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Omega-3 modulates anxiety and improves autistic like features induced by high fat diet but not valproate

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ABSTRACT

Background: continuous consumption of high-fat diet (HFD) during pregnancy and lactation could alter developmental complexity of higher brain functions and enhance appearance of social withdrawal and anxiety among offsprings. On the contrary, omega-3 fatty acids with its multi-neuro-protective effects could limit oxidative stress production and complications. We aimed to investigate the impact of HFD intake before and during pregnancy till weaning versus prenatal exposure to valproate (as a model of autism) on behavior and brain neurochemistry as well as the possible therapeutic rational of omega-3 intake.

Material and methods: After confirmation of G0 day of pregnancy, thirty Sprague–Dawley female rats were divided into: control group fed normal chow (10 kcal% from fat), group II (HFD) mothers supplied with diet of 60 kcal% from fat and group III (HFD-Omega) supplied with 60% HFD enriched with omega-3, group IV (Valp) pregnant females received sodium valproate once i.p, and group V (Valp-Omega) rats received valproate once i.p and diet enriched with omega-3. From day 7 till end of the study, offsprings were subjected to growth, neurodevelopmental assessment and behavioral tests using elevated plus maze and social interaction in open field. Levels of serotonin, GABA, neuropeptide Y, IL-6 and relative gene expression of syntaxin1A and FoxO1 gene were measured.

Results: Offsprings born to HFD and Valp-groups demonstrated growth retardation, social withdrawal together with disturbed levels of measured neurotransmitter that were improved in HFD group supplied with omega-3.

Conclusion: Omega-3 exhibited to be potential modulator of behavioral changes and autistic-like features induced by HFD.

1. Introduction

The prevalence of autism spectrum disorder (ASD) rises continually. In spite of the presence of many risk factors, some researches focused on neuropsychological drug used for treating epilepsy, valproate (VPA) (Mabunga, Gonzales, Kim, Kim, & Shin, 2015). Christensen et al. (2013) confirmed high risk of ASD development among offsprings born to mothers treated with VPA. However, genetic abnormalities and environmental factors remain to be exciting as one the causes of ASD.

Generally, chronic consumption of HFD could affect cognitive functions, attention, raise the incidence of depression, anxiety and induce low-grade neuro-inflammation (Duman, Li, Liu, Duric, & Aghajanian, 2012; Jacka, Cherbuin, Anstey, Sachdev, & Butterworth, 2015). About 80% of ASD cases have no clear etiology and the impact of HFD intake during pregnancy needs more investigations (Mariani et al., 2015). Consumption of HFD is associated with increased serum cytokine levels and contributes to the imbalance between pro-/anti-inflammatory cytokines in brain tissues (Lavin et al., 2011). Maternal obesity may influence changes in lipid peroxidation thus could alter hippocampal neurogenesis in offsprings (Kang, Kurti, Fair, & Fryer, 2014) born to HFD fed dams (60% of food caloric value is from fat), thus indicating the contribution of maternal obesity in development of ASD, anxiety-like symptoms and long lasting altered brain homeostasis (Krakowiak et al., 2012; Moss & Chugani, 2014; Reynolds, Inder, Neil, Pineda, & Rogers, 2014). Another case control study elucidated by Field (2014) linked the development of ASD to the intake of omega-3 deficient diet, adding another predisposing factor for development of ASD (Bu, Dou, Tian, Wang, & Chen, 2016). On the contrary, omega-3 could

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produce neuroprotective effect mediated via decreasing oxidative damage induced by HFD in different brain areas (De Mello et al., 2018).

Abnormal high levels of FoxO1 (Forkhead box transcription factors) in autistic patients could be responsible for macrocephalic appearance and glutamate/GABA neuron ratio imbalance. FoxO1 gene overexpression is positively correlated with symptoms of ASD (Mariani et al., 2015). Polter et al. (2009) found that FoxO1 and FoxO3a could influence distinct behavioral processes linked to anxiety and depression. Even more, FoxO1 knockout mice displayed reduced anxiety (Cattaneo et al., 2018). The state of hypersertonemia is the first biomarker in autism (Muller, Anacker, & Veenstra-VanderWeele, 2016) however, brain serotonin levels is still a matter of debate and the contribution of perinatal intake of HFD in altering serotonin, GABA and neuropeptide Y levels, as well as the role of FoxO1 gene needs more investigations. Due to the lack of direct estimation method for serotonin levels in human brain tissues, Muller et al. (2016) concluded decreased synaptic serotonin levels based upon increased peripheral platelet content. Another study reported decreased hippocampal serotonin levels with pre-natal exposure to valproate (Dufour-Rainfray et al., 2010) Thus, we aimed to test behavior changes and to estimate the level of neurotransmitters regulating anxiety in offsprings born to valproate-treated mothers (as a well-known model of autism) versus HFD fed dams, the diet regimen started one week before mating and continued during pregnancy and lactation. A recent meta-analysis found that supplementation of omega 3 could produce promising outcomes in treating patients with psychiatric diseases; indeed the current work investigated the effect of omega-3 supplementation during pregnancy and lactation on modulation of neurological changes induced by both valoproate and HFD intake.

2. Material and methods

2.1. Animals

Thirty Wistar adult female rats of body weight ranging from 150 to 200 gms were purchased and included in the current study. The present work was conducted in the animal house of national research center (NRC) under the Guide for the Care and Use of Laboratory Animals in NRC and all experimental details and investigations was conducted according to the ethical standards given in the Declaration of Helsinki guidelines.

2.2. Grouping and experimental design

After acclimatization to normal environmental conditions, rats were randomized into five groups. Females were kept with fertile male overnight for mating, following determination of the oestrus phase of their reproductive cycle. The presence of spermatozoa in vaginal smears indicated the first day of gestation (G0). Then, pregnant rats were housed singly and maintained at comfortable temperature (21 ± 2°C), humidity (30–35%) and under standard lighting conditions (12:12 h light–dark, lights on from 07:00 to 19:00 h), with ad libitum access to food and water. Female rats included in HFD and HFD-Omega groups were supplied with special diet regimens that continued all through the duration of study (one week before mating, pregnancy and lactation till weaning of their pups at post natal day (PND) 21).

The animals were divided to group I (n = 6): control pregnant rats received normal chow of 10% fat and group II (n = 6): Pregnant female fed with high fat diet of 60% calories obtained from animal source (HFD-group) (Tamashiro, Terrillion, Hyun, Koenig, & Moran, 2009) and group III (n = 6): Pregnant females supplied with HFD enriched with omega-3 (HFD-Omega group) (Shamseldeen et al., 2018), group IV (n = 6): Pregnant female received a single intraperitoneal injection of sodium valproate (VPA) (Sigma, Dublin, Ireland) (500 mg/kg) on gestational day 12.5 (G12.5) (Valp-group) (Wang et al., 2013), and group V (n = 6): Pregnant female received VPA that was given with the same dose and timing of pregnancy together with diet enriched with omega-3 given from G0 (Valp-Omega-group).

We recorded the presence of deaths among offsbring born to HFD fed dams and intake of omega-3 with high-fat diet reduced the number deaths (15% versus 35%). Omega 3 with Valp group did not show significant change in deaths compared to Valp group (30% versus 25%). However, no mortality was recorded among offsprings born to control group.

