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Nigella sativa Supplementation Attenuates Recognition Memory and Cellular Morphometric Impairments Induced by Toluene Administration in Mice

(Suplemen *Nigella sativa* Mengurangkan Penjejasan Ingatan Pengecaman dan Morfometri Sel Aruhan Pemberian Toluena kepada Mencit)

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ABSTRACT

Toluene exposure has been associated with detrimental effects on the central nervous system. Discovering natural products that can offer neuroprotection following toluene exposure is an essential alternative. *Nigella sativa* (NS), a popular natural supplement, is a good candidate due to its antioxidant and neuroprotective properties. The study aimed to investigate the protective potentials of NS against toluene, on recognition memory performance in the Novel Object Recognition (NOR) test and cellular morphometric measurements of hippocampal CA1 pyramidal neurons. Adult male ICR mice (n30) were randomly divided into five groups; G1: corn oil (CO), G2: toluene (TOL), G3: toluene and NS seed suspension (TOL-NSS), G4: toluene and NS oil (TOL-NSO), G5: toluene and thymoquinone (TOL-TQ). NS supplementations were administered orally once daily for 14 consecutive days while 500 mg/kg b.w. toluene was administered intraperitoneally from day 8 until day 14. Behavioural NOR test was conducted. Subsequently, mice were intracardially perfused and the brains were dissected, then histologically processed. The somatic size and shape of hippocampal CA1 pyramidal neurons were quantified according to specific morphometric parameters. Toluene reduced recognition memory performance of mice and somatic size of hippocampal CA1 pyramidal neurons. Contradictorily, TQ, NSO, and NSS improved mice recognition memory and somatic size of hippocampal CA1 pyramidal neurons. Somatic shape of hippocampal CA1 pyramidal neurons was unaffected by the different treatments. Although non-significant differences were observed, the results indicated the tendency for toluene to cause impairment, while NS supplementations improved mouse recognition memory performance and hippocampal neuronal neuron structure.

Keywords: Brain morphology; *Nigella sativa*; recognition memory; somatic morphometry; toluene

ABSTRAK

Pendedahan kepada toluena telah dikaitkan dengan kesan buruk pada sistem saraf pusat. Pencarian bahan semula jadi yang boleh menawarkan perlindungan saraf berikutan pendedahan toluena adalah alternatif yang penting. *Nigella sativa* (NS), suplemen semula jadi yang popular, adalah calon yang baik kerana mempunyai ciri antioksida serta berpotensi melindungi sistem saraf. Penyelidikan ini bertujuan untuk mengkaji potensi perlindungan NS melalui prestasi ingatan pengecaman dalam ujian Pengecaman Objek Novel (NOR) dan pengukuran morfometrik sel CA1 neuron piramid hipokampus. Tikus ICR jantan dewasa (n=30) dibahagikan secara rawak kepada lima kumpulan; G1: minyak jagung (CO), G2: toluena (TOL), G3: toluena dan biji NS (TOL-NSS), G4: toluena dan minyak NS (TOL-NSO), G5: toluena dan thymoquinone (TOL-TQ). Suplemen NS diberikan secara oral sekali sehari selama 14 hari berturut-turut manakala 500 mg/kg b.w. toluena diberikan secara intraperitoneal dari hari ke-8 hingga hari ke-14. Ujian NOR tingkah laku telah dijalankan. Selepas itu, perfusi intrakardiak dilakukan ke atas mencit dan otak yang dibedah, kemudian diproses secara histologi. Saiz dan bentuk somatik CA1 neuron piramid hipokampus dihitung mengikut parameter morfometrik tertentu. Toluena mengurangkan prestasi ingatan pengecaman mencit dan saiz somatik CA1 neuron piramid hipokampus. Sebaliknya, TQ, NSO dan NSS meningkatkan ingatan pengecaman tikus dan saiz somatik CA1 neuron piramid hipokampus. Bentuk somatik CA1 neuron piramid hipokampus tidak terjejas oleh rawatan berbeza tersebut. Walaupun perbezaan yang tidak ketara diperhatikan, keputusan menunjukkan kecenderungan toluena menyebabkan kemerosotan, manakala suplemen NS meningkatkan prestasi ingatan pengecaman dan struktur neuron hipokampus mencit.

Kata kunci: Ingatan pengecaman; morfologi otak; morfometri soma; *Nigella sativa*; toluena

INTRODUCTION

Air pollution exposure has been proven to cause negative impacts on human health. Toluene, a lipophilic organic compound with high volatile pressure, is one of the volatile organic compounds (VOCs) that has been classified by the United States Environmental Protection Agency (US EPA) as primary pollutants (Baghani et al. 2019). Unfortunately, it is widely used in various commercial products such as paint and printing inks and numerous industrial processes such as petrol production (Cruz, Torres-Flores & Galván 2019). Acute and chronic exposure to toluene has been shown to induce neurologic changes in animals and humans (Armenta-reséndiz et al. 2019; Braunscheidel et al. 2019; Demir et al. 2017; Dick et al. 2021; Soares et al. 2020; Werder et al. 2019) leading to deteriorating effects on the central nervous system. There is currently no medication for neuroprotection after toluene exposure. Treatment consists solely of respiratory and cardiovascular support (Agency for Toxic Substances and Disease Registry 2014).

