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Research Article

Effects of shisha smoke inhalation on some long-term memory forms in adult male mice

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ABSTRACT

Background: Shisha is a flavoured tobacco designed to be smoked in a water-pipe, but it effects on long-term memory has not been fully explored. This study was undertaken to evaluate the effect of Shisha smoke inhalation on some long-term memory models in adult male BALB/c mice. **Methods**: Twenty male mice were divided into 4 groups of five mice each. Group I (control): fresh air; group II: exposed to bonged Shisha; group III: exposed to unbonged Shisha; group IV: exposed to activated charcoal smoke only. Each group was exposed for thirty minutes daily for seven weeks. Long-term memory was assessed using elevated plus maze (EPM), novel object recognition test (NORT) and Barnes maze (BM). **Results:** There was statistically significant decrease ($P<0.05$) in novel object recognition in bonged Shisha group when compared with the control. There was statistically significant increase ($P<0.05$) in spatial learning and memory in bonged Shisha group when compared with control. There was statistically significant decrease ($P<0.05$) in acetylcholinesterase activity in bonged Shisha group when compared with control, but there was no statistically significant difference in anxiety related spatial memory in elevated plus maze when compared with the control. There was also increased in necrosis of hippocampal cells in bonged Shisha group and slight necrosis in unbonged and activated charcoal smoke when compared to control mice. **Conclusion:** The outcomes of this study suggest that bonged Shisha smoke is neurotoxic to the brain because of combined effect of various toxicants emanating from different Shisha smoke constituents used in the set-up.

INTRODUCTION

Shisha is a tobacco-based product designed to be smoked in a water-pipe. A water-pipe (hookah) consists of a head, glass, body, and hoses (Rastam *et al*., 2004). Shisha comes in a variety of sweetened fruit flavors and produces a mild aromatic smoke which is more pleasant to unseasoned palates, and this sweeter new product combined with the growth of social media have fueled the growth of use first in Arab countries, and world (Karem *et al*., 2019). Hookah products usually are combination of molasses, vegetarian glycerin, light leaf tobacco and flavorings. The tobacco is infused with glycerin to sustain its moisture and fermented in molasses to produce a tobacco base. Due to the varieties of flavors and attractive packaging, this tends to appeal to young people (Song *et al*., 2017), hence results in rising popularity of its consumption. There is also misconception that, smoking Shisha is relatively less hazardous than smoking cigarettes (Ramachandra and Yaldrum, 2015) with some believing that bubbling tobacco smoke through water may be safe (Al-sawalha *et al*., 2018), and most outlets offering Shisha remain unregulated (Cornwell *et al*., 2011). Therefore, the aim of this study was to evaluate the effects of Shisha smoke inhalation on some long-term memory form in adult male Mice.

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METHODS

Study Materials

Shisha clay pot, Aluminium foil paper, exposure chamber, activated charcoal, vacuum pump compressor (Model No. DN7174B55000, GEC Machine Ltd, Newcastle, United Kingdom), Bench centrifuge (Model No. 68-3856-23, Chadwell Heath, Essex, England), microplate reader (RT 006C Rayto, India), photometer, test tubes, cotton wool, strings, Ethylenediamine tetra

acetic acid bottles, masking tape, hand groves, light microscope, matches, plain bottles, mint flavour, banana flavor, apple flavor, methylated spirit, distilled water.

Experimental Animals

Twenty (20) apparently healthy adult male BALB/c mice between 7-10 weeks, weighing 25-35g, were acquired from the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria, where they were housed in standard laboratory cages and provided with feed and water *ad libitum.*

Experimental design

The mice were divided into four (4) groups of 5 mice each (n=5)

Group 1: Control (fresh air)

Group 2: Bonged Shisha (flavor + activated charcoal + water)

Group 3: Unbonged Shisha (flavor + activated charcoal)

Group 4: Activated charcoal smoke only (Suleiman *et al*., 2019)

Experimental Protocol

Shisha exposure was done according to method used by Forountanjazi *et al*. (2014). Briefly, the smoke produced by the Shisha apparatus was passed through an exposure chamber (13cm x 6cm x 6cm) containing the experimental mice through a vacuum pump compressor for 7 seconds and fresh air was allowed into the chamber through air inlet for 53 seconds at the pressure of 300 kpa. Although, each group was exposed to different constituent, the same procedure was deployed for all experimental groups. This was repeated for the duration of 30 minutes daily for seven weeks.

