalyzes the addition of oxygen to polyunsaturated fatty acids (PUFAs) such as arachidonic acid (Shimizu and Wolfe 1990). 5-LOX has been shown to play important roles in human pathology through its central role in leukotriene biosynthesis. Leukotrienes, as important lipid mediators, are active in low concentrations and induce immunomodulatory and proinflammatory effects. Inhibition of the expression or activity of 5-LOX has been shown to ameliorate neuroinflammation, restore normal synaptic plasticity, and improve learning and memory function in depressed rats (Luo et al. 2016).

Das et al. (2003) suggested that adequate prenatal and postnatal levels of various PUFAs, especially docosahexaenoic acid (DHA), an ω-3 fatty acid, are essential for the growth and development of the brain and effective at improving cognitive function. ω-3 is well accepted to be needed for the appropriate growth and development of the brain and proper synapse formation, as well as to improve cognitive function. Vit. B_{12} deficiency is usually concurrent with folate deficiency, which contributes to neurological abnormalities and birth defects (Saghiri et al. 2017). Vit. B_{12} deficiency is also inversely proportional to the homocysteine level, which is a known modulator of lipid metabolism. Vit. B_{12} supplementation has been associated with the normalization of the Hcy level and amelioration of impaired lipid metabolism (Jankowska et al. 2017). Indeed, human gut microbes are likely to present direct competition with their host for Vit. B12 (Degnan et al. 2014). Notably, individuals with high bacterial loads in their small intestines tend to have low Vit. B12 status (Albert et al. 1980; Brandt et al. 1977; Murphy et al. 1986).

Based on the fact that oxidative stress, impaired lipid metabolism, and decreased levels of ω-3 and Vit. B_{12} have been shown to be associated with the etiological mechanism of neuropsychiatric disorders (Hunaiti 2016), testing the effects of oral supplementation with ω-3 and Vit. B_{12} on ameliorating oxidative stress and lipid metabolic defects in a rodent model of autism induced by PPA neurotoxicity and identifying the involved enzymes are necessary for evaluating the use of ω-3 and Vit. B_{12} as a novel therapy but also by mediating fundamental biological processes in microbes, representing as such an attractive target for reshaping microbial communities.

Material and Methods

Animals A total of 35 young male western albino rats (80–120 g) were obtained from King Saud University Riyadh. Rats were randomly allocated to the following groups. The control group was given only phosphate-buffered saline. The oral buffered PPA-treated group ($n = 7$) was given a neurotoxic dose of PPA at 250 mg/kg body weight/day for 3 days (El-Ansary et al. 2012). The omega-3-treated group ($n = 7$) was orally given ω-3 at a dose of 200 mg/kg body weight/day for

30 days after the 3-day PPA treatment (Abdou and Hassan 2014). A third group of seven rats was given Vit. B_{12} (16.7 mg/kg/day) for 30 days after the 3-day PPA treatment (Abdulmajeed et al. 2015). A fourth group was given a combination of ω-3 and Vit. B_{12} for the same duration post PPA treatment. All groups were housed at a controlled temperature (21 ± 1 °C) with ad libitum access to food and water. All experiments were performed in accordance with national animal care guidelines and were pre-approved by the faculty ethics committee of King Saud University.

Ethics Approval All animal experiments were conducted with the approval of King Saud University.

Sample Collection

Brain Tissue Whole-brain tissue was collected and washed with cold normal saline and then homogenized in ten volume/weight of double distilled water. The homogenate was then centrifuged at 3000 rpm for 10 min. The resulting supernatant was used for various biochemical assays.

Fecal Sample Collection

The fecal pellets were collected in sterile containers from all the groups in study before and after treatment in the early morning and were immediately stored at − 80 °C for the microbiological analysis.

Biochemical Analyses

1. ***Spectrophotometric analysis***
   Lipid oxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) by the method of Ruiz-Larrea et al. (1994). A vitamin C assay was performed according to the method of Jagota and Dani (1982). GSH was assayed by the method of Beutler (1963), using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) with sulfhydryl compounds to produce a relatively stable yellow color. GST activity was assessed by the method described by Habig et al. (1974) based on the GST-catalyzed reaction between GSH, the GST substrate, and CDNB (1-chloro-2,4-dinitrobenzene).
2. ***ELISA analysis***
   Levels of phospholipase A2 and COX2 were measured using kits based on the sandwich ELISA principle, products of LSBio (Lifespan BioScience, Inc., North America), with a detection range of 3.12–200 and 0.156–10 ng/ml, respectively.

