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Neurohistological Degeneration of the Hippocampal Formation Following Chronic Simultaneous Administration of Ethanol and Acetaminophen in Adult Wistar Rats (*Rattus norvegicus*)

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ABSTRACT

Hippocampal formation is involved in learning and memory and has also been reported to be sensitive to neurotoxic insults. However, little has been reported on the chronic simultaneous intake of ethanol and acetaminophen despite their degrees of abuse and misuse as regards hippocampus. In this study, forty adult wistar rats of average weight 150±20.2 g were randomly distributed into four groups of treatments T_1, T_2, T_3 and control C (N = 10). For a period of six weeks, animals in group T_1 received 100 mg kg^{-1} b.wt. acetaminophen and 25% ethanol in 2% sucrose solution while group T_2 animals received 25% ethanol in 2% sucrose solution. T_3 animals were given 100 mg kg^{-1} b.wt. acetaminophen and group C animals were given only distilled water. The animals were sacrificed by whole body intracardiac perfusion fixation and the regions of hippocampus were dissected out using Paxinos stereotaxic coordinate method. Brain specimens were processed for routine histological techniques, sectioned at 6 μ and stained for nissl's substance. Significantly reduced neuronal density ($p>0.05$) of 44 and 38% neuronal loss in CA3 subfield, respectively in treatment groups T_1 and T_2 compared to control group was recorded. Also, marked degeneration of pyramidal neurons in the regions of CA1 and CA3 of treatment groups that received 100 mg kg^{-1} b.wt. acetaminophen and 25% ethanol in 2% sucrose solution as well as animals that received 25% ethanol in 2% sucrose solution, respectively with mild degenerative effects in the group that took 100 mg kg^{-1} b.wt. acetaminophen compared to the control group was also observed. These alterations observed following exposure to chronic simultaneous administration of ethanol and acetaminophen point to possibilities of higher memory impairments and learning deficits which are of very strong public health concern.

Key words: Adult brain, acetaminophen, hippocampus, pyramidal neurons, nissl's substance

INTRODUCTION

Acetaminophen is considered a Non-steroidal Anti-inflammatory Drug (NSAID), even though in clinical practice and in animal models it shows little anti-inflammatory activity (Botting, 2000). However, like NSAIDs, acetaminophen is used to treat pain and fever and it has become one of the most popular 'over-the-counter' non-narcotic analgesic agents (Posadas *et al*., 2010). The characteristic feature of alcohol use disorders is the consumption of dangerous amounts of alcohol despite the knowledge that problems occur during drinking (Crews and Nixon, 2009). Over the coast of Africa, more than half of the heavy duty workers are involved in both conscious and unconscious chronic abuse and misuse of acetaminophen as a pain reliever and ethanol being a central nervous system depressant. The brain is very susceptible to oxidative stress, in particular

the hippocampus which has limited Reactive Oxygen Species (ROS) scavengers and anti-oxidant capacity as mounting evidence suggests that ethanol exerts effects on learning and memory by altering cellular activity in the hippocampus and related structures (White *et al*., 2000). Just as high doses of acetaminophen have traditionally been associated with neurodegenerative disorders due to implications of oxidative stress (Pacheco *et al*., 2009) in the brain, severe cognitive impairment consistently occurs in chronic alcoholism regardless of the presence of associated thiamine deficits (Korsakoff syndrome) and it includes progressive and severe anterograde learning deficits, implicating impairment in hippocampal circuits (Herrera *et al*., 2003). The toxicity of acetaminophen depends primarily on the balance between the rate of formation of the hepatotoxic metabolite and the rate of glutathione synthesis in the liver. This study is expected to improve our understanding on the cellular integrity of hippocampus when assaulted chronically and simultaneously by ethanol and acetaminophen.

MATERIALS AND METHODS

This study was carried out in the year 2010. Forty adult healthy wistar rats of both sexes of average weight 200±3.23 g were maintained under standard laboratory conditions for an acclimatization period of 2 weeks in the animal holdings of Anatomy Department, Ladoke Akintola University of Technology Ogbomoso. During this course, the rats were fed with standard laboratory mouse chow (Ladokun feeds, Ibadan) and were given water *ad libitum*. Daily weights were taken and documented.

