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NEURODEGENRATION AND INFLAMATORY EFFECTS OF CONCURRENT USE OF CAFFEINE AND ALCOHOL ON HIPPOCAMPAL CA1 IN WISTAR RATS (*Rattus novergicus*).

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Abstract

A lot has been said about the neuroprotective effects of caffeine on neurodegenerative diseases at low dosage, but little has been said about its neurotoxic effects, especially when consumed with alcohol, on the CA1 region of the brain that is responsible for learning and memory and has been reported to be sensitive to neurotoxic insults. Therefore, this study aimed to determine the neurodegeneration and inflammatory effects of caffeine-alcohol consumption on the CA1 region of the hippocampus in male Wistar rats.

A total of twenty (20) adult Wistar rats weighing 120-140g were used in this study. The following experimental groups were designed for the study: control were given distilled water; caffeine-treated group were administered with 10 mg/kg/bw, Alcohol-treated group were administered with 30%v/v of alcohol; and the caffeine plus alcohol-treated group took 10 mg/kg/bw of caffeine plus 30%v/v of alcohol) and the administration were done for 30days after which the animals were euthanized using 0.1ml Ketamine Hydrochloride (intraperitoneally administered, then transcardiac perfusion was done using 0.9% Normal saline for about 2 minutes following 10% Neutral Buffered Formalin (NBF). The regions of the hippocampus were dissected out, weighed, and fixed in 10% Formol Saline for 24/48 hr.

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The levels of TNF-α and IL-1β significantly increased in the caffeine plus alcohol group compared with the control group. The percentage neuronal loss significantly increased in the caffeine plus alcohol group when compared with both the caffeine and alcohol groups and the pyramidal cell diameter significantly decreased in plus alcohol group when compared with the control. Caffeine plus alcohol, when compared with the control, significantly increased GFAP-positive astrocytes.

It was concluded that concurrent administration of caffeine and alcohol induced a neurotoxic response by neurodegeneration, increased oxidative stress, upregulation of proinflammation cytokines, and astrogliosis in the CA1 hippocampal subfield of Wistar rats.

1. INTRODUCTION

The popularity of so-called energy drinks has increased the attention paid to the influence of caffeine on the behavioral effects of ethanol, including ethanol use and abuse. Under the widely held idea that caffeine can somewhat counteract the intoxicating effects of ethanol, energy drinks with a high amount of caffeine are regularly consumed in combination with alcohol. The assumption that caffeine can mitigate the negative effects of ethanol in humans or rats have not received unanimous scientific support, and it is unclear exactly how caffeine and ethanol interact. Both ethanol and caffeine act on neurochemical processes connected to the neuromodulator adenosine; they share a biological substrate (López-Cruz *et al.*,2013). Alcohol and caffeine are two often used recreational substances (Crocq, 2003). Alcohol consumption is a severe health issue that has an impact on society and the economy on a global scale (Heinz *et al.,* 2013). However, caffeine consumption, even in excess, seems to be reasonably well tolerated because of the activating and attention-preserving qualities of methylxanthines, which can increase performance and productivity (Heinz *et al.,* 2013). The practice of mixing caffeine with alcohol may have its roots in the notion that caffeine might counteract alcohol's intoxicating effects (Heinz *et al.,* 2013). This theory has been validated by studies showing that caffeine reduces the effects of alcohol on human psychological functions such as information processing, memory, psychomotor performance, and others (Jacobson *et al.,* 2022). The most common way to characterize caffeine's direct activities is as an adenosine receptor antagonist that does not affect only the A 1 and A 2A subtypes of adenosine receptors in the brain (Nagy *et al.,* 1990). There are interactions between adenosine and ethanol, as shown by some published studies. By boosting adenosine release and lowering adenosine absorption, which occurs via a facilitative nucleoside transporter, ethanol can raise extracellular adenosine levels (Diamond *et al.* 1991). The extracellular adenosine level would rise as a result of this transporter's inhibition in the presence of ethanol, perhaps modulating some of the effects of the drug (Diamond *et al.,*1991). Secondarily, ethanol raises the levels of adenosine because the metabolism of ethanol produces acetate, which encourages the synthesis of adenosine (Kolahdouzan *et al.,* 2017). Additionally, high dosages of ethanol can have long-lasting consequences such as forgetfulness, decreased memory recall, and learning difficulties (López-Cruz *et al.,*2013). A few articles have concentrated on how caffeine and ethanol affect mice' memories (López-Cruz *et al.,* 2013; Anand *et al.,* 2012). In many ischemia scenarios, the co-administration of ethanol and caffeine is neuroprotective (Anand *et al.,* 2012).

The hippocampus is one of the oldest regions of the brain. It is composed of interconnected layers of the dentate gyrus and cornu ammonis. Each layer and subfields of the hippocampus is involved in different hippocampal

functions and tasks (Leutgeb *et al.*, 2012). The best-known hippocampal functions and tasks include the ability of the hippocampus to express current self-location in spatial and other cognitive dimensions (Samsonovich & Nadel, 2005) and its involvement in the storage and retrieval of memory (Tracy *et al.*, 2001). The hippocampus has been implicated in other areas such as learning and memory (Bartsch *et al.*, 2015). The hippocampus cornu ammonis 1 (CA1) is thought to be the area that is most sensitive and susceptible to insults (Tiwari & Chopra,2013). Additionally, it is among the three areas of the brain that are most vulnerable to oxidative stress and the first to experience functional impairment (Samsonovich & Nadel, 2005). Recently, there has been an increase in alcohol-related problems, especially among young drinkers. This has been a major concern because the early onset of alcohol abuse is a major risk factor for the onset of metabolic and degenerative disorders (Jacobson *et al.*, 2022) and chronic alcohol consumption induces the death of neurons in cognitively related brain regions, such as the cerebral cortex of adult rats. Some reports indicate that alcohol causes oxidative stress, provoking an inflammatory response in the hippocampus (Díaz *et al.*, 2016). Although it has been found that caffeine exerts neuroprotective effects in the hippocampus and cortex of rats, the synergistic effects of caffeine combined simultaneously with ethanol are limited in the literature hence, the reason for this study (Santos *et al.*, 2013).