   Levels of leukotriene B4 and prostaglandin E2 were measured using ELISA kits based on the competitive assay used for quantitative estimation, products of Cayman chemical (Cayman chemical company Ann Arbor, MI, USA), with a detection range of 3.9–500 and 7.8–1000 pg/ml, respectively.

Microbiological Analyses

Fecal Collection and Analysis

One gram of each fecal sample collected from each of the assigned groups in this study (control group, PPA group, ω-3, Vit. B_{12} and ω-3 + Vit. B_{12}) was homogenized in 10 ml sterile PBS solution (0.1 M, pH 7.2) using a sonicator for 30 s. The fecal solutions were then centrifuged at 5400 rpm for 3 min at 4 °C. Then, 1 ml of the fecal supernatant was serially diluted in 9 ml sterile PBS solution four times (Zhang et al. 2014).

Bacterial Culturing and Enumeration

Here, 100 μl of each of the prepared dilutions for every group of treated mice was plated on panel plates including nutrient agar (NA, Oxoid) plates, MacConkey (MAC) plates, blood agar (Bld) plates, and plates containing CCFA medium selective for Clostridia. The selective medium CCFA plates were incubated in an anaerobic jar with 5% CO_2 at 37 °C for 3 days, whereas the other culture media previously mentioned were incubated at 37 °C under aerobic conditions for 18–24 h. The experiment was repeated twice. The colony count per plate was recorded and tabulated as the average of the number of bacteria per plate.

Distinct colony types from each media used were selected, isolated, and purified on NA plates for preliminary identification, either microscopically through gram staining or through the use of different biochemical tests and selective media, namely eosin-methylene blue (EMB), the selective medium for the identification of *E. coli*; data not shown.

Statistical Analysis

The results of the present study were expressed as the means ± S.D. All statistical comparisons between the control group and the PPA, ω-3, B12, and, ω-3 + B12-treated rat groups were performed using one-way analysis of variance (ANOVA) tests with Dunnett's test for multiple comparisons. Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) was used for the statistical analyses. Significance was assigned at the level of $P < 0.05$. Receiver operating characteristic (ROC) curve analysis was also performed. The area under the curve (AUC), the degrees of sensitivity and specificity, and cutoff values were calculated. Pearson's correlations were performed between the measured parameters.

Results

Table 1 and Fig. 1 show the mean ± S.D. and the percentage change in the measured parameters in the five groups studied. PPA treatment induced a significant elevation in lipid peroxides, with an 11.72% increase, and 5-LOX, with a 100.62% increase, compared to control. On the other hand, PPA-treated rats expressed GSH, GST, and COX2 at a much lower level than control rats, showing a 22.65, 10.35, and 20.14% decrease, respectively. The ascorbic acid, leukotriene