2.3. Materials and reagents

2.3.1. Diet composition

Control diet contains 10% of calories derived from fat; Research Diets, New Brunswick, NJ, USA #D12450B) or HFD (60% of calories from fat; Research Diets New Brunswick, NJ, USA #D12492.

2.3.2. Omega-3

Omax3 (contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of 4:1 ratio) was purchased from Prevention Pharmaceuticals. 24 Arnett Avenue, Suite 107, Lambertville, NJ 08530. That was supplied at a dose of 25.2 mg/day for pregnant female (Shamseldeen et al., 2018).

2.3.3. Na-valproate

Valproic acid sodium salt was purchased from Sigma-Aldrich (Sigma, Dublin, Ireland) and dissolved in 0.9% saline solution, valproic acid was given once intraperitoneal at a dose of 500 mg/kg on gestational day 12.5 (G12.5) (Wang et al., 2013).

2.4. Procedure

After birth pups were assessed for determination of postnatal growth and maturation via determination of:

2.4.1. Weight gain that was measured on 7, 14 and 21 PNDs

2.4.1.1. Rats were daily observed for eye opening from days 12 to 16 and rated as follows. 0 both eyes closed; 1 one eye open; and 2 both eyes open (Schneider & Przewłocki, 2005).

2.4.2. Behavioral assessment and procedures using swimming performance

Rats were placed at the center of an aquarium filled with water (28–29 °C). Rats were evaluated for 10 s on PNDs 8, 10, 12, and 16. Swimming performance was evaluated according to head and nose position (angle) on water surface. Then it was rated as: 0 head and nose below water surface; 1 nose below the surface; 2 nose and top of head at or above water surface but ears still below it; 3 the same as two except for the presence of water line was at mid-ear level; and 4 the same as three except for the presence of water line at the bottom of ears. Finally, the tested pups were dried and returned to their cages (Castro, Tambasco, Paraíba, & Tambasco, 1999).

2.4.3. Behavioral assessment and procedures using elevated plus maze test

Plus maze consists of four arms (two arms without walls (open arms) and two enclosed by 30 cm high plastic walls (closed arms). The arms are attached to legs that it is elevated 50 cm (Walf & Frye, 2007).

Rats were placed in the intersection of the four arms of elevated plus maze and their behavior was recorded for 5 min. The recorded behaviors are the time spent in free arms, number of entries made on the open, number head dipping and stretched-attend postures.

2.4.4. Behavioral assessment and procedures using social interaction test

Before experiment, rats were housed separately overnight. Then they were examined in white apparatus that was 50 × 40 × 40 cm box. The examined rats were of the same age, gender, and weight. In the experimental room and after 60 min of habituation, one rat born to Valp-group was exposed to another one born to control group for

20 min (Wang et al., 2013). The percentage of engagement time spent was calculated. Mounting, grooming each other, and sniffing of any body part were taken as indicators of social engagement (Flagstad et al., 2004).

2.5. Biochemical investigations

Immediately before scarification, blood samples were withdrawn through retro-orbital route using capillary tubes in 10 ml eppendorf tubes. The animals (offsprings) were sacrificed by high dose of sodium pentobarbital (800 mg/kg injected intraperitoneal) (Zatroch, Knight, Reimer, & Pang, 2017) and the brain tissues were excised for biochemical estimation of: Hippocampal levels of serotonin. Mitochondrial complex-1 activity, brain H2O2, TAC (total anti-oxidant capacity) and IL-6 were measured, in addition to relative gene expression of syntaxin1A and FoxO1 gene.

2.5.1. Estimation of serum triglyceride levels

After centrifugation of blood samples (4,000 rpm, 4∘C, 20 min), serum triglyceride was determined enzymatically using Randox colorimetric reagent kits (Antrim, United Kingdom).

2.5.2. Estimation of serotonin, GABA and NPY levels

After dissection and separation of the brain tissues, the hippocampus was separated for estimation of serotonin levels, and the whole brain was estimated for levels of GABA and NPY by commercially available ELISA kits (MyBiosource, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm, using ELISA reader (Stat Fax 2300, United States).

2.5.3. Determination of brain NADH dehydrogenase activity (complex I)

Nicotinamide adenine dinucleotide (NADH)–coenzyme Q oxidoreductase enzyme specific activity was measured according to the method described by Birch-Machin, Briggs, Saborido, Bindoff, and Turnbull (1994) by monitoring the decrease in the absorbance at 340 nm due to oxidation of NADH Hþ. Complex I activity was expressed in nanomoles/minute/mg of protein. Finally, protein levels were detected by Moore, DeVries, Lipp, Griffiths, & Abernethy (2010).

2.5.4. Estimation of brain hydrogen peroxide

Hydrogen peroxide (H2O2) levels were measured by the method of Pick (1996), 100 u L of tissue homogenate was prepared in Tris − HCl buffer (20 mM, pH7.4) and 100 u L of assay solution (containing 0.2 ml phenol red,0.2 g/L and 0.2 ml of horse radish peroxidase, 20 U/mL in potassium phosphate buffer, 0.05 M, pH 7.0, and 9.6 ml of 0.9% NaCl) was taken and reaction was started by the addition of 10 u L of 1.0 N NaOH. Absorbance was recorded at 600 nm in a microplate using ELISA reader. Hydrogen peroxide standard curve was plotted by taking different concentrations of H2O2, ranging from 20 to 100 mmol in a total volume of 100 u l and processed in the same way.

2.5.5. Determination of brain TAC levels

Determination TAC was performed by use of ferric reducing ability of supernatant (Benzie & Strain, 1996. with modifications). Working reagent consisting of 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l 2,4,6-tri-pyridyl-s-triazine (TPTZ; Sigma, Poznan, Poland) in 40 mmol/l HCl and 20 mmol/l FeCl3 solution mixed in the ratio of 10:1:1, was prepared immediately before use. Working reagent was mixed with the supernatant and absorbance was measured at 593 nm against the working reagent alone. After exactly 10 min of incubation at room temperature, the absorbance was read again. The difference in absorbance at 0 and 10 min time was compared with standard curve. Results were recalculated per protein content of supernatants and expressed as umol/g protein (mean ± SEM). The changes in absorbance were directly related to the total reducing power of the electron-donating antioxidants present in examined samples.

Table 1
Primers Sequence of studied genes:

Gene	Sequence
Sntaxain A1	Forward primer:5′- GCT GCA GAA GCA AGA GAA CC -3' Reverse primer:5′- CAG CCA TAC AAA AAC CAC CA-3'
Foxo1	Forward primer: 5' -CGGCTCACTTTGTCCCAGAT- 3' Reverse primer:5' -TCTTGCCAGTCCCTTCGTTC- 3'
Beta actin	Forward primer: 5' - GACGGCCAGGTCATCACTAT -3' Reverse primer: 5' - CTTCTGCATCCTGTCAGCAA -3'

2.5.6. Determination of syntaxin1A and FoxO1 relative gene expression using quantitative real time PCR

Real-time PCR was performed for quantitative genes expression of (syntaxin1A and FoxO1). Tissue samples of all the studied groups were lysed and total RNA was isolated with RNeasy purification reagent (Qiagen, Valencia, CA).The purity of total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA was carried out with reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY, USA). Real-time PCR amplifcation and analysis were carried out using an Applied Biosystem with software version 3.1 (StepOneTM, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems). The data were analyzed with the comparative cycle threshold (CT) method. The expression of β-actin mRNA was used as an internal control in all samples. The primers used were shown in Table 1.

