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Research Article

Preventive Effect of *Nigella Sativa* Oil Extract on Neurobehavioural Deficit Induced By Prenatal Valproic Acid Exposure in Mouse Offsprings

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ABSTRACT

Women of childbearing age with chronic medical conditions such as epilepsy are often concerned about hazards from drug exposure during pregnancy and lactation. The avoidance of any medication after conception may often be unwise for maternal well-being. Exposure to Valproic acid (VPA) during intrauterine life has been shown to be responsible for a wide range of impairments on the offspring. Neurodevelopmental disorders such as maladaptive behaviours, speech and learning impairments has been recorded in children prenatally exposed to VPA. The main aim of the study is to evaluate the preventive effect of nigella sativa oil extract on motor, learning and memory impairment induced by intrauterine exposure to Valproic acid on mice offspring. Gravid mice were divided into four groups of six dam in each group, group 1 were administered Sodium Valproate only at a dose of 600mg/kg daily, groups 2 and 3 received Sodium Valproate at a dose of 600mg/kg/day + 0.2ml of Nigella sativa oil extract and 600mg/kg/day + FA 400µg respectively, group 4 dam were administered (0.9% saline), and served as control. The offspring were assessed for physical developmental millstones and put various behavioural tests. A significant improvement was observed in the offspring that had oil extract of N. sativa with VPA compared to those that had VPA only and VPA + FA, with no effect seen in the control group. In conclusion our study demonstrate that administration of nigella sativa oil extract improves learning and memory deficit on the offspring associated with prenatal exposure to Valproic acid in mice. It also shows the benefit of N. sativa oil extract in improving the muscular weakness, anxiety and autism like disorders associated with in-utero VPA exposure.

Keyword: Preventive; Nigella Sativa; Valproic Acid; Mice; Neuro-behavioural

INTRODUCTION

Women of childbearing age with chronic medical conditions such as epilepsy are often concerned about hazards from drug exposure during pregnancy and lactation. The avoidance of any medication after conception may often be unwise for maternal well-being [1]. Exposure to Valproic acid (VPA) during intrauterine life has been

shown to be responsible for a wide range of impairments on the offspring. Research findings have shown that birth defects a are significantly higher in offspring exposed to Valproic acid during pregnancy more than other anti-epileptic drugs (AEDs) currently in use [2]. The choice of AEDs to be used in treatment and prevention of recurrent seizure depend on age of the patient, tolerance, concomitant medications, efficacy and safety of the drug [3]. Recently magnetic resonance imaging studies showed that exposure to Valproic acid during pregnancy has been associated with significant left temporal atrophy [4]. Foetal Valproic syndrome (FVS) is commonly diagnose in the children of these mothers, which is characterised by minor and major congenital abnormalities and cognitive defect [2]. Neurodevelopmental disorders such as maladaptive behaviours, speech and learning impairments has been recorded in children prenatally exposed to VPA [5,6]. Gross motor deficits has been found in the first year of life in children with intrauterine VPA exposure [7]. Intellectual disability has also been found to increase in children expose to VPA during pregnancy at an average dose of 800mg (range 200-1200mg) compared to healthy control [8]. Based on the Griffiths mental development scale, 40% of the children expose to VPA had lower than normal developmental quotient score, compared to 12% of the children in control group [8]. In another study on 57 in school children exposed to VPA during pregnancy in Australia, using Wechsler intelligence scale for children fourth edition, shows significantly low verbal comprehension and working memory, with relatively intact perceptual reasoning and processing speech [9]. Verbal and non-verbal cognitive outcome was found to be reduced in children that were exposed to VPA during intrauterine life [10]. Studies has been done on animals, on the effect of VPA exposure during pregnancy on the postnatal neurogenesis and neuroanatomical changes. These changes are associated to reduce cell proliferation and increase apoptosis in the cortex of the embryo, 12 to 24 hours after exposure to VPA [11]. Folic acid deficiency is a known risk factor and folate supplements before and during early pregnancy may results in a decreased risk of neural tube defects [12]. Epigenetic mechanism has also been implicated in the delay mental development seen in children exposed to VPA during pregnancy, this is possible through histone deacetylase inhibition activity of VPA [13]. In a study by Schneider and Przewlocki, pregnant rats were exposed to 800mg/kg on the 12.5th day of gestation, the offspring are found to have delay in eye opening, abnormal response to non-painful and painful stimulus. They are noticed to have increase repetitive or stereotype behaviour and low social interaction [14]. Valproic acid has a potent effect on the foetal development in rats. About 50% reduction in litter size was recorded in Wistar rat exposure to VPA during pregnancy. However no significant affect was recorded on weight gain of the mother during pregnancy, and also on the offspring when measured after birth and weeks after delivery [15]. In another work VPA at a single dose of 600mg/kg administered to Wistar rat on embryonic day 12.5, lead to decrease in body weight of the offspring when measured on post natal day 23 and 180 [14].