Table 1 Mean ± S.D. of the measured parameters in the five studied groups

Parameter	Group	Mean ± S.D.	Percent change	*P* value[a]
Lipid peroxides	Control	0.50 ± 0.02	100.00	
	PPA	0.56 ± 0.02	111.72	0.003
	PPA + ω-3	0.51 ± 0.05	103.21	0.712
	PPA + B12	0.48 ± 0.03	96.33	0.609
	PPA + ω-3 + B12	0.55 ± 0.03	110.38	0.010
Ascorbic acid	Control	25.83 ± 1.40	100.00	
	PPA	24.02 ± 1.08	92.99	0.663
	PPA + ω-3	23.61 ± 2.76	91.43	0.500
	PPA + B12	26.40 ± 5.53	102.22	0.991
	PPA + ω-3 + B12	24.40 ± 2.90	94.48	0.813
GSH	Control	93.15 ± 3.27	100.00	
	PPA	72.06 ± 3.50	77.35	0.001
	PPA + ω-3	92.87 ± 1.81	99.69	1.000
	PPA + B12	100.05 ± 4.35	107.41	0.004
	PPA + ω-3 + B12	94.06 ± 4.24	100.98	0.969
GST	Control	200.38 ± 23.11	100.00	
	PPA	179.65 ± 18.20	89.65	0.047
	PPA + ω-3	159.13 ± 8.36	79.41	0.001
	PPA + B12	138.27 ± 2.53	69.00	0.001
	PPA + ω-3 + B12	171.44 ± 12.85	85.56	0.004
5-LOX	Control	293.88 ± 46.28	100.00	
	PPA	589.59 ± 126.69	200.62	0.001
	PPA + ω-3	402.66 ± 65.13	137.01	0.029
	PPA + B12	260.00 ± 34.58	88.47	0.794
	PPA + ω-3 + B12	298.14 ± 50.52	101.45	1.000
COX2	Control	81.66 ± 6.50	100.00	
	PPA	65.21 ± 5.24	79.86	0.005
	PPA + ω-3	77.41 ± 7.17	94.79	0.766
	PPA + B12	76.51 ± 8.00	93.69	0.634
	PPA + ω-3 + B12	77.05 ± 13.72	94.35	0.715
Leukotriene B4 (pg/g brain tissue)	Control	404.55 ± 9.39	100.00	
	PPA	391.82 ± 12.40	96.85	0.754
	PPA + ω-3	366.56 ± 29.25	90.61	0.030
	PPA + B12	381.33 ± 4.60	94.26	0.274
	PPA + ω-3 + B12	393.91 ± 45.72	97.37	0.847
Prostaglandin E2 (pg/g brain tissue)	Control	267.42 ± 51.26	100.00	
	PPA	244.75 ± 4.45	91.52	0.313
	PPA + ω-3	239.87 ± 8.25	89.70	0.169
	PPA + B12	233.53 ± 8.68	87.33	0.067
	PPA + ω-3 + B12	231.76 ± 24.02	86.66	0.051

Table 1 shows the results of the one-way ANOVA test between all groups with a multiple comparisons test (Dunnett test) to compare each group with the control group in all parameters