Assessment of memory

Elevated plus-maze for memory (EPM)

The elevated plus maze was used to evaluate anxiety related spatial memory (Itoh *et al.,* 1990). The elevated plus maze for mice consists of two perpendicular open arms (21.5 x 7.5 cm) and two closed arms (21.5 x 7.5 x 20 cm) which extends from a central 7.5 x 7.5 cm platform. The platform and floor were made from wood, and the lateral walls of the close arms was made of wood painted black. The maze was elevated 38 cm above the floor. On the 1st day (training), each mouse was place at the end of one open arm, facing away from the central platform. The latency of the mice to move from the open to the enclosed arms was recorded within 90s. Following entry into the arm, the mice were allowed to explore the apparatus for 30s. 24 hours later, the second trial (retention test) was performed, and the mice were observed for 90s. After each trial, the maze was wipe with a cotton wool dipped in 70% ethyl alcohol and allowed to dry to remove any olfactory cue.

Barnes Maze (BM)

The Barnes maze is a tool used to measure spatial learning and memory (Barnes, 1979). The testing apparatus is a circular platform with 2 diameter escape holes around the periphery of the platform. The procedure consists of three phases; habituation, training and probe trial. During the training, mice were placed in the Centre of the platform and received negative reinforcement, in the form of bright light, motivating them to escape to a dark box hidden underneath one of the holes (escape box). Each mouse was directed to the escape box and allowed to stay for 20 seconds, any mouse that was led by the experimenter to the escape box and allowed to stay for 20 seconds also. Latency to locate the escape box (primary latency), time taking to enter the escape box (total latency), number of head dips into incorrect holes before locating the escape hole (primary error), and number of head dips into incorrect holes for the whole experiment (total error) was taken. The same procedure was repeated after 24 hr. A probe phase was performed following a 48 hr delay, in which the mouse was assessed for remembering what had been previously learned. In probe trial each mouse was given 90 seconds. The mice are given 1-2 trials per day and testing was repeated over the course of 5 days and the location of the escape hole remains the same across trials. Latency to locate hole where platform was placed beneath the wooden holes, number of head dips into incorrect holes and correct hole were recorded and mice were given 1 to 2 trials daily, testing was repeated over the course of 5 days. After each trial the maze was wipe with a cotton wool dipped in 70% ethyl alcohol and allowed to dry to remove olfactory cues.

Novel Object Recognition Task (NORT)

NORT was done to assess non-spatial long-term memory according to the method described by Leger *et al.* (2013). A black colored open field box (36×50×36 cm^3) was used in this test. It consists of three phases: habituation, training and test sessions. In the habituation phase, each mouse was habituated to the empty open field by placing it in the open field arena and allowed to explore for five minutes twice in a day. After three days of habituation phase, training phase was performed by placing 2 similar objects (two small rubber boxes name A1 and A2) at the left and right position in the open field. Mouse were placed in the open field with their head positioned opposite to the

objects and allowed to explore freely for 10 min. After 24h, test session was conducted by replacing second rubber box with a novel object (a plastic box) with different colour and shape. Each mouse was allowed to explore for 3 min in the open field. The principle is that mice prefer to explore objects that they have not previously encountered over objects that are familiar. Preferences to explore the various objects are noted, and a tendency to explore the novel object over the familiar sample was interpreted as evidence of memory for the training exposure. Two days before the test, mice were allowed to explore the box twice for 5 minutes, to acclimatized. During the testing phase, each mouse was placed in the box for 2 minutes and left to explore objects freely. Exploration was defined as the mice sniffing, gnawing, or touching the object with the nose, whereas sitting and turning around the object were not considered as exploratory behaviors. To avoid the presence of olfactory cues, objects were cleaned after each trial. The following parameters were evaluated: (a) time spent by the mice in exploring the objects during either A1 or A2, (b) time spend on the novel.

$$\text{Percentage preference} = \frac{\text{time spent on the novel object}}{\text{time spent on the novel+time spent on familiar object}} \text{ X } 100\%$$

Hippocampus Dissection

Immediately after behavioural testing, the animals were sacrificed by light anaesthesia with a single intraperitoneal dose (75mg/2.5mg/kg) of diazepam/ketamine. The head was decapitated and the brain was extracted and placed over filter paper soaked with normal saline on a petri dish. The brain was divided into two halves and the right and left hippocampus was harvested. The hippocampus in the left side was used for histology, which were put into a plane bottle containing Bouin's fluid and the right half was used for acetylcholinesterase assay, which were put in an EDTA bottle containing 0.1M phosphate buffer at pH 7.5.

