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ORIGINAL ARTICLE

Role of Cytochrome C and Tumor Necrosis Factor-Alpha in Memory Deficit Induced by High Doses of a Commercial Solid Air Freshener in Mice

Michael Afighor[1], Benneth Ben-Azu[1,2], Abayomi M. Ajayi[1], Solomon Umukoro[*1]

[1]*Neuropharmacology Unit, Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Nigeria*

[2]*Department of Pharmacology, Faculty of Basic Medical Sciences,PAMO University of Medical Sciences, Port Harcourt, River States, Nigeria*

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KEYWORDS

Solid air fresheners; Memory deficit; Cytochrome C expression; Pro-inflammatory cytokines; Histomorphologic changes

ABSTRACT: The study evaluated the role of cytochrome C and pro-inflammatory cytokines in memory deficit produced by a commercial solid air freshener (SAF) in male Swiss mice. The animals were culled into 6 groups (n = 7): group 2-6 were exposed to SAF (10, 25, 50, 100 and 200 mg/kg) for 28 consecutive days through whole body exposure. Mice in group 1(control) were exposed to fresh air. Memory function was evaluated on day 28 using standard models. Mice brains were then processed for estimation of tumor necrosis factor-alpha and interleukin-6, cytochrome C expression and histomorphologic changes. The chemical constituents of SAF were also determined using GC-MS.SAF (100 and 200 mg/kg) produced memory decline and increased brain contents of tumor necrosis factor-alpha ($p < 0.05$) but not interleukin-6 ($p > 0.05$) relative to control. Increased cytochrome C immunopositive cells, reduced neuronal cells and cytoarchitectural changeswere observed in the hippocampus of mice exposed to SAF (200 g). MS GC-profiling showed the presence of 7 major volatile organic constituents in SAF. Increased release of tumor necrosis factor-alpha and cytochrome C expression might play a role in SAF-induced memory deficit in mice.

INTRODUCTION

Epidemiological studies have identified air fresheners (AF) as a major contributor to poor indoor air quality and a possible risk factor for about 2.7% of the global burden of human diseases [1, 2]. These fragrances are primarily used to mask bad odors, with the goal of inducing a pleasant environment [3]. Despite this good intention, air fresheners are known to release harmful chemical substances capable of impairing air quality and well-being of the regular users [2-3]. Air fresheners contain volatile organic compounds (VOCs), which are known to stimulate the olfactory nerve more strongly than unpleasant odors [3-5]. They are widely used in homes and public facilities across the globe [3]. The global market for the sale of AF has been estimated to be above ten billion US dollars [3]. Asia is known to be the fastest growing market for AF [3]. The use of these

[*]Corresponding author: umusolo@yahoo.com (S. Umukoro)
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consumer products has been reported to be on the increase in Korea [3].

Previous reports have shown that exposure to VOCs through frequent uses of AF in the homes cause dearaches and diarrhea in infants, and depression and headaches in their mothers [3, 6]. Epidemiological studies have also shown that about 20% of the normal population and 34% of asthmatic patients experienced headaches, breathing difficulties, or other health disorders due to AF exposure [7]. Studies have also linked emission of toxic substances from AF to injury of the vital organs of the body such as the brain, lungs, heart, and also the reproductive and immune systems [8]. The eyes, nose, and skin are the most common organs directly affected by AF [2]. Thus, the use of the term air freshener to describe these consumer products is rather deceptive, since they add more toxic chemicals into the environment [2]. Despite, the wide range of adverse effects associated with the use of these products, their chemical constituents are yet to be disclosed to the general public due to regulatory protections placed on consumer product ingredients [3]. Besides, it has been reported that less than 10% VOCs constituents are disclosed on AF labels [3].

Chemical substances released from AF have been reported to cause brain injury through induction of oxidative stress, which is a strong risk factor in the etiology of various mental health complications including mood disorders and cognitive impairments [9]. Studies have also shown that VOCs found in AF react rapidly with ozoneto produce deleterious oxidant molecules and other related oxidative products [3]. Abundant evidences have shown that VOCs including phthalates produced hormonal imbalance and neurotoxicity via oxidative damage and depletion of antioxidant enzymes [3, 5]. It has been suggested that decreased antioxidant status might render the brains of mice exposed to high doses of SAF more vulnerable to oxidative damage and perhaps contribute to its deleterious effects [10]. However, oxidative stress is also closely connected with neuro inflammation, which has been implicated in degeneration of neuronal pathways critical to learning and memory [11-13]. Moreover, cytochrome C, a soluble protein associated with mitochondrial electron transport system plays a crucial role in cell death and is known to be release in abundance in response to tissue damage [14].The mitochondrial pathway influences caspase activity by regulating release of cytochrome C [15]. We have shown in our previous studies that mice exposed to high concentrations of SAF had memory deficit and increased MDA accompanied by reduced antioxidant biomarkers [10]. This study is aimed at evaluating the role of pro-inflammatory cytokines and cytochrome C in memory deficit in mice exposed to SAF.