Autism spectrum disorder (ASD) is another well documented effect of VPA on children exposed during intra uterine life. ASD has been shown as most frequently diagnosed neurodevelopmental disorder in children of VPA treated mothers. The rate of ASD in children of VPA treated mothers is about eight times high than in the general population [16]. Studies have revealed that ASD risk in prenatal VPA exposure occurs even without associated congenital malformation [17]. Evidences has shown that increase deficit in spatial memory and learning are as a result of ASD [18].

Nigella Sativa L. (N. sativa) is a family member of the Ranunculaceae. It is an erect plant that is grown annually in the Mediterranean countries, such as Morocco, Algeria, Tunisia, Libya and Egypt and some North African countries. It is also cultivated in countries like Malaysia, Iran, India and China [19]. N. sativa seed also known as black cumin seed is the most used as medicinal part of the plant. There is now growing attention in the use of natural product in the treatment of many diseases both acquired and congenital. In a study conducted on male Wistar rats, it was found that black seed oil administered at a dose of 1ml/kg ameliorates scopolamine induced memory dysfunction [20]. Hydro alcoholic extract of Nigella sativa has been found to protective against scopolamine induced spatial memory deficits in adult rats, while also preventing learning and memory

impairment associated with hypothyroidism in juvenile rats [21]. [22].

In this study we hypothesize that Nigella sativa oil extract may protect against learning, memory and motor impairment due to prenatal Valproic acid exposure in mouse embryos. The main aim of the study is to evaluate the preventive effect of nigella sativa oil extract on motor, learning and memory impairment induced by intrauterine exposure to Valproic acid on mice offspring.

MATERIALS AND METHODS

Animals and treatment

The animal procedures used in the study were in compliance with the directive of University Putra Malaysia, institutional animal care and use committee (UPM/IACUC/AUP-R075/2015). Normal, pathogen free ICR mice of about 8 weeks old were used for the study. They were in a standard animal facility with temperature of 22 ± 2°C, with relative humidity of 60-75%, 12 hours light and dark cycle was maintained. The female mice were monitored for oestrous cycle phases. Female mice that were in their pro-oestrus phase were mated with male overnight (2 female + 1 male per cage). The following day, presence of sperm plug on the vagina of the female mice confirm that mating has taken place, 8:00am of that day was taken as day zero of pregnancy.

The gravid mice were divided into four groups of six gravid mice in each group. Sodium valproate syrup 200mg/5ml (Uither liquid manufacturing, France) and 100% oil extract of nigella sativa (HALAGEL, Malaysia) were administered on day 12.5 of gestation. Group 1 were administered single dose of Sodium Valproate only at 600mg/kg, groups 2 and 3 received Sodium Valproate at a dose of 600mg/kg + 0.2ml of Nigella sativa oil extract and 600mg/kg + folic acid (FA) 400µg respectively. Group 4 mice were administered (0.9% saline), and were used as the control. The body weights of the dams were monitored daily until delivery and average litter size per dam was recorded.

Physical assessment of the offspring

Physical assessment of the offspring was carried out as described by [23] The offspring were assessed for physical developmental millstones from postnatal day 1(PND) till weaning on PND 22. The parameters that were considered are weight, eye opening and hair appearance. The weight of the offspring was taken weekly until week 5, days for eye opening and appearance of hair on the body were all recorded.

Behavioural test

All the behavioural test were conducted when the offspring were between 5 and 8 postnatal except negative geotaxis which was performed 3rd week postnatal.

Surface righting test

The test was conducted as described by [23]. Each pulp was placed on the table with its back lying on the table and all the four limbs extended outward, the pulp was then released and the time it takes each pulp to right (for the pulp to turn and all the four paws are touching the table surface) was recorded. Failure to right after 30 seconds, the pulp was turned and latency time of 30 seconds was recorded. The test was conducted on PND 1, 7, 14 and 21.

Negative geotaxis

Six neonates (3male and 3 female) from the treatment groups and control were tested for Negative geotaxis to asses there vestibular system and motor co-ordination as described by [23]. To assess the effect of VPA on the motor coordination and evaluate the preventive effect of nigella sativa oil extract on it. As described by Takahashi et al. (2010) with some modification, briefly, the neonates were placed on an inclined grid surface 35° with the head looking downward. The time it takes each neonate to turn 180° and re-orientate itself to head looking upward position were recorded in seconds, the highest time allowed for each neonate was 60s. The assessment were started at PND 5 and repeated weekly for 3 times.