After acclimatization, the rats were randomly assigned into four groups (N = 10) such that T_1, T_2 and T_3 served as treatment groups, while C served as the control group. T_1 received 100 mg kg^{-1} body weight acetaminophen and 2% sucrose in 25% ethanol solution as their drinking water, T_2 took 2% sucrose in 25% ethanol solution as their drinking water while T_3 received 100 mg kg^{-1} body weight of acetaminophen and distilled water. The 2% sucrose in 25% ethanol solutions were replaced afresh daily at 18.00 h G.M.T. The rats in group C received distilled water *ad libitum*. All the animals were exposed for a period of 6 weeks.

Changes in body weights and volume of ethanol consumed were documented. Bottles containing absolute ethanol were obtained from Sigma Laboratory Ltd, San Francisco, USA, while acetaminophen was obtained from Emzor Pharmaceutical industrial limited, Nigeria.

At the end of administration, all the rats were sacrificed by whole body vascular intracardiac perfusion fixation. The thorax was carefully cut opened and the heart was exposed; a needle connected to the tubing from the fixative bottle was inserted into the left ventricle. The right atrium was cut open to drain out the blood and fixative. First 20-30 mL of saline was passed transcardially to flush out the blood and then perfuse with 10% formal calcium fixative. Fixation was monitored by the gradual discolorations of the tongue and eyeball. After fixation has been established, the brain specimens were further kept in 10% formal calcium fixative for 72 h. Regions of hippocampus were then dissected out using Paxinos stereotaxic coordinate method (Paxinos and Waston, 1998). The brain sections were processed for routine histological techniques sectioned at 6 μ and stained using Cresyl violet as described by Venero *et al*. (2000) for nissl's substance. Qualitative observations of CA1, CA2 and CA3 areas of hippocampus were done. Every 10th section was chosen from each animal. Using bright field compound Nikon microscope, YS100 (attached with Nikon camera), the slides were examined and photographed under 400X objective. For each slide, two areas of CA1, one area of CA2, two areas of CA3 were randomly selected. Using Image-pro Express software, count of neurons with prominent nucleolus within a measured rectangular area was performed in the selected regions. The absolute neuronal density per unit area of section for each region was estimated as described by Abercrombie (1946).

Statistical analysis: The data were analyzed using the computerized statistical package 'SPSS Version 11'. Mean and Standard Error of Mean (SEM) values for each experiment group was determined. The means were compared by analysis of variance at a level of significance of 95 and 99%. Independent samples t-test was performed on the counts of each area (CA1, CA2 and CA3) to determine if there is any statistically significant difference in absolute neuronal count between the control and treatment groups.

RESULT

Body weights: Treatment group T_1 and T_2 showed significantly decreased body weight ($p<0.05$) of Mean±SEM (138.7±6.63 and 144.8±14.53 g), respectively compared to the control group of Mean±SEM (213.3±5.59 g) as seen in Table 1. Treatment group T_3: At the end of the first week of acetaminophen administration, rats in this group presented an insignificant ($p>0.05$) decrease in body weight of Mean±SEM (168.4±3.24 g) compared to the initial weight of Mean±SEM (170.5±1.86 g). Continuous administration of acetaminophen during the 2nd, 3rd, 4th and 5th week showed a gradual increase in body weight to the end of the 6th week as Mean±SEM (209.6±10.20 g). However, this was insignificant ($p>0.05$) as compared to the control group of mean±sem (213.3±5.59 g) as seen in Table 1.

Feed consumption pattern: Ethanol was observed to severely depressed feeding as the two treatment groups T_1 and T_2 show a significantly ($p<0.001$) of Mean±SEM (200.0±9.3 and 200±8.6 g) compared to the control group of Mean±SEM (272.0±9.4 g) as shown in Table 2. There was no significant difference in the feeding pattern of T_3 compared to the controls.