[a] *P* value between each group and the control group

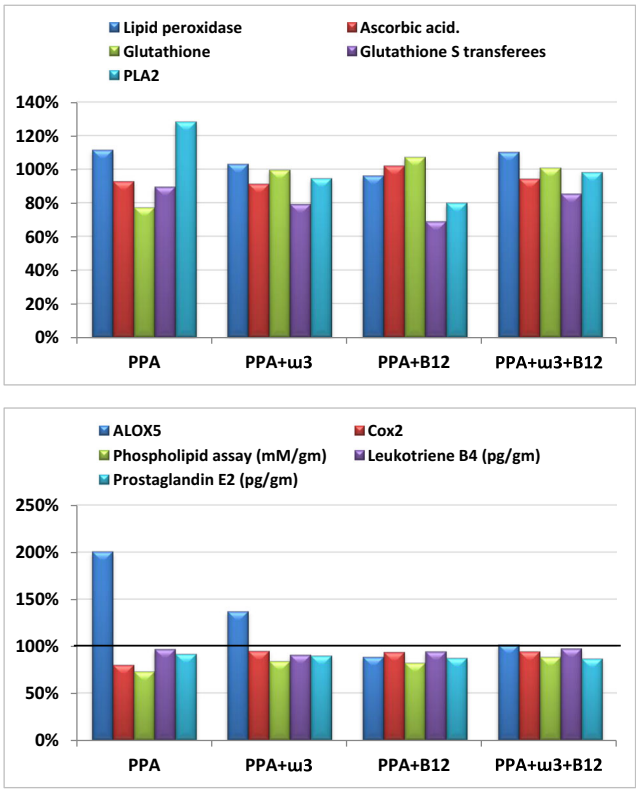

Fig. 1 Percentage change in all parameters in all groups compared to control

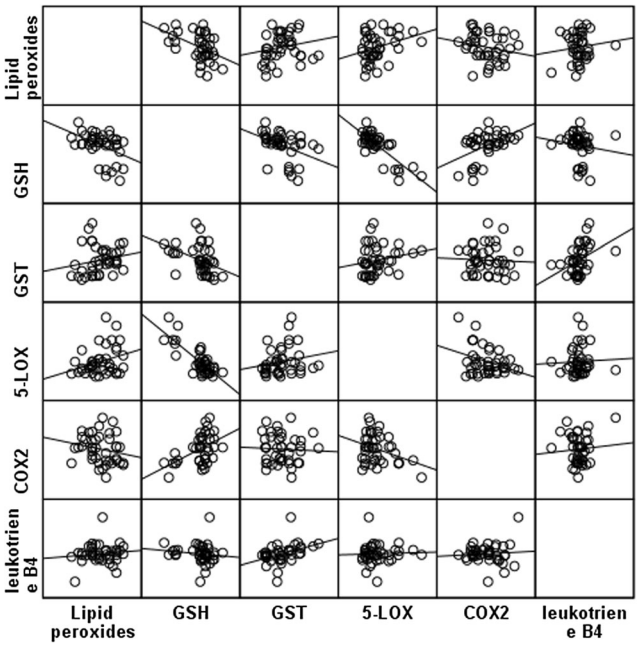

Fig. 2 Collective Pearson's positive and negative correlations between the measured variables

B4, and prostaglandin E2 levels did not change significantly with PPA treatment. The same table and figure illustrate the remarkable amelioration of most of the changes in marker expression with ω-3 and Vit. B_{12} treatment, both independently and in combination, to varying degrees.

Table 2 contains the Pearson's correlation coefficients between the measured parameters (Fig. 2). The lipid peroxide level, as a measure of oxidative stress, was negatively correlated with GSH expression ($P < 0.003$) and positively correlated with 5-LOX ($P < 0.049$). GSH was negatively correlated with GST ($P < 0.007$) and 5-LOX ($P < 0.001$) and positively correlated with COX 2 ($P < 0.001$). 5-LOX was negatively correlated with COX2 ($P < 0.029$).

Table 3 presents the cutoff values, AUC, sensitivity, and specificity of each of the measured parameters for the PPA-treated group and the PPA-treated groups supplemented with either ω-3 and Vit. B_{12} independently or in combination (ω-3 + Vit. B_{12}). Most of the measured parameters exhibited satisfactory AUCs, specificity, and sensitivity as a marker of PPA neurotoxicity and/or the therapeutic effect of ω-3 and Vit. B_{12}.

Fecal bacterial analysis from each of the animal groups in the study was performed and tabulated as an average of the bacterial count per plate. Data were compared between the groups before and after treatment with the different doses of ω-3, Vit. B_{12}., and both after PPA intake. Multiple bacterial strains were identified from the fecal matter in the control

Table 2 Pearson's correlation coefficients between the measured parameters

Parameters	R (Pearson's correlation coefficient)	Sig.	
Lipid peroxides with GSH	−0.442**	0.003	a
Lipid peroxides with 5-LOX	0.306*	0.049	b
GSH with GST	−0.409**	0.007	b
GSH with 5-LOX	−0.804**	0.001	b
GSH with COX2	0.488**	0.001	a
GST with leukotriene B4	0.400**	0.009	a
5-LOX with COX2	−0.338*	0.029	b

