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A COCKTAIL OF CAFFEINE AND ACETYLSALICYLIC ACID ADMINISTRATION IN COMBINATION WITH ETHANOL CAUSESOXIDATIVE STRESS AND INFLAMMATORY RESPONSE IN THE HIPPOCAMPAL CA1 OF ADULT WISTAR RATS

Olufunto Omodele Adeleye*[1] and Olatunde Owoeye[2]

[1]Department of Anatomy, Faculty of Basic Medical Sciences, LAUTECH, Ogbomoso, Oyo State,Nigeria.

[2]Department of Anatomy, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, OyoState, Nigeria.

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Corresponding Author: Olufunto Omodele Adeleye
Department of Anatomy, Faculty of Basic Medical Sciences, LAUTECH, Ogbomoso, Oyo State, Nigeria.

ABSTRACT

Ethanol, a psychoactive drug commonly used for recreational purposes carries a major health riskdue to its abuse, and this has been associated with early onset of degenerative disorders. Alcohol abusers are found to combine it with an analgesic containing a cocktail of caffeine and acetyl salicylic acid (CASA). The CASA is a common over- the- counter drug used for the treatment of body pains, cold, headache and fever, which also reverses the depressant effect of alcohol. Neurotoxicity has been reported with chronic consumption of CASA with alcohol, however its neurotoxic effects on the hippocampus is not fully elucidated. Therefore, this study was designed to investigate the structural and functional alterations of the hippocampus following chronic administration of ethanol and CASA in Wistar rats. Forty adult Wistar rats of both sexes (160.00±6.23g) were divided into four groups (n=10): Controlgroup (distilled water), CASA (31mg/kg), Ethanol (30% v/v) and CASA+ethanol (31mg/kg CASAand 30%v/v of ethanol). All substances were administered daily by oral gavage for 60 days. At theend of administration, the rats were sacrificed and brain samples obtained for biochemical and histological analysis. The hippocampal tissues were analyzed for Malondialdehyde (MDA), Nitric Oxide (NO), Reduced Glutathione (GSH), Superoxide Dismutase (SOD) and catalase by spectrophotometry by spectrophotometry. Pro-inflammatory markers: Tumor Necrosis Factor-α (TNF-α) and Interleukin 1-β (IL-1β) were assessed using ELISA. Glial Fibrillary Acidic Protein (GFAP) and B-cell lymphoma 2 (Bcl-2) activities were also assessed by immunohistochemistry. Data were analyzed using descriptive statistics and ANOVA at α0.05. Rats given ethanol and CASA plus ethanol significantly increased in oxidative level of MDA and NO respectively. There was significant decreased in GSH, SOD and catalase level when comparedwith control Rats given ethanol and CASA plus ethanol showed significantly increased TNF-α while in IL-1β the result showed significantly increased in all the treated groups. Immunohistochemical results showed significantly increased GFAP-positive astrocytes in all treated groups, and Bcl-2 immunopositively cells were significantly increased in the treated groups when compared to control. Chronic and combined use of ethanol with a cocktail of caffeine and acetyl salicylic acid induced increased oxidative stress, up-regulation of pro-inflammatory cytokines, astrogliosis and apoptosisin Wistar rats.

KEYWORDS: CASA, Ethanol, Hippocampus, Pyramidal neurons, Wistar rat.

1. INTRODUCTION

Drug users' abuse over-the counter products and prescription medicines when they are unable to obtain their usual illicit street drugs. Abuse of some prescription-only medicines, such as dihydrocodeine, is well known, and these medicines will have a "street" value. However, almostany substance can be misused. Misuse and abuse of prescription and over-the-counter medicines may result in hospital admission or to be contributory factor to the clinical presentation of thepatient. It is usual to categorize the drug misuser or abuser as young, often male, and aged between17 and 30 years. However, there are no social classes or age divisions among drug misusers.[1] Ethanol is a psychoactive drug and it is one of the oldest and most common recreationalsubstances.[2] It works in the brain primarily by increasing the effects of a neurotransmitter calledGABA.[3] This is a neurotransmitter presents in the brain and by facilitating actions of this GABA, ethanol suppresses the activity of the central nervous system.[3] Alcohol abuse has far-reaching consequences, both socially and economically, as well as causing serious pathological effects.[4-5] According to the World Health Organization[6], studies show that alcohol abuse is responsible forthe death of approximately 2.5 million individuals every year. Bava and Tapert,[7] indicate that theCenter for Disease Control and Prevention has identified alcohol abuse as a primary cause of preventable death. The prevalence of alcohol-related problems, particularly among young drinkers,has increased in recent years, and this is a major concern due to the link between early ons*et al*coholabuse and the development of metabolic and degenerative disorders.[8] The situation has worsenedas the concurrent use of other substances with alcohol has become more prevalent. Recently, research has indicated that the use of CASA, in particular, is commonly accompanied by alcohol.[9] There are concerns about the combined use of alcohol and CASA as research has suggested thatthe use of CASA may result in increased alcohol consumption due to the reduced depressant effectsof alcohol.[9]

A cocktail containing caffeine and acetylsalicylic acid (CASA) is a cheap local drug containing acetylsalicylic acid 760mg and caffeine 60mg. This drug is highly used by Nigerians for the treatment of pains, cold, headache and feverishness.[9] Caffeine, one of the components in this cocktail, is the most commonly used psychoactive substance in the world and is classified as a stimulant drug because of its ability to stimulate the central nervous system.[10] Although the Foodand Drug Administration considers it safe, excessive caffeine intake can lead to severe health problems, and in rare cases, even death.[11] The popularity of caffeine as a psychoactive drug is due to its ability to reduce adenosine transmission in the brain, which causes its stimulating effect.[12] According to Ferré, and O'Brien,[13] it is widely believed that caffeine antagonizes the intoxicating effects of alcohol, the molecular mechanisms underlying their interaction are incompletely understood. It is known that both caffeine and alcohol alter adenosine neurotransmission, but the relationship is complex.[13] Caffeine, which is a commonly used psychoactive substance worldwide, functions primarily as a stimulant by inhibiting adenosine receptors in the brain.[10] These receptors include A1, A2 (A and B), and A3, with A1 and A2A being the most prevalent in the brain.[13] The A1 receptor is found widely throughout the brain, with the highest concentration in the cortex, hippocampus, and cerebellum.[14]

The Central Nervous System (CNS) is a complex network, constituted by several types of cells including the glial cells. The glial cells include Oligodendrocytes which are specialized for the myelin formation. Astrocytes have multiple support functions to neurons, and Microglial cells playan important role in defense mechanism and inflammation; they also act as scavengers when tissueis destroyed. Some other types of cells in the CNS are Ependymal cells, which are epithelial cellsthat line brain ventricles and central canal of the spinal cord. They also assist in secretion and circulation of Cerebrospinal fluid. These cells are the endothelial cells which create a blood-brain barrier.[15] Glial cells were

discovered by the pathologist Rudolf Virchow in 1856. They representthe majority cell population in the CNS. There are about 12 to 15 billion neurons in the cerebral cortex and a billion neurons in the spinal cord. Whereas there are about 10 to 50 times more glialcells than neurons in the CNS. When they were discovered, glial cells have been recognized as brain glue as they were found to surround neurons and hold them in place.[16]

Later it was realized that glial cells play a number of other functions in the brain. Astrocytes are the most abundant type of glial cells, and present numerous projections that anchored neurons to their blood supply.[15] As the most abundant type of glial cells in the brain, Astrocytes provide metabolic and trophic support to neurons. They modulate synaptic activities and have a strong capacity to scavenge oxidants and suppress cellular apoptosis.