Neuronal density: Treatment groups T_1 and T_2 showed significantly reduced neuronal densities ($p<0.05$) of Mean±SEM (1059.3±36.97 and 1173.0±0.24 cm^{-2}) (Table 3), in the CA3 region of the hippocampal subfield compared to the control section with Mean±SEM (1609.2±7.01 cm^{-2}). The three areas of hippocampus CA1, CA2 and CA3 showed higher neuronal densities cm^{-2} in

Table 1: Body weight distribution at the end of experiment

		Groups			
Weeks	No. of rats	C	T_1	T_2	T_3
0	10	154.8±3.53	169.3±7.00	190.8±7.98	170.5±1.86
1	10	163.8±2.18	167.8±8.06	185.6±14.37	168.4±3.24
2	10	178.3±5.19	159.3±6.97	173.0±20.24	171.2±7.01
3	10	191.3±2.06	150.5±4.94	166.6±6.46	178.8±5.82
4	10	197.8±2.81	146.7±4.98	156.4±10.19	184.4±10.79
5	10	207.8±5.51	140.6±6.80	150.7±13.92	195.1±10.53
6	10	213.3±5.59	138.7±6.63*	144.8±14.53*	209.6±10.20

Values are presented as Mean±SEM. *Significance difference at $p<0.05$ when compared with control using t-test

Table 2: Feed consumed at the end of administration

Groups	N	Mean+SEM (g)	DOF	2-Prob
T_1	10	200.0±9.3*	10.2	0.004
T_2	10	206.0±8.4*		
T_3	10	240.0±10.6		
C	10	272.0±9.4		

*Significance difference at $p<0.001$ when compared with control using t-test

Table 3: Neuronal density per cm^{-2} of section of cortical areas

	Groups			
Cortical area	C	T_1	T_2	T_3
CA1	5754.8±110.53	5019.3±124.07	5129.8±137.98	5270.5±133.86
CA2	3363.8±122.18	3067.8±118.06	3105.6±164.37	3228.4±123.24
CA3	1878.3±115.19	1059.3±36.97#	1173.0±20.24#	1609.2±97.013#

Values are presented as Mean±SEM. *Significance difference at $p<0.05$ when compared with control using t-test. #Insignificance difference at $p>0.05$ when compared with control using t-test

Table 4: Percent Neuronal loss in the cortical areas

	Groups		
Cortical area	T_1	T_2	T_3
CA1	13	11	9
CA2	9	8	5
CA3	44	38	15

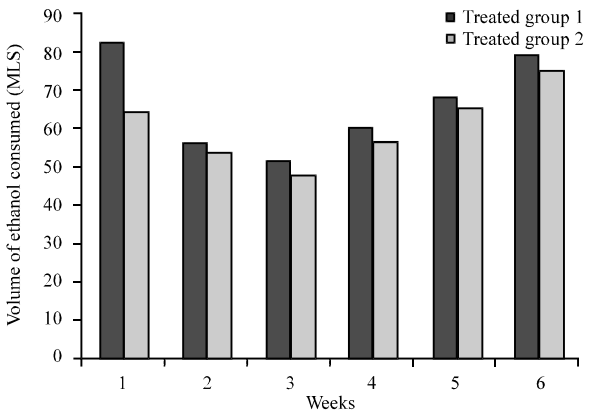

Fig. 1: Histogram of the ethanol consumption pattern

treatment groups T_3 than in treatment groups T_1 and T_2 compared to the control group. However, the percentage reduction in the neuronal density for CA1, CA2 and CA3 subfields are 13, 9 and 44%, respectively for T_1, 11, 8 and 38%, respectively for T_2 and 9, 5 and 15% for T_3 as shown in Table 4.

Ethanol consumption pattern: The two treatment groups T_1 and T_2 showed an initial decrease in volume of ethanol consumption in the first three weeks as shown in Fig. 1. However, gradual increase in the volume of ethanol consumed was seen from the end of the third week and throughout of the experiment.