***Correlation is significant at the 0.05 level; correlation is significant at the 0.01 level

[a] Positive correlation

[b] Negative correlation

Table 3 ROC-curve of all parameters in all groups

Parameter	Group	AUC	Cutoff value	Sensitivity %	Specificity %	*P* value
Lipid peroxides	PPA	0.990	0.515	100.0%	85.7%	0.002
	PPA + ω-3	0.602	0.530	42.9%	100.0%	0.523
	PPA + B12	0.694	0.485	57.1%	85.7%	0.225
	PPA + ω-3 + B12	0.990	0.515	100.0%	85.7%	0.002
Ascorbic acid	PPA	0.837	24.865	85.7%	85.7%	0.035
	PPA + ω-3	0.724	24.605	71.4%	85.7%	0.160
	PPA + B12	0.510	26.755	42.9%	85.7%	0.949
	PPA + ω-3 + B12	0.714	25.260	71.4%	71.4%	0.180
GSH	PPA	1.000	82.290	100.0%	100.0%	0.002
	PPA + ω-3	0.510	96.400	100.0%	28.6%	0.949
	PPA + B12	0.878	94.402	100.0%	71.4%	0.018
	PPA + ω-3 + B12	0.633	93.432	71.4%	71.4%	0.406
GST	PPA	0.837	198.650	100.0%	71.4%	0.035
	PPA + ω-3	0.939	179.100	100.0%	85.7%	0.006
	PPA + B12	1.000	149.650	100.0%	100.0%	0.002
	PPA + ω-3 + B12	0.878	187.100	100.0%	85.7%	0.018
5-LOX	PPA	1.000	385.885	100.0%	100.0%	0.002
	PPA + ω-3	0.939	336.655	100.0%	85.7%	0.006
	PPA + B12	0.735	277.595	71.4%	71.4%	0.142
	PPA + ω-3 + B12	0.531	281.305	71.4%	42.9%	0.848
COX2	PPA	1.000	73.291	100.0%	100.0%	0.002
	PPA + ω-3	0.653	77.479	71.4%	71.4%	0.338
	PPA + B12	0.653	77.251	71.4%	71.4%	0.338
	PPA + ω-3 + B12	0.612	70.453	42.9%	100.0%	0.482
Leukotriene B4 (pg/g brain tissue)	PPA	0.796	397.435	85.7%	85.7%	0.064
	PPA + ω-3	0.980	395.869	100.0%	85.7%	0.003
	PPA + B12	0.959	393.943	100.0%	85.7%	0.004
	PPA + ω-3 + B12	0.776	396.939	71.4%	85.7%	0.085
Prostaglandin E2 (pg/g brain tissue)	PPA	0.684	244.765	71.4%	71.4%	0.250
	PPA + ω-3	0.776	242.584	71.4%	85.7%	0.085
	PPA + B12	0.898	241.680	85.7%	85.7%	0.013
	PPA + ω-3 + B12	0.878	235.374	85.7%	100.0%	0.018

group, with the absence of *Clostridia* growth (Table 4); however, 1 *Clostridium* sp. colony was observed on the plate with the CCFA medium, a selective medium that characteristically labels *Clostridia* with yellow fluorescence, following PPA intake, which in turn caused a decrease in the bacterial number compared to that in the control group, with a bacterial count of 100 and 300 in the PPA and control group, respectively (Table 4).

Table 4 Colony count/plate of the fecal flora immediately after an orogastric dose of PPA (250 mg/kg body weight/day for 3 days)

Isolated organisms	Media and incubation conditions	Control	PPA
Staphylococcus and/or Bacilli (gram-positive cocci/rod or gram-negative rod)	NA/ aerobic 37 °C/24 h	300	100
Enterobacteriaceae (gram-negative rod lactose fermenters)	Mac/ aerobic 37 °C/24 h	0	0
Gram-positive/g-negative rod and cocci	Blood agar/aerobic 37 °C/24 h	100	11
Clostridium sp.	CCFA/anaerobic with 5% CO_2 3 days	0	1

Table 5 Colony count/ plate of the fecal flora from PPA-treated rats after treatment with omega-3 (200 mg/kg body weight/day), vitamin B12 (16.7 mg/kg/day), or the combination of omega-3 and vitamin B12