Though the modern medical system has advanced achievements, traditional and complementary medicines are continuously practiced by population worldwide (Azaizeh et al. 2010). Discovering natural products that can offer neuroprotection following toluene exposure is an essential alternative. *Nigella sativa* (NS) or also known as *Habbatus sauda*, a traditionally consumed natural supplement, is a good candidate.

Although NS is only often found in the Mediterranean countries, Western Asia, Middle East, and Eastern Europe (Khazdair et al. 2019), it is a widely used medicinal plant and considered as one of the greatest healing medicines in the Islamic literature (Sahak et al. 2016). NS is known to have antioxidant and neuroprotective properties, in addition to having antibacterial, antidiabetic, and anticancer effects (Beheshti et al. 2016). NS properties are most likely associated with oil found in the middle of the seed and/or the seed structure itself (Sahak et al. 2016). Chemical compositions of NS include thymoquinone (TQ) (Alhebshi et al. 2014; Bargi et al. 2017), p-cymene, carvacrol, 4-terpinol and thymol (Ahmad et al. 2013). TQ is considered the most prominent content of both volatile and fixed oil of NS seeds (Balbaa, Abdulmalek & Khalil 2017; Hossain et al. 2021).

To the best of our knowledge, comparison on the effects of *Nigella sativa* oil (NSO), *Nigella sativa* seeds (NSS) and its main bioactive constituent TQ against toluene toxicity has not been reported. Therefore, the two objectives of this study were: (1) to study the effects of neurotoxicant toluene and NS supplementations on recognition memory of mice by using the novelty of exploration, and (2) to investigate the neuroprotective property of NS supplementations against toluene toxicity on somatic development of hippocampal Cornu Ammonis 1 (CA1) neurons, which have been established as being involved in memory formation.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND ANIMALS

All experiments were carried out in accordance with the ethics and regulation approved by the Institutional Animal Care and Use Committee University Malaya (Ethics Clearance Number: S/03122018/01082018-01/R). A total of 30 adult (8 weeks old) male ICR mice with average weight of 20-40 g were used for the study. Mice were acclimatised for 14 days at room temperature, exposed to 12 h light/dark per day and had access to filtered tap water and food *ad libitum*.

The mice were randomly divided into 5 groups (n=6 per group). The treatment protocol for the experimental groups is as depicted in Figure 2(A) with all treatments carried out daily between 8.00 am and 10.00 am. Group 1 (G1/control) received oral administration of corn oil (CO) (10 mL/kg b.w.) for 14 days, and corn oil intraperitoneally from day 8 until day 14. Oral administrations of specific compounds were given to the other groups for 14 days: CO (10 mL/kg b.w.) to G2, NSS (10 mg/kg b.w.) to G3, NSO (0.1 mL/kg b.w.) to G4, and TQ (10 mg/kg b.w.) to G5. Apart from G1, the other groups were given toluene intraperitoneally (500 mg/kg b.w.) from day 8 to day 14.

The doses of NSS, NSO, and TQ used in this study were literature based (Al-Nailey 2010; Baghcheghi et al. 2018; Cheema et al. 2018). The toluene dosage used in this study was carefully selected based on our preliminary tests conducted prior to this study, and well aligned with previous studies (Chan et al. 2019; Seo et al. 2010). Intraperitoneal injection of toluene has been documented to cause the same behavioral symptoms as inhalation (Chan et al. 2019; Hsieh et al. 2020; Lee et al. 2020; Seo et al. 2010; Win-Shwe et al. 2012; Wu et al. 2018) and it is generally easier to administer. Thus, this treatment regimen was used in this study.

In previous reports that involved abuse-like toluene exposure, a dose of 750 mg/kg toluene caused alteration of 5-HT_{2A} receptor functions that is frequently associated with onset of hallucinations, as well as chronic cognitive deficits and social isolation in adult mice (Lee et al. 2020). Also, another study on volatile solvent abuse using rat models, demonstrated that 900 mg/kg toluene exposure resulted in significant increase in brain lipid peroxidation which is indicative of enhanced reactive oxidant species formation and significant decrease in crucial antioxidant glutathione marker (Abdel-Salam, Sleem & Morsy 2020). Meanwhile, acute administration of 300 mg/kg toluene had damaging but non-significant effects on the nonspatial

learning memory of mice (Win-Shwe et al. 2012). Moreover, it has been demonstrated that 500 mg/kg toluene in rats exhibited notably vacuolated neurons in the hippocampal area with normal cortex cellularity, and occasional degraded neurons (Abdel-Salam et al. 2021). In addition, a single dose of 500 mg/kg toluene significantly impaired memory performance of mice in Novel Object Recognition (NOR) (Chan et al. 2012). Therefore, in this study, a low-dose of 500 mg/kg toluene was used to examine the behavioural and morphometric changes of mice.

NOVEL OBJECT RECOGNITION (NOR) MEMORY TEST

The NOR test conducted was according to previously described protocol by Huang and Hsueh (2014). NOR test was conducted 24 h after the final treatment, for 4 days (day 15 - 18) in a square arena. The arena was placed at a fixed position to ensure consistency throughout the experiment. The objects to be discriminated were secured to the arena floor and placed at the back of the left and right corners of the arena, at 9 cm from the adjacent walls and 9.6 cm from each other (Figure 2(B)). They were made up of plastic play blocks of different colours and shapes, but with similar height and texture (Figure 2(C)). To ensure no location preference, the position of the novel object was counterbalanced between left and right for each subject. Objects were cleaned with 70% ethanol solution in between tests and animals, to further prevent bias due to any olfactory cues.

