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Nigella sativa Seed Oil Extract Prevents VPA Induced NTDS By Improving HDAC Activity and GADD45α Gene Expression.

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ABSTRACT

The universal occurrence of neural tube defects (NTDs) ranges from 0.5 to 12 per 1000 live births depending on the country with higher incidence in the developing countries; this accounts for 400,000 live births globally per annum. Valproic acid (VPA) is a well-established human teratogen that is frequently prescribed in the treatment of epilepsy, migraines, and bipolar affective disorder. Women of childbearing age with chronic medical conditions such as epilepsy are often concerned about hazards of drug exposure during pregnancy and lactation. Feeding neonate and juvenile with Nigella sativa extract at an early stage is associated with improved memory and learning in. The aim of this study is to investigate the effect *Nigella Sativa* oil extract on prevention of Sodium valproate induced neural tube defects through HDAC inhibition and GADD45α gene expression in mice. five groups of six dam in each group, group 1 were administered Sodium Valproate only at a dose of 600mg/kg daily, groups 2, 3 and 4 received Sodium Valproate at a dose of 600mg/kg/day + 0.2ml of Nigella sativa oil extract, Sodium Valproate at a dose of 600mg/kg/day + 0.1ml of Nigella sativa oil extract and 600mg/kg/day + FA 400µg respectively, group 5 dams were administered (0.9% saline), and served as control. Pregnant dams were sacrificed on gestational day 15 and the neural tissue of embryos were harvested for protein and gene study. Improvement in HDAC activity was found in the VPA +NS 0.2ml compared to VPA only group. It was also observed that the expression of GADD45α gene was reduced in the VPA+NS0.2 treated group compared to the VPA only group. In conclusion our study demonstrate that administration of nigella sativa oil extract improves HDAC inhibition associated with prenatal exposure to Valproic acid in mice. It also shows the benefit of N. sativa oil extract reducing the expression of GADD45α associated with in-utero VPA exposure, which prevents NTDs from the mice embryos.

Keywords: *Nigella sativa,* Oil extract, HDAC activity, Gadd45α Gene

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INTRODUCTION

Neural tube defects (NTDs) are the major congenital malformations of the central nervous system in humans. The commonest forms of NTDs found in humans are spinal bifida and Anencephaly which occur due to the absence of closure of posterior and anterior neuropore respectively [1]. The universal occurrence of neural tube defects (NTDs) ranges from 0.5 to 12 per 1000 live births depending on the country with higher incidence in the developing countries; this accounts for 400,000 live births globally per annum [2]. The aetiology of NTDs are complex, these involve a combination of genetic, nutrient, and environmental factors. Valproic acid (VPA) is a well-established human teratogen that is frequently prescribed in the treatment of epilepsy, migraines, and bipolar affective disorder [3]. The financial implications of managing neural tube defects pose a great burden on the families and patients affected. Women of childbearing age with chronic medical conditions such as epilepsy are often concerned about hazards of drug exposure during pregnancy and lactation. The avoidance of any medication after conception may often be unwise for maternal well-being. Proper prescription of drugs in pregnancy is a challenge and should provide maximal safety to the foetus as well as therapeutic benefit to the mother. Teratogens act with specificity in that they produce specific abnormalities at specific times during gestation. VPA is a histone deacetylase inhibitor; it is presently in clinical trials as an anticancer agent [4]. Microarray studies of VPA intake during pregnancy have demonstrated an increase in the expression of genes responsible for growth-arrest, such as gadd45b and gas5 in the head region of the embryo. These suggest that neural tube defects may arise from inhibition of cell growth and induction of apoptosis in the neuroepithelial cells [5]. Additionally, a study previously shows that embryonic p53 protein expression increases just 3 hours after maternal VPA administration, this was localized to the embryonic somites [6]. *Nigella Sativa* (NS) is a native annual flowering plant found in some part of Asia and southern Europe. The flower of NS contain some small black seeds, the seeds are the source of pharmacologically active component of NS [7]. These components include thymoquinone (TQ), ditimoquinone (DTQ) and nigellin, with thymoquinone been highest in concentration. Previous experimental studies have shown that the extract of NS seeds have anti-inflammatory and anticancer activities. It was also found that extract of NS is protective against oxidative damage in isolated rat hepatocytes [8]. Feeding neonate and juvenile with NS extract at an early stage is associated with improved memory and learning in rats [9]. The aim of this study was to investigate the effect *Nigella Sativa* oil extract on prevention of Sodium valproate induced neural tube defects through HDAC inhibition and GADD45α gene expression in mice.

