Research Paper
Atrazine-Induced Hippocampal Degeneration and Behavioral Deficits in Wistar Rats: Mitigative Role of Avocado Oil

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ABSTRACT

Background: Glutamate is essential to learning and memory as an excitatory neurotransmitter. This study evaluated the atrazine effect on the hippocampus and examined the mitigative role of avocado oil against the neuronal degeneration and behavioral deficits in Wistar rats.

Methods: Fifty adult male Wistar rats were divided into four groups of ten. Group 1 (controls) received 0.5 ml distilled water; group 2 received atrazine (215 mg/kg/d); group 3 received avocado oil (1 ml/ 250 g/d); group 4 received avocado oil (1 ml/ 250 g/d) 60 minutes before atrazine. Treatments were given by oral gavage over 28 days. Barnes maze and Y-maze tests were performed to assess the learning and memory. Histological and Glial Fibrillary Acidic Protein (GFAP)-immuno-reaction in the hippocampus were assessed, using Hematoxylin and Eosin (H&E) stain and anti-GFAP antibody. The glutamate and acetylcholinesterase levels were subsequently assessed.

Results: The learning and memory performance was significantly affected in group 2, but improved in group 4. In group 3, learning and memory performance was not different from group 1. In group 2, atrazine caused massive neurodegeneration and astrogliosis at Cornu Ammonis-1 (CA-1) and Dentate Gyrus (DG). Combined avocado and atrazine significantly reduced neuronal death and astrogliosis in CA-1 and DG areas. In group 2, glutamate level was high while acetylcholinesterase was low. In group 4, glutamate was low but acetylcholinesterase was high compared to those in group 2. Glutamate and acetylcholinesterase levels in group 3 was not significantly different from that of group 1.

Conclusion: Atrazine inhibited acetylcholinesterase and induced glutamate release. These were associated with excitotoxicity and neuronal degeneration in CA-1 and DG areas as shown by poor learning and memory. Treatment with avocado oil protected against high glutamate release, thus, mitigating neuronal degeneration and maintaining normal learning and memory in rats.

Keywords: Atrazine, Acetylcholinesterase, Glutamate, Hippocampus, Neurodegeneration

Introduction

Hippocampal degeneration is associated with neurodegenerative diseases, including cognitive impairment [1]. The degeneration is induced through various mechanisms, such as oxidative and metabolic stress, and impairment of long-term potentiation [2]. Degeneration of Cornu Ammonis-1 (CA-1) of the hippocampus impairs learning under certain conditions while degeneration of the Dentate Gyrus (DG) leads to associative memory defects [3, 4]. Hippocampus is vulnerable to

injury via exposure to certain environmental toxins, including insecticides, pesticides and herbicides. Atrazine is a well-known herbicide normally used on crops to prevent broadleaf weeds. It belongs to a family of chemicals known as triazines. The most common route of atrazine exposure is through ingestion of contaminated drinking water, while inhalation and dermal absorption are other routes of exposure [5]. Atrazine, like other triazines is well-known for endocrine-disrupting effects, and adverse developmental and reproductive outcomes [6]. This compound affects the hypothalamic-pituitary-adrenal axis, neuroendocrine hormones and major neurotransmitters [6-8]. Perturbation of dopaminergic neurons with atrazine results in anxiety-like behaviors. Further, developmental exposure to atrazine promotes functional and morphological lesions in the hippocampus and affects the hippocampus-dependent learning and memory in rodents [8].

Avocado oil is extracted from avocado fruit (*Persea americana Mill*.), containing high amounts of monounsaturated fatty acids and low amounts of polyunsaturated and saturated fatty acids. Also, it has large contents of oleic and palmitic acids [9, 10]. The antioxidant compounds present in avocado oil include: β-Sitosterol, α-Tocopherol, γ-Tocopherol, Campesterol, Estigmasterol, Sitoestanol and Campestanol [10]. Avocado oil is antihypertensive, and reduces glucose tolerance and insulin resistance. It also exerts a direct regulatory effect on lipid profile, among others. In the brain, avocado oil prevents mitochondrial dysfunction, decreases free radical levels and lipid peroxidation, and improves the oxidized glutathione ratio [11]. Supplementation of rat diets with avocado pulps results in high Docosahexaenoic Acid (DHA) in the hippocampus [12], which is critical for maintaining normal brain structure and function, and is considered neuroprotective [13]. Maternal supplementation with avocado oil improves memory in rats, particularly at adolescence and adult phases of development [12].