	Isolated Organisms	Media and incubation conditions	Control	Day 3	Day 15	Day 30
	Staphylococcus and/or *Bacilli* (Gram-positive cocci/rod or gram-negative rod)	NA/aerobic 37 °C/24 h	300	44	63	> 300
ω-3	*Enterobacteriaceae* (gram-negative rod lactose fermenters)	Mac/aerobic 37 °C/24 h	0	8	4	0
	Gram-positive/gram-negative rod and cocci	Blood agar/aerobic 37 °C/24 h	100	77	14	200
	Clostridium sp.	CCFA/ anaerobic with 5% CO_2	0	42	0	0
	Staphylococcus and/ or Bacilli (Gram-positive cocci/rod or gram-negative rod)	NA/aerobic 37 °C/24 h	300	27	200	> 300
Vit B_{12}	Enterobacteriaceae (gram-negative rod lactose fermenters)	Mac/aerobic 37 °C/24 h	0	4	0	0
	Gram-positive/gram-negative rod and cocci	Blood agar/aerobic 37 °C/24 h	100	9	10	180
	Clostridium sp.	CCFA/anaerobic with 5% CO2	0	27	0	0
	Staphylococcus and/or Bacilli (gram-positive cocci/rod or gram-negative rod)	NA/aerobic 37 °C/24 h	300	100	20	> 300
ω-3 + Vit. B_{12}	Enterobacteriaceae (gram-negative rod lactose fermenters)	Mac/aerobic 37 °C/24 h	0	2	0	0
	Gram-positive/gram-negative rod and cocci	Blood agar/aerobic 37 °C/24 h	100	35	4	100
	Clostridium sp.	CCFA/anaerobic with 5% CO_2	0	0	0	0

A higher number of *Clostridia* colonies were found primarily at day 3 after individual treatment with ω-3 and Vit. B_{12} (42 and 27 colony counts per plate) (Table 5). In contrast, treatment with the combination of Vit. B_{12} and ω-3 at the given doses inhibited *Clostridia* growth. A similar lack of *Clostridia* growth was observed throughout the treatment period.

The microbial profile of the last group treated with both ω-3 and Vit. B_{12} (Table 6) was mainly dominated by the presence of *Staphylococcus aureus*, identified as a bacterium with a grape-like structure when observed under the microscope following gram staining. On the other hand, a slight growth or even absence of enteric bacteria (gram-negative rod) was observed during the study period.

Discussion

Although the neurotoxic effects of PPA have been repeatedly recorded, the current study first aimed to ascertain the neurotoxic effect of PPA through the induction of oxidative stress and the impairment of lipid metabolism, which are two known etiological mechanisms of autism. Second, the study aimed to evaluate the possible therapeutic effect of ω-3 and Vit. B_{12} or their combination on the PPA-induced neurotoxicity in rat pups and to study the intestinal bacterial number and strain fluctuation in response to the dietary intake in study. The ω-3 polyunsaturated fatty acid, consisting of DHA and eicosapentaenoic acid (EPA), and Vit. B_{12} were selected because both play regulatory roles in central nervous system (CNS) enzyme activity as co-factors and are important in the correct metabolic function of these enzymes (Feng et al. 2012; Youdim et al. 2000;McCaddon et al. 2002).

Table 1 and Fig. 1 demonstrate the remarkable oxidative stress induced in the rat brain after PPA treatment. This oxidative stress can be observed through the significant increase in lipid peroxides together with the significant decrease in GSH expression. This observation is in accordance with our previous work in which oxidative stress was reported to be

Table 6 Summary of the dietary effects of the treatments on the bacterial growth in the present study

Bacterial number and strains	PPA	Omega 3	Vitamin B12	Omega 3 + Vit. B12
Gram-positive bacteria (cocci or bacilli)	↓	↑	↑	↓
Staphylococcus aureus	↑	↑	↑	↑
Enterobacteriaceae (gram-negative bacteria)	_	_	_	_
Clostridium sp.	↓	↑0 time End of treatment ↓	↓	↓

one of the persistent autistic features found in PPA-orally administered rat pups (El-Ansary et al. 2012). Moreover, GST expression was remarkably lower in PPA-treated rats than in control rats. The antioxidant effects of ω-3 PUFA reported in the present study are supported by the findings in the recent work of Mazereeuw et al. (2017), which showed antidepressive effects of ω-3 PUFA through the amelioration of oxidative stress. A reduction in oxidative stress, one of the major etiological mechanisms of autism, has been shown to alleviate autistic-like behaviors such as social impairment and repetitive behavior (Al-Amin et al. 2015). Weiser et al. (2016) proved that elevated dietary levels of ω-3 PUFA in pregnant mice were protective against maternal infection as environmental insults. Furthermore, these authors demonstrated that dietary supplementation with DHA can reduce autistic-like behaviors resulting from oxidative stress caused by maternal infection in mice (Weiser et al. 2016).