MATERIALS AND METHODS

Laboratory Animals

Six weeks old Swiss male mice (42) procured from the University of Ibadan Central Animal House were utilized in the study. The animals were kept in standard conditions with free access to food and water. Ethical approval (UI-ACURE 180070) was obtained from the Institutional regulatory body on the use of animal of the University of Ibadan.

Sources of chemicals

The commercial SAF was purchased from a supermarket in Ibadan, Oyo State, Nigeria. Biochemical reagents including trichloroacetic, sodium chloride, potassium carbonate, trichloroacetic, dithiobis-nitrobenzoic acid (Sigma Aldrich, USA), cytokine ELISA kits (Bio Legend MAX^{TM} Deluxe, UK) and cytochrome C immunohistochemistry antibody kit (Santa cruz, Germany) were used in the study.

Experimental design and SAF exposure

Experimental design of the study is presented in Figure 1. The animals were assigned randomly into 6 groups (n =7). Group 1 (control) had free access to natural air while mice in groups 2 to 6 were exposed to different amount of ground particles of SAF (10, 25, 50, 100 and 200g) respectively, through whole-body exposure in their home cages at 24 h/day for 28 days. The different doses of the SAF were placed into specific small containers with uniform holes on all sides to prevent the mice from having

direct contact with them. The containers were hung at the middle of the cage of each experimental group. The cages were kept in different places in order to prevent contamination [10].

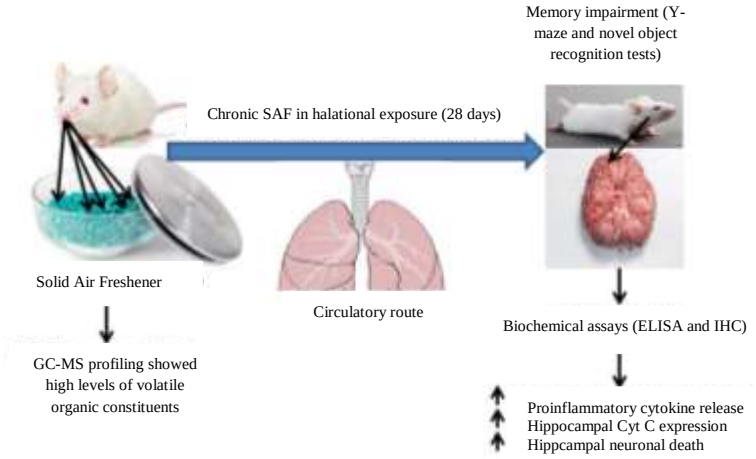

Figure 1. Schematic summary of the experiment

Chemical analysis of SAF

Gas chromatography-Mass spectrometry (GC-MS) technique was used to determine the volatile organic constituents in SAF. Gas chromatography (Agilent 6890) coupled to Mass spectrophotometry (5975) at the Department of Chemistry, University of Lagos; Lagos, Nigeria was used for the analysis. The SAF was extracted in n-hexane and acetonitrile (1:1) and 1 µL of it was injected at 1.2 mL/min of the helium carrier gas.

Behavioral assessments of cognitive function

Behavioral assessments of cognitive function of SAF were carried out on day 28 (30 min after the last exposure) using Y-maze and object recognition paradigms. Spatial working memory was measured using Y-maze test [10]. Index of spatial memory (%alternation) was determined [16]. The non-spatial working memory was assessedusing the object recognition test [17]. The test which consisted of training and test phases, were carried out on days 27 and 28 respectively. The discrimination index, which is used to indicate non-spatial working memory, was determined [17].

Tissues preparation for ELISA assay

After the behavioral tests, the animals were euthanized with ether anesthesia. The brains were then harvested, weighed, pulverized and centrifuged at 5400 *g* for 10 min at 4°C.The supernatants were separated and kept at −80°C until needed for the ELISA assay.

ELISA assay for brain proinflammatory cytokines

The procedures highlighted by the manufacturer were followed in estimating the brain contents of pro-inflammatory cytokines such as TNF-α and IL-6. Brain concentrations (pg/g tissues) of TNF-α and IL-6were extrapolated from the standard curve included in the assay kits.

Histomorphologic studies and histomorphometry

Coronal planes of formalin buffered and perfused brains of mice (n = 3/group) were sectioned using microtome (Leica, Germany).Each brain section was embedded and stained using H/E to show the histologic features of the prefrontal cortex (PFC) and CA1 region of the hippocampus. Leica microscope and camera with a computer interface (Magna Fire) was used to view and captured the slides. Analysis of histomorphometry was carried out to determine viability of the neuronal cells of the PFC and HC (CA1) [18].