Aim of the study: The present study evaluated the effect of atrazine on the hippocampal neurons and examined the mitigative role of avocado oil against atrazine-induced neuronal degeneration in the hippocampus, and behavioral changes in Wistar rats.

Materials and Methods

Animals: Forty male adult Wistar rats (weighing 250-260 g) were obtained from Tayo Animal Husbandry, Ogbomoso, Nigeria, and kept in the animal house of the College of Health Sciences, University of Ilorin, Ilorin State, Nigeria, on approximately 12hr of light and dark

cycles at room temperature. The rats were acclimatized for ten days before beginning the experiments, and had free access to food and water throughout the study period. Further, the rats were handled according to the guidelines for the care and use of laboratory animals as specified in the National Research Council publication [14]. The study protocol and treatment procedures were approved by the Ethics Committee of University of Ilorin, Nigeria (Registration #: UERC/ASN/2018).

Treatment: Atrazine powder, as the study treatment compound, was purchased under a trade name Atraforce (Atrazine 80% WP; Ilorin, Nigeria) and dissolved in distilled water. Avocado oil (Hemani Avocado oil, Pakistan) was purchased via the Jumiaapp store, Nigeria.

Experimental design: The rats were randomly distributed into four groups of ten each after ten days of acclimatization with the treatments administered by oral gavage for 28 consecutive days as follows:

▪ Group 1 (control) received distilled water (0.5 ml).

▪ Group 2 received atrazine (ATR; 215 mg/kg/d).

▪ Group 3 received avocado oil only (AVO; 1 ml/ 250 g/d).

▪ Group 4 received combined avocado oil and atrazine (AVO+ATR; avocado oil at 1 ml/ 250 g/d) 60 minutes before administration of atrazine (215 mg/kg/d).

The dosage of atrazine used in this study was based on a pilot study carried out by the authors while that of avocado oil was based on a previous study conducted by Ortiz-Avila et al. [9].

Behavioral tests: The behavioral tests were conducted between 8:00 a.m. and 12:00 noon. These tests commenced 24 hours after the completion of each of the study treatments.

Barnes maze test: This test was performed, using the modified technique as described earlier by Rosenfeld and Ferguson [15]. Briefly, a 20-hole circular table was placed in a lighted room. Nineteen of the holes were directly open to the ground, while the last hole led to a dark box attached. Initially, the rats were led from the center of the table to the attached dark box. Subsequently, the rats were allowed to locate the dark box on their own, starting from the center of the table. All of the rats completed a total of four trials before the final test. The activities of the rats were recorded, using a video camera and the images were subsequently analyzed. The parameters

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analyzed in this test included the latency to locate the correct hole and the number of errors made, i.e., number of incorrect holes visited by the rats.

Y-maze test: This test was performed according to a modified technique as described earlier by Kraeuter et al. [16]. Briefly, the rats were positioned in the Y-maze (consisting of three opaque-colored arms of equal length, interconnected at 120 degrees). The rats were allowed to explore the maze for seven minutes and the manner of alternations made by the rats was recorded. An alternation was defined as the consecutive entries into all three arms without going into the same arm twice in a row. The percent alternation for each rat was estimated as a ratio of the correct alternation over the total alternations, multiplied by 100.

Tissue collection: After the completion of the above behavioral tests, the rats assigned for histopathology examination (n=3) were anaesthetized by intraperitoneal injection of ketamine (50mg/ml, Pakson Pharma, PVT Ltd., India) before performing perfusion fixation with 4% Paraformaldehyde (PFA), according to the method described by Gage et al. [17]. The rats were subsequently decapitated, and the hippocampi excised in both hemispheres and post-fixed in 4% PFA for 18 hours.