However, when the capacity of these cells to eliminate the oxidants is overwhelmed; overproduction of reactive oxygen species (ROS) can cause their morphological and functional alterations. This may include the production of cellular CA^{2+} homeostatis and some active molecules tightly associated with neuronal activity.[17] In view of the crucial roles played by ethanol and CASA in the daily life of both healthy and unhealthy individuals, this study focused on structural and functional integrity of the hippocampus when chronically and simultaneously assaulted by ethanol and CASA. In view of the crucial roles played by ethanol and CASA in the daily life of both healthy and unhealthy individuals, this study focused on the oxidative and inflammatory activities of CA1 subfield of the hippocampus when chronically and simultaneouslyassaulted by CASA and ethanol.

2. MATERIALS AND METHODS

2.1 Animals: A total of forty (40) adult presumably healthy wistar rats of average weight 160±6.23gwere used in this study. The animals were obtained from central animal house of the Faculty of Basic Medical Sciences, the University of Ibadan, Ibadan. Animals were maintained under standard laboratory conditions during acclimatization and experimental periods of 12 weeks in theanimal holding of physiology department, University of Ibadan, Ibadan. During this period, the rats were fed with standard mouse chow and were given water *ad libitum.* Daily weights were taken and documented.

Ethical approval, numbered UI-ACUREC/141-1218/10 was obtained from University of Ibadan Animal Care and Use Ethics Committee. Every effort was made to minimize the number of animals used and to ensure minimal pain and/or discomfort to the animals.

2.2 Protocol of the Experimental Groups: After acclimatization, the rats were randomly assigned into four groups (n=10) which are the control group given distilled water, the CASA group which received 31mg/kg body weight of CASA, ethanol group which received 30%v/v of ethanol while CASA plus ethanol group received 31mg/kg body weight of CASA and 30%v/v of ethanol. The dosages utilized in the treatment were determined based on prior research, and the substances weregiven orally for a period of 60 days. Alterations in body weight were recorded.

2.3 Serum Ethanol Determination

In the next treatment (day 60), samples were drawn by cardiac puncture utilizing Becton & Dickinson Vacutainer blood collection tubes containing additives as sodium fluoride (15.0 mg) and potassium oxalate (12.0 mg [100/sp, 1000/ca]), according to manufacturer's instructions. Serum ethanol content levels were determined using the Dade International (formerly DuPont) ACA IV method. All reagents were supplied by the instrument's manufacturer. The ACA IV was

operated, maintained, and calibrated as specified by the manufacturer's instructions. The calibration range was 0 through 300 mg/dl.[18]

2.4 Assay of Lipid Peroxidation

After being diluted ten times in 0.15 M Tris-KCl buffer and deproteinized with 500 µL trichloroacetic acid (30%), 100 µL of supernatant will be used. The combination will be spun at 4000 rpm for 10 minutes at room temperature in a bench top centrifuge. 200 µL of the supernatantwill be taken out and placed in an eppendorff tube. Thiobarbituric acid (1%), 200 µL was then added, and the combination was heated at 80^{o}C for one hour. The tubes were cooled by setting them on ice; 200µ L was then taken out and placed in a microtitre plate, where absorbance was measured at 532 nm. An indicator of absorption for MDA (molar extinction coefficient 1.56 x 10^5/M/cm) was used to compute the outcome. In the tissues, the concentration of TBARS was quantified as mol MDA/ mg protein.[21]

2.5 Measurements of Nitric Oxide

Nitrite (NO^{2-}), a stable breakdown product of NO, was measured with the Griess Reagent System(Promega, Madison, WI).[18] Absorbance was measured with a 540 nm filter in a Beckman spectrophotometer. Results were expressed as micromoles of nitrite per milligram of protein (μMof NO^{2-}/mg of protein).[21]

2.6 ELISA to Quantify Proinflammatory Cytokines (IL-1β and TNF-α)

Following treatment, the animals were decapitated (n = 5/group), their brains were immediately removed and washed in cold SSI, and the hippocampus was dissected and homogenized in 3 mL of 0.1 M, pH 7.4, cold Phosphate Buffered Saline (PBS). Homogenates were centrifuged at 12,500rpm at 4^{0}C. The supernatant was obtained and stored at −70^{0}C until used for protein and proinflammatory cytokine measurements.[20] The concentrations of IL-1β and TNF-α in homogenates of the hippocampus of rats were quantified by a sandwich immunoassay procedure,as specified in the kit protocols (R&D Systems, Minneapolis, MN, USA).

2.7 Histological Examination

Histological Examination. Following treatment, the rats (n = 5/group) were anesthetized with sodium pentobarbital (40 mg/kg, ip) and then perfused with 200 mL of 4% paraformaldehyde. Thebrains were removed and post-fixed in the same fixative solution for 48 h and then embedded in paraffin. Coronal 5μm thick sections were taken from each brain at the level of the anterior temporal area, approximately −3.8 to −6.8 mm from the bregma.

2.8 Immunohistochemistry Using ImmPRESS™ HRP Polymer System

ImmPRESS^{TM} Polymerized Reporter Enzyme Staining System (Vector$^{®}$ Labs, USA), a non-biotin,enzymatic, one-step detection kit, offers extremely high sensitivity staining with very littlebackground interference in immunohistochemical applications. Horseradish peroxidase (HRP) micropolymers are conjugated to extensively cross-adsorbed, affinity-purified secondaryantibodies using a novel, proprietary method using the ImmPRESS^{TM} Reagent. Reagents supplied (#MP-7401; Vector$^{®}$ Labs, USA)**:** ImmPRESS™ (Peroxidase) Polymer Anti- Rabbit IgG Reagent (made in horse, ready-to-use). 2.5% Normal Animal (Horse) Serum for blocking (ready- to-use).

ImmPRESS™ Immunohistochemical Detection

ImmPRESS^{TM} Reagent is prepared for use. To achieve the best immunohistochemistry staining, the ImmPRESS^{TM} reagent doesn't need to be mixed or titrated. At room temperature, the staining technique is carried out. The

ImmPRESS^{TM} Reagent should be brought to room temperature before use for optimum efficiency. As a wash buffer, phosphate buffered saline (PBS) was utilized. The protocol was performed as previously described.[19,20]

2.9 Statistical Analysis

The results were expressed as the mean ± standard error (SEM) for all experiments. Statistical analyses were done using one-way ANOVA test and Bonferroni's post hoc test considering P <0.05 as significant. Graph Pad Prism 8.0 was also used.