2. MATERIALS AND THE METHOD

2.1 Chemicals and Drugs

Caffeine (1, 3, 7-trimethylxanthime, analytical grade, Shandong Xinhua Pharmaceutical Company, China) and absolute alcohol were purchased from Sigma-Aldrich, USA. The Assay kits for analysis were obtained from Randox Laboratories Ltd., United Kingdom. All other reagents were of analytical grades.

2.2 Ethical Approval

Ethical clearance was obtained from the Animal Care and Use Research Ethics Committee of the University of Ibadan with Ethical Clearance Number: UI-ACUREC/141-1218/10.

2.3 Animal Treatment

Twenty adults healthy Wistar rats of both sexes with an average weight of 200-220g were maintained under standard laboratory conditions for an acclimatization of 2 weeks in the animal holdings of the Anatomy Department of the University of Ibadan. During the study, the rats were fed mouse chow and water *ad libitum*. Daily weights were measured and documented. After two weeks of acclimatization, the rats were randomly divided into four experimental groups (n=5) such that the control group received distilled water, the caffeine group received 10 mg/kg/bw of caffeine, the alcohol group received 30%v/v of alcohol, and the alcohol plus caffeine group received 10 mg/kg/bw of caffeine plus 30%v/v of alcohol. All substances were administered orally to the rats for 30 days using a cannula. The doses for the treatment were calculated on the basis of previous reports (Paxinos & Watson, 2006).

2.4 Histological Examination

Following treatment, the rats were anesthetized and sacrificed by cardiac perfusion. The skulls were dissected and the brains were removed and fixed in 10% formocalcium solution for 48 h. Regions of the hippocampus were dissected using the Paxinos stereotaxic coordinate method (Dahchour *et al.*, 2005). The brain sections were then processed for routine histological techniques, sectioned using a Cambridge rocker microtome at 5 μm and stained using hematoxylin and eosin and Cresyl Fast Violet.

2.5 Immunohistochemical Preparation

Paraffin was removed from the sections (5 μm thick) and rehydrated according to conventional histological techniques (Santos *et al.*, 2013). The nonspecific binding sites were blocked by incubation in 2% IgG-free bovine

serum albumin (BSA, Sigma). Afterward, the specimens were incubated with 0.2% Triton X-100. The sections were incubated overnight at 4 to 8^{0}C with primary antibodies: glial fibrillary acidic protein (GFAP) (1: 500, Dako A/S, Denmark) to mark astrocytes. A modified unbiased stereological protocol was used to quantify GFAP labeling in the hippocampus. GFAP-labeled cells were counted in every sixth section at 400×. Taking (n=5) of rats per treatment, the results were presented as the average number of cells in each section. In all cases, labeled cells were focusing on the CA1 region of the hippocampus, and a cell was counted using ImageJ software if it was within or touched two cell diameters of the CA1.

2.6 Assay of Lipid Peroxidation (MDA)

The formation of lipid-soluble compounds was measured using an established method described previously (Pérez-Severiano *et al.*, 2004). Four milliliters of a chloroform-methanol mixture (2:1, v/v) was added to aliquots of 1 mL from previous homogenates. Samples were stirred and placed on ice for 30 min in the dark. The upper phase was discarded. The results were expressed as micromoles per milligram of protein (Díaz *et al.*, 2016).

2.7 Measurement of Nitric Oxide (NO)

Nitrite (NO^{2-}), a stable breakdown product of NO, was measured using the Griess Reagent System (Promega, Madison, WI) (Shoaib & Perkins, 2020). Absorbance was measured using a 540-nm filter in a Beckman spectrophotometer. Results were expressed as micromoles of nitrite per milligram of protein (μM of NO^{2-}/mg of protein).

2.8 Statistical Analysis

Data obtained from the study were expressed as Mean ±SEM and were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

3. RESULTS

3.1 Relative Brain Weight

After concurrent administration of caffeine plus alcohol, at the end of the experiment, the caffeine-alcohol group (as illustrated in figure 1 below) was the only experimental group with a significantly reduced brain weight compassed with the control at $p<0.05$ as revealed by Bonferroni's multiple comparison test, whereas the caffeine and alcohol groups alone had insignificantly reduced values compared with the control.

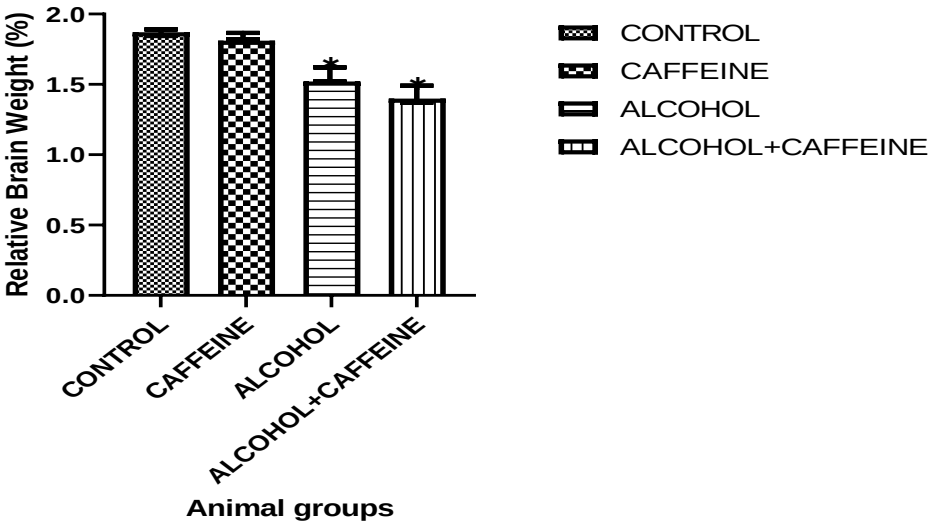

Figure 1: Chart showing Relative Brain Weight of the hippocampus of rats. The values show the Mean±SEM. * $p< 0.05$