MATERIALS AND METHODS

Seventy five (60 female and 15 male) Non gravid ICR mice were used for the study. The mice at 7 weeks of age were allowed two week of adjustment in the animal house of faculty of medicine and health science Universiti Putra Malaysia. In the first study, gravid mice were divided into five groups of six dam in each group, group 1 were administered Sodium Valproate only at a dose of 600mg/kg daily, groups 2, 3 and 4 received Sodium Valproate at a dose of 600mg/kg/day + 0.2ml of Nigella sativa oil extract, Sodium Valproate at a dose of 600mg/kg/day + 0.1ml of Nigella sativa oil extract and 600mg/kg/day + FA 400µg respectively, group 5 dams were administered (0.9% saline), and served as control. Pregnant dams were sacrificed on gestational day 15 and the embryos were harvested for physical examine and laboratory evaluation.

The neural tissue (brain and spinal cord) of the embryos from all the experimental groups and control were collected in 2ml eppendorf tube after dissection on ice and placed in dry ice, to maintain the protein integrity and then transfer to -80°c for storage before analysis.

Nuclear protein was extracted using, Nuclear extract kit (Active motif, USA) according to manufacturer protocol. Briefly, 200mg of the brain tissues sample from each group were homogenise in 600µl of hypotonic buffer for homogenization, and incubate on ice for 15 minutes. The homogenate was centrifuge at 8500xg at 4°c for 10min, the supernatant was removed and the pellet was re-suspended in 100µl of hypotonic buffer for cytoplasmic extract, the mixture was again incubated on ice for 15min, after which 1µl of detergent was added and centrifuge at 14000xg for 30sec at 4°c. The supernatant was removed and the pellet was again suspended in 10µl of complete lysis buffer, 1µl of detergent was added and vortex for 10sec, and then it was incubated on ice for 30min on a rocking platform. The samples were then vortex for 30sec and centrifuge at 14000xg for 10min at 4°c, the supernatant which contain the nuclear proteins was collected in pre-chilled tube and store at -80°c. The Concentration of the extracted nuclear protein was determine using BCA protein assay kit (Thermo scientific USA) following the manufacturer protocol.

Histone deacetylase HDAC activity was assayed using Epiquik HDAC activity/inhibition assay kit (Colormetric) (Epigentek, USA) based on the manufacturers protocol. Briefly, 50µl of biotinylated HDAC substrate (H3) was added to each strip well except the standard and blank wells, for the blank and standard wells only wash buffer was added. 1µl of HDAC assay standard at different concentrations (10, 5, 1, 0.5, and 0.1 µg/ml) was added to the standard wells, and the strip wells were covered with parafilm M and incubated for 45min. after then the content from each well were aspirated and washed with 150µl of wash buffer 2 times. Then 28µl of HDAC assay buffer was added to all the wells plus 2µl of the samples (10µg), for control and standard 2µl of HDAC assay buffer, and incubated for 60min at 37°c, after which the content from each well were aspirated and washed with 150µl of wash buffer 3 times. Then 50µl of capture antibody (H6) was added to each well and incubated for another 60min at room temperature on an orbital shaker at 50rpm (Orbital shaker Model 361 Fisher Scientific, USA), after then the content from each well were aspirated and washed with 150µl of wash buffer 4 times. Then 50µl of detection antibody (H7) was added to each well and incubated for 30min at room temperature, after which the content from each well were aspirated and washed with 150µl of wash buffer 5 times. 100µl of developing solution (H8) was added to each well and incubated for 10min away from light, after which 50µl of stop solution (H9) was added to each well and the colour turns yellow, the absorbance was read in a micro-plate reader (Gemini fluorescence micro plate reader, USA) at 450nM within 15min.

For GADD45α gene expression assay, neural tissues of the embryos from all the experimental and control groups were placed in 2ml eppendorf tube containing RNA stabilization solution (Thermo scientific USA) and placed on ice before transfer to -80°c for storage before use.