For the neurochemical assays, rats (n=7) were euthanized and sacrificed by cervical dislocation, and subsequently decapitated. This mode of euthanasia was utilized to avoid influence of anesthetic agents on the specific neurochemicals to be tested. Hippocampi in both hemispheres were excised and homogenized in glutamate assay buffer (100 μL, Sigma Aldrich Co., UK). For glutamate assay, the test was done, using 0.1 M phosphate buffer at pH 8.0. For acetylcholinesterase assay, approximately 20 mg of the tissue per ml was used after the tissue samples were homogenized in a Teflon Potter–Elvehjem homogenizer. The homogenates were centrifuged at 12,000 g for 10 minutes. The supernatants were then decanted into test tubes and used for measuring the glutamate and acetylcholinesterase levels.

Glutamate assay: To determine the glutamate concentration in the hippocampal samples, we used glutamate assay kits. The test was carried out according to the instructions provided by the assay kit manufacturer (Sigma Aldrich Co., UK). The absorbance was read at 450nm while the linear range of detection for this assay was between 2–10 nmol.

Acetylcholinesterase assay: The acetylcholinesterase activity was tested, using the colorimetric technique as described by Ellman et al. [18] and the absorbance was read at 412 nm, and changes in the absorbance were calculated per min.

Histopathological study: After fixing the tissue samples in 4% PFA for 18 hours, they were processed and mounted in paraffin embedded blocks. The tissue blocks were sectioned and stained with Hematoxylin and Eosin (H&E) for routine histo-architectural slides of the hippocampus, using the methods described by Jensen [19]. The stained tissue sections were examined under light microscopy (Olympus Model: XSZ-107BN, New Jersey, USA; and Amscope, MD500, CA, USA).

Immuno-histochemistry assay: After deparaffinization and antigen retrieval processes, sections were incubated for five minutes with two changes of Phosphate Bbuffered Saline (PBS) at pH 7.4, and hydrogen peroxide in methanol to block the endogenous peroxidase activity. Thereafter, sections were incubated in normal goat serum for 20 minutes at room temperature to suppress the background staining. Further, sections were incubated with polyclonal rabbit anti-Glial Fibrillary Acidic Protein (GFAP) serum (Sigma, Aldrich, UK; 1:500 in PBS) at 4°C for 24 hours. Sections were incubated with biotinylated mouse anti-rabbit solution for 30 minutes, and then with the Avidin–Biotin complex for 45 minutes. Visualization of the immunocomplex was achieved, using 3,3'-Diaminobenzidine (DAB). Sections were counterstained with Hematoxylin and examined under light microscopy at a scale bar of 25-180μm.

Morphometric analyses: The morphometric analyses were performed using H&E-stained and GFAP-immuno-stained sections on ImageJ software, version 1.52r. From each tissue block, five sections at six different visual fields were examined, at varying magnifications of 25-180μm. For H&E-stained sections, the number of Pyramidal Cells (PCs) were counted in the Cornu Ammonis (CA-1) for each of the groups. Additionally, the number of immuno-reactive GFAP astrocytes were counted in the CA-1 and DG areas. The qualifying criteria for GFAP-immuno-positive cells counting included clearly observed cell body with extended processes, per Kamphuis et al. method [20].

Statistical analyses: The data analyses were performed on Graph Pad Prism software, version 7. Statistical comparisons were made, using one way Analysis of Variance (ANOVA) with Tukey´s multiple comparison test. All results were expressed as the Mean±SD with the level of statistical significance set at P<0.05.

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Results

Changes in learning and memory status: As shown in Table 1, the outcome of Barnes maze test indicated a significant increase in the latency to locate the dark box and substantial errors were made by the ATR treated group compared to those in the control group. The combined AVO+ATR treated group displayed shorter latency to locate the dark box and committed fewer errors versus that of the ATR treated group. Further, the AVO+ATR treated group displayed longer latency to locate the dark box and committed fewer errors versus the control and AVO groups (Table 1).

Based on the Y-maze test (Table 2), there were fewer alternations in the ATR treated group than in the control and AVO+ATR treated groups. Similarly, the AVO+ATR treated group exhibited fewer alternations than the control one. The alternations in the AVO treated group was not significantly different from those noted in the control group.