Histological findings: Control sections revealed a normal neuronal cytoarchitecture in all the hippocampal subfields of CA1, CA2, CA3 and the Dentate Gyrus (DG) as shown in Fig. 2a-b. Treatment group T_1 sections showed degeneration of neurons with loss of nissl substance in the CA1, CA2 and CA3 layers as shown in Fig. 3a and b with severe degeneration of pyramidal

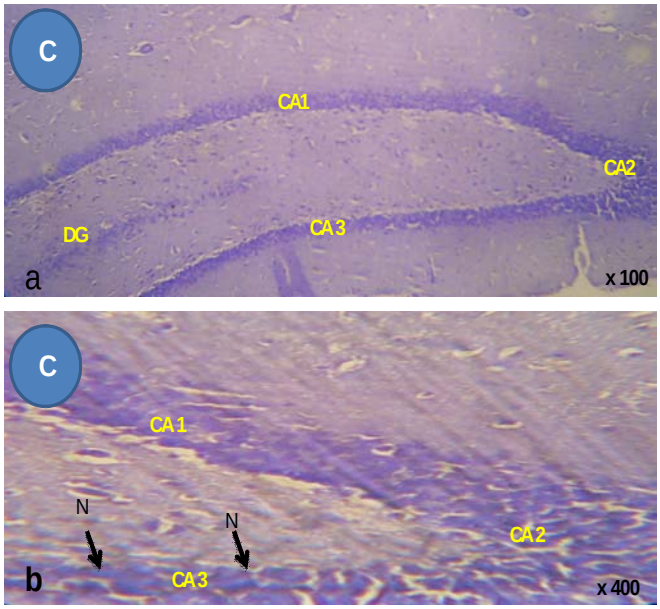

Fig. 2(a-b): Photomicrograph of the coronal section of hippocampus in the control group, showing the different subfields of the hippocampus: (a) CA1, CA2 and CA3 with the dentate gyrus (D.G.). (Cresy 1 Violet x100) and (b) CA1 region of the hippocampus presenting a normal morphology of Neurons (N) (Cresyl Violet x400)

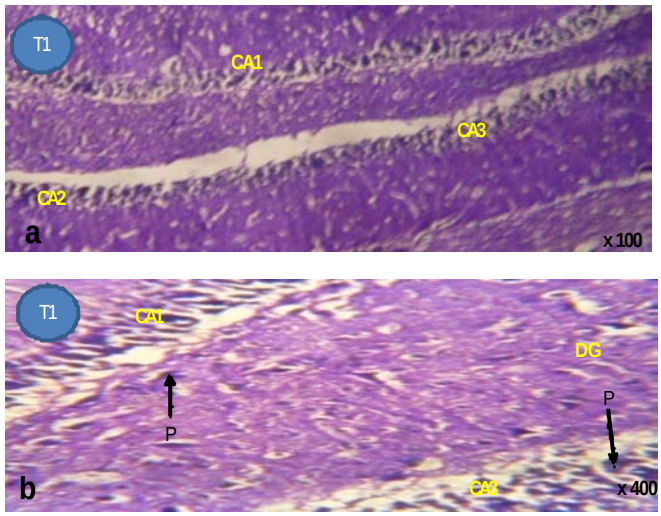

Fig. 3(a-b): Photomicrograph of the coronal section of the hippocampus in the treated group T_1, showing degeneration of neurons (N) with loss of nissl substance in the (a) CA1, CA2 and CA3 layers of the hippocampus (Cresyl Violet x100) and (b) CA1 and CA3 layers of the hippocampus with severe degeneration of pyramidal neurons(P) (Cresyl Violet x400)

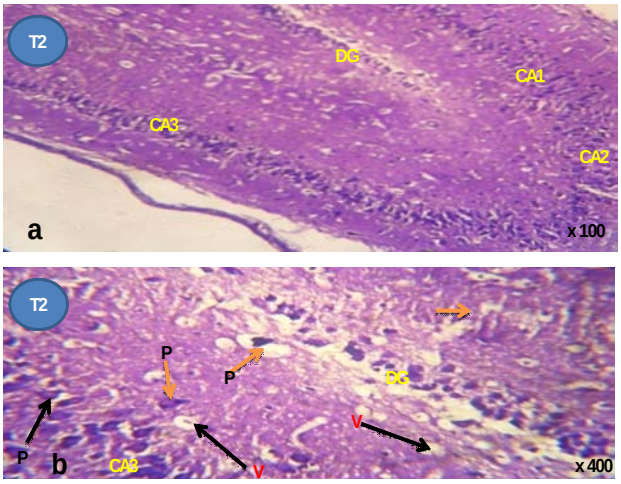

Fig. 4(a-b): Photomicrograph of the coronal section of the hippocampus in the treated group T_2, showing the (a) CA1, CA2 and CA3 layers of the hippocampus (Cresyl Violet x100) (b)Vacuolations (V), severe degeneration of neurons (P) around the Dentate Gyrus (DG) and CA3 layer (Cresyl Violet x400)