Total RNA was extracted using the single step chloroform/ phenol extraction method by TRIzol as described by Marselli et al. (2008). Briefly, brain tissue 100mg was homogenized in 1ml of trizol using power homogenizer. The homogenate was incubated for 5min at room temperature, 200µl of chloroform was added to the homogenate and vortex vigorously for 15sec and then incubated at room temperature for 3min. The sample was then centrifuged at 12,000 x g for 15min at 4°c (thermo fisher scientific USA). Following centrifugation the mixture separates into lower red phenol- chloroform phase, an intermediate phase and a colourless upper aqueous phase. The aqueous phase which contain the RNA was transferred into a new 2ml eppendorf tube. To precipitate the RNA from the transferred aqueous phase, 500µl of isopropyl alcohol was added and incubate for 10min at room temperature, and then centrifuged at 12,000 x g for 10min at 4°c. The RNA precipitate and formed a gel like pellet at the bottom of the tube, the supernatant was discarded and the pellet was washed by adding 1ml of 75% ethanol, vortexing and centrifuged at 7500 x g for 10min at 4°c, the ethanol was discarded and the pellet was allow to air dry for 5min. The pellet was dissolved by adding 50µl of DEPC water and incubating at 55°c for 15min, the RNA was then stored at -20°c before use. The concentration and purity of the RNA extracted from all the control and treatment samples were measured using spectrophotometer (Nanovue plus, GE healthcare Bioscience USA). The overall quality of the RNA extracted was assessed by electrophoresis on a denaturing agarose gel.

Total RNA was reverse transcripted to cDNA using Transcriptor first strand cDNA synthesis kit (Roche, USA). Based on the manufacturer's protocol. Briefly, the following component; 1µg of the isolated total RNA, 1µl of 25µM oligo dT primer and PCR grade water to make 13µl in total, in a 200µl tube. The mixture was incubated at 65°c for 10min and then placed on ice for 1min. Next step, 4µl of Transcriptor reverse transcriptase reaction buffer, 0.5µl of protector RNase inhibitor, 2µl of deoxynucleotide mix and 0.5µl of Transcriptor reverse transcriptase were added to the tube to make a total volume of 20µl, the content was mixed gently by pipetting up and down. The mixture was incubated using thermo-cycler (eppendorf USA) at 50°c for 60min, the reaction was inactivated by heating at 85°c for 5 and finally hold at 4°c, samples were kept at -20°c until PCR amplification. GADD45α gene, forward and reverse primers were design using probe finder version 2.50 for mouse (Universal probe library, Roche, USA). Two housekeeping genes were also designed for mouse, table below shows the primer sequence, size and annealing temperature (Tm).

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Table 1: List of primers design for amplification of GADD45A and the two housekeeping genes

Target gene	Primer sequence	Size	Tm
Gadd45α	Forward: 5' GCTGCCAAGCTGCTCAAC 3'	18	60
	Reverse: 3' TCGTCGTCTTCGTCAGCA 5'	18	60
Pgk1	Forward: 5' CAAGACTGGCCAAGCTACTGT 3'	21	59
	Reverse: 3' TGCTCTCAGTACCACAGTCCA 3'	21	59
Psmb2	Forward: 5' AACCTGGCTGACTGTCTTCG 3'	20	60
	Reverse: 3' CCTTCATGCTCGTCATAGCC 3'	20	60

Polymerase chain reaction was run using SYBR Green I master reagent (Roche, USA) according to the manufacturer's instruction. Briefly, PCR mix was prepared for the reaction by adding the following components; 5µl of 2x master mix, 0.5µl each of forward and reverse primers and 3µl of PCR grade water, to make a total of 9µl for each reaction, the components were mixed by gently pipetting up and down. 9µl of the mixture was pipetted into the each well of the LightCycler 480 multi-well plate, and 1µl of cDNA samples was added to make a total 10µl in each well. The plate was sealed with LightCycler 480 multi-well plate sealing foil, and centrifuge at 1500xg for 2min, after which the plate was placed in LightCycler 480 machine (Roche, USA) and PCR was performed. The crossing point (CP) value, slope and intercepts were recorded and used to calculate the copy number (input amount) for the gene expression.