Assessment of Acetylcholinesterase Enzyme

Brain acetylcholinesterase activity was assessed using mice acetylcholinesterase enzyme kits (CK-bio-14126 kit) with a method described by (Zatta *et al*., 2002). The brain was extracted from the skull and placed over filter paper soaked with normal saline on a petri dish. The right hippocampus was harvested and homogenized in 10 ml of a medium containing solution of 0.1M phosphate buffer at pH 7.5. The homogenate was centrifuged using bench centrifuge at 704 x *g* for 10 min at 4°C and the resultant cloudy supernatant was decanted. The supernatant was used for the determination of acetylcholinesterase activity. 5,5-dithiobis-2-nitrobenzoic acid was used to quantify the thiocholine produced from the hydrolysis of acetylthiocholine by AchE in brain tissue. The absorption intensity of DTNB adducts was used to measure the amount of thiocholine formed, which was proportional to the AchE activity. 0.6 ml of assay buffer was added into the vial of acetylthiocholine to form acetylthiocholine stock solution (retention mixture. 100µl of distilled water with 0.1% BSA (bovine serum albumin) into the acetylcholinesterase standard solutions. Serial dilutions were conducted to form 1000, 300, 100, 30, 10, 3, 1 and 0 mU/ml of the acetylcholinesterase standard. These dilution solutions and acetylcholinesterase-containing test samples were put into white bottom 96-well microplate. 50µl of acetylthiocholine reaction mixture was added to each well of the acetylcholinesterase standard, control and test samples to make the total acetylcholinesterase assay volume of 100µl/well. The reaction was protected from light and was incubated for 30 min in room temperature. The samples were then put in micro plate reader at wavelength of 450 nm and the absorbance decrease was recorded. Graph of absorbance was plotted against acetylcholinesterase enzyme concentration in mµ/ml using the standard curve described in the protocol. Values of acetylcholinesterase enzyme concentration above 1000 mµ/ml were considered as zero.

Histology of hippocampus

Tissue preparation was performed using routine tissue processing techniques outlined by Bancroft (2018). The hippocampus of the animals was fixed in Bouin fluid for 48 hours, then passed through ascending concentrations of ethanol (70%-90%), cleared in xylene, embedded in paraffin wax and sectioned at 5µm. Sections of the hippocampus were stained with hematoxylin and eosin stain. Photomicrographs were taken using amscope digital camera for microscope (DCM500), 5M pixels, made in Japan at the Histology unit of the Department of Human Anatomy, Ahmadu Bello University, Zaria.

Statistical Analysis

Data obtained were expressed as mean ± standard error of mean (SEM) and were analyzed using one-way analysis of variance (ANOVA). *Tukey's post-hoc* test was used to compare the level of significance between the test groups and the control. SPSS version 23 was