3.2 Biochemical Evaluation of Oxidative Stress

Figures 4 and 5 show the graphical data obtained from lipid peroxidation and nitric oxide, which are markers of oxidative stress. The results showed that the treated rats administered with a combination of caffeine and alcohol had the highest lipid peroxidation and also showed the highest value for nitric oxide in the hippocampus (435% and 574%) when compared to the control with 152% and, 318% values, respectively, while the groups with caffeine and alcohol only also showed significant values of (338% and 385% respectively) in lipid peroxidation and (398% and 443%) in nitric oxide, but the values obtained in the caffeine and alcohol groups only have low values when compared with the combination of caffeine and alcohol, although they have significant values.

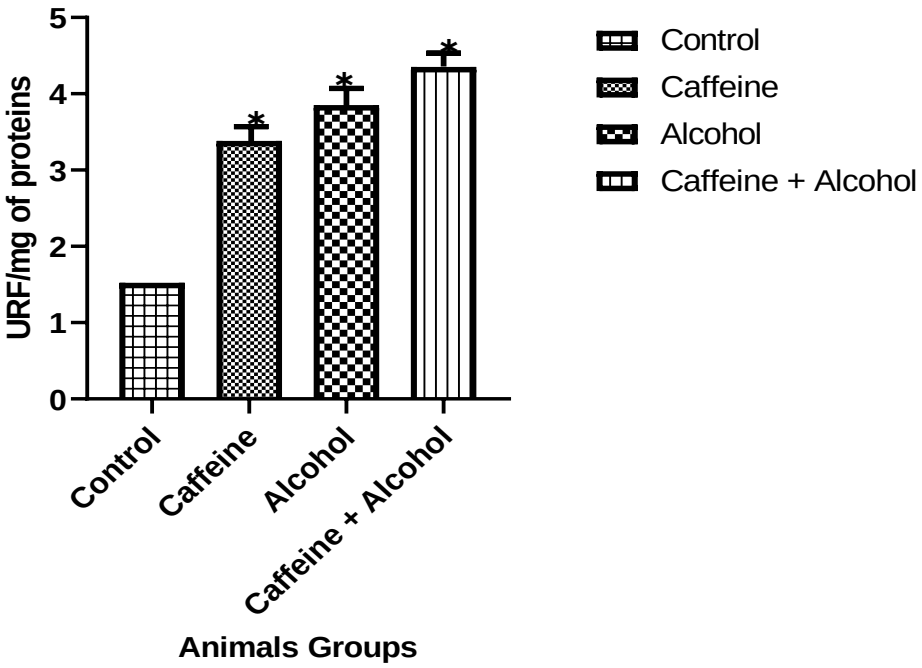

Figure 2: Chart showing the effect of the combination of alcohol and caffeine on lipid peroxidation in the rat hippocampus. The values show the Mean±SEM * p< 0.05

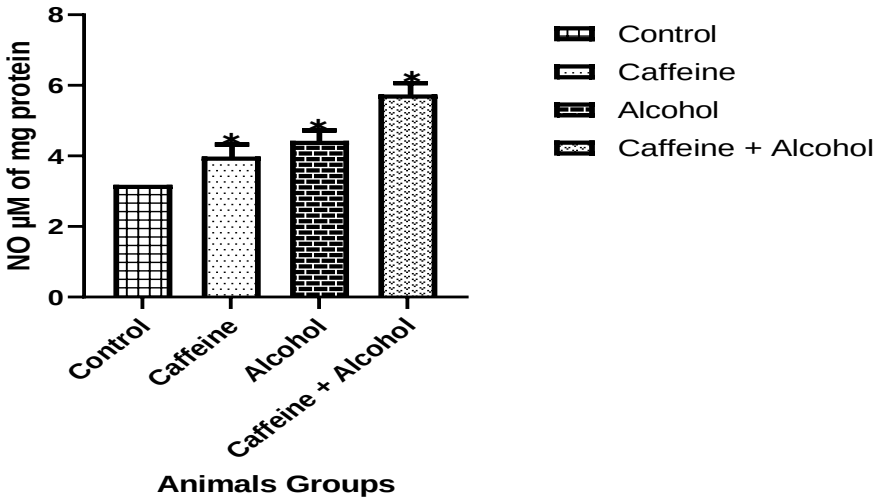

Figure 3: Chart showing the effect of the combination of alcohol and caffeine on Nitric Oxide (NO) in the hippocampus of rats. The values show the Mean±SEM. * p< 0.05.

Percentage of Neuronal Loss

The neuronal loss seen in the alcohol and caffeine + alcohol treated groups was significantly higher when compared with the caffeine group, but a significantly higher value was seen in the caffeine + alcohol group, as seen in figure 4 when compared to both caffeine and alcohol groups.

Pyramidal cell diameter

The pyramidal cell diameters seen in the alcohol and caffeine plus alcohol treated groups were significantly higher than those in the control and caffeine groups, but there was a higher value recorded in the alcohol group,while the caffeine plus alcohol group had the highest significant value, as seen in figure 5.