RESULT

Protein extraction and HDAC activity assay

The extracted nuclear protein from the frozen brain tissues shows a good concentration from BCA assay figure 4.7 shows the standard curve for the assay that was plotted using the absorbance vs known concentrations of the standard protein used in the assay.

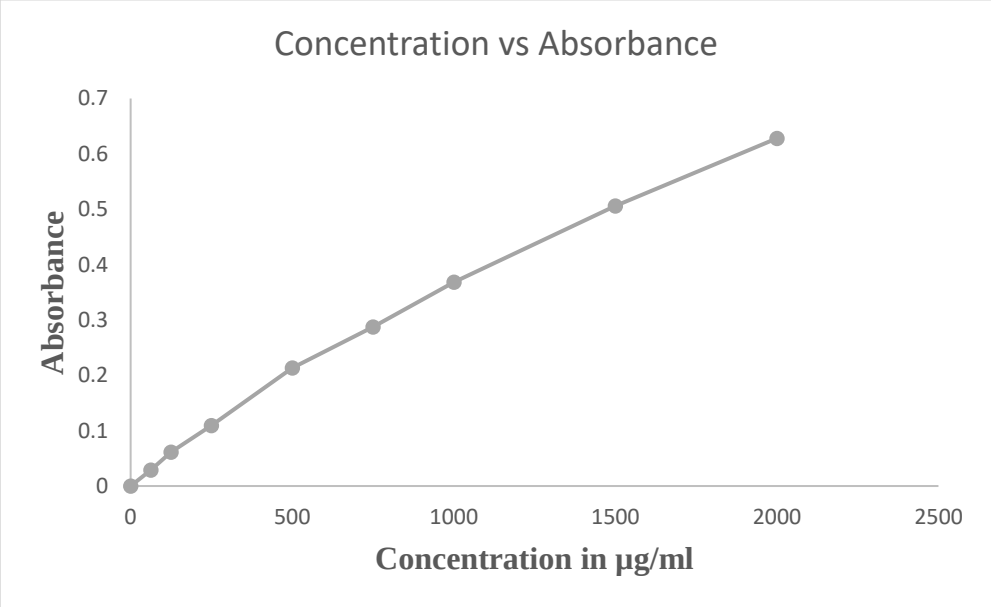

Figure 1: Standard curve from the BCA assay. Concentrations of the samples were determine from their absorbance on the standard curve.

The concentrations of extracted nuclear protein from individual samples were determined using the standard curve by extrapolating the absorbance of the samples at 562nm. Table 4.7 display the concentrations of the extracted nuclear protein.

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Table 2: Concentration of the extracted nuclear protein

Nuclear protein concentration in µg/ml					
Groups Animals	Control	VPA only	VPA+FA	VPA+NS 0.1ml	VPA+NS 0.2ml
Animal 1	177.9	375.4	303.3	212.3	240.6
Animal 2	478.8	372.2	920.9	441.2	221.7
Animal 3	268.8	99.5	240.6	318.9	359.7
Animal 4	215.5	243.7	237.4	250	779.8

VPA= Valproic acid, FA= Folic acid, NS= Nigella sativa,

For the HDAC activity assay, absorbance of the samples from control and treatment were estimated with spectrophotometer and HDAC activity was calculated using the formula given in the protocol.

$$\text{HDAC activity (OD/h/ml)} = \frac{\{\text{OD (control – blank) – OD (sample – blank)}\}}{\text{Reaction time (1 hour)}}$$

It was observed from the results obtained that the activity of the HDAC was reduced in the VPA only treated group compared to the control group, with significant improvement in HDAC activity in VPA + NS 0.2ml group. Some improvement in HDAC activity was also found in the VPA +NS 0.1 and VPA + FA groups but not significant, when compared to VPA only group, from one way ANOVA and turkey multiple comparism test $p < 0.05$. Figure 4.7 illustrate the activity of HDAC across the groups.