Determination of cytochrome C immnopositive cell expression

After several washes of the perfused brain regions as described above, immunohistochemistry was carried out and used to evaluate the expression of the immune positive proteins of cytochrome C (Cyt C) of the hippocampus and prefrontal cortex. The tissues for immunohistochemistry were deparafinized and hydrated with xylene and downward concentrations of alcohols. The slides were placed in citrate buffer tank after rinsing with saline and heated for 3-5 min in a water bath so as to retrieve antigens. After several washing PBS and blocking with milk solution at 25°C, the tissues slides for the different brain regions were incubated with Cyt C primary antibody (1:300) for 20-30 min at 25°C respectively. Incubation of tissue slides were carried out with one-step horseradish peroxidase (HRP) polymer for about 20-30 min, and rinsed with PBS 4-6 consecutive times. Droplets of freshly prepared 3, 3'-diaminobenzidine (DAB) reagents were released on each slide and incubated for a period of 6-10 min at 25°C. The photomicrograph of stained slides of each brain region were viewed and obtained with Leica camera connected to a computerized (Magnafire) Olympus microscope. Immunoreactive cells were identified and analyzed with imageanalyzer software [18].

Data analysis

Data were expressed as Mean ± S.E.M (standard error of mean). One-way analysis of variance (ANOVA)followed by Bonferroni *post-hoc* test for multiple comparisons using Graph Pad Prism software version 5 was used for data analysis. A level of $p < 0.05$ was considered as statistically significant.

RESULTS

GC-MS profiling of SAF constituents

The chromatogram of the GC-MS profiling of SAF and its major volatile organic constituents with their relative abundanceis presented in Figure 2 and Table1 respectively.

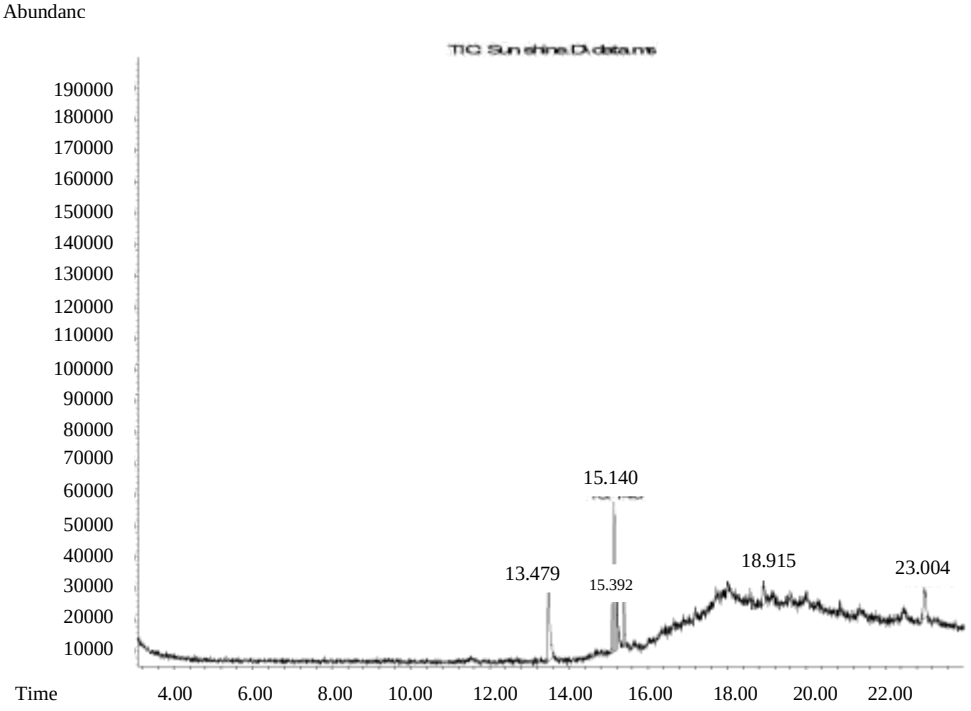

Figure 2. Chromatogram diagram of solid air freshener.

Table 1. Major volatile organic constituents (VOCs) in a commercial solid air freshener

S/N	Retention time	VOCs found in SAF	% (Abundance)	Molecular weight[a]	IUPAC name[a]
1	13.477	Hexadecanoic acid methyl ester	23.32	270.5 g/mol	methyl hexadecanoate
2	15.102	3-Methyl-3,5--(cyanoethyl)tetrahydro-4-thiopyranone	7.29	236.34 g/mol	3-[5-(2-cyanoethyl)-5-methyl-4-oxothian-3-yl]propanenitrile
3	15.140	13-Octadecenoic acid, methyl ester	36.75	296.5 g/mol	methyl (*E*)-octadec-13-enoate
4	15.196	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	4.99	153.14 g/mol	(*E*)-3-(2-amino-1*H*-imidazol-5-yl)prop-2-enoic acid
5	15.392	Guanidine, N,N-dimethyl-	10.30	87.12 g/mol	1,1-dimethylguanidine
6	18.915	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4(D-2-(2-(methylamino) acetamido)hydracyrlamido)-β-D)	6.83	Complex	Complex
7	23.004	4-Aminobutyramide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butyrnyl]-N',N'-bis(trifluoroacetyl)-	10.52	223.07 g/mol	2,2,2-trifluoro-*N*-methyl-*N*-(2,2,2-trifluoroacetyl)acetamide

a National Center for Biotechnology Information. Pub Chem Database accessed on July 13, 2019