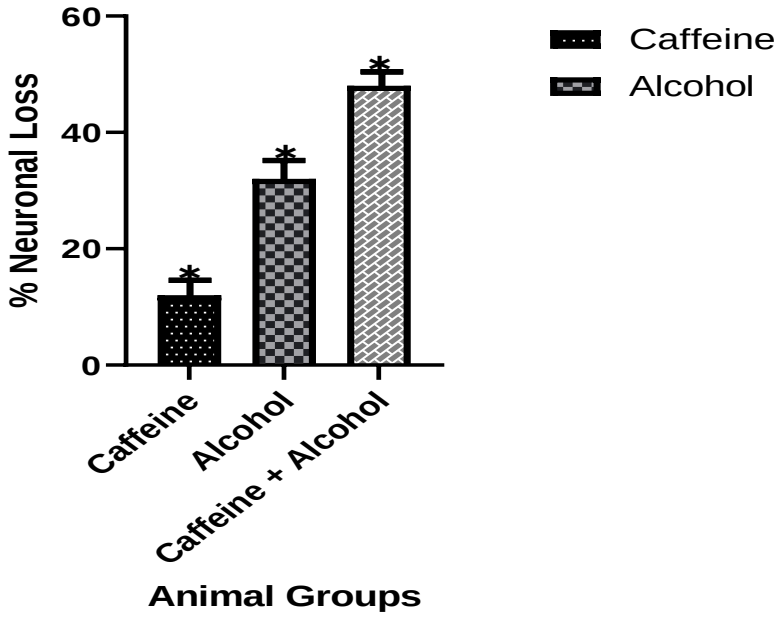

Figure 4: Chart showing the percentage neuronal loss in the hippocampal CA1 of rats. The values show Mean ±SEM. *p<0.05

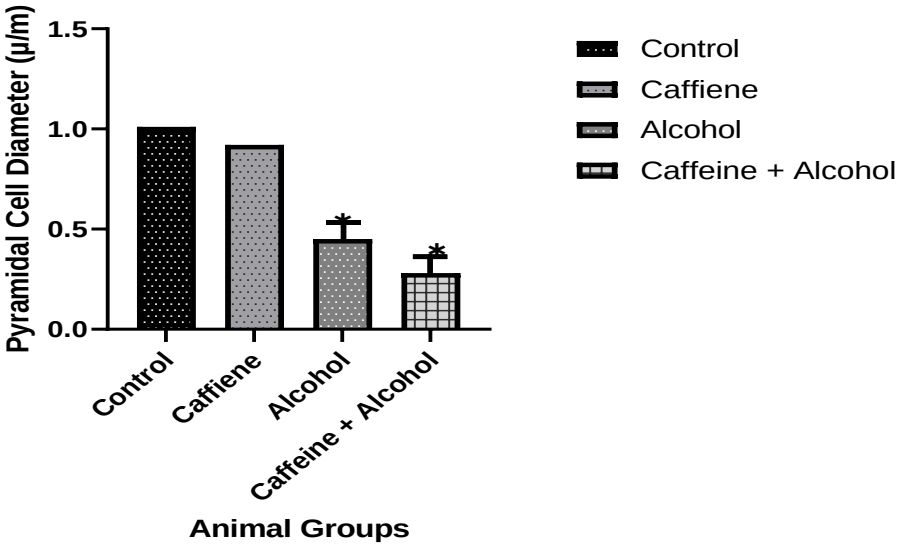

Figure 5: Chart showing cell diameter in hippocampal CA1 of rats. The values show Mean ±SEM. *p<0.05

3.3 Histological staining

3.3.1 Hematoxylin and Eosin (H&E) Stain

The figure below showed H&E-stained sections of the rat hippocampal CA1 subfield. The control group showed normal histological architecture; the caffeine group showed normal pyramidal neurons with few neuronal losses: the alcohol group indicated distorted, pyknotic and vacuolations in pyramidal neurons (arrow), whereas the alcohol + caffeine group showed wide spread vacuolations (arrow) and loss of axonal connections with several pyknotic neurons.

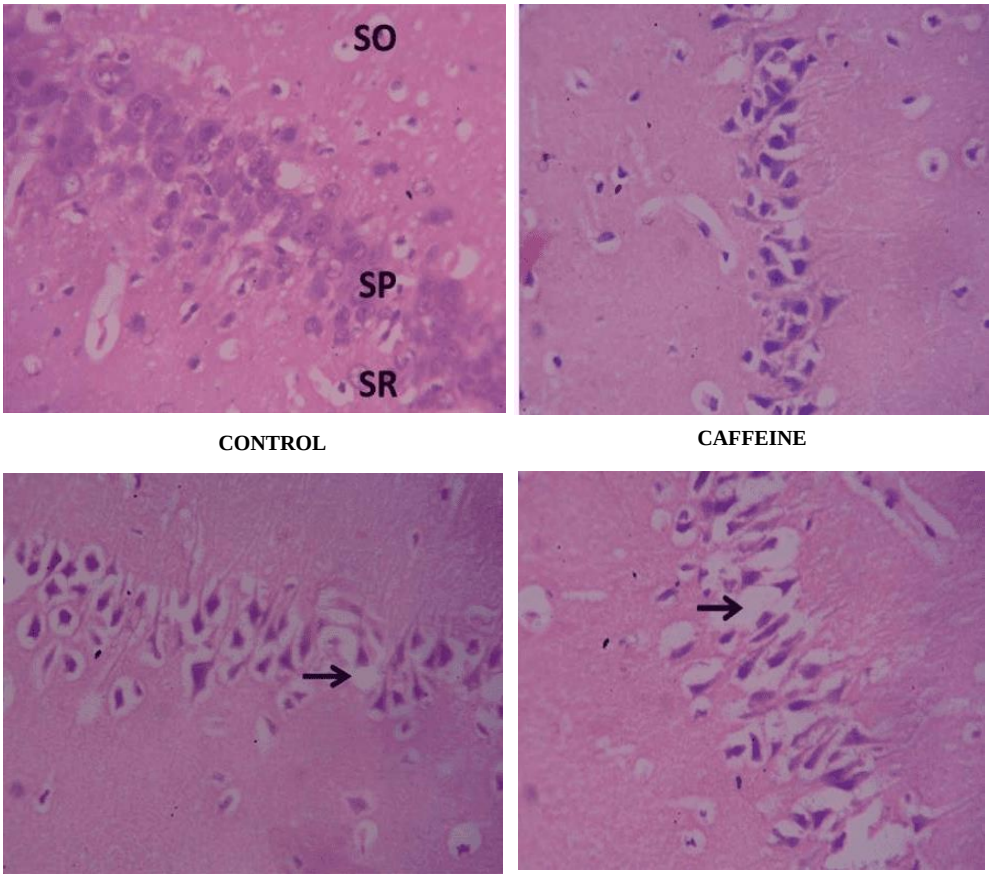

Figure 6: CA1 region in rat. Hippocampus; Rat neurons of Pyramidal layer (PL): Control group showed normal histological architecture. The Caffeine group showed normal pyramidal neurons with few neuronal losses. The alcohol group showed distorted and pyknotic and vacuolations in pyramidal neurons (arrow). Caffeine plus alcohol group showed widespread vacuolations (arrow), loss of axonal connections, and several pyknotic neurons. H&E stain, 400