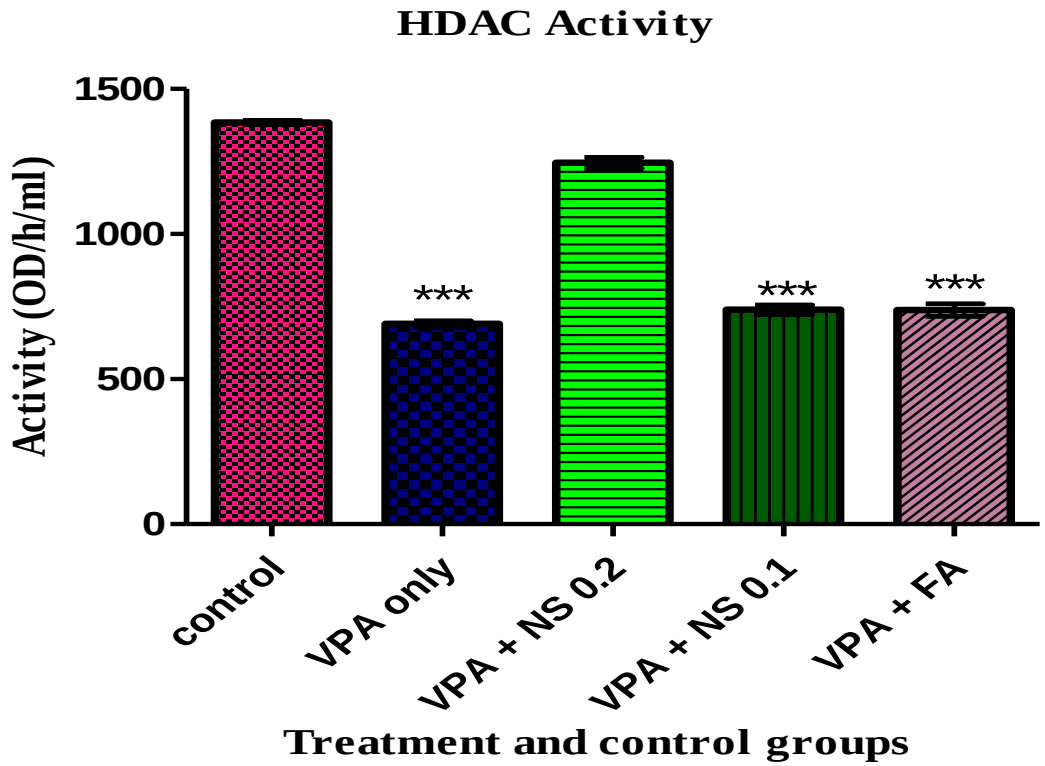

Figure 2: HDAC Activity assay. VPA = Valproic acid, NS = nigella sativa, FA = folic acid * Significant at P < 0.05.**

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RNA concentration and purity

The extracted RNA from embryo's brain samples shows good concentration and some purity, table 4.3 demonstrate the concentration and purity of the extracted RNA for treatment and control groups. Based on the spectrophotometer readings the RNA were of low purity, with little contamination, the OD $_{260/280}$ of some samples were less than 1.8. The agarose gel electrophoresis done on the RNA samples also showed that the RNA was not denatured and were found to be of good quality figure 4.8. The 28s and 18s bands were clearly demonstrated.

Table 3: Concentration and purity (A260/A280) value of the RNA

	Control		VPA only		VPA + FA		VPA + NS 0.1ml		VPA + NS 0.2ml	
	RNA Conc. ng/µl	A260/ 280	RNA Conc. ng/µl	A260/ 280	RNA Conc. ng/µl	A260/ 280	RNA Conc. ng/µl	A260/ 280	RNA Conc. ng/µl	A260/ 280
Animal 1	1465	1.56	4426	1.70	4942	1.68	1394	1.48	976.5	1.43
Animal 2	622.5	1.53	3028	1.84	4649	1.71	5809	1.65	2400	1.69
Animal 3	1881	1.62	1658	1.57	5033	1.67	4203	1.73	1673	1.58
Animal 4	3676	1.69	1211	1.56	4795	1.68	3789	1.66	2465	1.89

VPA= Valproic acid, FA= Folic acid, NS= Nigella sativa, RNA= Ribonucleic acid, Conc= Concentration, ng/µl= Nano gram / micro litre.