3.0 RESULTS

3.1 Serum Ethanol Concentrations

As shown in Fig. 1, the ethanol serum concentration in CASA group, which received 31mg/kg body weight of CASA and control group did not present detectable levels of ethanol, whereas in ethanol group which received 30% v/v ethanol, the mean levels of serum ethanol at end of the study were of 65 mg/dL. In CASA plus ethanol group which received CASA PLUS ethanol, the mean levels of serum ethanol at the end of the study were 30 mg/dL, therefore both the ethanol group and CASA plus ethanol showed a significant increase P< 0.005 which suggests that CASA combined with ethanol, enhances the clearance pattern avoiding intoxicating effects of alcohol consumption.

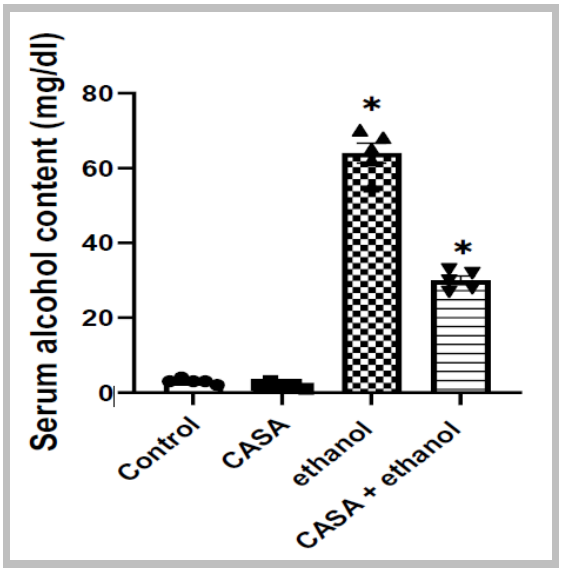


Figure 1: Showing Serum Ethanol Determination.

3.2 Relative Brain Weights of the Rats

Figure 2 below showed the relative brain weight in groups CASA, ethanol and CASA plus ethanol as recorded showed decreased P<0.05 of the Mean±SE 1.78±0.11 , 1.62±0.12 and 1.56±0.10) g values respectively when compared to the control with Mean±SE (1.82± 0.12) g. The relative mean brain was insignificant (P>0.05) for all the groups but showed increased values than the control groups and CASA plus ethanol group showed more decrease value when compared to all the three groups.

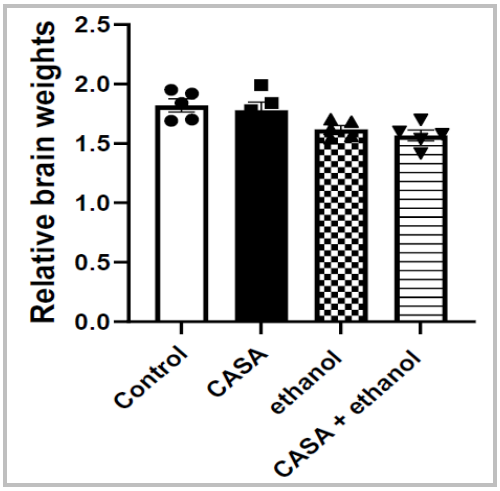


Figure 2: Histogram showing Mean brain weight distribution at the end of administration ofCASA and ethanol.

3.3 Oxidative Stress and Antioxidants Parameters Findings

The results obtained from lipid peroxidation measurements of both hippocampus is shown in figure3a. The result showed that rats that took ethanol and CASA plus ethanol had the highest malondialdehyde (MDA) level (210% and 154%) which is statistically significant when comparedto the control (80%) and CASA (87%) respectively ($p<0.05$).

The results from fig. 3b showed that NO level increased statistically in rats that took ethanol (559%) and CASA plus ethanol (665%) in the hippocampus ($p<0.05$) when compared to the control (56%) and CASA (101%).

The results obtained from SOD and CAT assays are shown in fig. 3c & 3d respectively. The resultsshowed that animals that were treated with ethanol and CASA plus ethanol had the lowest significant values (0.50±0.21 & 0.32±2.32) and (12.23±0.03 & 11.03±3.01) respectively and when compared with control and CASA groups (1.20±0.01& 1.82±1.00) although CASA group showeda higher value when compared with the control but it was not significant. The result from GSH ofhippocampus of rats as shown in fig. 3e showed significant decrease in ethanol and CASA plus ethanol treated groups with values (2.40±0.01 &2.03±0.43) when compared with the control and CASA groups with values (7.32±1.23 & 6.64±0.03).

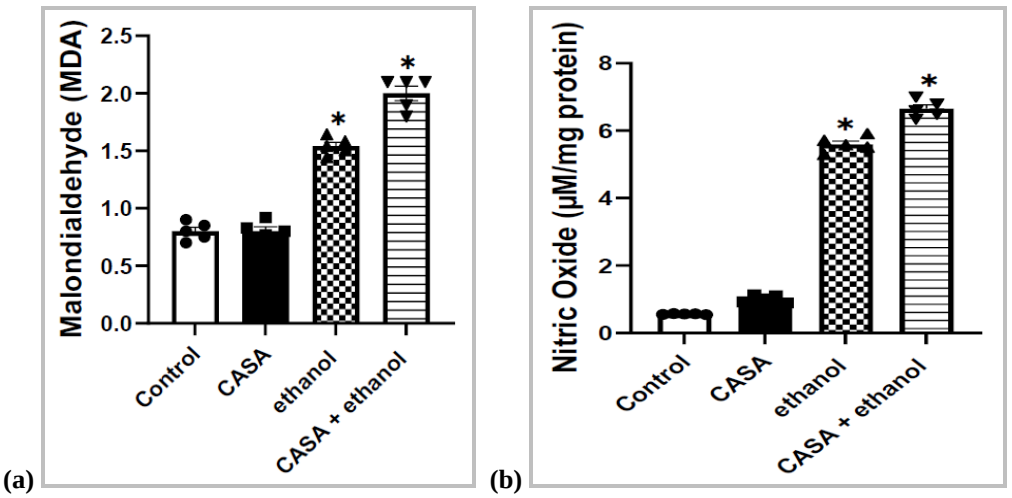
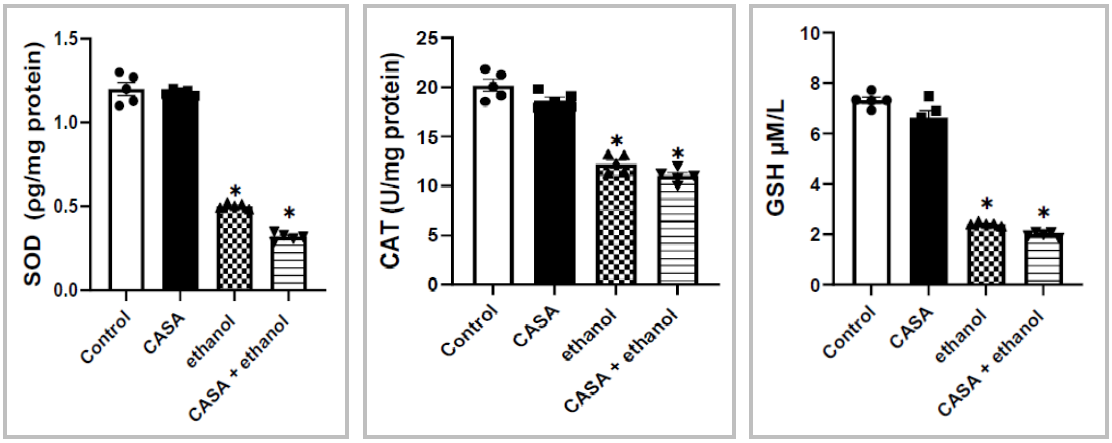