Variations in glutamate and acetylcholinesterase levels: As reflected in Table 3, the glutamate level was significantly increased in ATR treated group versus the control group. Conversely, the glutamate level in the AVO+ATR treated group showed a significant decrease compared to that of the ATR treated group. No statistically significant differences were detected when the glutamate level in the AVO treated group was compared with that of the control group. Neither was there a significant difference between the AVO+ATR treated and the AVO-treated groups. The acetylcholinesterase level in the ATR treated group was significantly lower than that of the control group (Table 3). Similarly, the AVO+ATR treated group showed a significant decrease in the acetylcholinesterase level compared to that of the control group. Conversely, the acetylcholinesterase level in the AVO+ATR treated group showed a significant increase compared to that of the ATR-treated group. There was no significant difference in the acetylcholinesterase level between the AVO treated and the control groups (Table 3).

Histopathological observations: In Figure 1, the H&E-stained sections of CA-1 from the control group showed closely packed pyramidal cells with normal neuronal processes and basophilic cytoplasm. In Atrazine treated group, numerous pyramidal cells were observed to have undergone degeneration with the stratum pyramidal cells
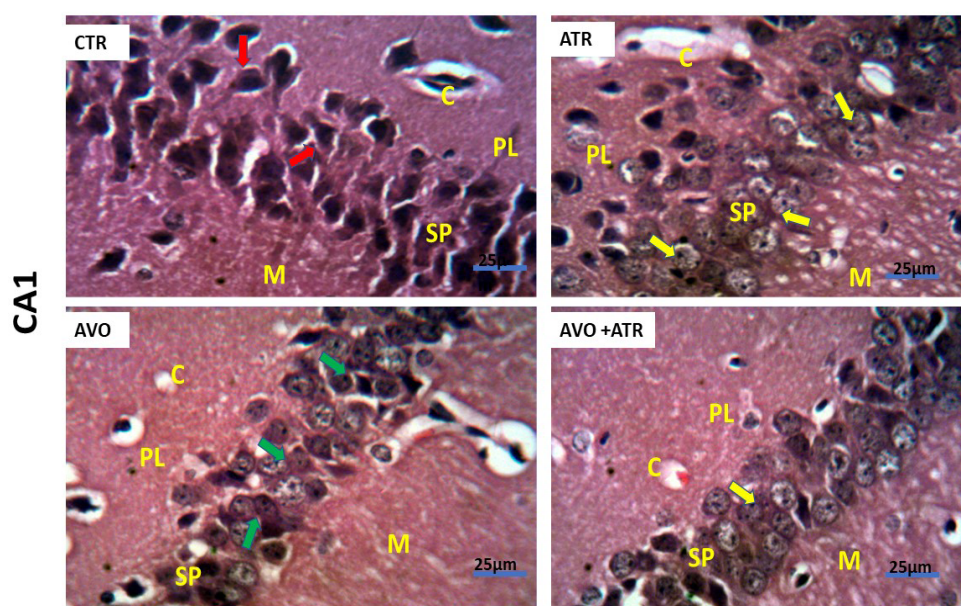

Figure 1. Photomicrographs of CA-1 sections from rat hippocampus (H&E stain).

The control group (CTR) showing Stratum Pyramidale (SP) and normal neuronal processes of Pyramidal Cells (PCs) (red arrows) and capillaries. ATR group showing numerous degenerated and shrunken PCs with dark-blue stained nuclei (yellow arrows) and dilated capillaries (C). AVO group showing organized SP with few shrunken PCs (green arrows) between normal PCs. AVO+ATR group showing organized SP, few shrunken cells with darkly stained nuclei and minimally-dilated capillaries (C). Scale bar: 25 μm.

CA-1: Cornu Ammonis 1; M: Molecular layer; PL: Polymorphic Layer H & E: Hematoxylin & Eosin; CTR: Control; ATR: Atrazine; AVO: Avocado oil; AVO+ATR: Avocado oil + Atrazine

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Table 1. Escape latency and number of errors in Barnes maze test (retention day)

Parameters	Mean±SD			
	Control	ATR	AVO	AVO+ATR
Latency to locate correct hole (s)	62.3±11.0	177.3±22.0[a]	74.1±8.0	96.8±13.0[a,b,c]
Number of incorrect holes visited	6.7±0.1	14.6±1.3[a]	8.5±0.7	10.8±1.0[a,b]

ATR: Atrazine; AVO: Avocado oil; AVO + ATR; Avocado oil + Atrazine; [a]P<0.05 vs control; [b]P<0.05 vs ATR; [c]P<0.05 vs AVO; n=10 rats, each group