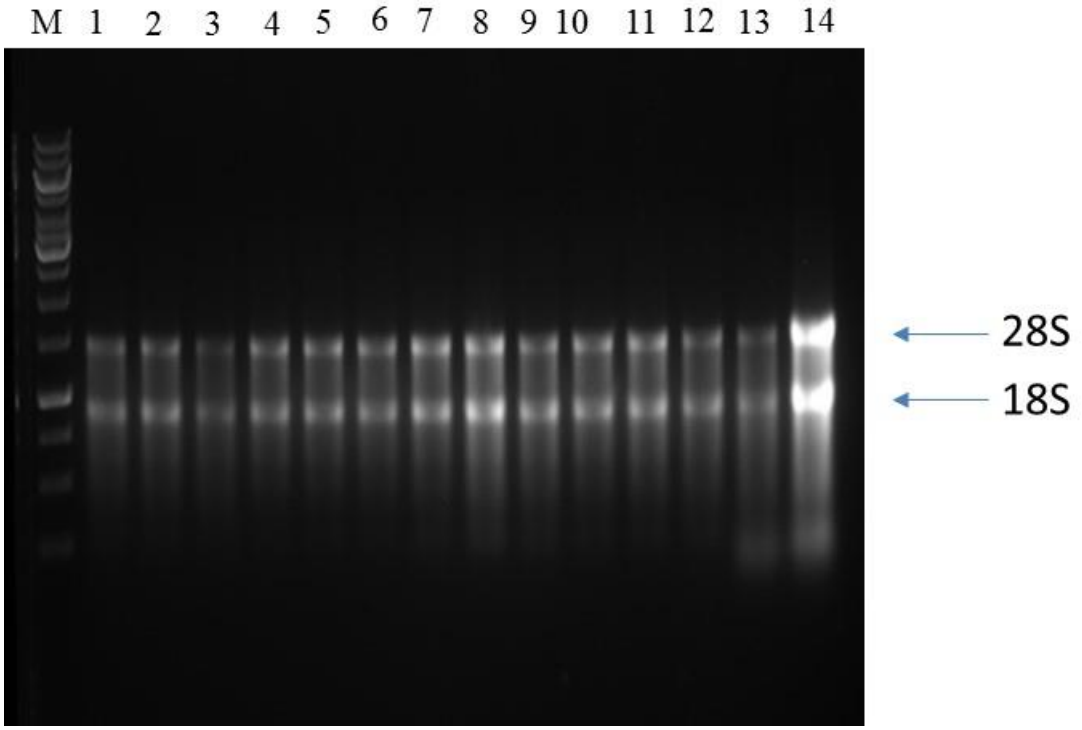

Figure 3: Agaros gel electrophoresis of rRNA. M= RNA Ladder; 1-14= RNA Samples.

Standard curve was generated from the values of the standard figure 4.9.

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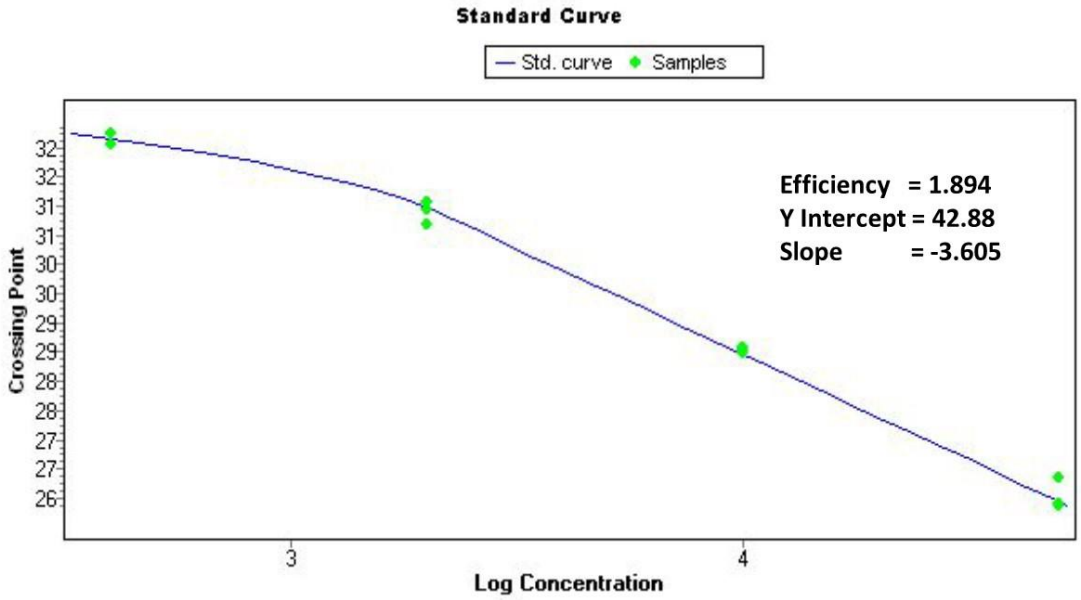