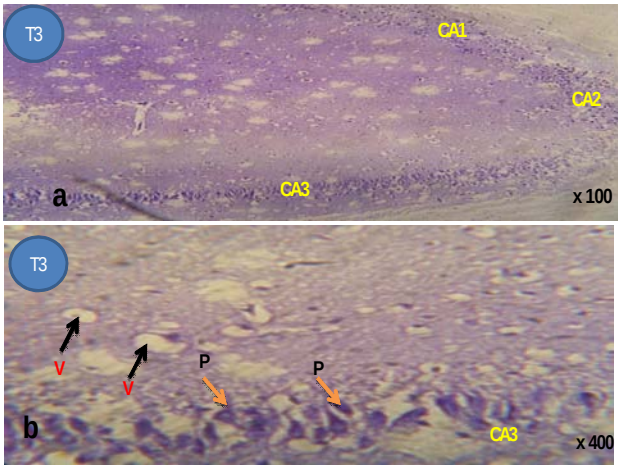

Fig. 5(a-b): Photomicrograph of the coronal section of the hippocampus in the treated group T_3, showing the (a) CA1, CA2 and CA3 layers (Cresyl Violet x100) and (b) vacuolations (V) (black arrows) with few degenerated neurons (P) Brown arrows) (Cresyl Violet x400)

neurons more noticeable in the CA1 and CA3 layers. Treatment group T_2 showed vacuolations, severe degeneration of pyramidal neurons around the Dentate Gyrus (DG) and CA3 layer as shown in Fig. 4a and b. Treatment group T_3 sections showed vacuolations many normal cells with few degenerated neurons around the CA3 layer as shown in Fig. 5a and b.

DISCUSSION

The results of this study revealed here that simultaneous intake of acetaminophen and ethanol clearly affects body weight as well as neuronal cellular distribution within the hippocampal formation. The significant ($p<0.05$) mean body weight decrease seen in the treatment groups T_1 and T_2 as shown in Table 1 can be correlated with the pattern of ethanol consumption as shown in Fig. 1, posting a pictorial representation of an initial decrease in volume of ethanol consumption in the first three weeks followed by gradual increase in the volume of ethanol consumed from the end of the third week and throughout of the experiment. The pattern of ethanol consumption when viewed in relation to the quantity of feed consumed Table 2 showed that ethanol depressed feeding significantly ($p<0.01$) in the treatment groups T_1 and T_2. The depressed feeding noticed here can be as result of a gradual process of ethanol dependence which is the need for continued alcohol consumption to avoid a withdrawal syndrome that generally occurs from 6 to 48 h after the last drink. Hence, gradually there appears to be more urge to drink ethanol than for the feed consumption. In other words the weight loss observed here is in conformity with the earlier reports of effects of alcohol on digestion, absorption, storage, utilization and excretion of essential nutrients such as vitamins, minerals and proteins (Lieber, 2003). Alcohol impairs nutrient absorption by damaging cells lining the stomach and intestines and disabling transport of some nutrients into the blood. Alcohol also inhibits the breakdown of nutrients into usable substances, by decreasing the secretion of digestive enzymes from the pancreas. There have been many reports claiming that the hepatotoxicity of paracetamol (acetaminophen) is increased in chronic alcoholics and that such individuals not only carry an increased risk of severe and fatal liver damage after acute overdosage but that similar serious liver damage may also occur with 'therapeutic' use (Andreu *et al*., 1999). In the original studies of the mechanisms of toxicity, paracetamol was found to cause liver damage through conversion by hepatic cytochrome P450 enzymes to a minor but toxic intermediate metabolite and this was subsequently identified as N-acetyl-p-benzoquinoneimine. Histological observations noted in the treatment groups T_1 and T_2 showing degeneration of the neuronal pyramidal cells in Fig. 3 and 4 most especially in the CA1 and CA3 layers of hippocampal subfields as a result of ethanol and acetaminophen intake, although more pronounced in T_1 and T_2, it was very mild T_3 as seen in Fig. 5. Neuronal degeneration has been reported to result in cell death which is of two types namely apoptotic and necrotic. Ethanol elicits a rapid increase in Reactive Oxygen Specie (ROS) with increased cellular oxidative stress occurs during the acute and chronic ethanol exposure of hepatocytes which is ultimately followed by apoptotic cell death in cultured neurons (Maffi *et al*., 2008). The proposed mechanisms of ethanol-induced ROS formation include oxidative protein modification and enzyme inactivation , antioxidant depletion and mitochondrial dysfunction, all of which may contribute to apoptotic cell death (Maffi *et al*., 2008). The discovery that alcohol also inhibits the ongoing genesis of neurons and glia has highlighted a new possible mechanism by which alcohol results in brain volume/tissue loss or neurodegeneration. Lack of cell generation may be a key mechanism of neurodegeneration (Nixon, 2006). Indeed, in many neurodegenerative diseases, the lack of ongoing cell generation by stem cells has been hypothesized to contribute to tissue loss (Armstrong and Barker, 2001). New neurons from neural stem cells are constitutively produced in at least two regions of the normal, adult brain. Acetaminophen is mainly metabolized, in the liver, via conjugation by sulphotransferase and UDP-glucuronosyltransferase and then excreted, but a small fraction of it is metabolised by CYP2E1 (Manyike *et al*., 2000). This P-450 isoenzyme is very abundant in the liver (Gonzalez, 2007) and is also expressed in the brain, although at much lower levels where it seems to play an important role in metabolizing some