Elevated plus maze

Six pulps (3 males and 3 females) were selected from each group for evaluation. Elevated plus maze was used to test the anxiety in the offspring on PND 35 as described by [25]. The apparatus is plus shaped consisting of two open and two closed arms measuring 30x5 cm and 30x5x15 cm respectively, same arms are opposite each other and the arms are elevated 35cm from the platform, the centre is 5x5 square. Testing was conducted between 09:00h and 12:00h. Each animal was placed at the

centre of the apparatus facing one of the open arm and allow to explore the plus maze for 180 seconds, a camera was placed above the setup for recording of the activity of each animal, and this was to avoid observer bias and interference from the observer. The apparatus was cleaned with 50% alcohol after each animal trail, this was to avoid effect of odour on the next animal. The video for each animal was analysed and the number of entry and the amount of time spent in each arm were recorded, entry into any arm was when all the four paws of the animal were in the arm.

Hanging wire test

The hanging wire test was conducted to evaluate muscles function and co-ordination among the offspring of the treatment groups and the control. The age of the animals at the time of the test was 5 weeks and the weight of the animals were averagely the same, test was conducted in a temperature and noise controlled room, and it was done in the morning between 09:00h and 11: 30h. The setup used was as described by [26]. Briefly, 55cm long 2mm thick plastic coated wire was suspended using 2 retord stands at a height of 35cm above the level bedding platform to avoid injury to the animals. Reaching and falling method was used, in which reaching and falling of each animal was recorded over 180 seconds. Six animals (3male and 3 female) from treatment groups and control were studied, each mouse was tested once, and the mouse was held by its tail and placed at the centre of the wire and allow to be suspended. The timer was stopped any time the mouse reaches the end of the wire or fall down, the timing is continued after re-suspending the animal until 180s. The scores increases from 0 to ten for reaches and reduces by 1from 10 for each fall of the mouse from the wire. So the higher score is 10 and the lowest is 0 for both form of test.

Morris water maze (MWM) test

The MWM test was conducted as described by [27]. A black circular container of 136 cm in diameter and 60cm height was used for the procedure. Water at 22-24^{0}c was filled to 40 cm depth, with a circular plastic escape platform 10cm in diameter at the centre of one of the quadrants. The platform was 1 cm above the water level during the training sessions and 1cm below during the other sessions, the pool was placed in a sound proof and dimly lit room. Automatic tracker was used on the mice with a digital video camera connected to a computer for recording. The mouse received acquisition training for 4 consecutive days, for each trails the mouse were placed in the water pool at the centre of each quadrant facing the wall of the tank. Each mice was allowed 120 seconds in which the mouse has to locate the platform and stay on it, the mice that failed to locate the platform was guided onto it with hand. The familiarization test was conducted on the fifth day with the platform hiding 1cm below the water level, the mouse were allowed 120 seconds to locate the platform, animal that failed to locate the platform was guided to it and the latency time was recoded as 120s. The parameters measured during both trails include: escape latency time (time spent to locate the platform in seconds), distance covered (cm) and speed of animals. Probe trail was performed after 2 hours rest from the familiarization test on the fifth day, the platform was removed during this trail and the mouse was allowed to swim for 30 seconds. The number of entry into the quadrant containing the platform, speed, time spent in the quadrant were all recorded for each mice.

RESULT

Litter size and gender proportion

The average number of pulps per dam in all the groups were 6.3 ± 1 for VPA only, 6.5 ± 1.1 for VPA + FA, 8.8 ± 1.5 for VPA + NS 0.2ml and 8.7 ± 1.6 for control group. There were significant reduction of litter size in VPA only and VPA + FA at $p < 0.05$, with no difference in litter size between control and VPA + NS 0.2ml groups figure 1. There was no significant difference in the proportion of male to female in all the treatment groups and the control. The average day for eye opening in new-born mice is 14 days postnatal, ANOVA multiple comparison for percentage of offspring with their eyes opened at day 15 PND among the groups shows significant difference between controls and the treatment group, $p > 0.05$ Figure 2.

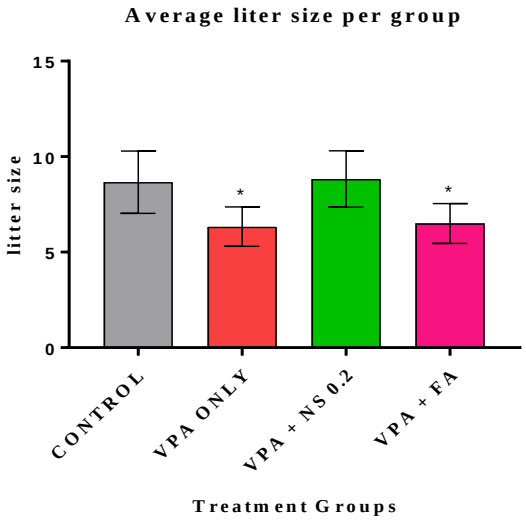

Fig. 1: Average litter size per dam in each treatment groups.
* Significant at $p < 0.05$.