SAF decreases spatial and non-spatial working memories

Figures 3A to 3B show the effects of SAF exposure on spatial and non-spatial working memories. Bonferroni post-hoc analysis revealed that lower doses of SAF (10, 25 and 50 g) exposure for 28 days, did not impair spatial and non-spatial working memories in mice. However, higher concentrations of SAF (100 and 200 g) reduced spatial [F (5, 36) = 7.304, $p < 0.0001$] and non-spatial [F (5, 36) = 13.80, $p < 0.0001$] working memories in mice (Figure 3).

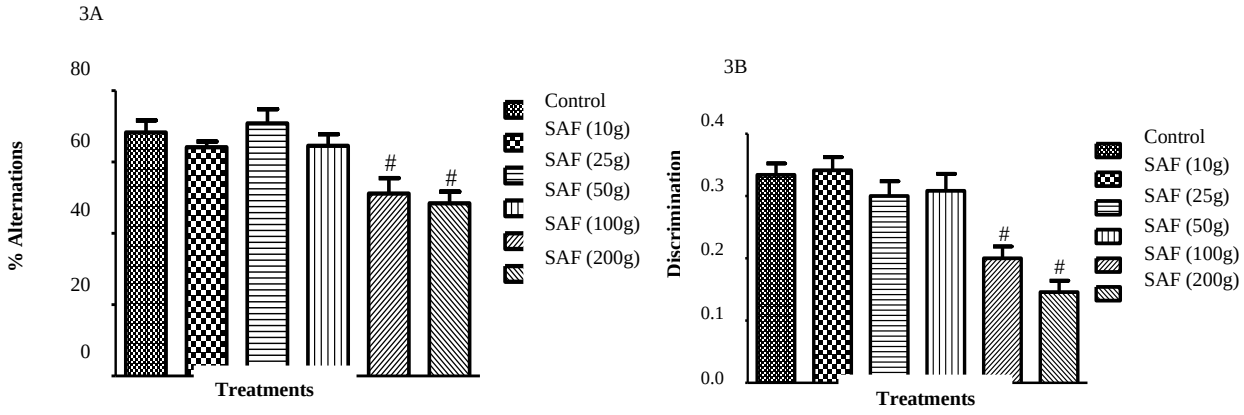

Figure 3. Effect of whole body exposure to solid air freshener (SAF) on spatial working (A) and non-spatial (B) memory in mice. Each column indicates the mean ± S.E.M (n = 7). $^{\#}p < 0.05$ vs control (One-way ANOVA followed by Bonferroni post hoc test).

SAF reduces brain TNF-α contents in mice

The effects of whole body exposure of SAF on TNF-α and IL-6 concentrationsare shown in Figure 4A-B. One way ANOVA in combination with Bonferroni post-hoc test showed that prolonged exposure of SAF (10, 25, 50, 100 and 200 g) did not produce any effect on IL-6 level when compared with control [F (5, 36) = 4.229, $p = p = 0.1613$] (Fig. 3A). However, higher concentrations of SAF (100 and 200 g) significantly [F (5, 36) = 1.693, $p = 0.0040$] increased brain concentration of TNF-α in comparison with control (Figure 4B).