NOR test consisted of (a) Habituation phase for two days (day 15 - 16): Mouse was placed in the middle of the arena and allowed to explore the empty arena for 10 min each day; (b) Familiarisation phase for 10 min (day 17): Mouse was allowed to explore a set of two similar objects (A＋A) or (B＋B) placed inside the arena. Half of the mice were familiarised with the set of two object A, while the other half were familiarised with the set of two object B. Mouse was placed at the opposite end of the arena, far from the objects and facing the wall to prevent coercion; (c) Test phase for 5 min (day 18): Mouse expressed its natural exploratory behaviour of favouring novel object. One of the objects in the set used previously in the familiarisation phase was replaced by a novel object; hence, replacement with object B for original set (A+A) or object A for original set (B+B). Animal behaviour was video recorded.

Object exploration time was defined as the length of time of mouse directing its nose at a distance of 2 cm or less from the object, with active vibrissae, sniffing, and/or pawing the object. Just sitting or standing on the object was not included as object exploratory behaviour. Object exploration time and locomotor activity were measured using a semiautomatic behavioural tracking software, OptiMouse (Ben-Shaul 2017).

One-way analysis of variance (ANOVA) followed by post hoc Dunnett's t-test was used to determine the discrimination index (DI) in the experiment. For the assessment of the object recognition memory performance, the percentage of object preference (OP%) and DI during the test phase were calculated (Figure 1). A positive DI value indicated more time exploring the novel object. A DI value of zero indicated equal time spent with both objects, while a negative DI value indicated more time investigating the familiar object (Denninger, Smith & Kirby 2018).

BRAIN GROSS MORPHOLOGY AND MICROSCOPICAL STUDIES

Twenty-four h after the NOR test phase, mice were deeply anesthetized via intraperitoneal route with 80 mg/kg b.w. ketamine and 8 mg/kg b.w. xylazine cocktail. Harvested brain tissues were placed in 10% formalin solution for 48 h post-intracardiac perfusion. The weight, length and width of the brain were measured before subsequent histological processing. The process started with dehydration of the brain tissue in a series of alcohol solutions and finally Nissl's staining of paraffin embedded 10 µm coronal sections. Neurohistological component of the study focused on the hippocampus, a dorsolaterally located structure responsible for memory (Meira et al. 2018). The neuronal cells making up its CA1 region were morphometrically studied. Six (6) consecutive best-stained slides were selected for every group. Only 2 best sections from the 6-8 brain tissue sections per slide were selected for future analysis. The difference between the selected samples were ensured to be at least 30 µm so as to minimize the possibilities of analyzing the same cells. Slide examination of slides and image acquisition were performed using inverted microscope equipped with Leica Application Suite (LAS) Version 4.0 microscope imaging software (Leica Microsystems (Switzerland) Limited).

Somatic development of hippocampal CA1 neurons was assessed by the neuronal somatic size and shape. Somatic size of neurons was analysed accoding to somatic area and somatic perimeter, while somatic shape was analysed based on the somatic circularity, somatic aspect ratio, and somatic roundness. Measurements of the somatic properties were conducted using ImageJ analysis software. For each of the image acquired, 20 neuronal somas with clear nucleus and nucleolus were traced. Neurons were distinguished from glia by the visible presence of a nucleolus, a well-defined nuclear envelope, and nongranular cytoplasm (Meitzen et al. 2011). Data obtained were analysed using Statistical Package for the Social Sciences (SPSS) statistical software (SPSS for Windows, version 23.0). Data were analysed using one-way ANOVA,

$$OP\% = \frac{\textit{time spent exploring novel or familiar object}}{\textit{total time spent exploring both novel and familiar object}} \times 100\%$$

$$DI = \frac{\textit{(time spent exploring novel object)-(time spent exploring familiar object)}}{\textit{total time spent exploring both novel and familiar object}}$$

FIGURE 1. Novel Object Recognition (NOR) test data analysis formula. OP% is the percentage of object preference and DI is the discrimination index