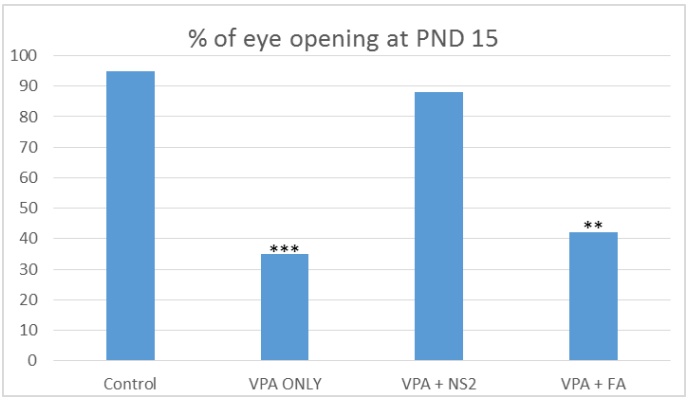

Fig. 2: percentage of offspring with eyes opened at PND 15.
VPA + FA (**) and VPA only (***) were statistically significant at $P < 0.05$.

Physical assessment of the offspring

The weight of the offspring at birth shows reduced birth weight in the offspring exposed to VPA only and VPA + FA 0.9 ± 0.14 and 1.1 ± 0.12 while no significant difference was observed between the control and VPA + NS 0.2mls groups at $P < 0.05$ The weight gain over the first three weeks after birth also shows statistically significant reduction in weight gain among the offspring exposed to VPA + FA and VPA only during intrauterine life, and normal weight gain in offspring of control and VPA + NS 0.2mls (figure 3).

Kinked tail was the prominent gross anomaly that was observed in most of the offspring of VPA only and VPA + FA treated mothers, while those of VPA + NS 0.2ml and control shows no kinked tail among the offspring, Figure 4 and Figure 5 shows representative pictures of kinking types as found in the treatment and control groups.

Righting reflex

The pulps from all the groups placed on their back and allow to turn with all the paws touching the table surface, one way ANOVA and Dunnett's post hoc test reviled that there was a significant difference in the time it takes the pulps to turn between the control, VPA only and VPA + FA, while no significant difference with VPA + NS0.2ml from PND 1 to 2 weeks after delivery (figure 6). At the 3rd
week of delivery no significant difference was observed among the groups.

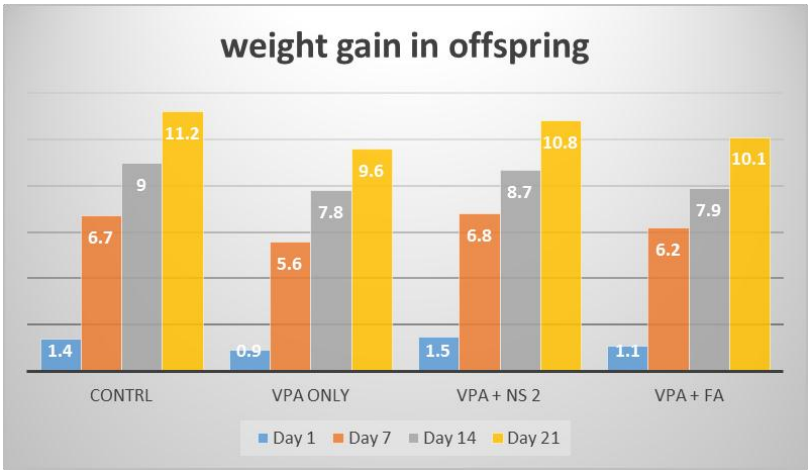

Fig. 3: weight gain in the offspring from treatment and control groups in the first three weeks of life.

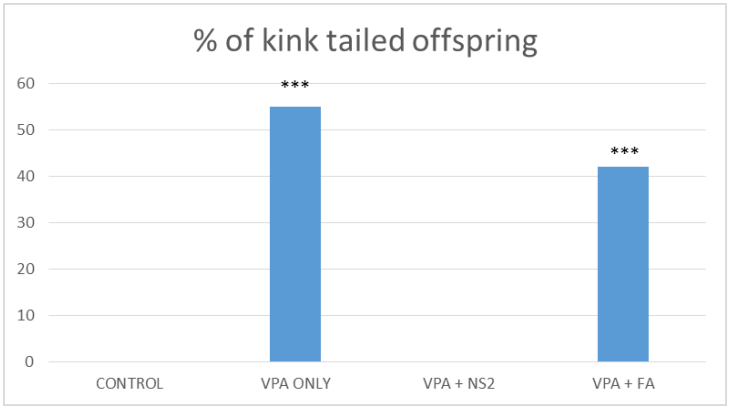

Fig. 4: percentage of the offspring from treatment and control groups with tail kinking.