Table 2. Alternation of rats in Y-maze test

Parameter	Mean±SD			
	CTR	ATR	AVO (1 ml/250 g)	AVO+ATR
Alternation (%)	71.8±13.0	47.9±9.0[a]	76.1±11.0	62.8±8.0[a,b,c]

ATR: Atrazine; AVO: Avocado oil; AVO + ATR; Avocado oil + Atrazine; [a]P<0.05 vs control; [b]P<0.05 vs ATR; [c]P<0.05 vs AVO; n=10 rats, each group

disorganized. Also, many PCs were dead and shrunk with darkly stained nuclei. Additionally, the blood capillaries appeared dilated and congested (Figure 1, ATR). The AVO treated group showed organized stratum pyramidal cells with few shrunk cells noted (Figure 1). The group treated with AVO+ATR presented organized stratum pyramidal cells and few evidence of degeneration compared to the group treated with ATR only. Characteristically, there were few shrunk cells in this group with darkly stained nuclei and minimally-dilated capillaries (Figure 1).

In Figures 2 and 3, the Glial Fibrillary Acidic Protein (GFAP) immuno-stained sections of CA-1 and Dentate Gyrus (DG) in the control group, showed few immuno-reactive, star-shaped astrocytes between pyramidal and granule cells. The Atrazine (ATR) treated group revealed a substantial number of immuno-reactive astrocytes in both CA-1 and DG zones compared to those in the control group (Figures 2 and 3). In the AVO treated group, the GFAP immuno-reactivity was minimal in CA-1 and DG areas of the hippocampus (Figures 2 and 3). Further,

the GFAP immuno-reactivity was mild in CA-1 and DG areas in the AVO+ATR treated group compared to those found in the control group (Figures 2 and 3).

Morphometric assessment: The number of pyramidal cells in the ATR group was significantly decreased compared to that of the control (Figure 4). Inversely, the quantity of GFAP immuno-positive cells increased significantly in the ATR group compared to that of the controls (Figure 5). The number of pyramidal cells in the AVO+ATR treated group was significantly increased compared to those treated with ATR only (Figure 4). On the other hand, the number of GFAP immuno-positive cells decreased significantly in the AVO+ATR treated group, compared to those treated with ATR only. The number of PCs and GFAP immuno-positive cells in the AVO treated group was not significantly different from those in the controls (Figures 4 & 5).

Table 3. Changes in hippocampal glutamate and acetylcholinesterase levels in rats

Parameters	Mean±SD			
	Control	ATR	AVO	AVO+ATR
Glutamate (µmol/g)	38.8±5.1	46.7±4.2[a]	40.6±3.8	41.8±6.4[a,b]
AchE (U/min/mg)	0.12±0.005	0.068±0.002[a]	0.10±0.004	0.09±0.002[a,b]

ATR: Atrazine; AVO: Avocado oil; AVO + ATR; Avocado oil + Atrazine; [a]P<0.05 vs control; [b]P<0.05 vs ATR; [c]P<0.05 vs AVO; n=7 rats, each group

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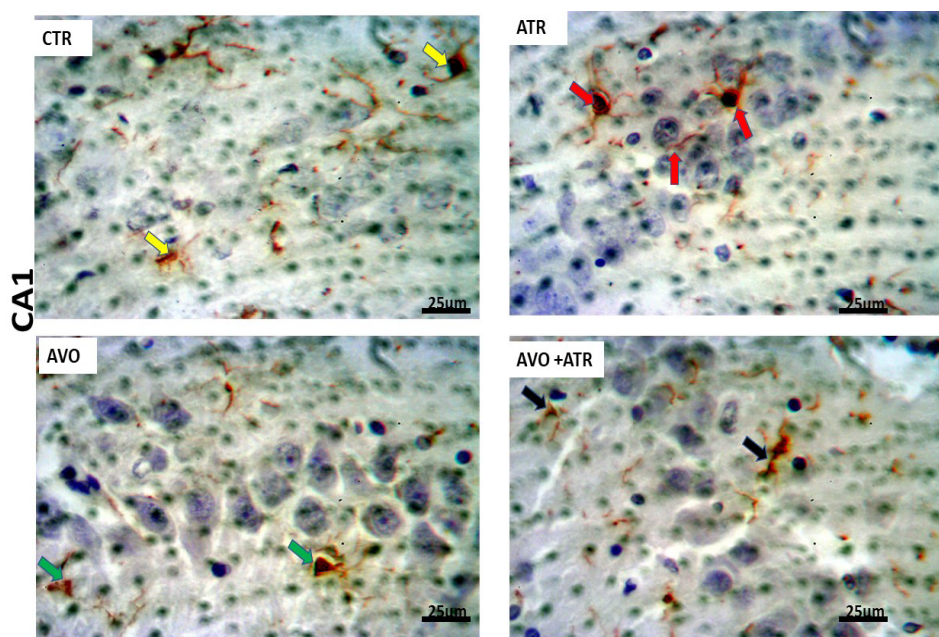
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Figure 2. Photomicrographs of CA-1 from rat hippocampal sections (GFAP-immuno-stained sections).