2.6. Statistical methods

Data were coded and entered using the statistical package SPSS version 25. Data was summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons Benferroni post hoc test (Chan, 2003a). For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5 (Chan, 2003b). Correlations between quantitative variables were done using Pearson correlation coefficient (Chan, 2003c). P-values less than 0.05 were considered as statistically significant.

3. Results

3.1. The growing pups born to both Valp and HFD group showed marked and continuous decrease in growth and body weight that was observed by changes in PND7, 14 and 23

Body weight of rats at PND 7 showed significant decrease in HFD and Valp group compared to control group. HFD-Omega group showed significant increase in body weight compared to HFD but significant decrease compared to control group. Valp-Omega group showed significant increase in body weight compared to Valp group but significant decrease compared to control group.

Also at PND14, body weight of rats in HFD group and Valp group showed significant decrease in compared to control group. HFD-Omega group showed significant increase in body weight compared to HFD but didn't show significant difference compared to control group. Valp-Omega group did not show significant change in body weight compared to Valp group but showed significant decrease compared to control group.

Moreover at PND23, changes in body weight of rats born to HFD and Valp group showed the same manner of significant decrease as in PND 7 and 14 compared to control group (16.62 ± 2.02 versus 35.50 ± 3.73 g and 22.2 ± 3.73 versus 35.50 ± 3.73 g respectively). HFD-Omega group showed significant increase in body weight

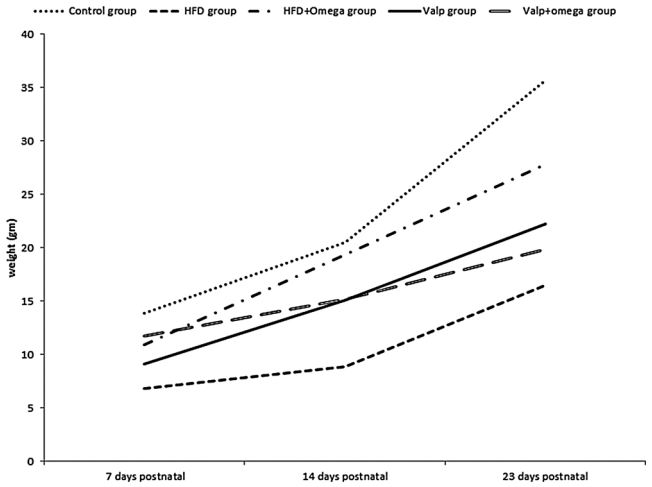

Fig. 1. Line curve for changes in body weights for pups born to control, Valp, Valp-Omega, HFD, HFD-Omega groups over days of early postnatal life till weaning. It shows marked growth retardation among pups born to HFD-group and more improvement with intake of omega-3 in HFD-omega than in Valp-omega group.

compared to HFD (27.71 ± 3.8 versus 16.62 ± 2.02 g) but significant decrease compared to control group (27.71 ± 3.8 versus 35.50 ± 3.73 g). Valp-Omega group did not show significant change in body weight compared to Valp group but significant decrease compared to control group (19.86 ± 1.79 versus 35.50 ± 3.73 g). HFD group showed growth retardation compared to Valp group that was demonstrated in recorded body weight mean values at PND7, 14 and 21 (Fig. 1).

3.2. Recording changes in swimming, eye opening and survival in different groups

At PND13, one eye opened in all rats of control group while both eye were closed in all rats of all other groups. At PND15 both eyes opened in control group while both eyes were closed in HFD group and one eye opened in all other groups. At PND16 both eyes were open in all groups except HFD group in which only one eye was open.

At PND8, the angle of swimming was 3 in all rats of control group but was 0 in HFD group and was 1 in HFD-Omega group and in valp group and 0 in Valp-Omega group. At PND10, the angle of swimming was 3 in all rats of control group but was 1 in HFD group and was 2 in HFD-Omega group and was 3 in Valp group and in Valp-Omega group At PND12, the angle of swimming was 4 in all rats of control group but was 2 in HFD group and was 4 in HFD-Omega group and was 3in Valp group and in Valp-Omega group. At PND16, the angle of swimming was 4 in all rats of all groups except in HFD group it was 3. So swimming performance was reduced in all groups compared to control. Omega 3 treatment improved swimming performance in HFD group.

3.3. Data of elevated plus maze and social interaction tests indicated the presence of severe anxiety in both HFD group and Valp group with more improvement in HFD-omega than Valp-omega group

Percent of time spent in open arm showed significant decrease in HFD compared to control group. However, values in HFD-Omega group showed significant increase compared to HFD but did not show significant change compared to control group. Percent of time spent in open arm showed significant decrease in Valp group compared to control group. Valp-Omega group didn't show significant change in percent of time spent in open arm compared to Valp group but showed significant decrease compared to control group. Valp group showed significant decrease in percent of time spent in open arm compared to HFD group.

Percent of frequency of open arm entry showed significant decrease in HFD compared to control group. In response to intake of omega-3 data showed significant increase compared to HFD but it wasn't significant compared to control group. Percent of frequency of open arm entry showed significant decrease in Valp group compared to control group, however in Valp-Omega group, there wasn't significant change in % of frequency recorded compared to Valp group but significant decrease was observed compared to control group. Valp group showed significant decrease in percent of frequency of open arm entry compared to HFD group.

Head dips showed significant decrease in HFD compared to control group, and significant increase in HFD-Omega group compared to HFD but did not show significant change compared to control group. In Valp group head dips showed significant decrease compared to control group. Valp-Omega group didn't show significant change in head dips compared to Valp group but showed significant decrease compared to control group. Valp group didn't show significant change in head dips compared to HFD group.

Stretched attend posture showed significant increase in HFD compared to control group and significant decrease in stretched attend posture in HFD-Omega group compared to HFD but did not show significant change compared to control group. Stretched attend posture showed significant increase in Valp group compared to control group, however intake of Omega didn't result in any significant change compared to Valp group but caused significant increase compared to control group Valp group didn't show significant change in stretched attend posture compared to HFD group (Table 2).

Time of engagement (seconds) indicating social interaction was recorded and the results showed significant decrease in HFD compared to control group, increased time of interaction in pups born to HFD-Omega group presented by significant increase in its percentage compared to HFD could indicates nero-protective role of omega-3. However percentage of engagement time showed significant decrease compared to control group. Social interaction time (seconds) showed significant decrease in Valp group compared to control group and didn't show significant change in Valp-Omega group compared to Valp group but showed significant decrease compared to control group. Valp group showed significant decrease in social interaction time compared to HFD

Table 2
Estimated parameters of elevated plus maze and social interaction test in control, HFD group, HFD-Omega group, Valp group and Valp-Omega group.