Vit. B_{12} demonstrates antioxidative properties and is involved in the biosynthesis of myelin and phospholipids, which are critically important compounds during brain development. Vit. B_{12} also exhibits anti-inflammatory and anti-apoptotic effects (Kikuchi et al. 1997; Masuda et al. 1998; Zhang et al. 2008 and Das 2008). These mechanisms could explain the observed beneficial effects of Vit. B_{12} in the present study, either independently or in its combination with ω-3 PUFA. This benefit can find more support in the recent study conducted by Moosavirad et al. (2016) in which Vit. B_{12} and ω-3 or their combination was effective at ameliorating the toxic effect of lead and restoring lead-induced cognitive loss.

The synthesis of leukotrienes from AA is initiated with 5-LOX in concert with 5-LOX-activating protein (FLAP). Although FLAP does not have catalytic activity, it activates the ability of 5-LOX to react with AA. Leukotriene A4 (LTA4) is either conjugated with reduced glutathione by leukotriene C4 (LTC4) synthase to yield LTC4 or is converted into leukotriene B4 (LTB4) by LTA4 hydrolase. LTB4 and LTC4 are exported from the cell by specific transporter proteins. Exported LTC4 is the parent compound of cysteinyl leukotriene (LTD4), which undergoes conversion to leukotriene E4 (LTE4) by sequential amino acid hydrolysis. The amount of LTB4 and cysteinyl leukotriene (LTD4 and LTE4) depends on the distal enzymes LTA4 hydrolase and LTC4 synthase, respectively. LTA4 and LTB4 (non-cysteinyl leukotrienes) are structurally different from the cysteinyl leukotrienes (Cys-LT) as they lack the cysteine moiety present in the Cys-LT (LTC4, LTD4, and LTE4) (Kuhn et al. 2015). Table 1 and Fig. 1 demonstrate that in spite of the twofold increase in 5-LOX expression in the PPA-treated rat pups compared to that in the control group, LTB4 levels were non-significantly changed. This difference may be explained on the basis that LTC4 synthase might have a lower Km and higher affinity for LTA4 than LTA4 hydrolase. Moreover, the non-significant change in LTB4 in spite of the remarkable increase in 5-LOX activity can be attributed to the fact that enzymatic hydration products (LTB4) are primarily less reactive metabolites that can be conjugated and excreted. A great analogy between human, mouse, and rat LTC4S has been reported. Human and mouse LTC4S have highly similar catalytic characteristics to rat LTC4S, with recorded Km and Vmax values of 18.8 ± 2.9 μM and 56.2 ± 5.6 nM/min/mg protein, respectively, when LTA4 was used as the substrate (Schröder 2007). These two suggested mechanisms are also supported by the significant decrease in GSH and GST, two components critically required for either LTB4 conjugation and excretion or the biosynthesis of LTC4 from LTA4 by LTC4S (Seidegård and Ekström 1997). Moreover, interestingly, LTA4 hydrolase is inhibited by its substrates, a process that limits the production of LTB4 in LTA4S-containing cells (McGee and Fitzpatrick 1986). Under conditions of essential fatty acid deficiencies, such as PPA neurotoxicity (El-Ansary et al. 2016), the production of 5-LOX metabolites results in the inhibition of LTA4 hydrolase, decreasing basal LTB4 production below what would be expected from AA acid depletion (Stenson et al. 1984; Cleland et al. 1994). This explanation is supported by the most recent study by Zakharov et al. (2017), which reported that patients with brain damage had lower LTB4 levels than healthy controls. Table 1 and Fig. 1 also present the ameliorating effect of ω-3 and Vit. B_{12}, with Vit. B_{12} being the most effective followed by treatment with the combination of Vit. B_{12} and ω-3 and treatment with ω-3 alone, which was less potent, resulting in a 37% increase in 5-LOX activity compared to that in control untreated rats.