*** Significant at P < 0.05.

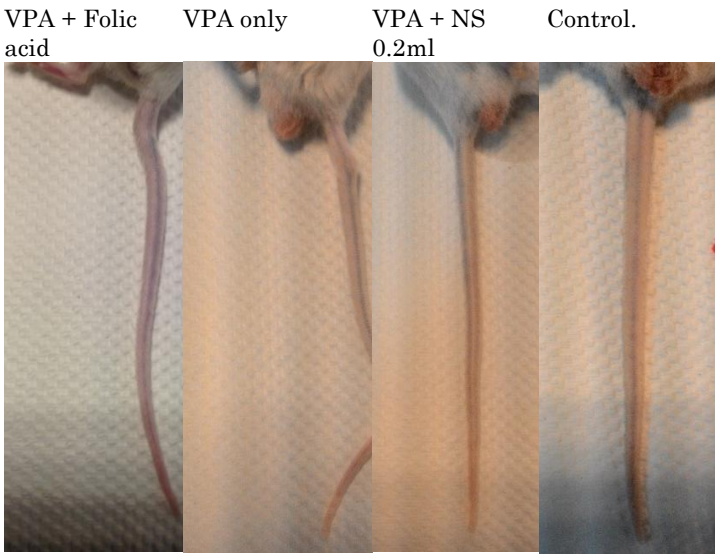

Fig. 5: Tail kinking as shown in the offspring of the treatment groups

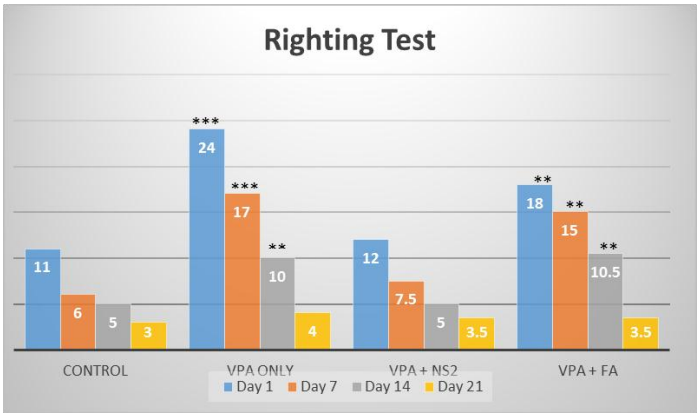

Fig. 6: Righting test over the first 21 days of live

Numbers on the bar are the mean time it takes the pulps to right. ***statistically significant at P < 0.05

Negative geotaxis

The test result for negative geotaxis show that at PND 21 all the selected pulps from control and treatment groups, with significant difference in the latency time among the treatment and control groups. One way ANOVA test was significant at P < 0.05. Tukey's multiple comparison test shows significant difference between control and VPA only and VPA + FA at p < 0.05. A significant difference was also observed between VPA+ NS with VPA only and VPA + FA at p < 0.05, while no difference was seen between control and VPA + NS2 groups figure 7.

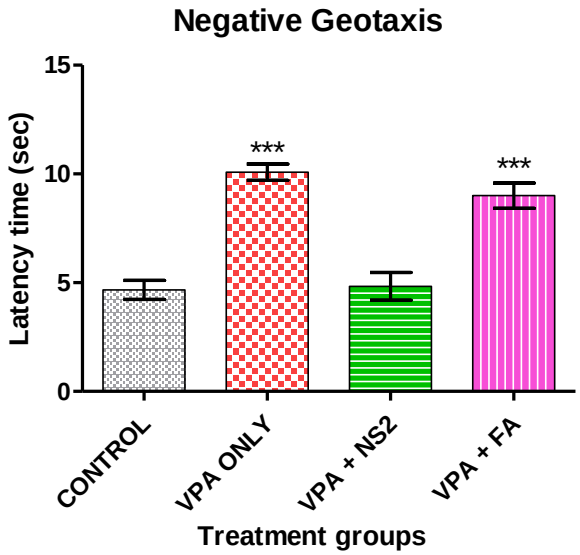

Fig.7: latency time for the pulps turn head upward in negative geotaxis

*** Significant at p < 0.05.

Elevated plus maze

The effect of prenatal exposure to VPA on anxiety in the offspring and the preventive effect of N. sativa on the increase anxiety was evaluated. A significant reduction in entrance into open arm by the offspring exposed to VPA only and those that had folic acid as prevention (VPA + FA) was observed p < 0.001. However, no significant decrease in entrance into open arm was observed in the offspring of that had NS as prevention (VPA + NS) and control group (figure 8). A significant reduction in time spent in the open arm by the offspring exposed to VPA only and those that had folic acid as prevention (VPA + FA) was also observed p < 0.05. However, comparison between control and VPA + NS shows no significant decrease in the time spent in the open arm (figure 9).