	Control group	HFD group	HFD-Omega group	Valp group	Valp-Omega group
% of time spent in open arm	32.00 ± 2.85	22.15 ± 1.41*	31.41 ± 2.94 #	19.07 ± 1.49 *#$	20.14 ± 1.17*$
% of frequency of open arm entry	27.00 ± 1.92	21.23 ± 1.69 *	27.82 ± 2.56 #	15.87 ± 2.20 *#$	17.00 ± 2.42 *#$
Head dips	24.70 ± 2.70	20.00 ± 2.35 *	25.88 ± 3.08 #	17.88 ± 2.92 *$	19.43 ± 3.11 *$
Number of Stretched attend posture	2.90 ± .85	5.85 ± 1.52 *	3.18 ± .73 #	5.50 ± 1.59 *$	4.36 ± .84 *#
Interaction time (seconds)	174.00 ± 4.97	74.69 ± 3.38 *	139.29 ± 4.55*#	63.44 ± 3.74 *#$	66.57 ± 3.74 *#$

Data presented by mean ± SD.
*: statistically significant compared to corresponding value in control group ($P < 0.05$).
#: statistically significant compared to corresponding value in HFD group ($P < 0.05$).
$: statistically significant compared to corresponding value in HFD-Omega group ($P < 0.05$).

group (Table 2).

3.4. Anti-oxidant effects of omega-3 intake during pregnancy could modify HFD- but not valproate-induced neurodevelopmental alteration

Serum triglycerides (mg/dl) showed significant increase in HFD compared to control group (113.98 ± 1.60 versus 77.20 ± .98). HFD-Omega group showed significant decrease in serum triglycerides compared to HFD (88.26 ± 1.63 versus 113.98 ± 1.60) but showed significant increase compared to control group (88.26 ± 1.63 versus 77.20 ± .98). Serum triglycerides did not show significant change in Valp or Valp-Omega group compared to control group.

Brain H2O2 (n mol) showed significant increase in response to HFD intake and valproate compared to control group (15.13 ± 1.49 versus 4.41 ± .12 and 20.54 ± .83 versus 4.41 ± .12 respectively) Valp group compared to control group (20.54 ± .83 versus 4.41 ± .12). Intake of omega-3 together with HFD caused significant decrease in brain H2O2 compared to HFD (7.86 ± .85 versus 15.13 ± 1.49) but showed significant increase compared to control group (7.86 ± .85 versus 4.41 ± .12). However, in Valp-Omega group omega-3 didn't show significant change in brain H2O2 compared to Valp group (18.99 ± 2.49versus 20.54 ± .83 3) but showed significant increase compared to control group (18.99 ± 2.49 versus 4.41 ± .12). Valp group showed significant increase in brain H2O2 compared to HFD group (20.54 ± .83 versus 15.13 ± 1.49).

Local brain content of TAC (m mol) showed significant decrease in HFD and valproate compared to control group (15.62 ± 2.68 versus 43.22 ± 4.18 and 12.26 ± .16 versus 43.22 ± 4.18 respectively). HFD-Omega group showed significant increase in brain TAC compared to HFD (24.46 ± 1.76 versus 15.62 ± 2.68) but it still significant decreased compared to control group (24.46 ± 1.76 versus 43.22 ± 4.18). Valp-Omega group didn't show significant change in brain TAC (m mol) compared to Valp group (12.76 ± .87 versus 12.26 ± .16) but showed significant decrease compared to control group (12.76 ± .87 versus 43.22 ± 4.18). Valp group showed significant decrease in brain TAC compared to HFD group (12.26 ± .16 versus 15.62 ± 2.68).

Brain complex 1 (microU/m) showed significant decrease in both HFD and valp group compared to control group (17.17 ± 1.99 versus 26.12 ± 1.49 and (9.95 ± 1.31 versus 26.12 ± 1.49 respectively). HFD-Omega group showed significant increase in brain complex 1 compared to HFD (20.54 ± .51 versus 17.17 ± 1.99) but showed significant decrease compared to control group (20.54 ± .51 versus 26.12 ± 1.49). Valp-Omega group didn't show significant change in brain complex 1 compared to Valp group (10.95 ± 2.32 versus 9.95 ± 1.31) but showed significant decrease compared to control group (10.95 ± 2.32 versus 26.12 ± 1.49). Valp group showed significant decrease in brain complex1 compared to HFD group (9.95 ± 1.31versus 17.17 ± 1.99).

Brain IL-6 (pg/ml) showed significant increase in HFD compared to control group (61.87 ± 7.79 versus 12.78 ± .23), and combined intake of HFD and omega caused significant decrease in brain IL-6 compared to HFD (29.85 ± 1.45 versus 61.87 ± 7.79) but it still significantly higher than control group (29.85 ± 1.45 versus 12.78 ± .23). Local IL-6 showed significant increase in Valp group compared to control group (102.16 ± 5.85 versus 12.78 ± .23), however Valp-Omega group didn't show significant change in brain IL-6 compared to Valp group (97.71 ± 3.17 versus 102.16 ± 5.85) but showed significant increase compared to control group (97.71 ± 3.17 versus 12.78 ± .23). Valp group showed significant increase in brain IL-6 compared to HFD group (102.16 ± 5.854 versus 61.87 ± 7.79) (Fig. 2)

3.5. Omega-3 could modify relative gene expression of brain FoxO1 and syntaxin 1 A in HFD + omega group but not in Valp-Omega group

HFD intake could significantly increase relative gene expression of brain FoxO1 compared to control with mean values of (4.50 ± .25 versus 1.00 ± .00). Combined intake of omega-3 and HFD could decrease FoxO1 relative expression compared to HFD (2.11 ± .03 versus 4.50 ± .25). Brain FoxO1 relative expression showed significant increase in Valp group compared to control group (6.59 ± .21 versus1.00 ± .00). Valp-Omega group didn't show significant change in FoxO1 relative expression compared to Valp group (6.21 ± .84 versus 6.59 ± .21) but showed significant increase compared to control group (6.21 ± .84 versus1.00 ± .00). Valp group showed significant increase in brain FoxO1 compared to HFD group (6.59 ± .21 versus 4.50 ± .25).

HFD intake could significantly decrease relative gene expression of syntaxin compared to control with mean values of (.35 ± .02 versus1.00 ± .00). Combined intake of omega-3 and HFD could increase syntaxin relative expression compared to HFD (.64 ± .08 versus .35 ± .02). In Valp group, syntaxin relative expression showed significant decrease compared to control group (.18 ± .03 versus 1.00 ± .00). However, adding omega-3 to Valp group didn't show significant change in brain syntaxin relative expression compared to Valp group (.22 ± .02 versus .18 ± .03) but showed significant decrease compared to control group (.22 ± .02 versus1.00 ± .00). Valp group showed significant decrease in syntaxin compared to HFD group (.18 ± .03 versus .35 ± .02) (Fig. 3)

3.6. Disturbance in neurotransmitters secondary to intake of HFD and valproate was partially corrected with the intake of omega-3

HFD-Omega group showed significant increase in brain serotonin (ng/mg) compared to HFD (37.02 ± 1.02 versus 25.54 ± 2.96) however both HFD and HFD-Omega group showed significant decrease compared to control group. Brain serotonin showed significant decrease in Valp group compared to control group (19.26 ± 1.53 versus 39.58 ± 1.40). Valp-Omega group didn't show significant change in brain serotonin compared to Valp group (21.09 ± 2.35 versus 19.26 ± 1.53) but showed significant decrease compared to control group (21.09 ± 2.35 versus 39.58 ± 1.40). Valp group showed significant decrease in brain serotonin compared to HFD group (19.26 ± 1.53 versus 25.54 ± 2.96) (Fig. 4).