3.3.2 CRESYL FAST VIOLET (CFV) STAIN

The histological outline of the hippocampal CA1 region of rats is presented in figure 7. The hippocampal outline of the rats in the control group showed normal morphology of neurons. The hippocampal profile of the rats in the caffeine group showed few degenerating neurons with loss of Nissl's substance. The hippocampal CA1 region of rats in the alcohol group showed that the neuronal outline was with loss of neuronal connections and Nissl's substance with complete loss of cytoplasmic content and nuclear material (arrows). The caffeine plus alcohol group showed a neuronal outline with markedly distorted neuronal connections, loss of cytoplasmic contents and Nissl substance (black arrow), neuronal vacuolations (blue arrow) loss of perinuclear Nissl granules, degeneration

of axonal systems, loss of connections to adjacent neurons and atrophy of neurons; these observed characteristics showed features of apoptosis and/or necrotic cell death.

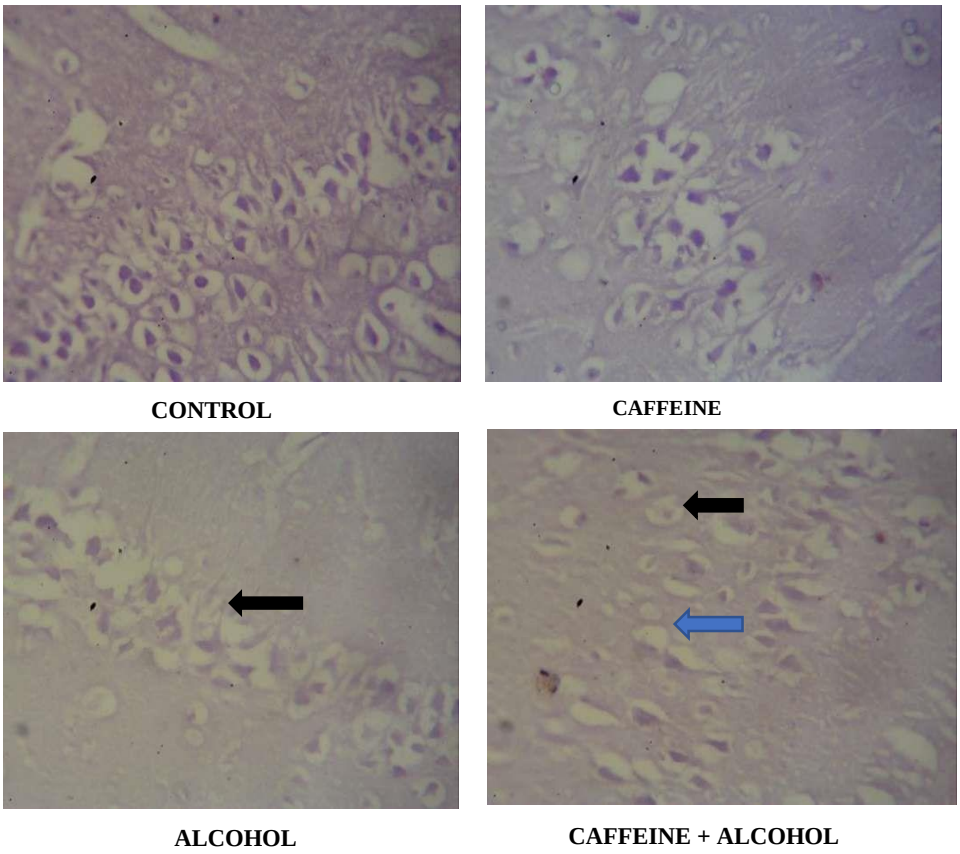

Figure 7: CA1 region in rat. Hippocampus; Rat neurons of Pyramidal layer (PL): Control group presents with normal morphology of neurons. Caffeine group showing few degenerating neurons with loss of Nissl substance. The alcohol group showed that the neuronal outline was with loss of neuronal connections and Nissl's substances with complete loss of cytoplasmic content and nuclear material (arrow). The caffeine + alcohol group showed that the neuronal outline was with markedly distorted neuronal connections, loss of cytoplasmic contents and Nissl substance (black arrow), neuronal vacuolations (blue arrow) loss of perinuclear Nissl granules, degeneration of axonal systems and loss of connections to adjacent neurons and atrophy of neurons. CFV stain, 400.

3.4 Evaluation of Proinflammatory Cytokines

The level of TNF- α in the hippocampus showed that treated rats administered alcohol combined with caffeine had the highest significant value (31.30 ± 0.02) with respect to other groups. The group treated with alcohol also presented with a higher significant value (24.23± 0.01) when compared with the control (17.01± 0.01) and caffeine group (18.34 ± 0.02). There was no significant difference in caffeine compared with the control group as shown in figure 8.

The level of IL-1β showed in treated rats administered with caffeine combined with alcohol had the highest significant value (33.76 ± 0.02) with respect to other group. These group treated with alcohol also presented with a higher significant value when compared to control and caffeine groups as seen in figure 12 below.