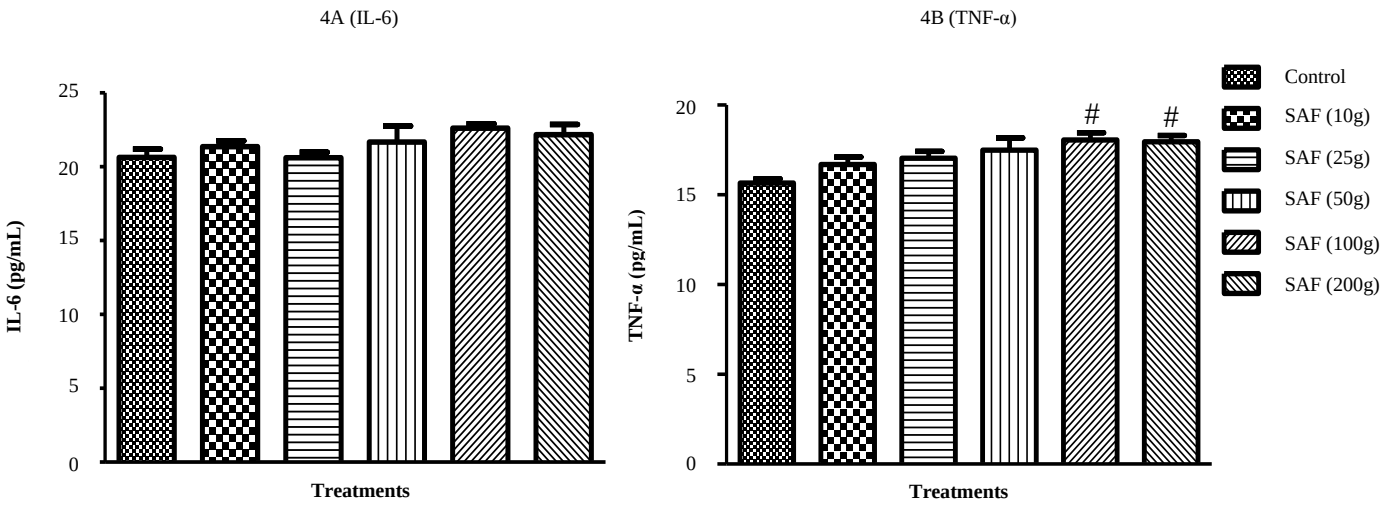

Figure 4. Effect of whole body exposure to solid air freshener on interleukin-6 (A)and tumor necrosis factor-α (B) concentration in mice brains. Each column indicates the mean ± S.E.M (n = 7). $^{\#}p< 0.05$ relative to control (one-way ANOVA followed by Bonferroni post hoc test).

Prefrontal cortical and hippocampal neurons of mice exposed to SAF

The effect of SAF on histologic and histomorphologic (density of viable cells) changes in the prefrontal cortical and hippocampal neurons is shown in Figure 5A-B and 6A-B. Hematoxylin and eosin staining revealed that at lower concentrations of SAF (10-100 g), it did not produce significant effect on the cytoarchitectureand neuronal cell viability of the PFC and HC of mice (Figure 6A-B). However, exposure to high concentration of SAF (200 g) altered cytoarchitectural features and reduced the population of viable neuronal cells [F (5, 36) = 3.455, p = 0.0364] of the CA1of the HC, but not those of the PFC [F (5, 36) = 0.4610, p = 0.7979] (Figure 6A-B).

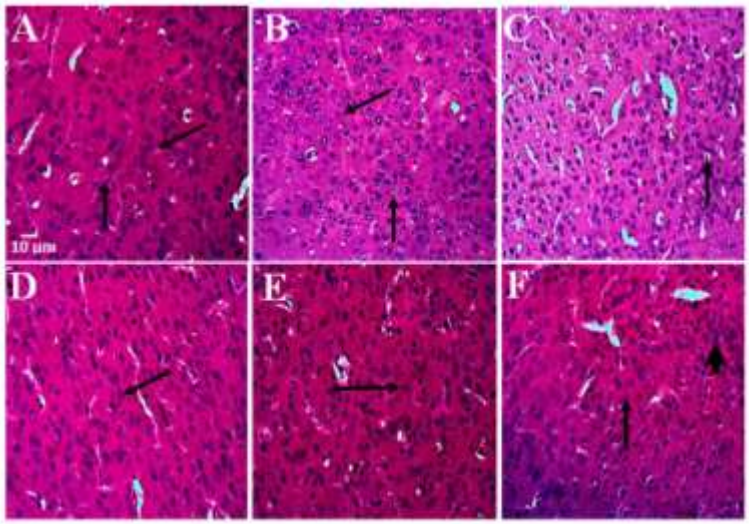

Figure 5A. Representative stained sections of the effect of long-term whole body exposure of air freshener on histological changes of the prefrontal cortex of mice. A = control, B = SAF 10 g, C = SAF 25 g, D = SAF 50 g, E = SAF 100 g, and F = SAF200 g. Slides A, B, C and D showed normal multiple neuronal cells with hyperchromatic nuclei, Slide F shows a few ghost neuronal cells and normal cells. Arrow indicates normal neurons while arrowhead shows dark neurons. Calibration bar = 0.01 mm (10 µm) for all figures; H/E stain: x400.