Brain GABA (n mol/mg) showed significant decrease in HFD compared to control group (3.56 ± .17 versus 9.13 ± .99). HFD-Omega group showed significant increase in brain GABA compared to HFD (5.10 ± .31 versus 3.56 ± .17) but showed significant decrease compared to control group (5.10 ± .31 versus 9.13 ± .99). GABA levels showed significant decrease in Valp group compared to control group (2.59 ± .16 versus 9.13 ± .99). In addition, Valp-Omega group didn't show significant change in brain GABA compared to Valp group (2.85 ± .28 versus 2.59 ± .16) but showed significant decrease compared to control group (2.85 ± .28 versus 9.13 ± .99). Valp group showed significant decrease in brain GABA compared to HFD group (2.59 ± .16 versus 3.56 ± .17).

Neuropeptide Y (pg/mg) showed significant increase in HFD compared to control group (24.53 ± 2.78 versus 12.02 ± 1.00), that was significantly decreased with combined HFD and omega-3 intake compared to HFD alone (16.31 ± .09 versus 24.53 ± 2.78). Brain neuropeptide Y showed significant increase in Valp group compared to control group (45.59 ± 1.34 versus 12.02 ± 1.00). However, in Valp-Omega group it didn't show significant change compared to Valp group (44.40 ± 1.64 versus 45.59 ± 1.34) but showed significant increase compared to control group (44.40 ± 1.64 versus 12.02 ± 1.00) Valp group showed significant increase in brain Neuropeptide Y compared to HFD group (45.59 ± 1.34 versus 24.53 ± 2.78) (Fig. 4).

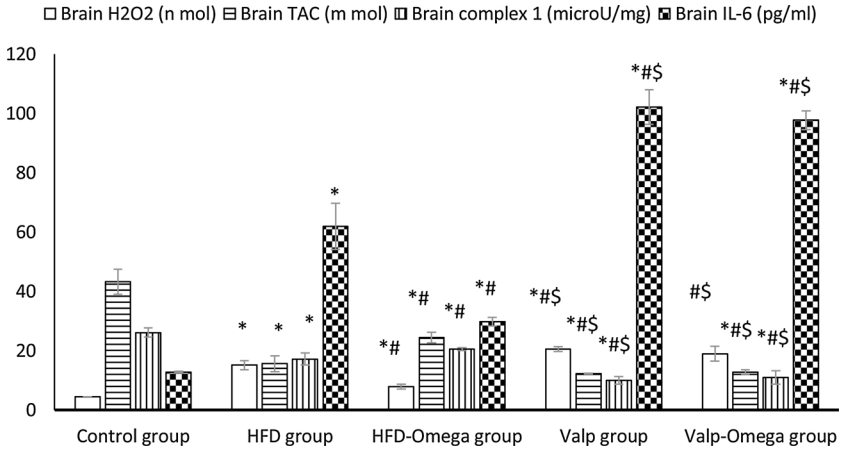

Fig. 2. Mean values ± standard deviation of Brain H2O2 (n mol), TAC (m mol), complex 1 (microU/mg) and IL-6 (pg/ml) in offspring's born to control, HFD, HFD-Omega, Valp and Valp-Omega groups. *: statistically significant compared to corresponding value in control group (P < 0.05). #: statistically significant compared to corresponding value in HFD group (P < 0.05). $: statistically significant compared to corresponding value in HFD-Omega group (P < 0.05).

4. Correlations

Mean values of FoxO1 showed inverse correlation with percent of time spent in open arm (r = -0.915, p < .001), percent of frequency of open arm entry (r = -0.894, p < .001), Head dips (r = -0.715, p < .001) and social interaction time (r = -0.964, p < .001) but there was direct positive correlation between FoxO1 relative expression and Stretched attend posture(r = 0.601, p < .001) (Fig. 5(1,2,3,4,5)

In addition, the results showed inverse correlation between neuropeptide Y and percent of time spent in open arm (r = -0.873, p < .001), percent of frequency of open arm entry (r = -0.892, p < .001), head dips (r = -0.675, p < .001) and social interaction time (r = -0.889, p < .001) but there's direct positive correlation between neuropeptide Y and stretched attend posture(r = 0.506, p < .001) (Fig. 6 (1,2,3,4,5).

The results showed inverse correlation between brain serotonin and IL-6 **(r = -0.963, p < .001)** (Fig. 7).

5. Discussion

Prenatal exposure to valproate (or valproic acid, VPA) could induce ASD and anxiety like symptoms; currently autistic like features was also induced by continuous intake of HFD during pregnancy and lactation. Both VPA and HFD could alter fetal serotonergic system via modulating syntaxin A1 and FoxO1 gene expression.

Some studies reported association of maternal obesity with the high incidence of ASD (Kang et al., 2014). However, the current work examined effects of HFD intake (60% of calories were from animal source) on development of the common manifestations of ASD; social withdrawal (Lainhart et al., 2002; Pickles et al., 2000) and anxiety (Micali, Chakrabarti, & Fombonne, 2004; Pickles et al., 2000). VPA model of autism could accelerate generation of hyperexcitable neurons linked to pathophysiology of ASD (Mabunga et al., 2015). Kim et al. (2014) studied the effect of prenatal exposure to valproate, using three-chamber social approach assay and the results of their work showed reduction in sociability. Ornoy (2009) suggested that VPA may create specific embryonic changes to different organs however; fetal brains are more sensitive to ROS than any other organ. That was observed in the current work with increased brain H2O2 both in Valp group and HFD group compared to control. The immature embryonic antioxidant mechanisms ((Mabunga et al., 2015) may increase susceptibility to oxidative stress induced by VPA (Zhang et al., 2012).

Prolonged intake of VPA could induce phosphorylation of FoxO1, subsequent nuclear export and ubiquitination (Wu & Shih, 2011). In the present work, VPA was given once during prenatal period and the results recorded significant increase in FoxO1 gene in Valp group compared to control. FoxO1 could regulate metabolic changes in response to oxidative stress and nutritional status (Webb & Brunet, 2014). Higher levels of FoxO1 in rodent brain exposed to prenatal stress are associated with enhanced vulnerability to develop stress related disorders (Cattaneo et al., 2018). In the current work increased ROS in response to Valp and HFD group is moreover associated with higher levels of FoxO1. Indeed, increased ROS levels could function as feedback regulator of FoxO transcriptional activity (Essers et al., 2004). Thus it may represent a target for treating stress-induced mental disorders. Under conditions of oxidative stress, FoxO proteins undergo nuclear translocation, deacetylation and trapping. Finally, it could increase transcription of cell cycle arrest genes and genes code for antioxidant