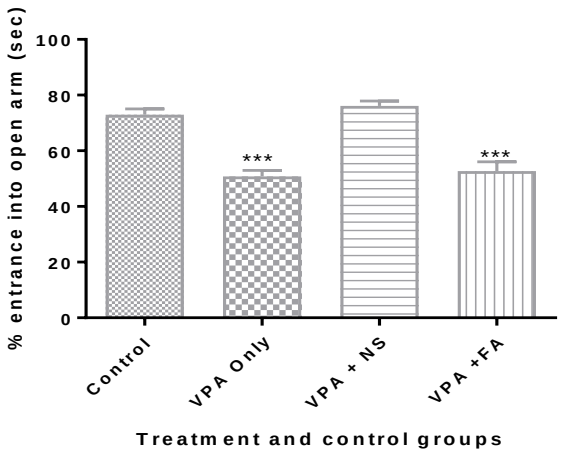

Fig. 8: Percentage entrance into the open arm by the animals.
*** Significant reduction in entrance at $p < 0.001$ based on one way ANOVA and Dunnett's multiple comparison test

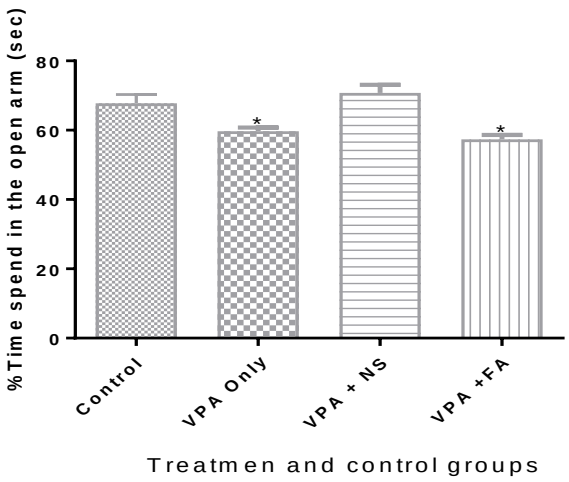

Fig. 9: Percentage time spend in the open area by the animals.
* Significant reduction in time spent at $p < 0.05$ based on one way ANOVA and Dunnett's multiple comparison tests

Hanging wire test

The effect of prenatal exposure to VPA on muscle strength and coordination, and the preventive effect of NS oil extract on the offspring were evaluated using reaches of the animal. One way ANOVA and Dunnett's multiple comparison test shows that there were significant decrease in the number of reaches by the offspring exposed to VPA only and VPA + FA prenatally $p < 0.001$ (figure 10). Meanwhile there was no significant decrease in the value for falls in VPA + NS2 in the offspring compared to the control. One way ANOVA and Dunnett's multiple comparison test shows that there were significant increase in the number of falls by the offspring exposed to VPA only and VPA + FA prenatally $p < 0.001$ (figure 11). Meanwhile the little increase in falls for VPA + NS2 offspring from that of control is not statistically significant.

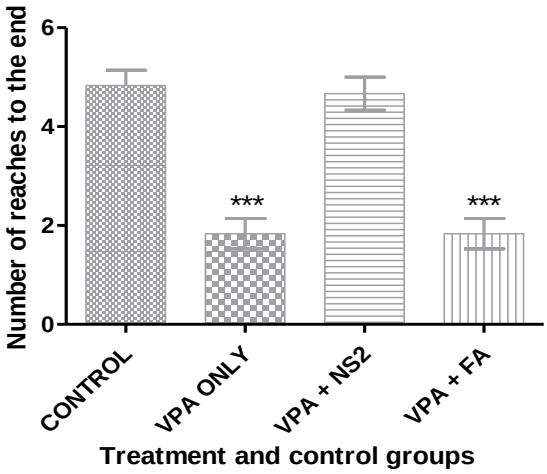

Fig. 10: Number of time the offspring reaches either end of the wire.
*** Significant decrease in reaching the ends at $p < 0.001$ based on one way ANOVA and Dunnett's post hoc test.

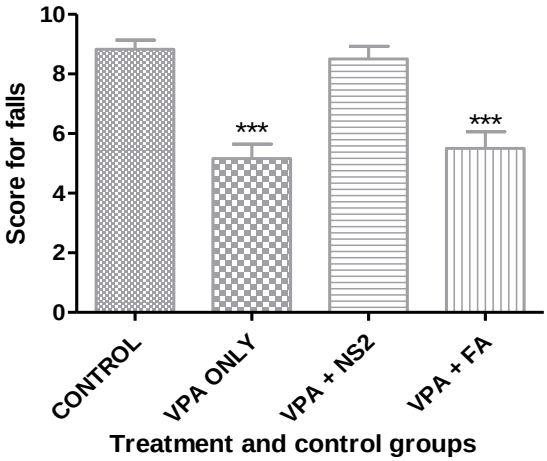

Fig. 11: Inverse number of time the offspring falls from the wire.
*** Significant increase in falls at $p < 0.001$ based on one way ANOVA, Dunnett's post hoc test