Figure 3: Effects of the combination of CASA and ethanol on (a) lipid peroxidation and (b) concentration of nitrites in the rat hippocampus. The values show the mean ± SEM (n=5) (one-way ANOVA with Bonferroni's post hoc test, *p<0.05.



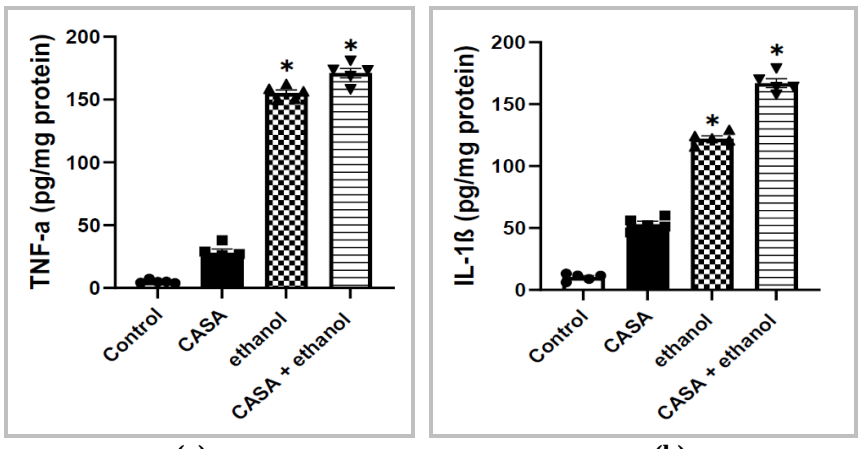
(c) (d) (e)

Figure 4: Effects of the combination of CASA and ethanol on (a) lipid peroxidation and (c, d, e) concentration of antioxidants in the rat hippocampus. The values show the mean ± SEM(one-way ANOVA with Bonferroni's post-hoc test *p<0.05).

3.4 Evaluation of Proinflammatory Cytokines

In order to understand the effects caused by the combination of CASA and ethanol on the inflammatory response on the brain of rats, the concentration of IL-1β and TNF-α were also evaluated. The concentration of cytokines which were gotten from the homogenized tissues of hippocampus of rats were shown in fig. 5a & b. The level of TNF-α (fig.5a) showed that rats givenethanol and CASA plus ethanol had the highest significant concentration of TNF-α with values (155.36±2.40 & 171.23±3.75) respectively when compared to control and CASA groups with values (5.196±0.61 & 28.07±2.93) P<0.05.

The level of IL-1β (fig.5b) from the treated groups, CASA, ethanol and CASA showed significantly increased values (53.24±2.29, 122.31±2.18, 167.32±3.56) when compared with the control (10.35± 0.25) p<0.05.



(a) (b)

Figures 5: a, b Showing the concentration of TNF-α and IL-1β respectively in the hippocampus of treated groups.

3.5 GFAP Immunoreactivity

In order to understand the effects caused by the combination of CASA and ethanol on the inflammatory response in the hippocampus of rats, the immunoreactivity to GFAP were evaluatedin the area of the brain examined.

Fig. 6a showed immunoreactivity to GFAP (brown color) across the four groups of the study. Theresults showed that, for CASA group, ethanol group, and CASA plus ethanol group, the immunoreactivity to GFAP in the CA1 subfield of the hippocampus increased when compared to the photomicrographs of the control group. Nonetheless, mixture of CASA plus ethanol producesincreased immunoreactivity which was distributed in greater proportion compared to the groups treated separately with either CASA or ethanol. The number of GFAP-immune positive cells in theCA1 subfield of the hippocampus showed that co-administration of CASA plus ethanol gives rise to greater immunoreactivity to GFAP, when compared to the control, CASA and ethanol only fig. 6b although the expression in CASA group were lesser than that in ethanol and CASA plus ethanol,yet when compared with the control it was significant.

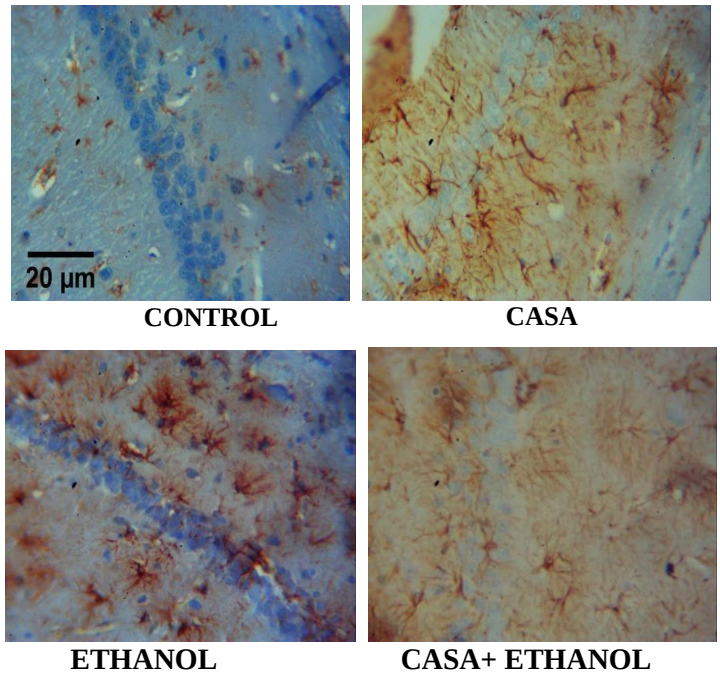


Figure 6a: The combination of ethanol and CASA caused inflammatory responses in the CA1of the hippocampus of rat. These photomicrographs showed the immunoreactivity to GFAP(brown color) of the CAI subfield of the hippocampus of rat. Mag. X 400.

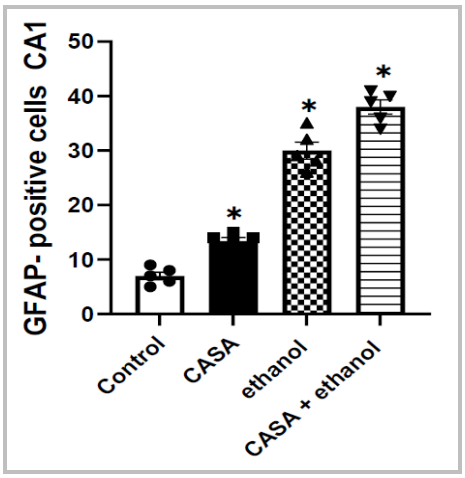


Figure 6b: Quantitative data of the number of GFAP positive cells in CA1 region of the hippocampusof the treated groups. The values show the mean ± SEM (one-way ANOVA with Bonferroni's post-hoc test *p<0.05).