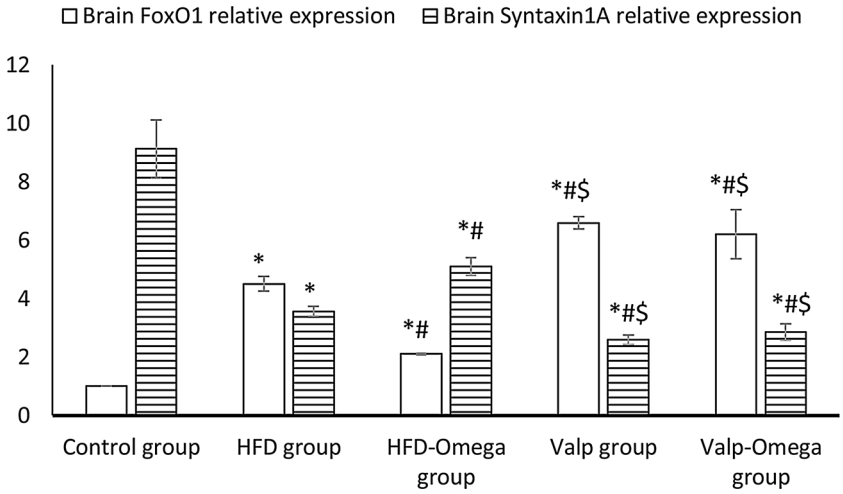

Fig. 3. Mean values ± standard deviation of brain FoxO1 and Syntaxin1A relative gene expression in offspring's born to control, HFD, HFD-Omega, Valp and Valp-Omega groups. *: statistically significant compared to corresponding value in control group (P < 0.05). #: statistically significant compared to corresponding value in HFD group (P < 0.05). $: statistically significant compared to corresponding value in HFD-Omega group (P < 0.05).

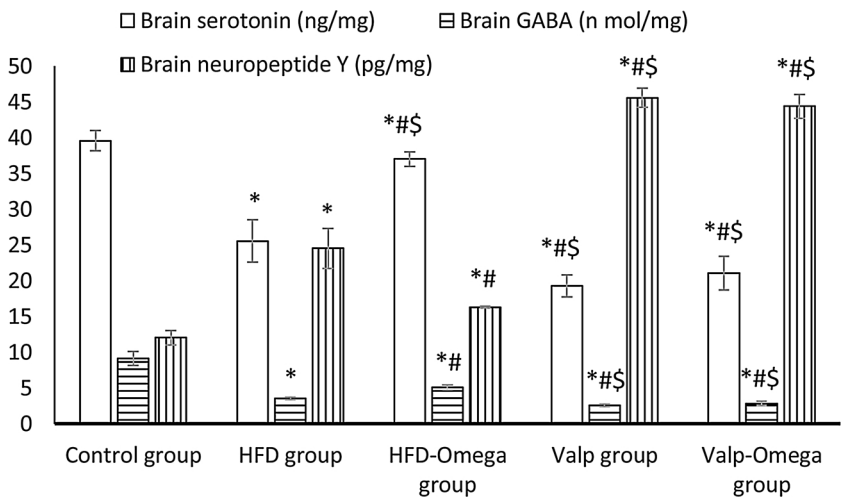

Fig. 4. Mean values ± standard deviation of brain serotonin (ng/mg), GABA (n mol/mg) and neuropeptide Y (pg/mg) in offspring's born to control, HFD, HFD-Omega, Valp and Valp-Omega groups. *: statistically significant compared to corresponding value in control group ($P < 0.05$). #: statistically significant compared to corresponding value in HFD group ($P < 0.05$). $: statistically significant compared to corresponding value in HFD-Omega group ($P < 0.05$).

activity such as superoxide dismutases (Klotz et al., 2015).

Rodent offspring born to HFD fed mothers exhibit neural inflammation as evidenced by persistent higher microglial activation in hippocampus till adulthood (Bland et al., 2010). Exposure to increased pro-inflammatory cytokines may lead to the perturbations in fetal serotonergic system (Sullivan et al., 2010). Sullivan et al. (2010) reported maternal intake of 32% of HFD which simulates western diet resulted in perturbations of fetal serotonergic system towards disruption of serotonergic system development. Serotonin controls impulsive aggression and social aggression. Depleting brain serotonin levels results in shift from cooperative behavior towards aggressive one. In addition, tryptophan supplementation in schizophrenic patients could decrease social anxiety (Patrick & Ames, 2015). The changes in serotonin receptor density and serotonin synthesis with age suggest involvement of serotonin in all aspects of brain development. Alteration of these normal patterns of change could affect somatosensory cortex and nearly most of brain functions (Chugani, 2002).

With the presence of conflicting results about the levels of brain serotonin in ASD patients, the present study investigated the effect of valproate model of autism and 60% intake of HFD during pregnancy and lactation on brain serotonin levels of offsprings after weaning. In our results, Valp-group and HFD-group showed decreased levels of serotonin and increased brain IL-6 with the presence of strong negative correlation between them.

Syntaxin 1A is a key component needed for vesicle fusion and serotonin release (Südhof, 2004). Syntaxin 1A could specifically regulate serotonin transport (SERT) activity through subcellular redistribution (Ciccone, Timmons, Phillips, & Quick, 2008). During early stages of brain development syntaxin 1A levels are involved in the pathogenesis of autism by influencing serotonergic system (Nakamura et al., 2008). In thalamocortical neurons, syntaxin 1A could alter relative subcellular distribution of SERT and regulate its functional expression. Even more, In WT/1A oocyte 5HT-induced inward currents were not evident with co-expressing SERT and syntaxin 1A (Quick, 2003). Currently, decreased syntaxin 1A expression was associated with significant decrease in brain serotonin levels, also Nakamura et al. (2008) reported serotonin levels could be indicator for pathogenesis of autism throughout prenatal and postnatal brain development. In the present work exposure to HFD and valproate during pregnancy resulted in increased pro-inflammatory cytokines and decreased syntaxin 1A expression that was observed just after weaning in neonatal brain tissues and associated with decreased brain serotonin levels. Offsprings born to HFD-fed mothers exhibited increased anxiety that could be related to development abnormal neural circuits involved in behavioral regulation. Developmental dys-regulation of brain neurotransmitter especially serotonin has been implicated in functioning autism, in a previous study, rats exposed to valproate demonstrated increased repetitive-stereotyped movements that was observed by repetitive tendency to re-enter the same explored arm in Y-maze (Zhang et al., 2012).

Another regulatory factor for anxiety, FoxO1, which is expressed neocortex, hypothalamus and different parts of murine brain is predominantly present in hippocampus (Fukunaga, Ishigami, & Kawano, 2005; Zemva et al., 2012). Polter et al. (2009) reported mice lacking brain FoxO1 exhibited an anxiolytic behavioral altitude, thud indicating that FoxO1 is implicated in anxiety-like behavior regulation. In our work, levels of FoxO1 gene expression showed a strong positive correlation with percentage of time spent and percent of frequency of open arm entry indicating association between FoxO1 gene and severity of anxiety. Nuclear expression of FoxO1 gene could stimulate transcription of orexigenic neuropeptide Y and Agouti-related protein that was hypothesis supported by (Kim et al., 2006). In the present work, increased whole brain expressions of FoxO1 gene was present in HFD-group and Valp-group together with increased levels of neuropeptide Y. The latter was strongly correlated with percentage of time spent and frequency of open arm entry. Even more, percentage of engagement time spent in social interaction test in pups born to both Valp-group and HFD-group was decreased together with delayed eye opening, low body weight, delayed motor development and attenuated co-ordination elucidated by swimming test suggest the existence of complex neuronal dysfunction. In agreement with our results, Hinnebusch, Miller, and Fein (2017)) reported delayed mental development in some children with autism below the age of 12 months.