Morris water maze

There was significant difference in the latency time for the mice to locate the platform and escape from the water, by using one way ANOVA at $p < 0.05$. Tukey's post hoc multiple comparison test shows significant difference in the latency time between control and VPA only and also between VPA + NS and VPA only (figure 12). There was no significant difference in the speed at which the mice moves during the trail using one way ANOVA $p < 0.05$, (figure 13). There was significant difference in the number of entrance into the new zone (the zone that contained the escape platform) during the probe trail using one way ANOVA $p < 0.05$, while no significant difference was seen among the groups with Dunnett's post hoc test, (figure 14).

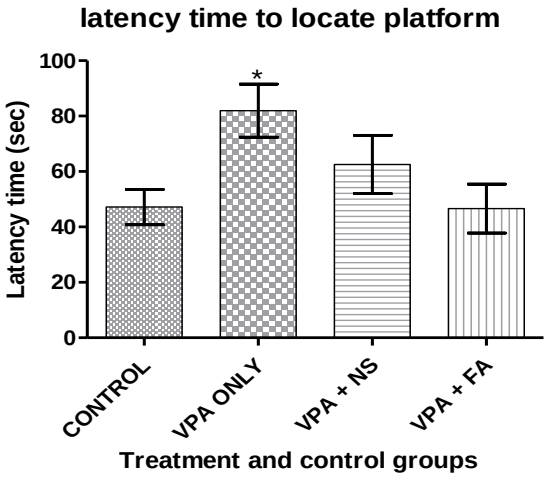

Fig. 12: Latency time in seconds for the animal to locate the escape platform.
*Significant increase at $p < 0.05$ based on one way ANOVA Tukey's post hoc test.

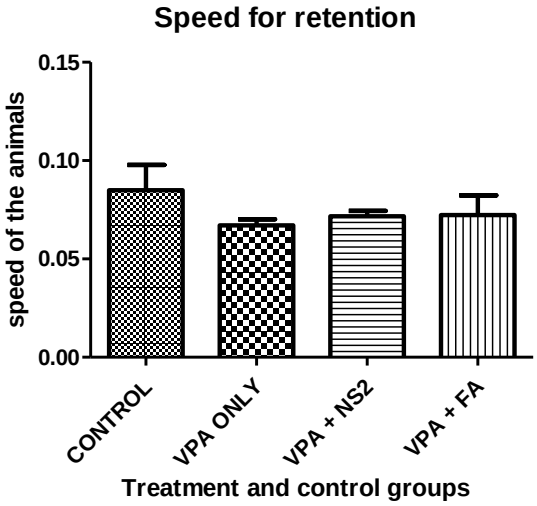

Fig. 13: speed at which the animal move to locate the escape platform.
*Significant increase at $p < 0.05$ based on one way ANOVA Tukey's post hoc test

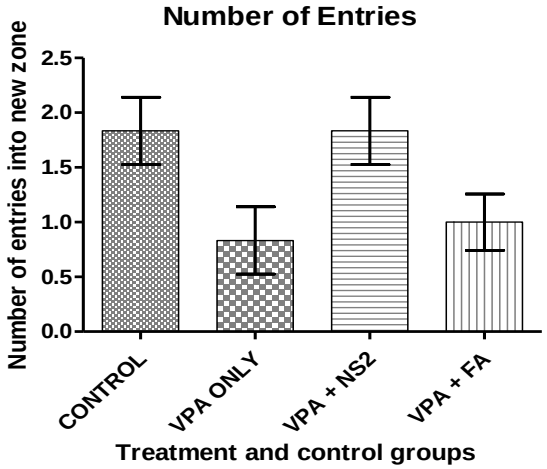

Fig. 14: number of entries into the new zone (Quadrant that was containing the escape platform).
Significant increase at $p < 0.05$ based on one way ANOVA

DISCUSSION

The neuro-behavioural deficit associated with prenatal Valproic acid exposure has been well documented, our study was on the preventive effect of Nigella sativa oil extract on these deficits. We found that there was a significant decrease in litter size per dams in the group that were exposed to VPA only compared to the control group, and the group that had VPA + NS 0.2ml shows a remarkable improvement in the litter size with no much improvement seen VPA + FA group. The finding was not in consistence with what was reported by Podgorac et al. (2016) in which 400mg/kg/day of VPA was the highest dose used in there study, while in our study it was 600mg/kg/day of VPA that was administered. Their study also shows that VPA at dose lower than 200mg/kg/day has no significant effect on the litter size. Morphological changes in the ovaries and significant decrease in serum estradiol level has been reported in rodents exposed to VPA at dose of 300mg/kg twice daily [29,30] which can result in the decrease in litter size associated with VPA exposure. We also found, in an earlier study, that pregnant mice exposed to 600mg/kg/day shows about 30% absorbed site on the uterus (unpublished data) which can also be the reason for decrease litter size in VPA only group. Eye opening on PND 15 was monitored and found to be significantly improved 88% by administering N. sativa oil extract with VPA compared to the groups that had VPA only and VPA + FA, 35% and 42% respectively, but not as good as those in the control group that has 95% of the offspring with their eyes opened. Delayed eye opening on PND 14 was reported by Roullet et al. (2010).