3.6 Neuronal neuclei protein (NeuN) immunoreactivity

To determine the effect of the consumption of CASA and ethanol on the immunoreactivity to NeuN in the CA1 of the hippocampal subfield. It was observed that anti-NeuN antibody (brown color) showed less intense reaction to the group treatment with ethanol and CASA plus ethanol, been distributed uniformly in the CA1 subfield of the hippocampus whereas the control group and the group treated only with CASA showed more immunoreactivity to NeuN antibodies with less neuronal protein loss. These indicated that there was significantly less neuronal loss in the control and CASA group when compared to the groupstreated with ethanol and CASA plus ethanol where there was high loss of neuronal proteins.

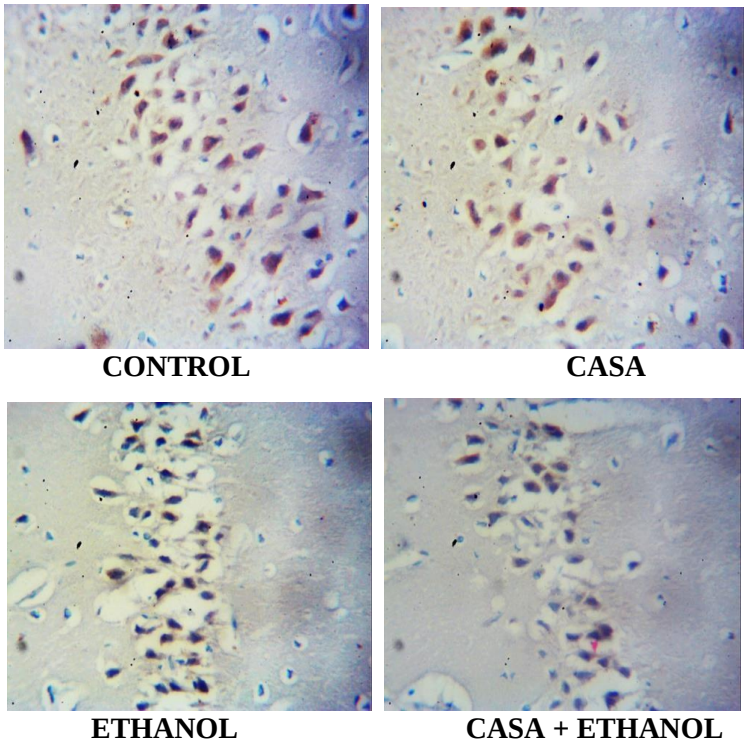


Figure 7: Photomicrograph showing immunohistochemical staining (NeuN) of rat hippocampal CA1 region of the various groups. CASA plus ethanol showed a less intense reaction with high neuronal loss, whereas in the control group and the group treated only with CASA, there showed a more intense reaction to NeuN. Magnification X400.

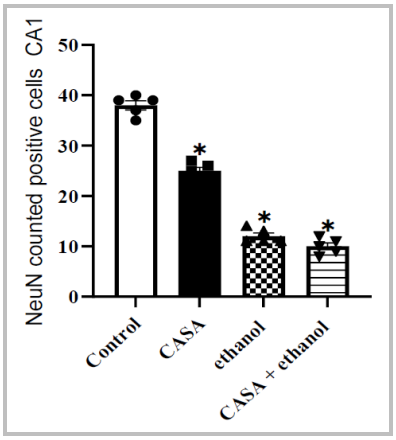


Figure 7b: Quantitative data of the NeuN immunoreactivity in CA1 of the hippocampal of rats. The values show the mean ± SEM (n=5) (one-way ANOVA with Bonferroni's post hoc test, *p<0.05.

3.7 B-CELL LYMPHOMA-2 (BCL-2) IMMUNOREACTIVITY

The results from the Bcl-2 immunohistochemistry demonstrated that the Bcl-2 protein level and immunoreactivity were higher in rats treated with ethanol and CASA plus ethanol for 60 days when compared to the rats in the control group and in CASA (Fig. 8). According to the quantitative results of Bcl-2 immunohistochemical staining, there were significant increase difference in the number of Bcl-2-positive cells in ethanol and CASA plus ethanol treated groups. Bcl-2 protein expression was higher in ethanol and CASA plus ethanol treated groups in the CA1 area of the hippocampus which were significant while in control and CASA treated group decreased numberof Bcl-2-positive cells were seen when compared to the ethanol and CASA plus ethanol treated groups. This suggests that there was apoptosis in the cells of rats in ethanol and CASA plus ethanoltreated groups.

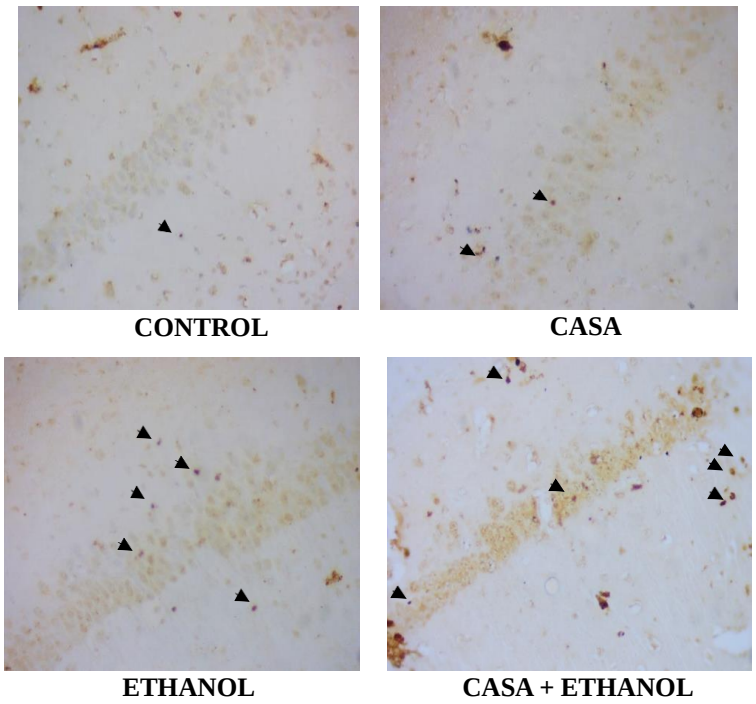


Figure 8a: Photomicrograph of immunohistochemical staining for Bcl-2 in the CA1 subfieldof the hippocampus (arrows indicate Bcl-2 immunopositively cells). Mag. x400.