Marked decrease in levels of glutamic acid decarboxylase estimated in postmortem brain specimens of ASD patient may be the cause of suppression of the GABA-ergic system (Fatemi et al., 2002).in the current work, teratogenicity-mediated effects of valproate was elucidated by decreased brain levels of GABA and increased syntxin1A expression thus promoting inhibition of GABA reuptake leading eventually to increased GABA levels (Fan, Fan, Bao, & Pei, 2006). The imbalance between excitatory and inhibitory neurons existed towards generation of hyper-excitable neurons (Lin, Gean, Wang, Chan, & Chen, 2013) and is deeply implicated in pathophysiology of ASD. Taken together, the high level of anxiety in both Valp-and HFD-group was comparable to control group, however the intake of HFD one week before mating, and pregnancy till lactation wasn't efficient to induce the same severity of neurodevelopmental changes present in pups born to Valp-group.

A diet rich in omega-3 PUFA (DHA and EPA) could reduce triglycerides levels in pups born to HFD-group, in addition omega-3 could provide neuro-protection with its anti-inflammatory and anti-apoptotic properties (Zendedel et al., 2015). Our results was in agreement with De Andrade et al. (2017) they reported that the intake of high-fat diet causes excessive production of IL-6 from activated microglia and

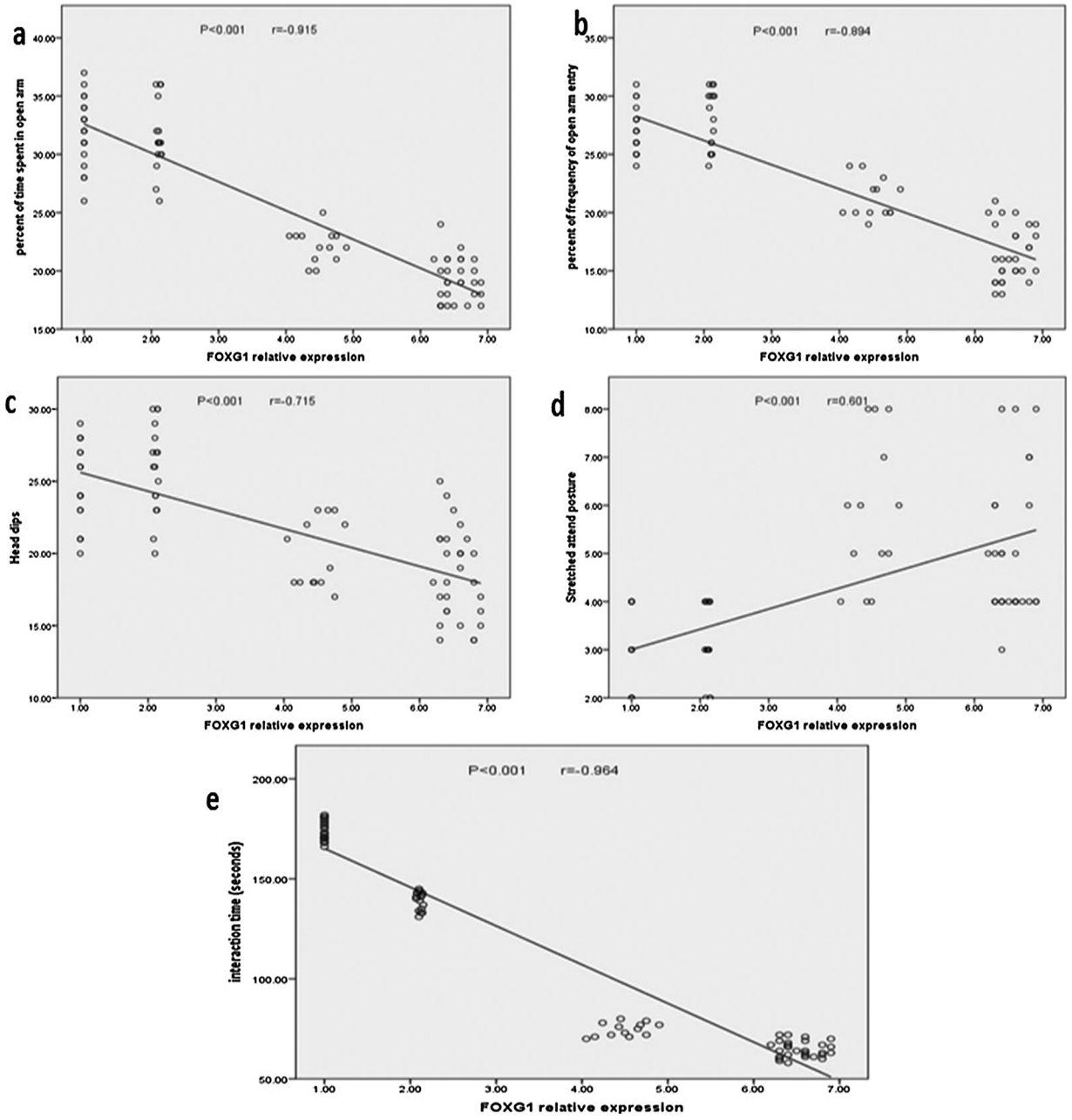

Fig. 5. (1,2,3,4,5): Scatter dot showing **a:** negative correlation between FoxO1 relative expression and percent of time spent in open arm, **b**: negative correlation between FoxO1 relative expression and percent of frequency of open arm entry**, c:** showing negative correlation between FoxO1 relative expression and head dips, **d:** positive correlation between FoxO1 relative expression and stretched attend posture, and **e:** negative correlation between FoxO1 relative expression and social interaction time.

subsequent activation of astrocytes, however omega-3 could suppress HFD-induced neuroinflammation and reduce levels of oxidative stress and IL-6 in whole brain. On the other hand, omega-3 with it's the anti-oxidant effects could decrease levels of H2O2 in Valp-Omega-group. However, this decrease wasn't enough to lower anxiety symptoms in Valp-group compared to HFD-group.

Some data reported Omega-3 deficiency with a wide range of mental disorders, including attention deficit hyperactivity disorder, depression, schizophrenia and ASD **(**Agostoni et al., 2017). In addition, with the ability of the fetus to get its omega-3 requirement from his mother, DHA accumulates in fetal brain especially during the third trimester which is time needed for brain growth. Thus, indicating that omega-3 supplementation during pregnancy could modulate the serotonin system development. In specific, EPA increases presynaptic release of serotonin by reducing synthesis of prostaglandin E2 and DHA regulates serotonin receptor function by increasing membrane fluidity (Patrick & Ames. 2015).