There was significant reduction in birth weight and weight gain by the offspring at PND7 and PND14 that was observed in offspring exposed to VPA prenatally in comparison to the control group. The weight at birth was found to be improved by N. sativa in the offspring of VPA + NS2 group, with no significant difference at PND14. This can be due to the altered feeding ability in the offspring exposed to VPA prenatally as reported in VPA model of autism in rodents [32]. Podgorac et al. (2016) also reported reduction in birth weight in offspring prenatally exposed to VPA at doses of 200 and 400mg/kg/day, also a 16.5% reduction in weight was found by Roullet et al. (2010) in VPA exposed offspring compared to the control group. We reported absence of tail kink in the offspring of dams that were given N. sativa as prevention, as 0% tail kink was also found in the control group, the VPA only and VPA + FA group had 55% and 42% of tail kinking respectively. Our finding was slightly higher than 34% that was reported by Saft et al. (2014). Though we use same dose, the route of administration of VPA in their study was intraperitoneal and it was oral in our own study. Only 9% of tail kink was reported in another study in which 500mg/kg/day was administered IP on GD 12.5 (Favre et al. 2013, [34])

The righting reflex of the pulps was asses over the first 3 weeks of life, we found a significant increase latency time for the pulps to turn in those exposed to VPA only and VPA +FA prenatally, till the second week of life. With no significant difference between control and VPA + NS groups in latency time, and at the 3rd week of life in all the groups. This is in agreement with what was reported by Wagner et al. [35]. The delay in righting can be due to reduce GABAnergic neuronal makers associated with prenatal VPA exposure [36]. These can also be the reason for the increase latency time negative geotaxis seen in the VPA only and VPA +FA groups, while the improvement seen in the group that had N. sativa with VPA can be due to the protective effect of N. sativa has on neuron mitochondrial membrane potential depolarization [37].

Anxiety associated with exposed to VPA prenatally was significantly reduced by administering N. sativa with VPA to the dams in this study which was contrary to Increase in depression like behaviour that was reported by Nakasato et al. (2008) with IP administration of VPA at 800mg/kg/day, which make the animal to disengage from coping with stress stimuli. Anxiety like behaviour was reported to be increased in animals with VPA associated hyperactivity of the amygdala function, when administered at 500mg/kg on rat model for autism [39] and in mouse model [40].

The hanging wire test was used to evaluate muscle strength of the animal by examining the number of fall and reaches by each animal within 180s. A significant effect of Valproic acid

treatment was observed in the group that were exposed to VPA only prenatally, with significant improvement in the reaches and fall in the group that had N. sativa with VPA. Sex of the animal did not significantly affect reaches and falls of the animals in our study. There was a reported decrease in griping strength among Nrf2 knockout female mice that were exposed to VPA [41]. Factors that can affect the performance of the animals in the test include volition, weight and fatigue of the animals [42].

Morris water maze was conducted to evaluate learning and memory among the offspring from the treatment groups and the control, these was to evaluate the preventive effect of nigella sativa oil extract on the impairment of learning and memory in offspring that were exposed to VPA prenatally. In the visible platform paradigm, the latency time for the animals to find the platform above the water level was found to be significantly lower in the control and VPA + N. sativa treated group than in VPA only and VPA + FA treated groups. These was in consistent with the significant prolonged escape latency time in offspring exposed to VPA in-utero which was reported to be improved by administration of DHA [43]. The delay in locating the platform by VPA only treated mice may be due to reported vision deficit associated with long time treatment of VPA [44]. The latency time was also found to be increased in VPA exposed offspring of rats compared to their control, which revealed a disturbance in learning and memory ability [45]. In contrast to our finding, no significant difference was observed in VPA rats compared to control group, which revealed that only slight impairment in spatial learning and memory in Morris water maze [39].

In conclusion our study demonstrate that administration of nigella sativa oil extract improves learning and memory deficit on the offspring associated with prenatal exposure to Valproic acid in mice. It also shows the benefit of N. sativa oil extract in improving the muscular weakness, anxiety and autism like disorders associated with in-utero exposure to VPA. We hope to further the study, by examining the molecular mechanism of these preventive effects.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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