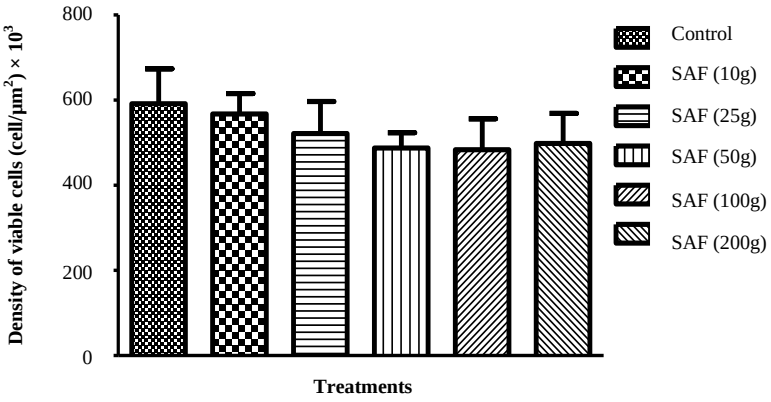

Figure 5B. Effect of long-term whole body exposure of solid air freshener on density of viable neuronal cells of the prefrontal cortex of mice. Each column indicates mean ± S.E.M of 3 mice per group.

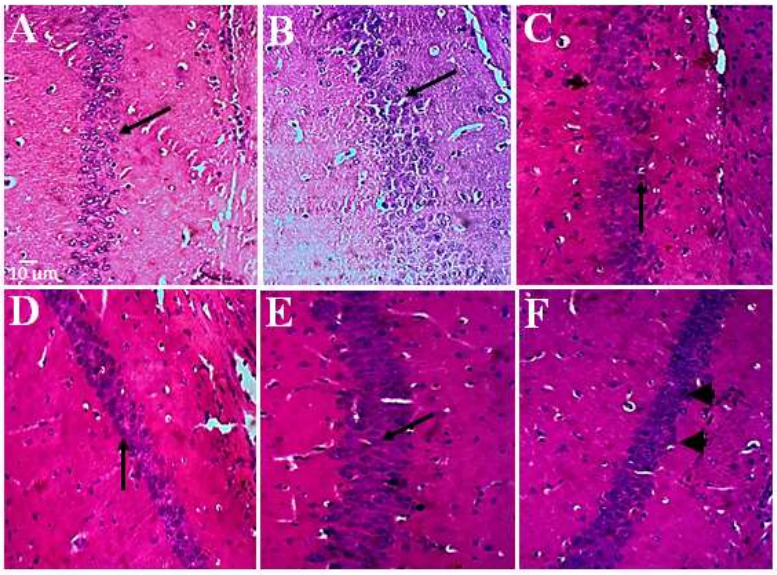

Figure 6A. Representative stained sections of the effect of long-term whole body exposure of air freshener on histological changes of the hippocampus (CA1) of mice brain. A = control, B = SAF 10 g, C = SAF 25 g, D = SAF 50 g, E = SAF 100 g, and F = SAF200 g. Slide A, B, C and D showed normal multiple neuronal cells with hyperchromatic nuclei. Slide F shows a ghost neuronal cells and evidence of pyknotic cells. Arrow shows normal neurons while arrow head indicates dark neurons.Calibration bar = 0.01 mm (10 µm) for all figures; H/E stain: x400.

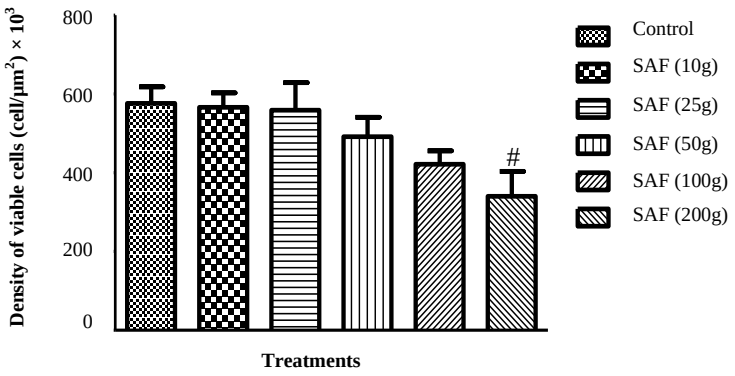

Figure 6B. Effect of long-term whole body exposure of solid air freshener on density of viable neuronal cells of the hippocampus of mice. Each column represents the mean ± S.E.M of 3 mice per group.$^{\#}P< 0.05$ vs control (one-way ANOVA followed by Bonferroni*post-hoc* test).

Effect of SAF on prefrontal cortical and hippocampal

cytochrome C immnopositive expression in mice

The photomicrographs and cytochrome C immune positive cell expression in PFC and HC (CA1) of mice exposed to SAF are presented in Figure 7 and 8. Prolonged exposure of high concentrations of SAF (100 and 200 g) did not produce significant changes in cytochrome C immune positive cell expression in PFC ($p<0.05$) [F (2,6) = 0.1519, p = 0.8623] of mice (Figure 7).As presented in Figure 8, SAF (100g) did not produce significant changes in CA1 neurons. However, increased expression of cytochrome C immunopositive cells in the CAI neurons in mice exposed to 200 g of SAF occurred (Figure 8).