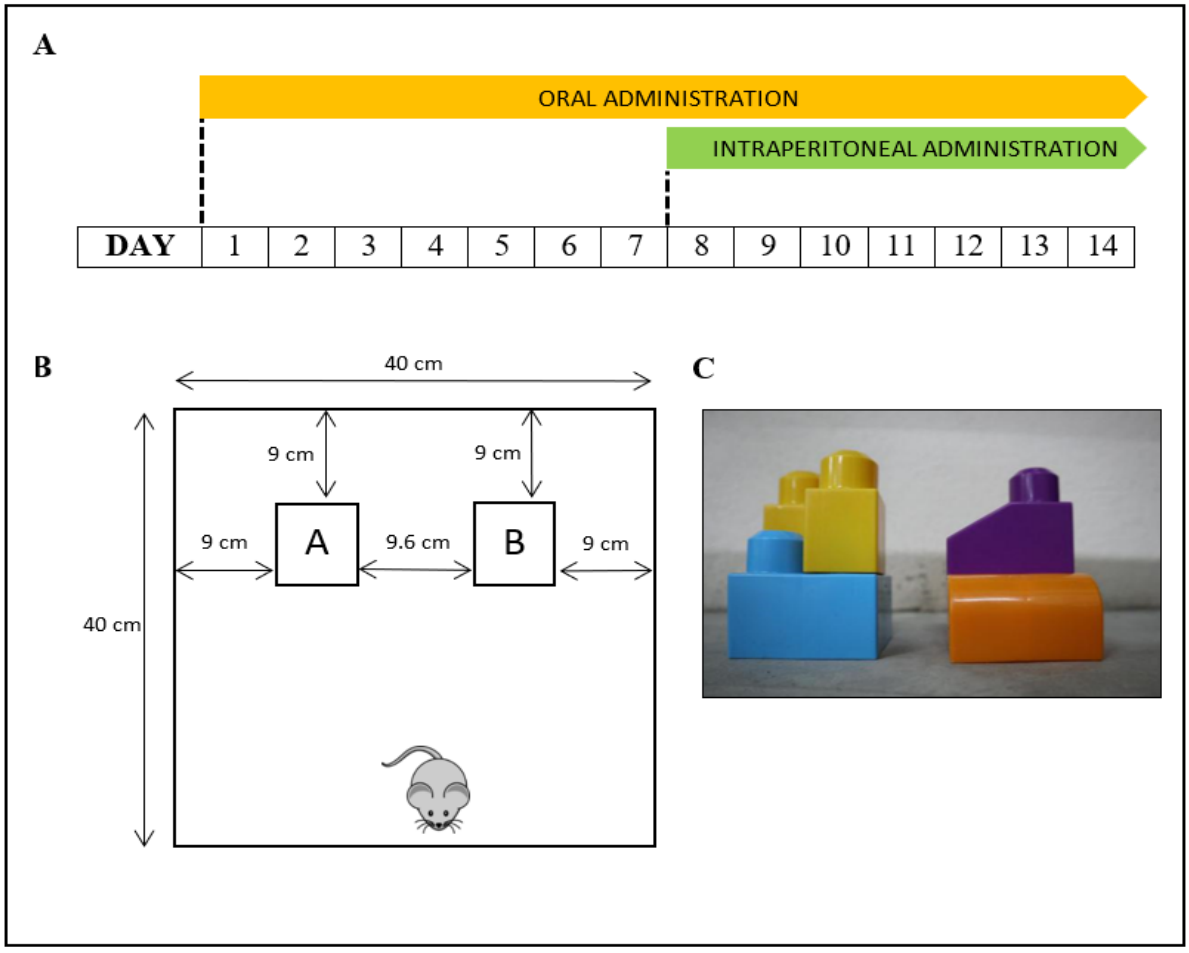

FIGURE 2. Experimental design of the study. (A) Treatment protocol of 5 experimental groups and route of administrations. Except for the control group, the other groups received intraperitoneal administration of toluene (500 mg/kg b.w.) on day 8 to 14 while receiving oral administration of compounds at specific dosages, (B) Schematic diagram of the apparatus in the Novel Object Recognition (NOR) test. Structural objects were placed in left and right corners at the back of the arena. (C) Structural objects made up of plastic play blocks used in the NOR test

followed by Tukey's post hoc test, and presented as mean ± standard error mean (SEM). A value of $p \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

EFFECT OF *Nigella sativa* SUPPLEMENTATIONS AND TOLUENE ON RECOGNITION MEMORY PERFORMANCE

In this study, the control group G1 demonstrated the natural exploratory behaviour of mice, which favoured the novel object in comparison to the familiar object. G2 showed higher inability to recognize the novel object, suggesting that toluene induced deficiency in recognition memory of mice. The percentage of object preference (OP%) of G2 mice treated with toluene only demonstrated lower ability to display the natural exploratory behaviour; i.e., mice did not favour the novel object (Figure 3(A)). Supporting a similar tendency was G2 mice having the lowest discrimination index (DI) (Figure 3(B)).

These findings are supported by a study that reported despite not showing statistically significant differences, mice treated with acute toluene exposure of 300 mg/kg b.w. showed poor discrimination between novel and familiar object and had higher preference towards the familiar object, as compared to its control group (Win-Shwe & Fujimaki 2012). Similarly, mice exposed to 1,000 ppm toluene showed reduction in the recognition index, though reduction was only significant in the groups of mice exposed to 2,000-6,000 ppm toluene (Montes et al. 2017). Prolonged exposure to high doses of air freshener containing toluene caused significant memory dysfunction in mice, while no changes were observed in lower doses (Umukoro et al. 2021). As opposed to the present study, the study by Chan et al. (2019) administered toluene in mice 30 min before the NOR test. Rats subjected to the NOR test 30 min after exposed to toluene also showed significant reduction in the recognition index (Huerta-Rivas et al. 2012).

Although exposure to 500 mg/kg toluene did not reach statistical significance in recognition memory performance of mice, it showed a tendency to have negative effects. In relation to human studies, memory performance of healthy employees in an automobile painting sector who were exposed to 50 ppm toluene for an extended period of time showed no variation when compared to the control group (Kantachai, Kaewkaen & Janthakhin 2020). A cross-sectional examination of workers in the printing industry in Surabaya also found no significant association between toluene exposure (mean concentration of 1.23 ppm) and malondialdehyde levels despite health complaints of coughing, slight headaches, and shortness of breath experiences reported by the workers (Ayu et al. 2020). Previous studies on humans and animals demonstrated similar trends that toluene has negative but insignificant effects in memory performance when exposed to environmental and occupational toluene exposure and that behavioural effects of toluene are time and dose-related.