PUFAs could act directly on syntaxin (Darios & Davletov, 2006) inducing up-regulation of its levels leading eventually to vesicle fusion and serotonin release by exocytosis (Darios, Connell, & Davletov, 2007). In this context, the present results showed increased gene expression of syntaxtin 1A as well as serotonin levels in groups supplied with omega-3 that was associated with decreased levels of FoxO1 gene. Polter et al. (2009) confirmed that serotonin and antidepressants could inhibit phosphorylation of FoxO1 gene in mice brain and they hypothesized that the lack of FoxO1 gene could induce an anxiolytic behavioral phenotype. FoxO1 gene was significantly decreased in HFD-Omega group compared to HFD-group, which may indicate

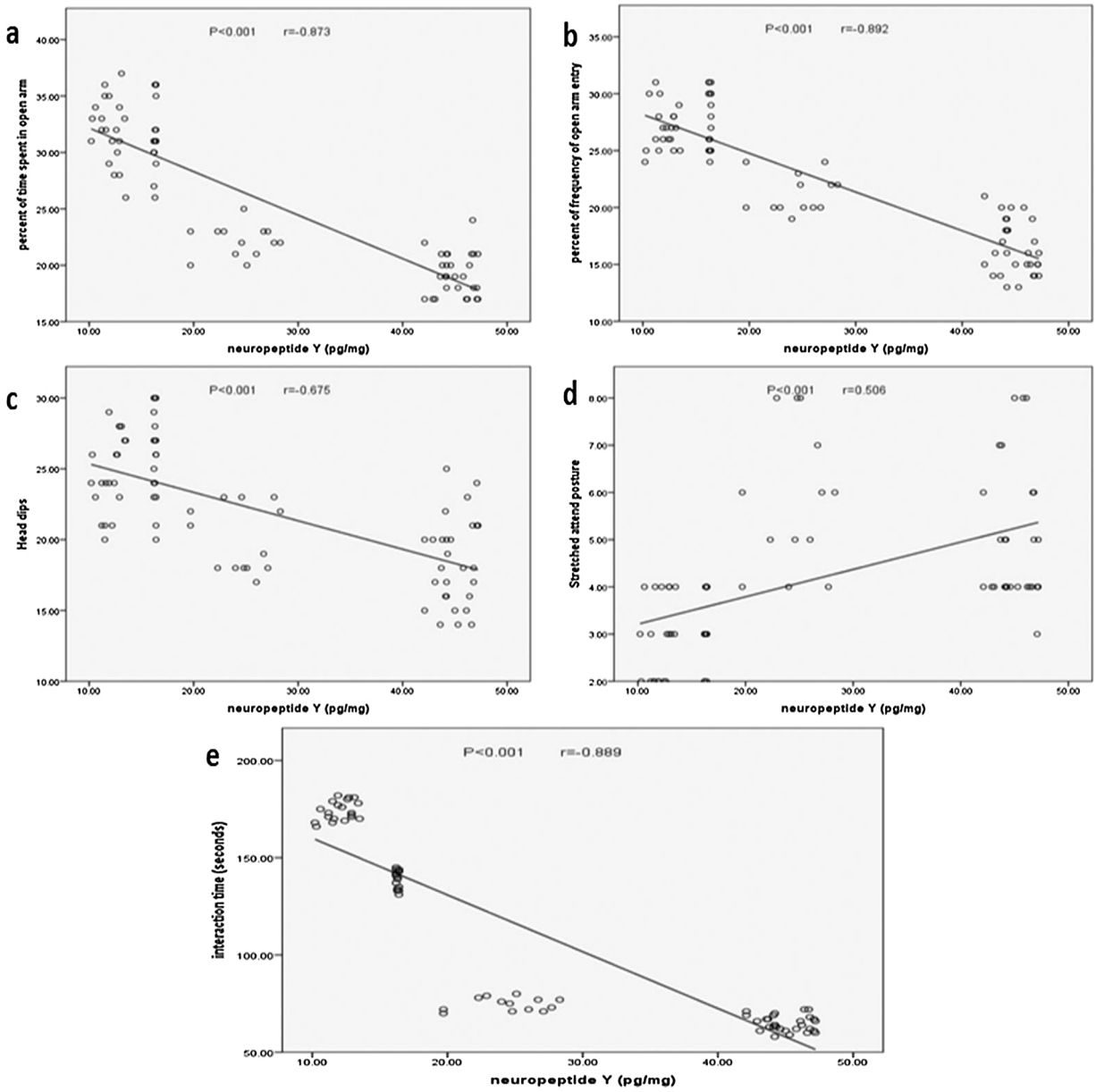

Fig. 6. (1,2,3,4,5) Scatter dot showing **a:** negative correlation between neuropeptide Y and percent of time spent in open arm, **b:** negative correlation between neuropeptide Y and frequency of open arm entry, **c:** negative correlation between neuropeptide Y and head dips, **d:** positive correlation between neuropeptide Y and stretched attend posture, **e:** negative correlation between neuropeptide Y and interaction time.

multifactorial actions of omega-3 in protection against HFD-induced altered neurodevelopmental changes. On the other hand this decrease in FoxO1 gene wasn't significant in Valp-Omega group compared to Valp group. Based on reported etiological abnormalities of fatty acid metabolism in autism, the efficacy of using omega-3 fatty acids in treating other psychological disorders including depression, and the current results, we recommend maternal intake of omega-3 during pregnancy, even more, omega-3 could be added to therapeutic area where the proposed treatment is to reduce the symptoms of ASD.

6. Conclusion

Currently, the state of anxiety and social withdrawal was markedly reduced in HFD-Omega but not VPA-Omega group. Therefore, omega-3 intake during pregnancy dampened the effect of prenatal intake of HFD either by lowering TGs levels and state of neruro-inflammation or by exerting up-regulation of syntaxin1A and modulation of serotonin levels. Therefore, omega-3 supplementation seemed to have a favorable impact on fetal sociability and communication that could mitigate symptoms of ASD.

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Disclosure statement

The authors declare no potential conflict of interest either financial or non-financial.

Ethical statement

The present work was conducted in animal house of national

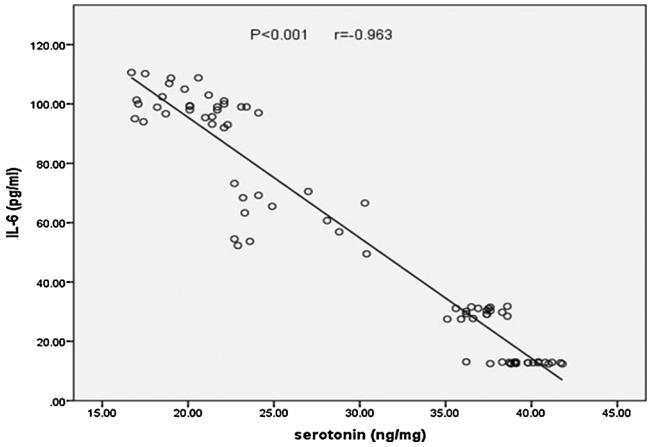

Fig. 7. Linear correlation curve showing strong negative correlation between brain levels of IL-6 and serotonin in all studied groups.

research center under the Guide for the Care and Use of Laboratory Animals in NRC (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington DC, USA).

Data statement

All data are available

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