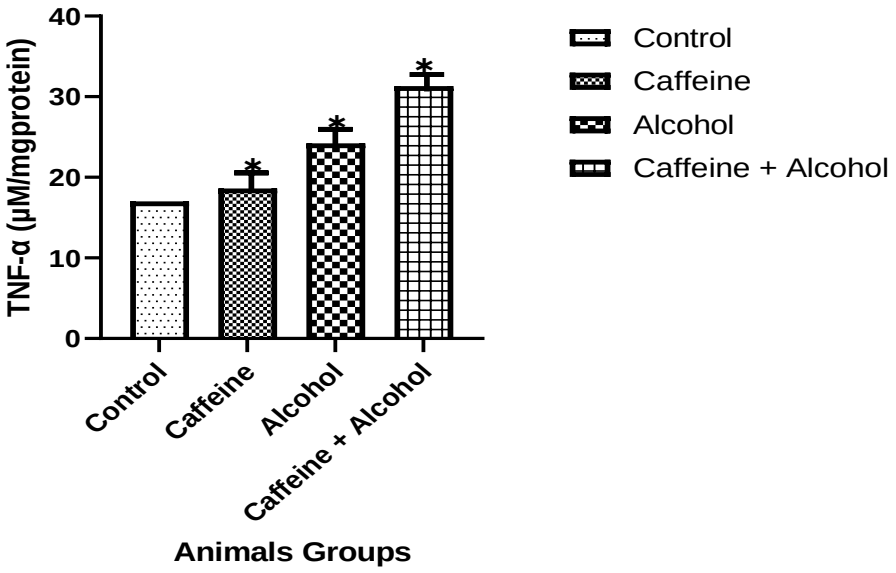

Figure 8: Serum concentration of TNF-α in the hippocampus of rats. The values show Mean ±SEM. *p<0.05.

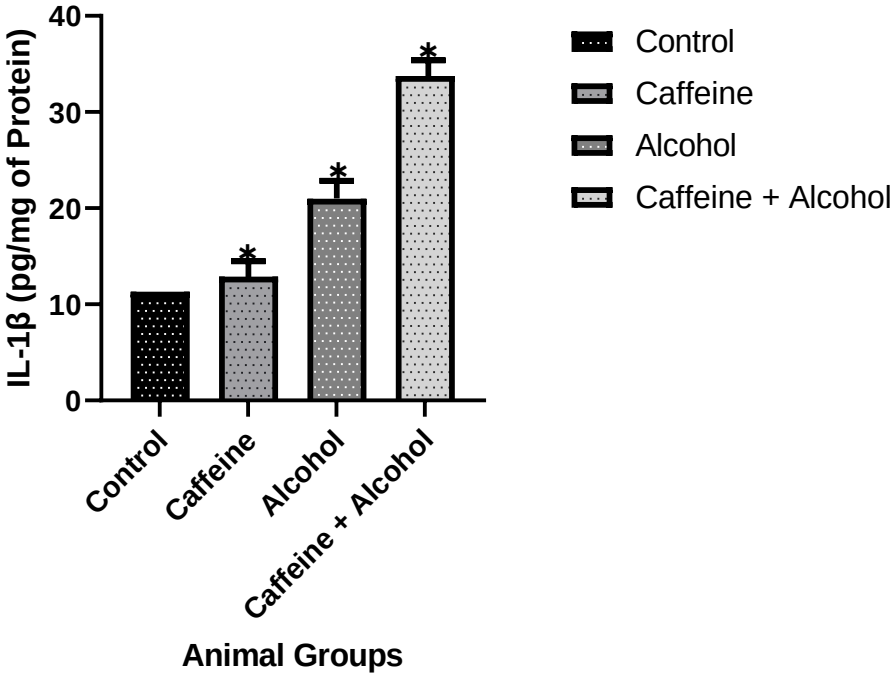

Figure 9: Serum concentration of IL-1β in the hippocampus of rats. The values show Mean ±SEM. *p<0.05.

3.5 Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity

To understand the effects caused by the combination of caffeine and alcohol on the inflammatory response in the hippocampal CA1 region of rats, the immunoreactivity to GFAP and the concentrations of TNF- α and IL-1β were measured in the brain region of interest. Figure 10 shows immunoreactivity to GFAP (brown color). The results showed that rats treated with caffeine, alcohol, and caffeine plus alcohol showed increased immunoreactivity to GFAP in the CA1 region of the hippocampus when compared with that of the control group. However, the immunoreactivity to GFAP in groups treated with alcohol and caffeine plus alcohol showed a higher proportion distribution when compared with both control and caffeine groups although that of the caffeine group

was higher when compared with the control group. The proportion of distribution of the immunoreactivity in the caffeine plus alcohol group was higher when compared with the other groups. Therefore, the number of immune-positive cells in the CA1 subfield showed that consuming a mixture of caffeine and alcohol induces higher immunoreactivity to GFAP when compared with control, caffeine and alcohol, as shown in figure 11 (one-way ANOVA with significance of $p<0.05$).