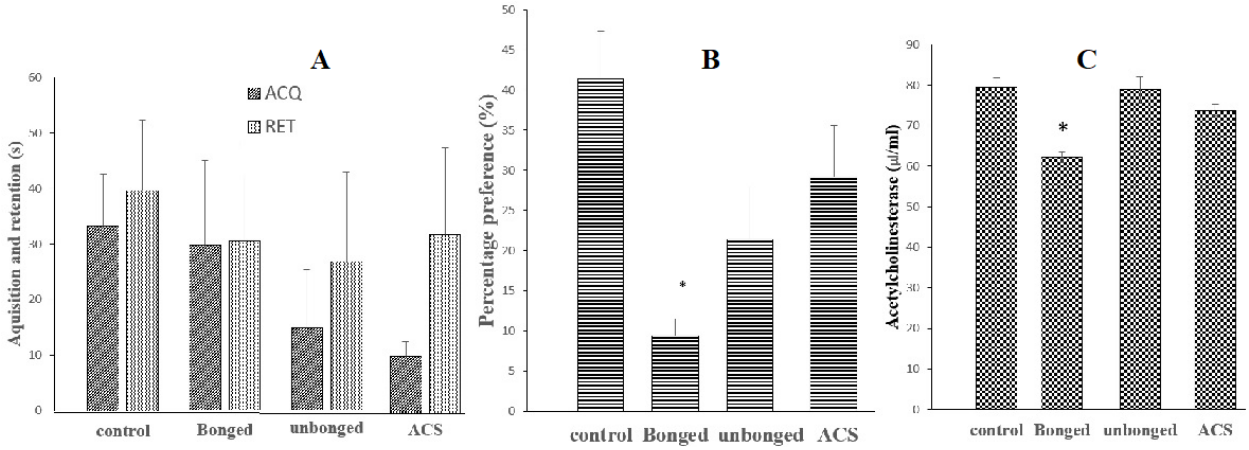

Fig. 1. (A): Effect of *Shisha* smoke inhalation on anxiety related spatial memory of mice in elevated plus maze during training (acquisition) and the retention. ACQ: Acquisition, RET: Retention, ACS: Activated charcoal smoke. **Fig. 2. (B):** Effect of *Shisha* smoke inhalation on non-spatial long-term memory of mice in percentage preference (NORT). ($p < 0.05$), (n = 5) * indicate statistical difference when compared to the control. ACS: Activated charcoal smoke. **Fig. 3. (C):** Effect of *Shisha* smoke inhalation on acetylcholinesterase activity of mice on long-term memory ($p < 0.05$), * indicate statistical difference when compared to all the control. ACS: Activated charcoal smoke

Table 1: Effect of *Shisha* smoke inhalation on Visuo-spatial long-term memory using Barnes maze

	T L Day1(s)	T E Day 1 (s)	T L Day 2 (s)	T E Day 2 (s)
Control	60.40 ± 11.62	29.00 ± 4.39	52.40 ± 9.06	23.40 ± 3.43
Bonged Shisha	42.80 ± 11.35	19.40 ± 5.68	21.60 ± 4.06*	49.00 ± 8.30
Unbonge Shisha	36.20 ± 6.39	27.00 ± 5.65	31.80 ± 5.26	31.40 ± 7.44
ACS	29.00 ± 3.96	27.00 ± 5.65	52.20 ± 7.73	39.00 ± 6.67

used for the analysis. Values of $P < 0.05$ were considered statistically significant.

Ethics

Ethical approval on guidelines for care and use of laboratory animals in scientific research, was obtained from Ahmadu Bello University Committee on Animal Use and Care with approval No: ABUCAUC/2021/048.

RESULTS

Neurobehavioral Assessments

Effect on anxiety related spatial memory using elevated plus maze

The result shows no statistically significant difference (0.94) [F (3, 16)] in the transfer latency (acquisition and retention) in the bonged, unbonged Shisha group and activated charcoal smoke when compared to the control (Figure 1).

Effect of Shisha smoke inhalation on visuo-spatial long-term memory using Barnes maze

The result presented in table 1 and 2 shows statistically significant different between bonged Shisha group (21.60 ± 4.06, p = 0.02) when compared to control (52.40 ± 9.06), [F (3, 16)] in latency to locate the escape hole in day two and number of head dips into incorrect hole during the probe phase. However, there was no statistically significant difference (0.15) in unbonged *Shisha* group and activated charcoal when compared to the control.

Table 2: Effect of *Shisha* smoke inhalation on Visuo-spatial long-term memory using Barnes maze during a one-day probe trial

	L LE (s)	NHC	NHI
Control	40.80 ± 11.43	1.20 ± 0.37	5.20 ± 0.86
Bonged *Shisha*	12.00 ± 12.00	0.20 ± 0.20	7.80 ± 0.86*
Unbonged *Shisha*	33.80 ± 15.20	1.20 ± 0.58	4.40 ± 0.51
ACS	18.00 ± 12.00	0.40 ± 0.24	4.40 ± 0.75

Value with superscript * indicates statistical significantly different when compared to unbonged and ACS. ($p < 0.05$). LLE: Latency to locate the escape holes, NHC: Number of head dip into correct holes, NHI: Number of head deep into incorrect holes

Effect of Shisha smoke inhalation on non-spatial long-term memory of mice in percentage preference using Novel Object Recognition Test (NORT)

The result shows significant decrease seen in percentage preference in bonged Shisha group (9.4±2.16, p = 0.01), [F (3, 16) when compared to control; however, there was no statistically significant difference