The unexpected decrease in COX2 and PGE2 expression in response to PPA-intoxication ($P < 0.005$) (Table 1 and Fig. 1) may be related to the observed alteration in the gut microbiota of the treated rats. COX-2 is well known to have a critical role in the adaptive cytoprotection response in gastrointestinal (GI) mucosal cells. When the GI is inflamed (e.g.) in response to toxins of pathogenic bacteria overgrowth, large amounts of PGs are produced at sites of injury by rapidly induced COX-2 expression, which usually aids in the healing process of the injured gut. Under this condition, inhibition of COX-2 should be avoided in patients who are vulnerable to GI inflammation (e.g., autistic patients) (Parente 2001). Table 1 and Fig. 1 also demonstrate the effects of independent or combined treatment with ω-3 and Vit. B_{12}. The three therapeutically treated groups did not demonstrate a significant difference in COX-2 expression when compared to control-untreated rat pups, but COX-2 expression in all of the therapeutically treated groups was significantly different from that in the PPA-treated rats ($P < 0.018$).

Our findings are supported by the work of Tabbaa et al. (2013), which showed that after intravenous injection of *Escherichia coli* LPS in animal models, fish oil, a major source of ω-3, effectively restored the intestinal integrity and decreased LPS-induced inflammation (Liu et al. 2012;

Oliver et al. 2012; Titos et al. 2011). Moreover, fish oil induced the synthesis of PGs, an important COX-2 product. PGs have modulatory effects on GI inflammation through the induction of resolvin D1 and protectin D1, which reduce the macrophage pro-inflammatory response to LPS associated with *E. coli*. This modulatory effect was associated with an enhanced production of tumor necrosis factor-α (TNF- α) to assist in the clearance of the pathogenic bacteria (Palmer et al. 2011; Weylandt et al. 2012).

Studies of the gut microbiota of mammals have shown that several bacterial species, predominantly belonging to the phyla *Bacteroidetes* and *Firmicutes*, are found and that their presence is highly influenced by the host diet (Ley et al. 2008) (Hooper and Gordon 2001; Sonnenburg et al. 2010). However, this change occurs within a short period of time (1–4 days after diet intake) (Hooper and Gordon 2001; Sonnenburg et al. 2010). The variability in bacterial types screened from the animal groups in this study pre- and post-treatment was in accordance with previous findings, showing various types of bacteria and a complete absence of *Clostridium sp.* in the control group. However, *Clostridia* growth was found to be induced with PPA intake on day 3 and reached its highest number following individual treatment with ω-3 and Vit. B_{12}. *Clostridia* growth then decreased or disappeared once again after more doses of ω-3 and Vit. B_{12}. The fecal flora from those treated with the combination of ω-3 and Vit. B_{12} did not show *Clostridia* growth at any time point along the treatment period. These results indicated that dietary intake alters the gut microbiota in a relatively short amount of time. On the other hand, the last group of rats treated with ω-3 and Vit. B_{12} showed that the intestinal composition of the rats in this study mainly included *Staphylococcus aureus* following ω-3 dietary intake. This observation suggests that higher intake of polyunsaturated fatty acids alters the gut microbiota, resulting in a microbiota mainly dominated by the gram-positive bacteria *Staphylococcus aureus*, potentially related to an increase in carbohydrate production in the gut environment promoting the colonization of these gram-positive cocci. Furthermore, the influence of dietary supplementation on *Clostridium sp*. growth and the restoration of a healthy microbiota shown in this study suggest that these dietary supplements could be considered as promising alternative treatments for *Clostridium difficile* disease and other intestinal dysbiosis (Borody et al. 2004; Bakken et al. 2011). Studies related to the intake of a specific dietary component have demonstrated that bacteria may respond to a specific dose of a nutrient either by decreasing or increasing in number or even by being masked by other species. Fats, proteins, carbohydrates, and probiotics all induce changes in the gut microbiota with effects observed on host immunity and metabolic markers. A high unsaturated fat diet has not been reported, from human studies, to induce a significant alteration in the gut bacterial profile; however, mouse studies have reported increases in *Actinobacteria* (*Bifdobacterium and Adlercreutzia*), lactic acid bacteria (*Lactobacillus and Streptococcus*), and *Staphylococcus aureus*, as observed in this study. Thus, a healthy microbiota is critical for maintaining the metabolic lifestyle of the host.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Ethical Approval All procedures performed were in accordance with the ethical standards of the institutional research committee, Ref No.:4/67/352670.

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