The control group (CTR) shows slight immunoreactivity (yellow arrows) in the star-shaped astrocytes in close contact with pyramidal neurons. The ATR group presents extensive immunoreaction (red arrows). The AVO group displays minimal immunoreactivity (green arrows). The AVO+ATR group display mild immunoreactivity (black arrows). Scale bar: 25 μm.

CTR: Control; ATR: Atrazine; AVO: Avocado oil; AVO+ATR: Avocado oil + Atrazine; GFAP: Glial Fibrillary Acidic Protein; CA-1: Cornu Ammonis
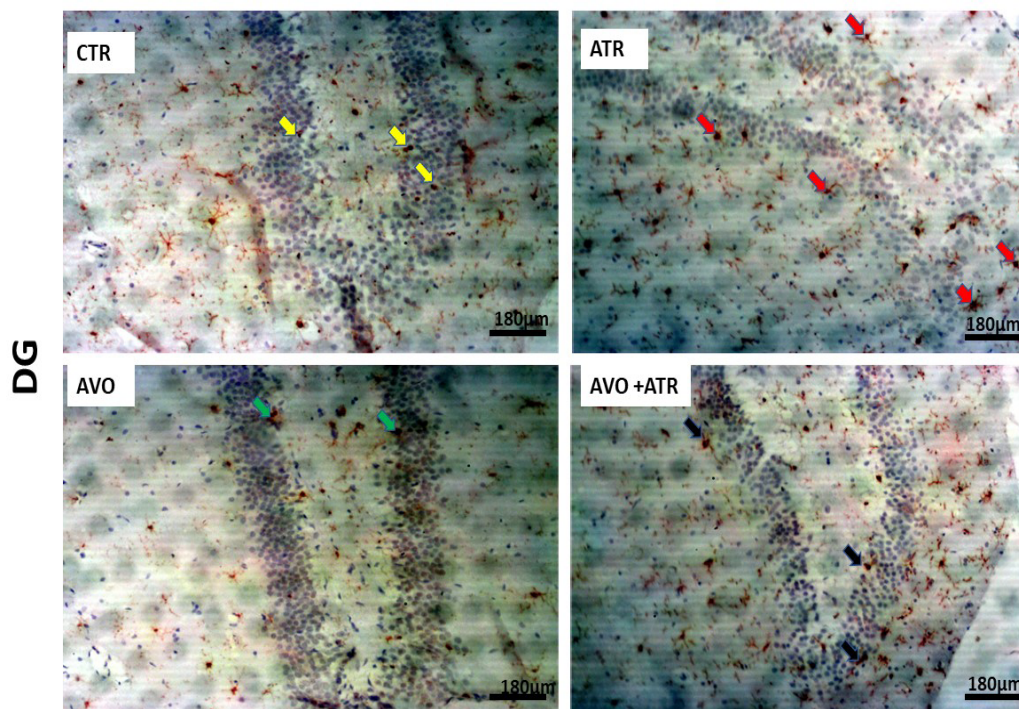

Figure 3. Photomicrographs of Dentate Gyrus (DG) from rat hippocampal sections (GFAP-immuno-stained sections).

The control group shows slight immunoreactivity (yellow arrows). ATR group displays immense immunoreaction (red arrows). AVO group presents minimal immunoreactivity (green arrows). AVO+ATR group shows moderate immunoreactivity (black arrows). Scale bar: 180 μm.