The tendency of decreased memory performance in toluene treated subjects might be due to the effects of toluene on neurotransmitters involved in memory. Prominent reduction in the expression level of NR1 and NR2 messenger ribonucleic acid (mRNA) after a single toluene exposure (Win-Shwe et al. 2012) and a destructive effect on short and long-term memory performance in the NOR test in rats treated with MK801, a noncompetitive NMDA receptor antagonist (Chan et al. 2019) were observed. NMDA receptor converts certain neuronal activities into structural and functional changes of the synapse, which are essential for memory formation (Liu et al. 2019).

Meanwhile, NS supplementations showed the tendency to alleviate the memory impairment effects by toluene as indicated by higher OP% in G3 (TOL-NSS), G4 (TOL-NSO), and G5 (TOL-TQ) in comparison to G1 and G2 (Figure 3(A)). Thus, NS supplementations did not hinder natural exploratory behaviour of the mice. Although the results showed non-significant differences between the groups, the mice of G5 demonstrated the highest DI (0.33 ± 0.20) (Figure 2(B)) followed by G4 (0.12 ± 0.12), G3 (0.05 ± 0.23), G1 (0.04 ± 0.14), and the lowest DI observed in G2 (0.02 ± 0.13). Among the three NS supplementations, TQ seems to best alleviate the toluene-induced memory impairment effects (Figure 3(B)).

It has been established that TQ has the ability to enhance cholinergic function and attenuate oxidative stress (Baghcheghi et al. 2018). Supplementation of TQ (10 mg/kg b.w.) significantly improved the memory performance of global cerebral ischemia-reperfusion injured rats in NOR test (Hussien et al. 2020) and Alzheimer-induced rats in Y-maze test (Fanoudi et al. 2019). The ameliorating effects of NS against toxicity is probably due to its antioxidant properties and protection against acetylcholinesterase activity. Learning and memory performance of rats in the Morris Water Maze test were improved after administered with 0.5 mL/kg b.w. of NSO (Asrar et al. 2020) and 400 mg/kg b.w. of NSS ethanolic extract (Tahmasebi et al. 2020). Treatment with hydro-alcoholic extract of NSO (200 and 400 mg/kg b.w.) also increased memory performance of rats in the passive avoidance task (Toktam et al. 2011).

EFFECT OF *Nigella sativa* SUPPLEMENTATIONS AND TOLUENE ON BRAIN GROSS MORPHOLOGY MEASUREMENTS

All toluene-treated groups (G2, G3, G4, G5) demonstrated the tendency to have lesser brain weight, compared to the control group, G1. However, mice brain in G3 (TOL-NSS),

G4 (TOL-NSO), and G5 (TOL-TQ) which also received NS supplementations weighed more than G2 (TOL) that did not receive any (Figure 4(A)). Similarly, all toluene-treated groups showed decreased length and width of brains compared to the control group (Figure 4(B)). However, these effects are not statistically significant.

Similar to present result, rabbits exposed to 1000 ppm of toluene for 8 h each day for 14 days resulted in no significant changes in brain weight/body weight (Abouee-Mehrizi et al. 2020). Another study on rat model of chronic intermittent toluene also showed no significant changes in brain weight following exposure to 3,000 ppm toluene (Duncan et al. 2012). Similarly, prenatal exposure of 650 mg/kg toluene to rat pups resulted in no significant changes in brain weight. It is proposed that despite the aberrant proliferation and migration of neurons, the insignificant reduction in brain weight of the rats might be due to the enhanced formation of glia or neuropil (Gospe Jr. & Zhou 2000).

MORPHOMETRIC ANALYSIS OF THE EFFECTS OF *Nigella sativa* SUPPLEMENTATIONS AND TOLUENE ON HIPPOCAMPAL CA1 PYRAMIDAL NEURONS

Histological examination of Nissl-stained sections focused on the hippocampal CA1 pyramidal cells since they are the main structural feature that are accountable for memory and learning, and are most susceptible to oxidative insult (El-Safti et al. 2017). Figure 5(A) shows the intact morphology of the hippocampus with densely stained neurons that made up its three main regions (CA1, CA3 and DG regions). Figure 5(B) focuses on the CA1 region of the hippocampus. The pyramidal neurons of CA1 region demonstrated a normal profile with pale nucleus and prominent Nissl granules in the cytoplasm.

Somatic area and somatic perimeter of CA1 pyramidal neurons did not show significant differences in all treatment groups. One-way ANOVA demonstrated non-significant effect of toluene and NS treatments on somatic area and somatic perimeter of CA1 pyramidal neurons between all groups ($p \geq 0.05$). However, as seen from Figure 5(C), G2 showed the smallest somatic area of 20.4 μm^2 while G4 showed the largest somatic area of 25.75 μm^2. G2 also showed the smallest somatic perimeter of 16.45 μm while G4 showed the largest somatic perimeter of 18.50 μm (Figure 5(D)).

Somatic circularity, somatic aspect ratio, and somatic roundness of CA1 pyramidal neurons did not show significant differences between the treatment groups. One-way ANOVA indicated non-significant effect of toluene and NS treatments on these parameters of CA1 pyramidal neurons between all groups ($p \geq 0.05$). G4 showed the largest somatic circularity of 0.922 μm, while G3 showed the smallest somatic circularity of 0.901 μm (Figure 5(E)). G3 showed the largest somatic aspect ratio of 1.33, while G4 showed the smallest somatic aspect ratio of 1.28 μm (Figure 5(F)). G4 showed the largest somatic roundness of 0.797 μm, while G3 showed the smallest somatic roundness of 0.770 μm (Figure 5(G)).