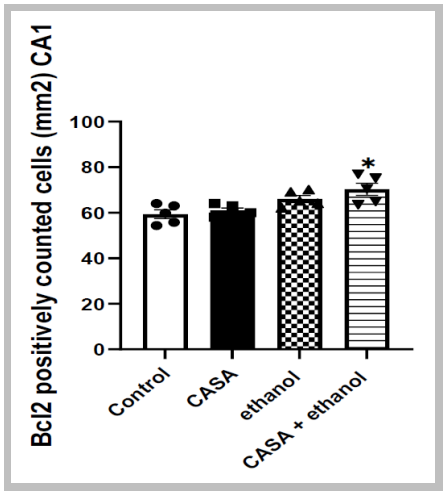


Figure 8b: Quantitative data of Bcl-2 immunoreactivity in CA1 region of the hippocampus of rats. The values show the mean ± SEM (n=5) (one-way ANOVA with Bonferroni's post hoc test, *p<0.05.

4.0 DISCUSSION AND CONCLUSION

4.1 Discussion

The ethanol serum determination result gotten in this study corresponds with the work done by.[18] Ethanol was detected in the group that received only ethanol, with a mean serum ethanol levelof 65 mg/dL at the end of the study. However, in the group that received both CASA and ethanol, the mean serum ethanol level was lower, measuring 30 mg/dL at the study. The control and CASA administered groups showed no evidence of ethanol in their systems, therefore both ethanol and CASA plus ethanol groups showed a significantly decrease in serum levels, which suggest that CASA combined with ethanol increases the clearing pattern thereby causing avoidance of toxicological effects for the consumption ethanol. These findings provide support for the hypothesis that the common belief that caffeine counteracts the intoxicating effects of ethanol is accurate. Additionally, the study showed that caffeine can mitigate the impact of ethanol on various psychological factors in humans, including psychomotor performance, information processing and memory.[13]

For the effects of ethanol and caffeine, the central nervous system is a major target organ.[22] According to reports, mixing alcohol with energy drinks (which commonly contain caffeine, plant-based stimulants, simple sugars, and other additives) is a common practice in the United States, particularly among young people.[23] Consuming alcohol and caffeine together may increase the rate of alcohol-related injuries.[24] According to reports, both laboratory animals and humans' excitability is regulated by the glutamatergic system.[25] We investigated and gotten to know that majority of Nigeria communities and individuals frequently combine ethanol and alabukun® (a common example of CASA) to reduce the depressant effects of ethanol.[9] As a result, there is growing concern that consuming ethanol alongside caffeine which is one of the ingredients in CASA may promote the death of neurons through apoptosis.

Oxidizable substrates are greatly concentrated in the brain (PUFA, catecholemics, etc.). The brainis more susceptible to oxidative damage because some areas of it have an excess of catalytic transition metals, a high oxygen demand, and relatively few anti-oxidant enzymes.[26] As a result, brain damage brought on by oxidative stress has a great chance of impairing regular CNS activities.[27] The mean brain weight in ethanol and ethanol plus CASA groups showed significantly decreased values when compared to the control. The mean brain weight was insignificant for CASA group when compared to the control group. Previous investigators have reported that many neurotoxins have been implicated in reduced brain weight.[28] Oxidative stresshas been linked to brain impairment.[29] Neurotoxins have been implicated in reduced brain weights by some investigators.[28] The significant decrease in the brain weights in ethanol and CASA plus ethanol treated rats in this study might be attributed to neurodegenerative and atrophicchanges resulting in brain damage induced by increased lipid peroxidation leading to oxidative damage of the hippocampal and dentate tissue in the ethanol and CASA plus ethanol treated rats. Report from the literature has indicated that Reactive Oxygen Species (ROS) are generated continuously in the nervous system during normal metabolism and neural activity. Oxidative stressis induced when the delicate balance between ROS, superoxide; hydrogen peroxide, hydroxyl radicals and singlet oxygen and body antioxidant system is lost.[30]

The generation of reactive oxygen species (ROS) has been considered to be a primarily event in the light of a variety of stressful conditions.[31] Moreover, it has also been generally accepted that active oxygen produced under stress is a detrimental factor which ultimately results in lipid peroxidation, enzyme inactivation and oxidative damage to DNA.[32] Free radicals produced duringmetabolism react with polyunsaturated fatty acids of cell membrane leading to impairment

of mitochondrial and plasma membranes. Ethanol could dissociate readily to release free glutamate. The diminution of glutamate produces ammonium ion that may be toxic unless detoxified in the liver through the reactions of the urea cycle.[33]

The weight recorded in wet brains of treatment group ethanol and CASA plus ethanol when compared to the control and CASA group is due to the combinatory effects of CASA and ethanol as findings have revealed that alcohol impairs nutrient absorption,[34] and also cause brain damage.[35]

Lipid peroxidation (LPO) increased significantly in ethanol and CASA plus ethanol treated groups compared with the control and the group treated with CASA in this study. Malonaldehyde (MDA) is the major aldehyde metabolite of lipid peroxidation. The elevation of lipid peroxidation observed in ethanol and CASA plus ethanol treated rats might be due to increase glutamate level which resulted in excitotoxic neuronal cell death through the activation of N-methyl-D aspartate (NMDA) and non- NMDA glutamatergic receptors in the CNS.[36] Additionally, it has been noted that increased lipid peroxidation could also be induced by alteration in redox potential of the concerned cell and thus enhancing lipogenesis.[37] Lipid peroxidation has been known to be an autocatalytic mechanism resulting in oxidative damage of cellular membranes.[38] The significant increase in MDA level in the CASA plus ethanol treated rats in this study is similar to the report of Diaz *et al*.[21] that administration of energy drink in combination with alcohol causes oxidative stress, Also, it was reported,[39] that chronic administration of ethanol significantly increased MDA level in rat hippocampus and this significant increase can eventually induce apoptosis which is a cell mediated death. Although, no report has documented its level in CASA and combination of ethanol plus CASA but it has been reported that ethanol combined with energy drink (which contain caffeine; one of the components of CASA) presented with highest lipid peroxidation levels in the hippocampus when compared to the control,[21] which supports this study that CASA plus ethanol presented the highest levels of lipid peroxidation when compared with the other groups.