CTR: Control; ATR: Atrazine; AVO: Avocado oil; AVO+ATR: Avocado oil + Atrazine; GFAP: Glial Fibrillary Acidic Protein; DG: Dentate Gyrus
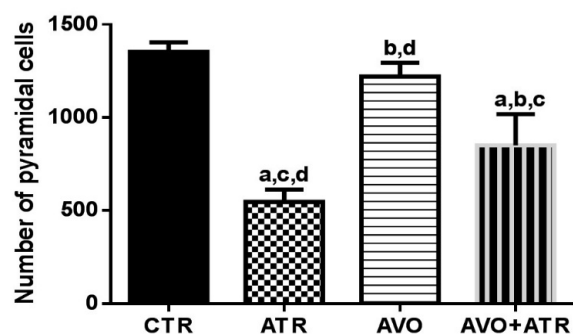
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Figure 4. Number of pyramidal cells in CA-1 of rats' hippocampus. Values are Mean±SD.

CTR: Control; ATR: Atrazine; AVO: Avocado oil; AVO+ATR: Avocado oil + Atrazine; CA-1: Cornu Ammonis. [a]P<0.05 vs control; [b]P<0.05 vs ATR; [c]P<0.05 vs AVO; [d]P<0.05 vs AVO+ATR

Discussion

Glutamate is an excitatory neurotransmitter in the central nervous system, and plays an important role in learning and memory [21]. Under abnormal conditions, glutamate may behave as neurotoxin (excitotoxicity), leading to a variety of neurodegenerative disorders including impaired cognition. Long-term potentiation, i.e., the mechanism that triggers learning and memory development, is influenced by glutamate-gated cation channels like N-Methyl D-Aspartate Receptors (NMDARs) [2]. In this study, ATR treated group showed a significant increase in the glutamate level compared to the control group. However, the combined AVO and ATR treatment significantly decreased the glutamate level. This finding indicates that ATR can induce abnormal glutamate increased level, resulting in excitotoxicity. Further, AVO treatment might have induced reduction of glutamate level by inhibiting the glutamate release in the rats.

Acetylcholinesterase (AChE) is a cholinergic enzyme distributed in nervous system with the task of hydrolyzing Acetylcholine (ACh) into acetic acid and choline [22]. This enzyme is inhibited by many toxins including pesticides [23]. The major role of AChE is to terminate neuronal transmission among synapses to prevent the activation of nearby ACh receptors. Inhibition of this enzyme may lead to a decreased breakdown and subsequent accumulation of acetylcholine, resulting in excitotoxicity and impaired memory [22, 24]. In this study, ATR treated group showed a significant decrease in AChE levels compared to the controls. However, co-treating ATR and AVO significantly increased AChE levels compared to that noted in the ATR treated group. This outcome suggests that ATR can inhibit AChE with the resultant excitotoxicity and neuronal degeneration, as found in the histopathologic observation
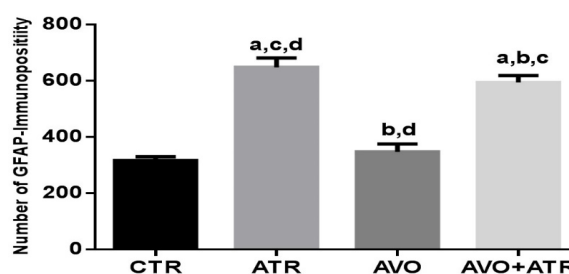

Figure 5. Number of GFAP-immuno-positive cells of the study groups. Values are the Mean±SD.

CTR: Control; ATR: Atrazine; AVO: Avocado oil; AVO+ATR: Avocado oil + Atrazine; GFAP: Glial Fibrillary Acidic Protein. [a]P<0.05 vs control; [b]P<0.05 vs ATR; [c]P<0.05 vs AVO; [d]P<0.05 vs AVO+ATR

of this study [23]. Further, we may conclude that treatment with combined AVO and ATR leads to the up-regulation of AChE activities.

Impaired learning and memory are some of the highlights of neurodegenerative diseases, and it is known that hippocampal neurodegeneration leads to impaired learning and spatial memory [2, 25]. The degeneration disrupts neuronal connectivity, which in turn affects the memory decoding and storage of learned information [26].