The centrally located soma of a neuron contains the neuron's genetic information, directs protein synthesis, and holds the task of manufacturing all the other organelles (National Institutes of Health 2022). Morphological dysregulation in the neuronal soma may have substantial consequences on the brain circuit function, leading to certain neurodevelopmental disorders (Paramo et al. 2021; Sathe et al. 2017). No significant changes in somatic size and somatic shape of hippocampal CA1 pyramidal neurons between the treatment groups were demonstrated in the present study.

To date, there are no reports on studies involving morphometric analysis on the somatic size and shape of CA1 hippocampal pyramidal neurons after toluene and NS supplementations. However, neurons in group TOL manifested lower somatic size than the control group. This is supported by studies on rats administered with toluene, reporting loss of normal architecture in the cytoplasm, mitochondria, and nuclei of cells (Salem & Kelada 2020) and shrunken hippocampal neurons and darkly stained cytoplasm (Shaffie & Shabana 2019). Moreover, rats injected with 0.5 mL/kg toluene demonstrated evident shrinking of neuron soma, degeneration and vacuolization of neuropils, and detachments in the pia mater (Meydan et al. 2012). Although the pathophysiological mechanisms underlying toluene neurotoxicity are still not fully understood, these findings could be attributed to the compound's high lipophilicity, which allows it to easily incorporate into cell membranes and cause structural distortions in neurons and their lipid-rich organelles (Moawad, Abd El Fattah & Alsemeh 2021)

A limitation of this study is that morphometric analysis of the neuronal soma in this study was conducted by manually tracing individual cells. Manual quantification of neuronal morphology is time-consuming and prone to observer bias (Billeci et al. 2013). However, in the current study, only one researcher was involved in analysing neuronal morphology using only the ImageJ software. Morphometric analysis of neuronal soma is also highly subjective since there is no definite boundaries between soma and the origin of its dendrites and axon. An automated soma segmentation method to characterise neuronal soma morphology employing a three-dimensional virtualisation technique would allow more distinct observation of morphological alterations in cell bodies between treated groups (Luengo-Sanchez et al. 2015). Besides that, a unique high-throughput neuronal morphology analysis