Figure 4: Standard curve for GADD45α gene expression

Using the values from the generated standard curve, linear function of logarithmic concentration and Cp, the input amount was calculated using the formula;

$$Y = Mx + C$$

Where,
Y= Crossing point value (obtained from the TR-qPCR result)
M = Slope
x = Copy number
C = Intercept

Using the above formula above to Calculate the copy number (x), which is the Input amount in log10 was determine by making x subject of the formula as below.

$$x = Y - C/M$$

The input amount in log 10 for sample from treatment and control groups as determine from the generated crossing point value, intercept and slope are shown in table 4.4.

Table 4: Input amount in log 10 for GADD45α gene

Control	VPA Only	VPA + NS0.1	VPA + NS0.2	FA
0.587886	0.94213	1.393846396	0.805166976	0.753891
1.157263	0.898203	0.787601619	0.821860961	0.893236
1.168964	1.595547	1.05479413	0.742704634	1.185649
0.707061	0.77927	0.991766739	0.90112543	1.227911

VPA= Valproic acid, FA= Folic acid, NS= Nigella sativa

The input amount indicates the expression of GADD45α gene for samples from treatment and control groups. It was observed from the results obtained that the expression of GADD45α gene was reduced in the

VPA+NS0.2 treated group compared to the VPA only group, but one way ANOVA shows no significant difference in the expression GADD45α gene across the groups. Dunnett's multiple comparism test also shows no significant difference among the treatment groups when compared to the control group $p < 0.05$. Figure 4.10 illustrate the GADD45α expression across the groups.