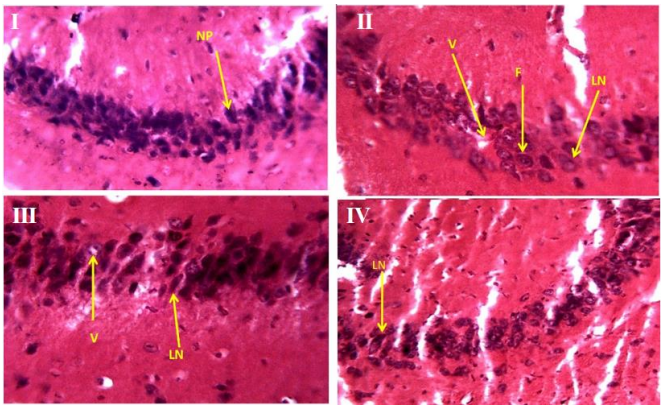

Plate I: Photomicrograph of group 1 (control group) showing a normal hippocampus structure; Conu ammonis three (CA3) under H&E stain at x400. Key: NP= Normal Pyramidal cells

Plate II: Photomicrograph of group 2 (Unbonged *Shisha*) showing Cornu ammonis (CA3) with loss of nucleus of large pyramidal cells and fragmentation of the nucleus and vacuolations of the cells. H&E stain, x400. Key: CA= Cornu ammonis, F=fragmentation, LN=loss of nucleus, V= vacuolation

Plate III: Photomicrograph of group 3 (Unbonged *Shisha*) showing Cornu ammonis (CA3) with moderate loss of nucleus of large pyramidal cells and fragmentation of the nucleus and vacuolation of the cells. H and E stain, x400. Key: CA= Cornu ammonis, LN=loss of nucleus, V=vacuolation

Plate IV: Photomicrograph of group 4 (ACS) showing conu ammonis three (CA3) with fragmentation of neuclei of the pyramidal cells under H&E stain at, x400. Key: LN= Loss of Nucleus

($p > 0.10$) in unbonged *Shisha* group and activated charcoal smoke when compared to the control (Figure 2)

Effect of Shisha smoke inhalation on Acetylcholinesterase activity on long-term memory

The result shows statistically significant decrease in AchE activity in bonged *Shisha* group (62.21 ± 1.22, $p = 0.01$), [$F (3, 16)$] when compared to control. However, there was no statistically significant different (0.07) in Unbonged *Shisha* group and activated charcoal when compared to the control (Figure 3).

DISCUSION

The present study investigated the effect of Shisha smoke inhalation on long-term memory in adult male mice by evaluating AchE, anxiety related special memory, visuo-spatial long-term memory, and histology of hippocampus.

During acquisition and retention in elevated plus maze for memory, no significant difference was seen among the groups. This is in agreement with the finding of Itoh *et al*. (1990) who reported that, mice in the plus maze escape from the open arm to the enclosed arm because of fear and anxiety as a result of their fear of open and elevated places.

In the Barnes maze (BM) paradigm, there was a significant decrease in number of head dips into incorrect holes in the bonged Shisha group when compared to the control during the probe trial. Latency to locate the escape box decrease in bonged and unbonged Shisha group when compared to the control, this may be due to decrease in oxygen content in the brain, which lead to hypoxia, as a result of CO produce by Shisha. Hypoxia causes degeneration of neuronal cells of the hippocampus which causes impairment of memory. This is in line with the findings of Meo *et al*. (2017), who reported that hypoxia, prolonged the reaction time and increase in error rate as a negative impact of cognitive function due to the reduced oxygen content in the brain. Exposure to formaldehyde and acetaldehyde may cause significant damage to the heart and central nervous system (CNS). Forden and Carrillo (2016) demonstrated that ninety percent of the CO and seventy-five to ninety-five percent of the polycyclic aromatic hydrocarbon, emitted from hookah comes from the charcoal. The group exposed to bonged Shisha may have been affected by the added oxidants (formaldehyde, acetyldehyde and acrolein) used in flavouring the Shisha, because it also contributes to the mainstream smoke. This agreed with the findings of Harrison *et al*. (2009) who posted that barnes maze is less stressful when compared with water maze after measuring corticosterone level. Sharma *et al*. (2015) also showed that rodents tend to commit more error as training increases due to the less aversive nature of the barnes maze. Therefore, Shisha causes impairment of long-term memory.