Hippocampus is a brain structure that plays an important role in learning and memory. It is vulnerable to a variety of injuries that lead to neurodegeneration and atrophy among other defects [2]. The CA-1 area contains pyramidal cells which play a vital role in identifying information obtained from CA-3 neurons, and contributes to incremental value learning under certain circumstances [2, 3]. The dentate gyrus plays a crucial role in associative memory [4]. From the histopathological observations of this study, ATR treatment alone resulted in the degeneration of numerous pyramidal cells in the CA-1 area. However, the combined AVO+ATR treatment reduced the degeneration and other disruptive effects of ATR. The evidence for the degenerative effect of ATR on CA-1, and the restorative effect of AVO were observed when the number of pyramidal neurons were counted and compared with other groups in this study. These results suggest that exposure to ATR promotes neuronal degeneration and disruptive alterations in CA-1 area of the hippocampus, based on the excitotoxicity induced by excessive stimulation of glutamate receptors associated with high glutamate concentration [21, 25]. Conversely, the AVO+ATR treatment ameliorates the neuronal degeneration and disruptive changes likely

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through the inhibition of excessive glutamate release and its subsequent toxicity.

An increase in GFAP response is the hallmark of reactive gliosis, which is associated with astrocytes in neurodegenerative diseases due to neural injury [27]. The current study revealed an increase in GFAP immuno-reaction in CA-1 and DG areas of the ATR treated rats. However, in the AVO+ATR treated rats, there were few neurons with GFAP immuno-reactivity. This finding indicates that exposure to ATR promotes astrogliosis in response to damages caused in CA-1 and DG areas. Further, the AVO treatment is likely to minimize the resultant astrogliosis by preventing tissue damages when treated concurrently with ATR.

This study demonstrated that ATR treatment alone leads to impaired learning and memory performance as evident by the results obtained from the Barnes maze and Y-maze tests. The impaired learning and memory in ATR-treated rats could be linked to massive neurodegeneration found in the rats' CA-1 and DG areas. The CA-1 area of hippocampus plays a vital role in processing of information received from the CA-3 area and contributes to incremental value learning under certain circumstances. Also, DG area plays a crucial role in associative memory [2-4]. Consistently, the learning and memory performance was better in the AVO+ATR treated rats likely because the neurodegeneration in the CA-1 and DG areas was not as bad as that found in the group treated with ATR only.

Conclusions

This study demonstrated that atrazine induced a significant increase in the cerebral glutamate level leading to excitotoxicity, which caused neuro-degeneration in the CA-1 and DG areas of the hippocampus in rats. Excessive astrogliosis, and impaired learning and memory performance were also observed in the rats treated with atrazine. However, treatment with avocado oil kept the glutamate levels in the hippocampus low, likely by the inhibition of its neuronal release. The resultant effect of low glutamate level in the hippocampus or possibly in other areas of the rats' brain was reduced toxicity and neurodegeneration, while improving their learning and memory performance.

Limitations of the study: The current study has some limitations. First, the study did not consider the effects of age or gender as only adult male Wistar rats were used to evaluate the effects of atrazine in hippocampus. Second, the study did not consider CA-2 and CA-3 of

the hippocampus as only CA-1 and dentate gyrus of the hippocampus were considered. Third, cognitive function was evaluated without tests, measuring anxiety-like behavior, depression-like behavior and other relevant behavioral tests in the Wistar rats.

Recommendations for future studies: Further studies involving animal models of different age and gender with a complete battery of behavioral tests are recommended to accurately establish the effect of atrazine in the hippocampus.

Ethical Considerations

Compliance with ethical guidelines

The experimental procedures and animal care were based on the requirements and approval of the Ethics Committee of University of Ilorin (Registration #: UERC/ASN/2018) and in agreement with the recommendations of the National Research Council Guidelines for the Care and Use of Laboratory Animals (NRC Publication 2011).

Authors' contributions

Designed the experiments, reviewed literature, participated in the experiments and reviewed the final draft of the manuscript: Nathaniel Ohiemi Amedu; Participated in the experiments, analyzed the results, wrote the first draft of the manuscript; Michael Olim Obu; Reviewed and approved the final draft of the manuscript prior to submission to this Journal: Nathaniel Ohiemi Amedu, Michael Olim Obu.

Conflict of interest

Authors declares no conflict of interest.

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