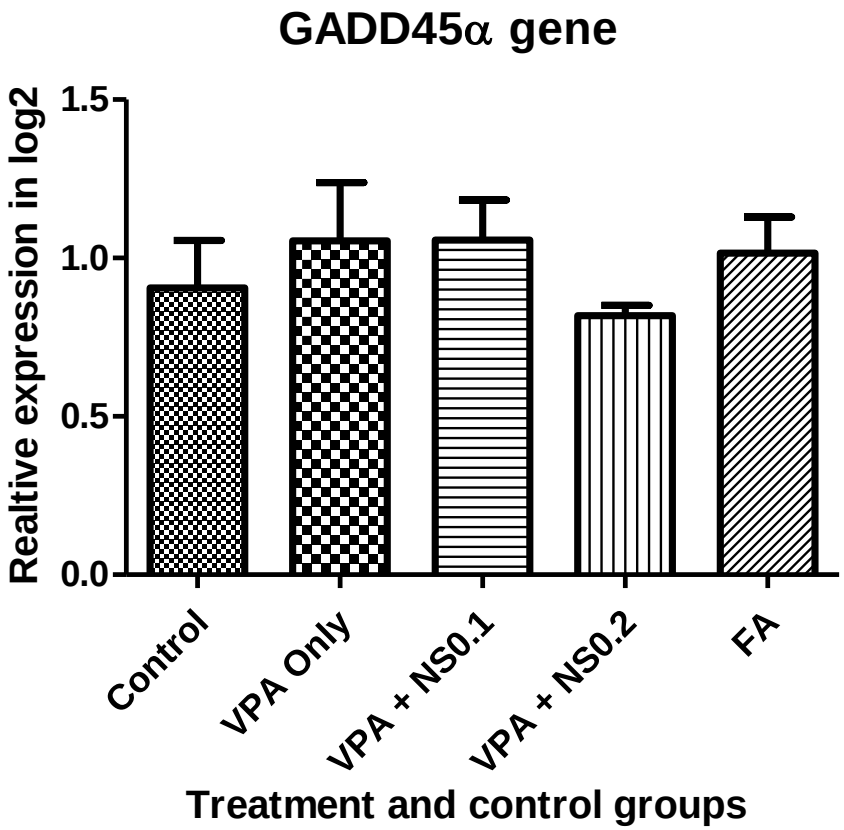

Figure 5: GADD45α expression across the groups

DISCUSSION

Epigenetic modifications are responsible for chromatin dependent regulatory mechanism and are vital for the regulation of gene expression during embryonic development. Epigenetics comprises of changes in gene and protein expression levels, not due to DNA sequence, but due to genomic imprinting , histone modification, DNA methylation and non-coding RNA [11,12]. Histone acetylation as an important way of epigenetic modification, it regulates the state of chromatin to decide inhibition or activation of gene expression [13]. In the progression of embryonic development, it is important for some specific gene to be expressed accurately. Inappropriate expression of these genes can change trajectories in the developmental process and result in congenital malformation [14]. Histone acetylation is critical phenomenon in epigenetics. Inhibition of HDAC activity in cells leads to hyper acetylation of the histone in the nucleus, which causes impairment in transcriptional regulations and cell cycle progression [15]. It has been an established fact that from various work that HDAC inhibitors can cause neural tube defect when administered during early embryogenesis [16–18]. VPA is a potent histone deacetylase (HDAC) inhibitors. The inhibition of histone deacetylation will lead to modification of chromatin structure and foetal gene expression [19,20]. In our study we found a significant reduction in the activity of HADAC in the neural tissue of embryos of VPA treated mice compared to the embryos from the control in which the activity of the HDAC was not impaired. A study by Wu and his group on mice, also found that administration of sodium valproate (NaVP) to mice during pregnancy leads to inhibition of histone deacetylase in the embryos, which in turn leads to various form of cardiac malformations [21]. Preventive treatment with 0.2ml of nigella sativa oil extract shows significant improvement in HDAC activity in the embryos compared to those exposed to VPA only. Treatment with 0.1ml nigella sativa oil extract show little improvement

in HDAC activity but not statistically significant, which is same as results found in treatment with 400µg of folic acid. Histone over acetylation caused by treatment with Trichostatin has been implicated in neural tube defects in chick embryo [22]. Intraperitoneal injection of VPA at 175mg/kg significantly potentiate amphetamine induced behavioural sensitization in mice [23]. Some researchers has found that Teratogenicity of 20 derivatives of VPA that has various types of structural modifications have striking correlation with potency to inhibit HDAC activity, there by leading to foetal malformation in mouse model.

Genes of the GADD45 family, are known to play an important role in the response of cells to the DNA damage resulting in cell proliferation blockage and cell death by apoptosis. Since GADD45 is a HDAC inhibitor activated gene, in this work we focused on the involvement of GADD45α gene expression in embryos exposed to HDAC inhibitor (VPA) in-utero. Previously reported studies indicate that the knockout of GADD45 gene does not eliminate HDAC inhibitor induced cell circle arrest, but it increases the sensitivity of cells to various genotoxic stress as well as to HDAC inhibitors induced apoptosis. This implies that GADD45 gene is not apparently involved in HDAC inhibitors induced suppression of cell growth. There are two opposing views on the contribution of GADD45 gene in the regulation of apoptosis. First approach was that GADD45 gene are capable of playing a pro-apoptotic role. Secondly, ectopic expression of GADD45 was found to sensitize cancer cells to apoptosis induced by etoposide, UV and gamma radiation [24–26]. However, our results shows increase expression of GADD45α gene in the embryos that were exposed to VPA only, VPA + NS 0.1ml and VPA + FA during intra uterine life compared to the control and VPA + NS 0.2ml which shows decrease in the expression of the gene.

CONCLUSION

In conclusion our study demonstrate that administration of nigella sativa oil extract to pregnant mice prevents neural tube defect on the offspring associated with prenatal exposure to Valproic acid. The prevention is done through improving HDAC inhibition activity of VPA. It also shows that no significant benefit for N. sativa oil extract in preventing the increase expression of GADD45α associated with in-utero exposure to VPA.

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