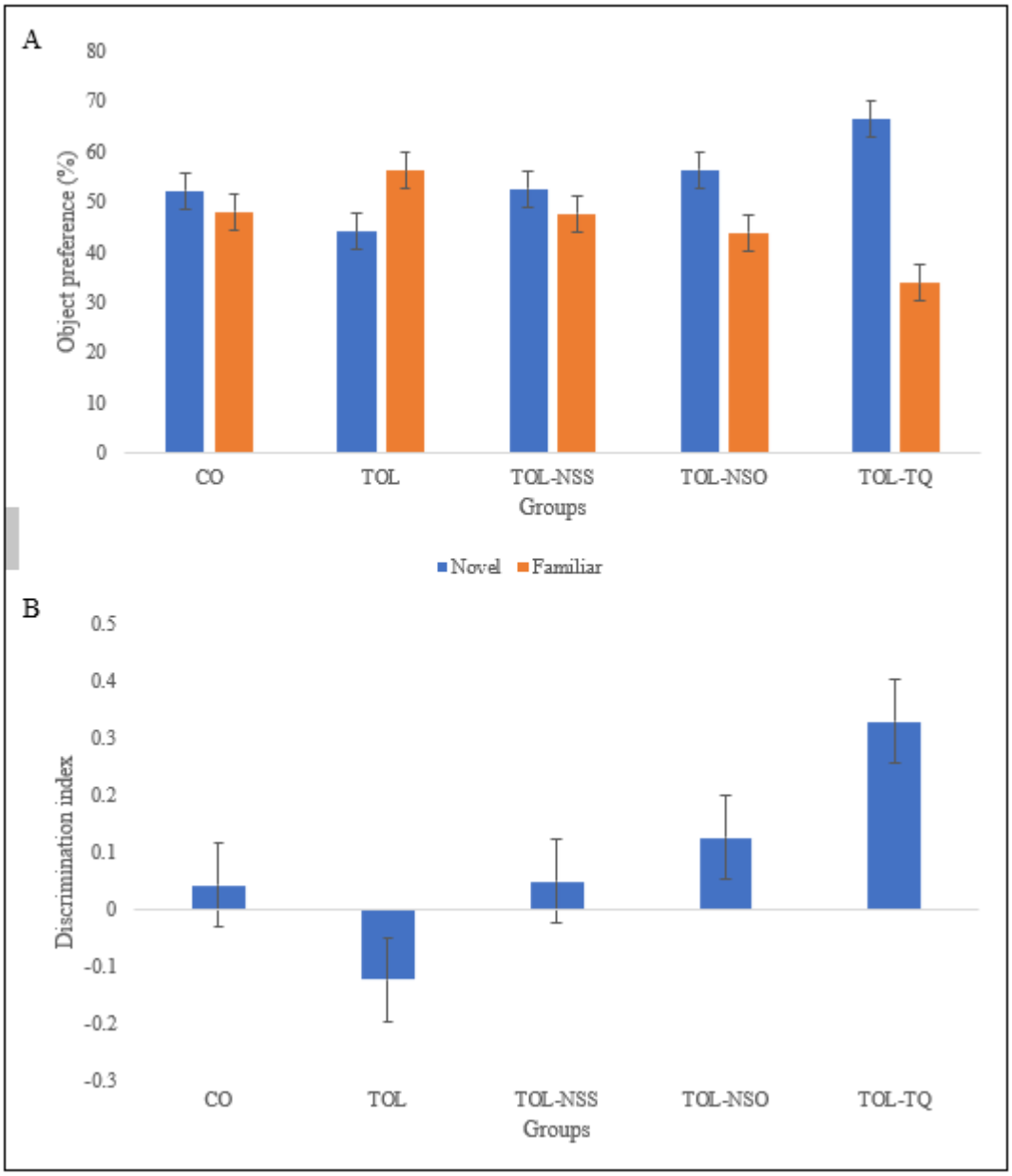

FIGURE 3. Behavioural Novel Object Recognition (NOR) test data. (A) Percentage of novel and familiar object preference during test session, and (B) Discrimination index (DI) during test session. CO: Control/Corn oil; TOL: Toluene; TOL-NSS: Toluene and *Nigella sativa* seeds; TOL-NSO: Toluene and *Nigella sativa* oil; TOL-TQ: Toluene and Thymoquinone

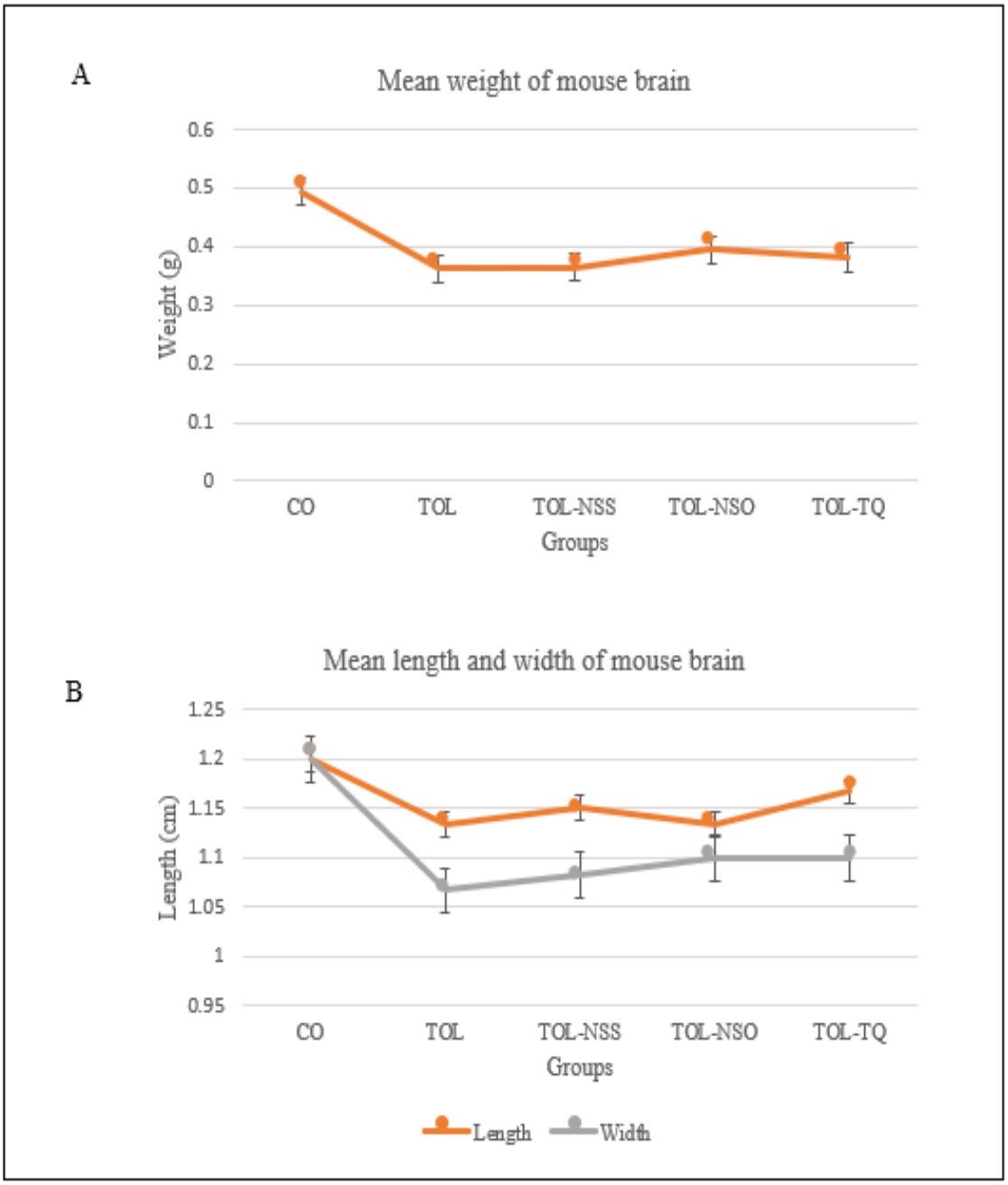

FIGURE 4. Gross morphological data of mouse brain. (A) Mean weight of mouse brain, and (B) Mean length and width of mouse brain. CO: Control/ Corn oil; TOL: Toluene; TOL-NSS: Toluene and *Nigella sativa* seeds; TOL-NSO: Toluene and *Nigella sativa* oil; TOL-TQ: Toluene and Thymoquinone