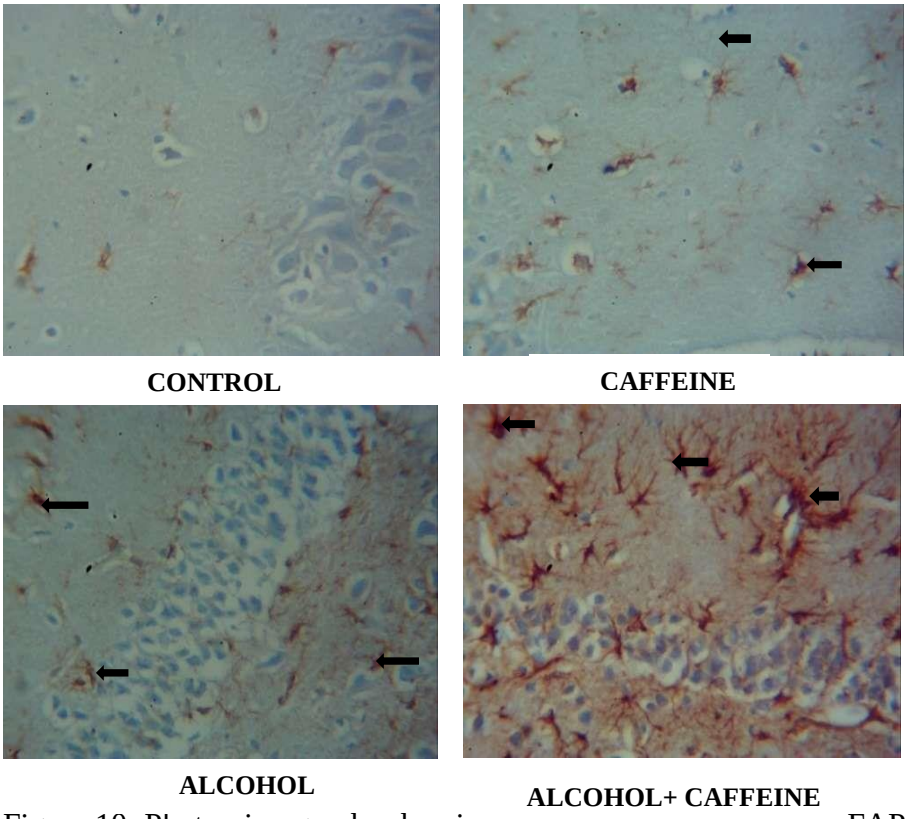

Figure 10. Photomicrographs showing the immunoreactivity to GFAP (brown color) of the CA1 subfield of the rat hippocampus across the groups. GFAP 400.

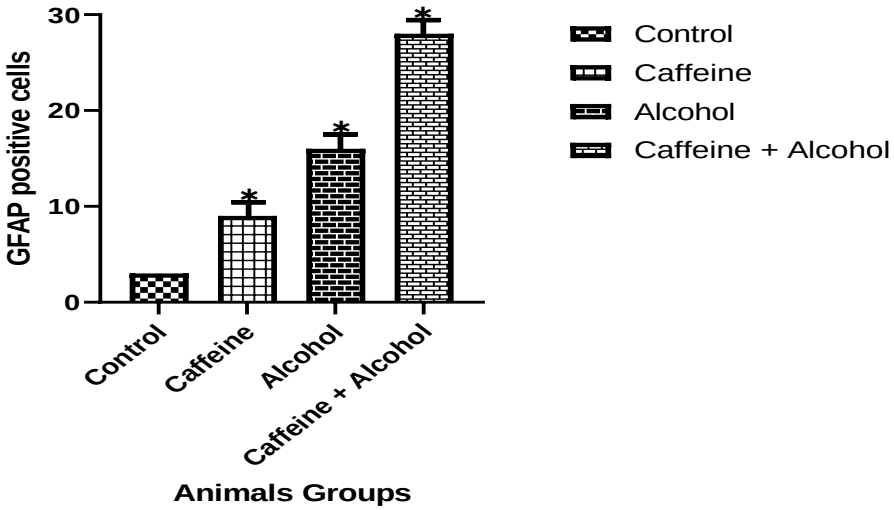

Figure 11: Quantitative data of the number of GFAP-positive cells in the CA1 region of the hippocampus of the treated groups represented as Mean±SEM. * $p<0.05$.

4. Discussion and Conclusion

In recent years, there has been an excessive increase in the abuse and use of psychoactive drugs by the general public, including the use of minor tranquilizers. However, there is a dearth of information to determine the impact of such drugs on the hippocampus. Co-administration of caffeine and alcohol for a consecutive period of 30 days showed that the combination of these two drugs had deleterious effects on the region of the brain studied. It was found that the combination of the two drugs actually causes oxidative stress, inflammation, and apoptosis in the CA1 region of the hippocampus, the area believed to be more vulnerable to neurotoxins. It has been extensively studied that caffeine at a very low dose improves learning and memory (Ruggiero *et al.,* 2022). The recognition of inflammation and neurodegeneration as common mechanisms of disease in neurological and neuropsychiatric diseases has brought to an increasing interest of the neuroscience community in caffeine-alcohol consumption (Du *et al.,* 2014). In this study, we chose a high dose of caffeine (10mg/kg) for the main reason that a lower dose of caffeine has been shown to be safe (Ruggiero *et al.,* 2022), therefore, it would be of interest to extend the present study to higher doses of caffeine and alcohol, especially in the area of the brain responsible for learning and memory. The main finding of this study includes a significant overall degeneration and inflammation in the pyramidal neurons of the CA1 region of the hippocampus of rats treated with a combination of both drugs for a duration of 30 days. These effects were markedly significant in the group of rats administered both caffeine and alcohol simultaneously. The distortion and neuronal loss of the hippocamp of the rats co-administered with caffeine and alcohol may be related to some alteration in some neuronal functions associated with the hippocampus that could be detrimental and injurious to the functions of learning and memory because it has been noted that hippocampal CA1 is one of the three brain areas that are most vulnerable to oxidative stress and various neurotoxins, which may lead to the decline in the functions meant to be carried out (Fakunle *et al.,*2013).

It was shown from this study that alcohol and a combination of caffeine and alcohol confer adverse effects on brain weight and the popular cell population of hippocampal CA1. The distorted brains reported in this study are in conformity with the earlier findings of Fakunle *et al.*, (2013) which proved that chronic intake of alcohol accounted for brain weight loss. The relative brain weight loss recorded showed that there was a significant decrease in groups administered with alcohol and the one that took a combination of caffeine and alcohol when compared to the control and also when compared to the caffeine group as shown in figure 1, studies have shown that alcohol leads to the impairment of the absorption of nutrients and structural brain shrinkage has been implicated in the chronic consumption of ethanol (Nutt *et al.,* 2021).