Nitric oxide (NO) is a molecule synthesized from the amino acid, arginine by nitric oxide synthase (NOS) in the brain upon demand, a cognitive condition for which NO activity is required. Neurons synthesize NO as a response to activation of N-methyl D-aspartate (NMDA) receptors by the excitatory amino acid glutamate.[40] Nitric oxide has a double-edged knife in relation to pathophysiology in that both the paucity and abundance of NO induces various diseases.[41] Furthermore, previous studies have indicated that direct toxicity of NO is enhanced by reacting with superoxide radical such as peroxynitrite which is capable of oxidizing cellular structure which consequently results in lipid peroxidation, a process leading ultimately to membrane damage.[42] The increase in NO level in the treated rats in this study occurred concomitantly with increase in lipid peroxidation (LPO) in treated rats. In other words, the relationship associated with elevation of NO and lipid peroxidation level in the treated rats in this study may be another explanation of excitotoxicity and cell death induced by CASA and ethanol. It has also been reported that several mediators of systemic vasodilatation such as NO is associated with some particular diseases.[43] The activity of NO in the brain has been implicated in the pathological conditions such as epilepsy, stress, diseases and side effects of some therapeutic agents.[44]

Superoxide dismutase (SOD) is the first enzyme implicated in the antioxidant defense. The treated rats in this study showed a discernible decline in SOD activity. This decrease in SOD levels in the rats treated with ethanol and CASA plus ethanol is probably because of overproduction of reactive oxygen species (ROS) and it is known that overproduction of ROS can harm biological membranes.[45] Past study has shown that a decline in SOD activity can

raise ROS levels even further, which ultimately causes catalase to become inactive.[46] Superoxide and other ROS have also been found to be produced by mitochondria when glutamate receptors are activated.[47] During oxidative stress, the superoxide generated can damage iron-sulfur centers, permanently disabling enzymes that contain iron.[48] In this study, rats treated with ethanol and CASA plus ethanol showed a significant increase in the catalase activity in contrast to CASA and control groups. Catalase activity rose in the CASA group but not statistically significantly more so than in the control group. Previous studies on the CAT activity in the rat hippocampus have similarly shown a reduction in CAT activity after dietary monosodium glutamate treatment.[49] This study's observation of a considerable decline in catalase activity might be explained by the decreased availability of NADPH. In several parts of the brain, oxidative damage is linked to reduction in catalase activity.[50]

Our result with the considerable drop in hippocampus catalase levels shown in the ethanol and CASA treated animals is consistent with.[51] A reduction in catalase activity is also seen in catalase after intraperitoneal monosodium glutamate administration in adult rats.[52,53] Depletion or inactivation of catalase activity is linked to increased free radical generation as shown in our study.Rats treated to CASA plus ethanol showed a considerable drop in catalase levels in the pyramidal and granular cell layers. This decrease may have been caused in part by the increased generation of superoxide anions that followed CASA-ethanol administration.

The tripeptide glutathione (GSH), which is made up of glutamate, cysteine, and glycine, is essential for shielding cells against xenobiotics and oxidative damage.[53] Through a number of non-enzymatic interactions with superoxide, nitric oxide, hydroxyl radicals, and peroxynitrite, GSH exerts its antioxidant effects.[54] GSH serves as a scavenger of free radicals. [53] Cysteine, the precursor to GSH, is in a state of equilibrium that is disturbed by glutamate toxicity. As a result of this disturbance, intracellular GSH levels are reduced and cells are less able to defend themselves against oxidative damage and injuries, which can lead to cell death or damage to the cells.[55] GSH and other enzymes' active sulfhydryl groups can be removed by lipid peroxidation.[56] In the current investigation, rats given ethanol and CASA plus ethanol showed significant increase in lipid peroxidation. Increased reactive oxygen species (ROS) can deplete the GSH pool, and falling cellular GSH levels make oxidative stress even worse.[32]

Catalysis, metabolism, and transport are all significantly influenced by GSH, which functions as an intracellular reductant.[57] It guards cells against free radicals, peroxides, and other harmful substances.[54] According to other studies, GSH can reduce oxidative stress by either facilitating the removal of substances that cause peroxidation in the cell membrane or by improving the efficiency of the detoxifying enzymes by increasing the availability of nicotine amide dinucleotide phosphate (NADPH). [58] Lipid peroxidation starts when the GSH concentration falls to 20% of its initial level and that there is an indirect link between GSH and lipid peroxidation.[59] It has been hypothesized that monosodium glutamate causes lipid peroxidation, which depletes tissue levels of GSH, which is consistent with our finding.[52] The increased consumption or utilization of the antioxidant system in scavenging the ROS (free radicals) produced by the ethanol and CASA treatments may be the cause of the significant reductions in pyramidal and granular GSH levels seen in the rats treated with ethanol and CASA plus ethanol in this study.

It has been demonstrated that long term, chronic alcohol consumption causes reactive astrocytosis and promotes the release of proinflammatory mediators, mainly in brain region such as the hippocampus.[60] These results clearly showed the presence of reactive astrogliosis induced by ethanol and CASA, besides the increase in IL-1β and TNF-α, in the brain region of study. Furthermore, the effect of CASA on neuroinflammation has not been

described, although report, that consuming caffeine in high concentrations causes an inflammatory response and death in neurons of neonatal rats and in cell cultures.[21] High immunoreactivity to GFAP was observed in rats treated only with CASA, although the group administered with both CASA and ethanol showed higher immunoreactivity to GFAP with respect to the other experimental groups. Possibly, a synergistic effect between ingredients of CASA with alcohol is able to aggravate the release of glutamate; however, this is still unclear, this suggests that reactive astrogliosis and the release of proinflammatory factors like IL-1β and TNF-α are a signal of the growing inflammatory response in the CA1 region. Cytokines are considered to be regulators of the intensity and duration of the inflammatory process. [61] These promote the activation of various signaling pathways in response to cellular stress, including iNOS, responsible for the increasing concentrations of NO, which reach toxic levels and lead to the development of oxidative stress and neuronal death in vitro.[62] Administration of these drugs for 60 days could promote chronic glial activation accompanied by the increased release of proinflammatory cytokines such as IL-1β and TNF-α, thus triggering proliferation of glial cells.[63] Furthermore, regulating the expression of iNOS in astrocytes produces high concentrations of NO which facilitate the generation of oxidative stress and neuronal dysfunction.[21] The ethanol induced inflammatory response, together with the CASA, could be responsible for the NO production by iNOS activity, thereby worsening brain inflammation,[64] because NO is an important source of ROS, which contributes to oxidative stress process and death in neurons.[65] Just as seen in this study, the groups given ethanol and CASA plus ethanol presented a significant increase in the formation of free radicals and lipid peroxidation, compared with the control group and the group treated with CASA only. For the group treated with CASA only, the levels of lipid peroxidation and NO do not show a significant difference as compared to CASA plus ethanol suggesting that CASA plus ethanol; and not the consumption of CASA only, causes neurotoxic effects. Therefore, it is necessary to combine CASA and ethanol to trigger oxidative stress and an inflammatory response. This consequently contributes to death in neurons of the hippocampal CA1.

Astrocytes are ubiquitous cells that are found throughout the brain tissue which perform essential homeostatic functions that could directly influence tissue integrity, neuronal survival and functional outcome following Traumatic brain injury (TBI).[66] These essential functions include, maintenance of extracellular ion and fluid balance, clearance of extracellular glutamate, water transport, production of pro – or anti-inflammatory cytokines and chemokines, production of growth factors, production of glucose and other energy metabolites and free radical release or free radical scavenging.[67] It has been indicated that astrocytes respond by producing changes in gene expression, cellular hypertrophy and cell proliferation in proportion to the severity of the injury.[66] Significant increased numbers of GFAP positive astroglial cell have been demonstrated in human following brain injury.[68] Increased expression of GFAP has been reported to represent astroglial activation and gliosis during neuronal degeneration.