In NORT, there was significant decrease seen in percentage preference in bonged Shisha group when compared to the control. This result indicates impairment of spatial memory shown by percentage preference. The reduction in discrimination ratio was probably caused by the effect of Shisha which caused a decrease in the oxygen content in the brain and increased the level of nicotine and CO concentration, causing neuronal damage leading to reduced cognitive function. Mice exposed to Shisha potentiated serotonin depletions and exhibited reductions in novel object recognition performance. This is in agreement with work of Meeter *et al*. (2006) whose studies has been shown that disruption of the normal 5-HT levels when exposed to Shisha results in cognitive deficits. This result contributes to the growing literature showing learning or memory deficits in animals exposed under varying conditions of Shisha exposure.

Photomicrograph of group 1 (control) showed a normal hippocampus structure; conu ammonis three (CA3) under H&E stain at x400. Photomicrograph of group 2 (bonged Shisha) showed Cornu ammonis (CA3) with loss of nucleus of large pyramidal cells and fragmentation of the nucleus and vacuolations of the cells, Plate V: Photomicrograph of group 3 (Unbonged Shisha) showed Cornu ammonis (CA3) with few loss of nucleus of large pyramidal cells and fragmentation of the nucleus and vacuolation of the cells and group show only fragmentation of pyramidal cells, this probably due to decrease in the oxygen supply to the brain by the effect of hypoxia which causes necrosis of hippocampus cells, this finding is in agreement with the work of Shaimaa *et al*. (2013) who found hippocampus cells necrosis when working on histological and functional study on hippocampus formation in normal and abnormal diabetes rats.

Acetylcholinesterase activity, there was statistically significant decreased in the bonged *Shisha* group when compared to control. This showed that chronic exposure to bonged *Shisha* affect acetylcholinesterase activity in the brain of the mice, this is in agrees with the work of Hadidi *et al*., (2015) who demonstrated that chronic administration of nicotine (a major constituent found in *Shisha*) in the hippocampus decreased the AchE activity, this consequently decreases accumulation of acetylcholine in the brain. This may be explained by the conversion nicotine to its active, cotinine as it passes through the water. Aki *et al.* (2010) who posted that *Shisha* smoke is an efficient means of delivery harmful toxicant to the body when they are been converted to their active form. *Shisha* also results in changes in blood counts and decreases the cerebral flow rate in the anterior, middle and posterior cerebral arteries (Meo *et al*., 2017). The probable mechanism in line with *Shisha* smoking and cognitive impairment is the presence of significant number of possibly cytotoxic compounds in *Shisha* smoke including carbon monoxide, ketones, nitrosamines, aldehydes, dihydroxyl benzene. These compounds may impair the neuronal and cellular membrane function of the hipocampus. Besides these toxic compounds also promote oxidative damage to neuronal or glial cell organelles (Al-Sawalha *et al*., 2018). It has also been reported that, *Shisha* produces high amount of carbon monoxide (CO). Carbon monoxide is a cellular poison which binds to haemoglobin two hundred to three hundred times more tightly than oxygen and it inhibits the release of oxygen from haemoglobin to tissues, causing hypoxia (Meo *et al*., 2017).

Hypoxia causes difficulty in performance decrement in concentration and faulty judgment. Study has shown that hypoxia prolongs the reaction time and increasing in error rate has a negative impact on cognitive function. Carbon monoxide exposure is also associated with memory impairment and elevated oxidative stress, oxidative stress has been associated with neuronal damage and subsequent impaired spatial learning and memory (Meo *et al*., 2017).

Neergard *et al*. (2007) reported that long-term *Shisha* smokers absorb more toxicant (nicotine) than short-term *Shisha* smokers. The foetus is more at risk than the adult to the effects of certain carcinogens, such as polycyclic aromatic hydrocarbons. 75 – 95% of the polycyclic aromatic hydrocarbon emitted from *Shisha* comes from the charcoal (Aslam *et al*., 2015). A study using hookah mixture in a waterpipe found that a single smoking session delivered approximately fifty times the quantities of carcinogenic 4- and 5-membered ring polycyclic aromatic hydrocarbon as a single 1R4Fcigarette smoke (WHO, 2006)

CONCLUSION

In conclusion, the outcomes of this study suggest that bonged *Shisha* smoking is neurotoxic to the brain as a result of combined effect of various toxicants emanating from different *Shisha* smoke constituents used in the set-up. During the time of this investigation, bonged *Shisha* showed memory impairment and vaculation, loss of nucleus and fragmentation (necrosis) of cells of the hippocampus, were as, unbonged and activated charcoal smoke did not show potential of memory impairment but cause slight necrosis of the cells of hippocampus.

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