compounds like acetaminophen and ethanol. CYP2E1 is the most important P-450 isoenzyme involved in acetaminophen metabolism, although other isoforms like CYP1A2 or CYP3A might also be involved. It has long been known that induction of P-450 enhances acetaminophen-mediated liver toxicity. Acetaminophen metabolism by CYP2E1 produces a chemically reactive metabolite, NAPQI that might bind to sulfhydryl groups in cellular proteins, including mitochondrial proteins, inducing oxidative stress and leading to cellular damage and death (James *et al*., 2003). Acetaminophen-induced neuronal death exhibits the hallmarks of apoptotic death, such as DNA fragmentation and degradation, suggesting activation of the mitochondrial pathway and ROS production in AAP-mediated neuronal toxicity. The significantly reduced neuronal density ($p<0.05$) in T_1 and T_2 and an insignificantly reduced neuronal density ($p>0.05$) in T_3 in all the subregions CA1, CA2 and CA3 when compared to the control group as seen in Table 3 may have been caused by cell death due to toxic effects of ethanol and acetaminophen although this was more marked in T_1 than in T2 group with much more reduced effects in T_3 and this points to the fact that when ethanol and acetaminophen are chronically combined the aftermath effect is more deleterious than when either drug is used singly. In structures with a compact volume, estimation of the total numbers of neurons is possible such as the hippocampus, lateral geniculate body and even the superior colliculus. Neuron counting from histological sections has been used to estimate the total number or cell density in brain regions. These objective cytometric methods have been used to give the report of total cell numbers within a given region and is unaffected by change in the volume of the structure or cell size, distinguish two adjacent areas within the human prefrontal lobe and determine the effects of experimental treatments on different brain regions This study had used computer Image-Pro Express software and the sections were stained with nissl's stain which revealed the pyramidal neurons characteristically which were counted with the aid of a digital image software on a computer. The results confirmed the morphological observations. The loss of these neurons may underline adverse effects of chronic simultaneous intake of acetaminophen and ethanol resulting in impairment of the functions of CA3 as an extensive excitatory recurrent connection of region in encoding and retrieval of associations, including autoassociative completion of a single pattern, or associative retrieval of the next pattern in a sequence while the activities of CA1 involved in matching of CA3 output with afferent input from entorhinal cortex will also be impaired.

CONCLUSION

With the loss of pyramidal neurons majorly in the CA1 and CA3 areas of hippocampal formation following chronic simultaneous consumption of acetaminophen and ethanol it could be concluded that most especially, the chronic abusers of these drugs are most likely to suffer great learning and memory deficits.

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