The findings from the present study have shown various histopathological changes in the hippocampal CA1 subfield in Wistar rats following the administration of caffeine plus ethanol. The histopathological changes in the CA1 subfield of the hippocampus in caffeine plus alcohol-treated rats appeared to be in the form of neurodegenerative and atrophic changes in the pyramidal neurons, where some pyramidal cells appeared pyknotic and, shrunken with neuronal loss, vacuolization, and axonal loss connection. The increased significant neuronal cell loss was observed in the three treated groups, although the neuronal loss observed in alcohol and caffeine plus alcohol was higher than that seen in the caffeine treated group alone and was highest in the caffeine plus alcohol group. According to Kamar et al. (2020), there was a total reduction in the number and length of total dendrites after treatment with caffeine and taurine.

In addition, the pyramidal cell diameter was significantly reduced in both alcohol and caffeine plus alcohol groups compared with the control and caffeine groups alone. Although the group that reduced cell diameter, it was not significant compared with the control. The various histopathological presentations in the caffeine plus alcohol-treated sections may be attributed to the deleterious effect of alcohol combined with caffeine because there appeared to be no changes in the histological outline of the group treated only with caffeine, although the

deleterious effect was the neuronal cell loss shown in the alcohol-treated group only, but it was not as significant as the one seen in the group treated with both drugs. This suggested that caffeine in combination with alcohol and not caffeine alone has a greater deleterious effect on the CA1 of the hippocampus. This contradicts a study by Han *et al.,* (2007), which showed that chronic consumption of caffeine could inhibit hippocampal-dependent learning and memory through inhibition of hippocampal neurogenesis.

The main finding of this study includes significant overall degeneration and inflammation in the CA1 subfield of the hippocampus of rats treated with a combination of caffeine and ethanol for 30 days. This effect was markedly significant in the CA1 region of the hippocampus of rats in the caffeine plus alcohol group. The distortion and neuronal disruption of the hippocampus of rats co-administered with both drugs may be related to some alteration in some neuronal functions associated with the hippocampus that could be detrimental to normal well-being. The observed alterations in the CA1 of the rats in caffeine plus ethanol occur because of the higher and/or marked neurotoxic effect(s) of the combined effect of both drugs

Studies have shown that alcohol confers neurotoxic effects on several regions of the brain (Jacobson *et al.,* 2022; Fakunle *et al.,*2013). In this study, it was observed that oral administration of 30% v/v ethanol for 30 days conferred neurodegenerative and inflammatory characteristics on the neurons in the CA1 region of the hippocampus of alcohol-treated rats. Pathological cell death is known as necrosis and could occur from extrinsic factors such as toxins and trauma (Nutt *et al.,* 2021). In cellular necrosis, there is marked disruption of cellular membranes, and the structural and functional integrity of the cell is altered.

It has been reported that the combined use of alcohol and caffeine exerts marked effects on behavior and causes a decreased content of 5-HT and 5-HIAA in many regions of the brain (Heinz *et al.,* 2013).

However, this study showed that a higher dose of long-term consumption of caffeine plus alcohol could impair memory, as seen in the results

Therefore, this study found a marked significant distortion in the neuronal outline of the neurons in the CA1 region of the hippocampus of the alcohol, caffeine, and caffeine plus alcohol group compared with the control and caffeine and alcohol treated groups, respectively. Rats administered alcohol during the study period displayed a comparatively slight alteration in the neuronal outline of the neurons in the CA1 region of the hippocampus.

To understand the effects caused by the combination of alcohol and caffeine on the inflammatory response in the Hippocampus of rats, the immunoreactivity to GFAP and the concentration of IL-1β and TNF-α were evaluated in the CA1 region of the hippocampus. Figure 10 shows the immunoreactivity to GFAP in the four study groups. The results indicate that, for animals treated with caffeine, alcohol and caffeine plus alcohol, the immunoreactivity to GFAP increased when compared with the photomicrographs of the control group. In particular, the increased GFAP immunoreactivity in the caffeine plus alcohol group was distributed in greater proportion compared with the groups treated separately with caffeine or alcohol. The quantitative data for GFAP-positive cells show that the administration of caffeine mixed with alcohol increased the number of reactive astrocytes compared with the control group (Figure 11. Likewise, the group administered both drugs produced astrogliosis greater than that produced by the caffeine and alcohol groups (one-way ANOVA with significance of $P < 0.05$). Similarly, the number of GFAP-immune positive cells indicates that consumption of caffeine with alcohol induces greater immunoreactivity to GFAP, when compared with consumption of caffeine, alcohol, and the control alone (Figure 11 (one-way ANOVA with significance of $P < 0.05$).

The concentrations of cytokines determined from the homogenates of the Hippocampus of rats after 60 days of consumption of caffeine and alcohol as well as the combination of caffeine plus alcohol are shown in Figures 8 and 9. The levels of IL-1β clearly show that rats that were given a combination of caffeine and alcohol recorded

the highest concentration of IL-1β, when compare to the other groups (one-way ANOVA with significance of $P < 0.05$). Similarly, TNF-α levels from rats treated with caffeine and alcohol were also significantly higher in the brain region of interest when compared with the control, alcohol, and caffeine- treated groups, respectively (one-way ANOVA with significance of $P < 0.05$). To determine the effect of concurrent consumption of caffeine and alcohol concentration of nitrite (stable metabolite from NO) in the hippocampus, the tissues were examined at 60 days of administration. NO concentration was evaluated indirectly by measuring nitrite (NO^{2-}) from the homogenates of the Hippocampus of the different groups. Figure 4 shows that NO^{2-} levels in the hippocampus of the caffeine plus alcohol group were significantly higher than those in the control group (one-way ANOVA with significance of $P < 0.05$).

The data obtained from the lipid peroxidation measurements are shown in Figure 3. The results indicate that rats that consumed the combination of caffeine and alcohol presented the highest lipid peroxidation levels when compared with the caffeine and alcohol groups, whereas the control group showed statistically reduced levels when compared with the treated groups (one-way ANOVA with significance of $P < 0.05$).

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