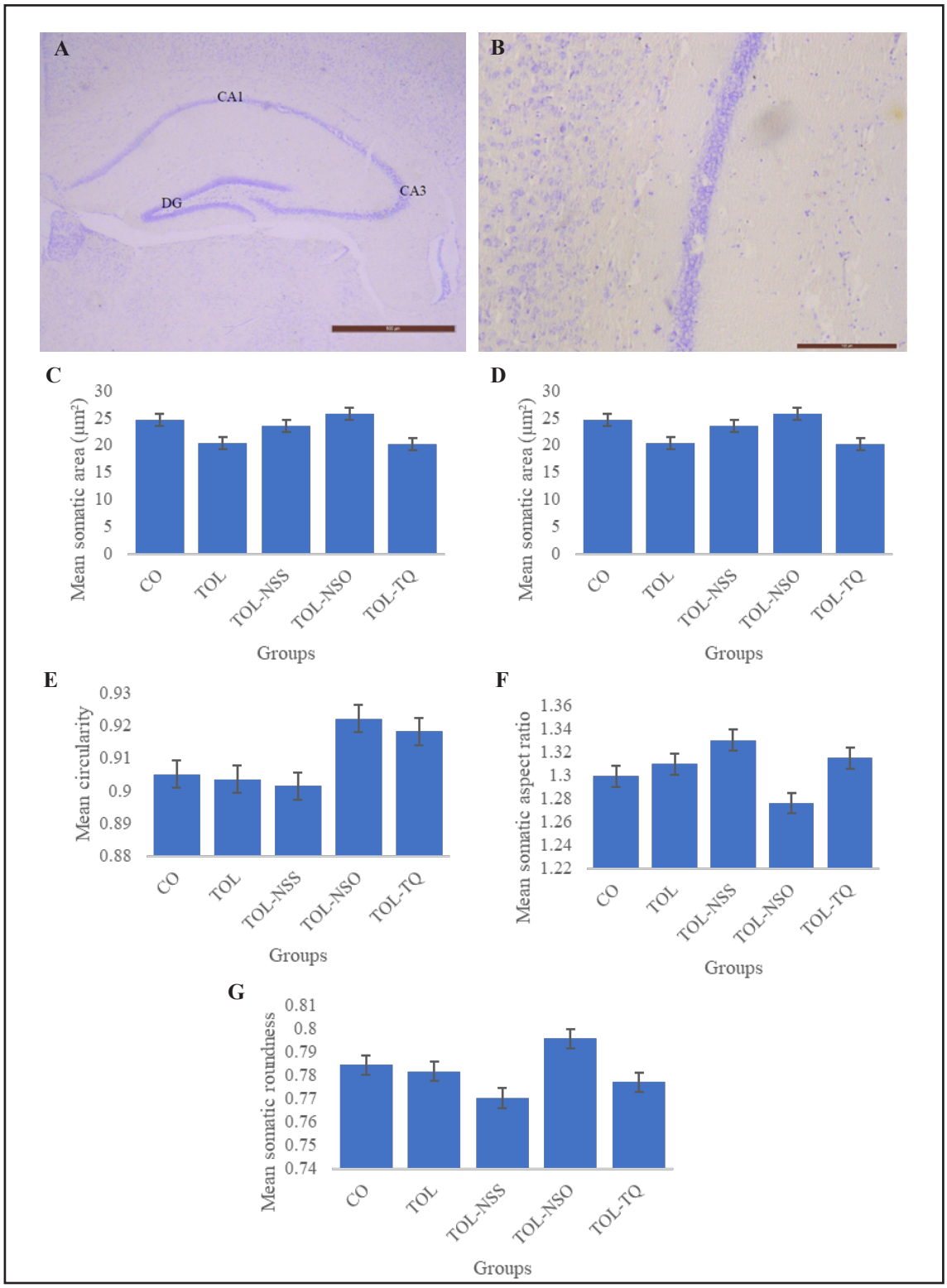

FIGURE 5. Histological and cellular data. (A) Section of brain right hemisphere showing the hippocampus area at 40× magnification. Scale bar, 500 µm. (B) Hippocampal CA1 region at a 200× magnification. Scale bar, 100 µm. (C) Mean somatic area of CA1 pyramidal neurons, (D) Mean somatic perimeter of CA1 pyramidal neurons, (E) Mean somatic circularity of CA1 pyramidal neurons, (F) Mean somatic aspect ratio of CA1 pyramidal neurons, and (G) Mean somatic roundness of CA1 pyramidal neurons. CO: Control/Corn oil; TOL: Toluene; TOL-NSS: Toluene and *Nigella sativa* seeds; TOL-NSO: Toluene and *Nigella sativa* oil; TOL-TQ: Toluene and Thymoquinone

framework (ANMAF) is an alternative technique that can be applied to improve morphological characterisation in future studies (Tong et al. 2021).

CONCLUSION

In conclusion, the non-significant effects of toluene toxicity on memory and cellular structure in this study suggests that low-dose environmental toluene exposure may not cause significant harm to health. Yet, neuroprotective property of NS supplementations against its neurotoxicity was still observed based on the recognition memory performance and cellular morphometric measurements, specifically on the somatic development of hippocampal CA1 pyramidal neurons. Consequently, as there is currently no medicinal treatment for neuroprotection following toluene exposure, the results suggest that NS supplementations especially TQ are promising neuroprotective agents. However, further research should be done to elucidate the exact mechanism of actions of the three different NS forms (NSS, NSO, and TQ), in exerting maximum neuroprotective properties.

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