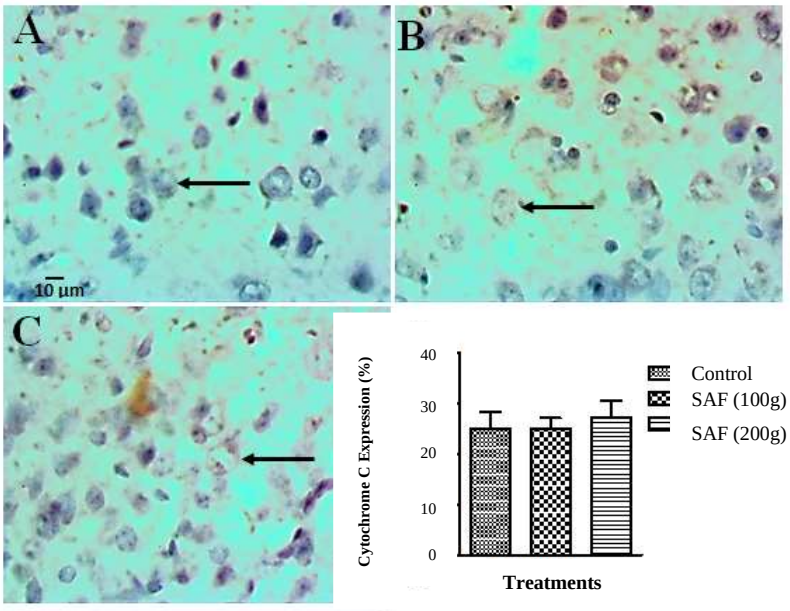

Figure 7. Representative photomicrographs and densitometric immunopositive cell expression of the effect prolonged solid air freshener exposure on cytochrome C in the prefrontal cortex of mice. A = control, B = SAF 100 g, and C = SAF200 g. Vertical arrow indicates high immunopositive cell expression while horizontal arrow shows low immune positive cell expression. Densitometric bar represents mean ± S.E.M of 3 animals per group.

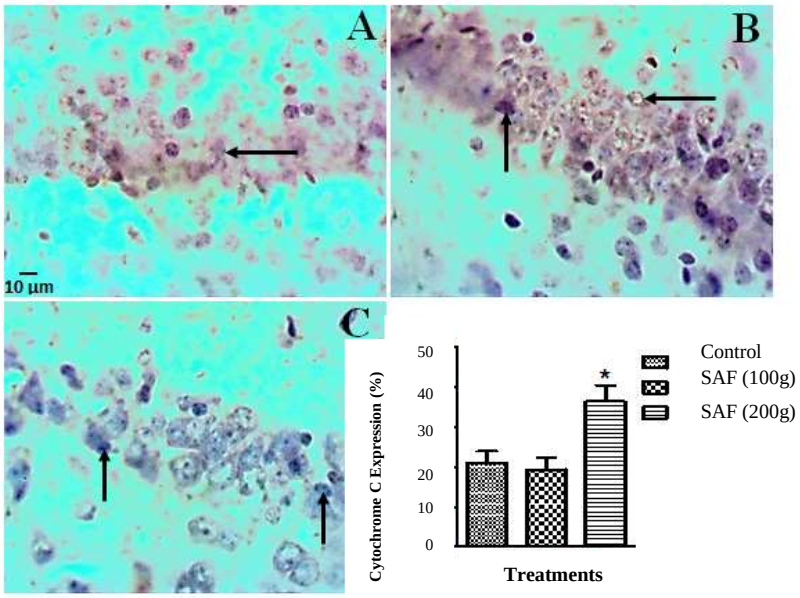

Figure 8. Representative photomicrographs and densitometry immunopositive cell expressions of the effect prolonged solid air freshener exposure on cytochrome C in the hippocampus (CA1) of mice. A = control, B = 100g, and C = SAF 200 g. Vertical arrow indicates high immuno-positive cell expression while horizontal arrow indicates: Low immunopositive cell expression. Densitometric bar represent the mean ± S.E.M of 3 animals per group. $^{\#}P < 0.05$ vs control (one-way ANOVA followed by Bonferroni*post-hoc* test).

DISCUSSION

The results of this study showed that high dose of SAF reduced percentage alternation in mice subjected to Y-maze paradigm. This screening procedure depends on rodents' tendency to explore unfamiliar environment and is routinely utilized to detect hippocampal-dependent spatial memory [16, 17].Rodents prefer to explore a different arm of the maze instead of previous arm and the hippocampus is the major portion of the brain implicated in this task [16, 17].The correct sequence of arm entry or alternation is used as a measure for intact memory [16, 17]. Wrong alternation arm choices therefore indicates memory deficit. Our findings that high dose of SAF impairs alternation behavior suggest the need to sensitize consumers of the inherent danger pose by prolonged exposure to this deodorant and other related products. The effect of SAF on memory function after prolonged exposure was further evaluated in mice using the NORT. This test was originally described by Ennaceur and Delacour [18] and it relied on rodents' natural tendency to explore novel objects in comparison to familiar ones. Thus, rodents tend to spend lesser time in exploringfamiliar objects in comparison to novel ones [18-20]. The NORT is known to be less stressful and ppropriate test for memory following environmental insults or genetic manipulations[19, 20].The discrimination index (DI), which has been described as the differences in period spent in exploring novel objects and familiar counterparts is routinely used for assessing memory in NORT [19, 20]. Animals with reduced DI are known to have memory deficit whereas cognitive enhancers caused increase in DI [18, 21]. The decrease in DI in mice due to prolonged exposure to SAF further confirms its adverse effects on memory. These findings are in accordance with earlier investigations, which have implicated the presence of toxic chemical substances with memory impairing effect in AF [11, 10].