Furthermore, an association between nitric oxide and expression of GFAP, explaining the role of the former in the expression of the latter has been described by other investigators. GFAP expression has been reported to be sensitive to oxidative stress in that GFAP transcription in cultured glia is increased by oxidative stress in response to hydrogen peroxide and cysteamine.[69] Moreover, oxidative stress is associated with increased expression of GFAP in response to brain injury.[69] Astrocytes have multiple roles in the CNS, including maintenance of the integrity of the blood – brain barrier, uptake and recycling of glutamate, GABA maintenance of the extracellular ionic milleu and neuronal metabolic support.[70] Astrocytes are the major glial cell population in the CNS. They play important physiological roles in brain functions.

Astrocytes react to various neurodegenerative insults rapidly, leading to vigorous astrogliosis.[71] Consequently, activation of astrocytes has been implicated in the pathogenesis of a variety of neurodegenerative diseases including Alzheimer's disease, acute traumatic brain injury and inflammatory demyelinating diseases.[72] Previous investigation revealed that activated astrocytesalso express inducible nitric oxide synthase (iNOS) to produce an excessive amount of NO, a molecule implicated virtually in all reported neuroinflammatory and neurodegenerative conditions.[71] This corroborates this study as the expression of NO was high in the groups treated with CASA, ethanol and CASA plus ethanol but when CASA and ethanol were co-administered, the NO quantitative level become higher and this also agreed with previous investigators that NOinduces the expression of GFAP in astrocytes, this revealed that the number and size of GFAP – astrocytes in the CNS increase following brain injury and in various pathological conditions.[70] The inflammatory response in the brain involves reactive astrocytes and the production of various proinflammatory mediators, such as proinflammatory cytokines and deleterious free radicals, which are associated with stress activated signal transduction pathways, leading to death in neurons.[71] These disorders display an accumulation of derivative products from the increase of oxidative stress, leading to widespread damage of lipids and protein.

The higher level of increase expression of astrocytes in ethanol and CASA plus ethanol treated rats compared with control and CASA in this study may be attributed partly to increased level of NO. Also, tissue damage induced by ethanol and CASA plus ethanol groups invariably may contribute to the increased expression of astrocytes in ethanol and CASA plus ethanol treated rats relative to the control and CASA group. Also, the combination of CASA and ethanol causes increased astrogliosis in the hippocampal CA1 when compared with the administration of CASA or ethanol alone. Diaz et al.[21] reported that long term, chronic ethanol consumption causes reactive astrocytosis and increase the release of proinflammatory mediators especially in the hippocampus. The results of this study showed the presence of reactive astrogliosis induced by ethanol, aside from the increase in IL-1β and TNF-α, in the brain region of study. Furthermore, the effect of CASA on neuroinflammation has not been reported, although Diaz et al.[21] reported that consuming caffeine in high concentrations causes an inflammatory response and death in neurons of neonatal rats and in cell cultures. However, in the brains of the animals administered with only CASA, low immunoreactivity to GFAP when compared to ethanol and CASA plus ethanol groups although the result of the CASA increased when compared with the control groupbut it was not significant. This can also be said that the group administered with CASA plus ethanolshowed higher immunoreactivity to GFAP when compared to the other experimental groups. It ispossible that there is a synergistic effect between ingredients of the CASA and ethanol which mayalso be able to aggravate the release of astrocytes; although, this is still not understood, thereby suggesting that astrocytosis and the release of proinflammatory factors such as IL-1β and TNF-$\alpha$are a reflex of the increase inflammatory response in the area of the brain studied.

Neurons include unique proteins that are indicative of their development into postmitotic cells, according to studies on the proteome of neural tissue and the use of immunocytochemistry on nervous system organs.[73] Since more than 20 years ago, NeuN-specific monoclonal antibodies have been used in immunohistochemistry investigations to evaluate the functional status of neurons in both healthy and pathological situations.[73]

NeuN protein production in cells can be affected by nervous system damage in a variety of ways,according to studies. For instance, motoneurons in the facial nerve nucleus can significantly lose their NeuN immunoreactivity as a result of axonal damage.[73] Since NeuN protein is particular tonervous tissue, it stands out from other neuronal differentiation

marker proteins present in other cell types as a commonly utilized universal marker for examining the differentiation of stem cells into neurons.[74] NeuN immunoreactivity has been noted in primary cell cultures of mouse, rat, and human brains.[75] Anti-NeuN antibodies have also been linked to spotting pathogenic alterations in existing neuronal populations. NeuN immunoreactivity in neurons has been found to wane or disappear in several investigations due to various pathogenic processes.[73]

For instance, neuronal nuclei staining completely vanished in rats' striatum after ischemic damage, decrease in its immunoreactivity was noted in brain injury, and disappearance of NeuN protein was reported from nearly dead or damaged pyramidal cells in the hippocampus.[73]

While reduction in NeuN immunoreactivity seen in this study's groups treated with ethanol and CASA plus ethanol suggests a reduction in NeuN associated with neuronal death in the affected region of the brain, the loss that was seen in the examined locations may have resulted from subsequent brain injury.Furthermore, the findings of earlier research by Diaz et al.[21] that co-administration of ethanol and energy drinks promotes neuronal loss in the hippocampus are supported by the decreased neuronal loss in the CASA group and ethanol alone as opposed to ethanol plus CASA.

We also evaluated death in cells as to be caused by the combination of CASA and ethanol in the hippocampus of rats, using Bcl-2 which is an anti-apoptotic protein. Decreased immunoreactivity of Bcl-2 were observed in the groups given ethanol and CASA combined with ethanol in the hippocampal CA1 subfield. It has been reported that chronic alcohol consumption in rats induces death in neurons by apoptosis in different hippocampal and cerebral cortex regions; thus, demonstrated by the increase in immunoreactivity of Bcl2. [76] This could explain our finding showing that the increase in lipid peroxidation correlates significantly with an increase of Bcl-2 expression. Therefore, the overexpression of Bcl-2 could be a defense mechanism, conferring resistance to oxidant injury and preventing apoptosis. It has also been established that inflammatory factors, oxidative stress and excitotoxicity represent the main causes of neuronal apoptosis.[77]

4.2 CONCLUSION

The results obtained from this study following treatment of Wistar rats with 30%v/v of ethanol for ethanol group, 31mg/kg/bw of CASA for CASA group and 30%v/v and 31mg/kg/bw for CASA plus ethanol group respectively for 60 consecutive days demonstrated various changes in hippocampal subfields CA1 in Wistar rats investigated. This study concluded that exposures of Wistar rats to chronic doses of ethanol and CASA for sixty consecutive days resulted in compromise of neuronal integrity and impairment of CA1 pyramidal cell layers functions in rats investigated.

In view of the results obtained from the current study, the present data have conclusively demonstrated that the hippocampal CA1 is hugely at risk in Wistar rats and consequently the principal function of this part of the brain are being compromised following long term simultaneous intake of CASA and ethanol more than when either of the drugs is taken.

Conflicts of Interests

The authors declare that they have no conflict of interests.

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