In our previous studies, we have reported that prolonged exposure to SAF resulted in increseased cetylcholinestrease activity, elevated MDA content accompanied by reduced endogenous antioxidant molecules in brains of mice [10]. Besides, there are reports, which have also shown that chemical substances emitted from AP depleted antioxidant armory of body cells [11, 15]. Glutathione, for example, is a well-known potent endogenous compound that provides the cellular defense mechanism against injurious effects of

oxidant molecules [15].Thus, low antioxidant status renders the brain cells to be more vulnerable to oxidative damage in animals exposed to environmental toxicants [12]. Studies have also shown that VOCs found in AF react rapidly with ozone to produce more deleterious oxidant molecules and related products [5, 22]. However, oxidative stress is connected with neuro inflammation, which have been implicated in degeneration of neuronal pathways relevant in learning and memory [12, 13, 21 and 23].To this end, the effects of prolonged exposure to SAF on proinflammatory cytokines, cytochrome C and histomorphologic features were evaluated in mice. Our results showed that prolonged exposure to high dose of SAF increased brain TNF-α content and up regulated cytochrome C expression but did not significantly alter IL-6 level. Cytochrome C is a soluble protein associated with the inner mitochondrial membrane, where it acts as a component of the electron transport chain [24]. Cytochrome C is normally restricted within the mitochondria but its exodus into the cytoplasm triggers apoptosis [12]. It is release in abundance in response to tissue damage or to toxic stimuli [12, 24].The mitochondrial pathway has been implicated in the regulation of caspase activity by modulating cytochrome C release through mechanism related to Bcl-2 anti-apoptotic proteins [15, 24]. This study shows that prolonged exposure to high dose of SAF produced an increase in the hippocampal immune positive cells of cytochrome C in mice. However, prefrontal cortex neurons were not significantly affected, suggesting that hippocampal mitochondria are more susceptible to SAF.

Studies have implicated neuronal apoptosis, a type of programmed cell death, in the pathophysiology of neurodegenerative disorders [15, 24]. Several pathological events including loss of neuronal cells, altered microenvironment and decreased levels of hippocampal neurogenesis orchestrate functional deficits such as memory deteriorations [20]. Our findings showed that prolonged exposure to high dose of SAF induces histomorphological changes in mice brains. Specifically, high dose of SAF (200g) produced a significant cytoarchitectural changes and reduced hippocampal neuronal cell viability, but no effect on the PFC neurons. Overall, this finding suggests that the deleterious effects of SAF on the integrity of the hippocampal neurons might be contributing to its memory-impairing activity in mice.

The adverse effects of AF have been linked to the presence of over 100 different types of chemical substances including VOCs (limonene, linalool, ethanol, formaldehyde, benzene, toluene, and xylene) and semi-volatile organic substances (phthalates) [3]. GC-MS analysis of the commercial SAF used in our study revealed the presence of hexa decanoic acid methyl ester, 3-Methyl-3,5-(cyanoethyl)tetrahydro-4-thiopyranone, 13-Octadecenoic acid, methyl ester, imidazole, 2-amino-5-[(2-carboxy)vinyl], Guanidine, N,N-dimethyl, glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4(D-2-(2-(methylamino) acetamido)hydracyrlamido)-β-D) and 4-aminobutyramide,N-methyl-N-[4-(1-pyrrolidinyl)-2-butyrnyl]-N',N'-bis(trifluoroacetyl) as the major constituents. However, further investigations are necessary to confirm the relevance of these chemical constituents in the memory-impairing effect of this SAF in mice. Meanwhile, studies carried out in Germany, United States of America and Korea showed a slight variation in the chemical compositions of different types of AF produced by manufacturers across the globe [25, 26].Taking together, these findings suggest that different brands of AF emit different toxic chemicals with diverse degrees of deleterious effects.

CONCLUSIONS

Increased release of tumor necrosis factor-alpha and over expression of cytochrome Cimmunopositive cells might play a role in memory deficits induced by prolonged exposure of mice to high dose of SAF.

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Conflict of interests

Authors have no conflict of interests to declare.

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