

Modelling Rohypnol [Flunitrazepam] and Alcohol Abuse and Resultant Effects on Hippocampal Histoarchitecture and Biochemicals in Wistar Rats

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Abstract: Rohypnol and alcohol abuse is often reported among young people. Rohypnol is often abused voluntarily to erase memory of indulgence. It is also used in perpetuating crime such as in rape cases when the perpetrator administers certain doses of rohypnol to make the victim lose the memory of such event. Interestingly, young people often abuse alcohol and rohypnol. While the former is often used as a social drug, the latter is combined in certain instances to erase or reduce the memory of such. A number of literatures exist on the effects of alcohol on heath, brain and mind; but not a good number of investigations have considered the effects of rohypnol on hippocampal structure as well as the nature of its effects when combined with alcohol. This particular investigation is aimed at modelling rohypnol and alcohol abuse and resultant effects on hippocampal histoarchitecture and biochemicals in Wistar rats. Forty eight [n=48] adult male Wistar rats were divided into six groups, A-F. Group A served as the control Group B received the low dose of flunitrazepam; Group C received the high dose of flunitrazepam; Group D received the low dose of alcohol; Group E received the high dose of alcohol; Group F received the low dose of flunitrazepam and alcohol. Experiment lasted 21 days and animals were sacrificed by cervical dislocation. Results showed that the two substances did not produce extensive structural disruption of the hippocampus. However, specific regions of the hippocampus showed morphological aberrations, resulting in mild heterogeneity. Also, the enzyme chemistry of the hippocampus cytochrome C oxidase and G6PDH enzymes was altered.

Keywords: Memory, Rohypnol, Alcohol, Hippocampus, Date Rape, Abuse

1. Introduction

Flunitrazepam, trade name Rohypnol, is a central nervous system depressant in a class of drugs called benzodiazepines used in some countries to treat severe cases of insomnia. (Geller 1991). The chemical structure is the fusion of a benzene ring and a diazepoxide ring. Some examples of benzodiazepine are; Oxazepam, Diazepam, flurazepam, triazolam and lorazepam. Flunitrazepam is referred to as a “date rape drug”. Flunitrazepam was discovered by Roche as part of the benzodiazepine work led by Leo Sternbach; the patent application was filed in 1962 and it was first marketed in 1974 [1]. Flunitrazepam is illegal in the USA but is available in Mexico. In some

instances, it is used as sedative before surgery in part because it causes amnesia [2]. People administered with this drug do not remember the surgery and circumstances surrounding it [3]. It is referred to as a date rape drug because when the drug is given to a person in a club, it can facilitate a sexual assault. Street names for rohypnol include Roofies, Rophies, Rope, Roach, Roach-2, Roche, Roopies, Robutal, Row-shay, Ruffles, Wolfies, La Rocha, Lunch Money Drug, R-2, Mexican Valium, Circles, Pingus, Forget Me Pill [4].

Ethanol also commonly called alcohol, ethyl alcohol, and drinking alcohol, is the principal type of alcohol

found in alcoholic beverages, produced by the fermentation of sugars by yeasts (Mckenzie, 2001). It is a neurotoxic, psychoactive drug, and one of the oldest recreational drugs. It can cause alcohol intoxication when consumed in sufficient quantity. Ethanol is a volatile, flammable, colourless liquid with a slight chemical odour. It is used as an antiseptic, a solvent, a fuel, and due to its low freezing point, the active fluid in many alcohol thermometers. The molecule is a simple one, being an ethyl group linked to a hydroxyl group [5].

The hippocampus is a small region of the brain that forms part of the limbic system and is primarily associated with memory and spatial navigation. The hippocampus is located in the medial temporal lobe of the brain, underneath the cortical surface. Its structure is divided into two halves which lie in the left and right sides of the brain. The organ is curved with a shape that resembles a seahorse, and its name is derived from a coupling of the Greek words "hippo" for horse and "kampos" for sea. The "hippocampus" contains CA1 - CA3. CA means Cornu Amonis (Ammon's horn). The "hippocampal formation" includes CA, dentate gyrus ("tooth-like bump"), entorhinal cortex, subiculum, pre- and parasubiculum [6].

The hippocampus deals with the formation of long-term memories and spatial navigation. In diseases such as Alzheimer's disease, the hippocampus is one of the first regions of the brain to become damaged and this leads to the memory loss and disorientation associated with the condition [4]. The hippocampus can become damaged through oxygen deprivation [7]. Research has been done to show effectiveness of flunitrazepam and alcohol on memory, learning or coordinated activities, such research include the research carried out by a group of scientists showing effect of flunitrazepam on sleep and memory. Flunitrazepam's effect on sleep and memory was investigated in healthy volunteers by polysomnography (PSG) and memory testing. The results of this study suggested that impairments in memory resulted from the dose of FNZ, and that there is a possibility of a relationship between memory disturbance and REM sleep suppression caused by this benzodiazepine. Also, flunitrazepam in combination with alcohol engenders high levels of aggression in mice and rats [4].

The aim of this research was to prove the effect that flunitrazepam and ethanol used both individually or collectively has on the hippocampal functions of the juvenile mice.

2. Materials and Methods

2.1. Grouping and Treatments of Animals

The 48 male Wistar were divided into six groups. The groups were labelled as groups; A, B, C, D, E and F. These groups were administered different percentage of the drug. The administration was as follows:

Table 1. Table showing the Groups A-F of experimental animals and the administered substances.

GROUPS	DOSAGE
A	The control group, fed with food and water only
B	Low dose of flunitrazepam
C	High dose of flunitrazepam
D	Low dose of alcohol
E	High dose of alcohol
F	Low dose of flunitrazepam and alcohol

Flunitrazepam and ethanol were administered through oral gavage method. After drug administration for 21 days, animals were sacrificed by cervical dislocation. Brain tissues were excised after surgical dissection of the skull. Homogenates were prepared for the enzymes assays while other samples were fixed in formal saline for histological and histochemical demonstrations following the formalin fixed paraffin embedded histological methods.

2.2. Haematoxylin and Eosin Staining Technique for General Histoarchitecture [8]

Deparaffinizing sections were hydrated and placed in Hematoxylin for 8 - 15 minutes, then rinsed in tap water until water runs clear. Decolorizing was done in 1% acid alcohol, 3 - 6 quick dips followed by dipping in Bluing Agent, 3 - 5 long dips. Staining in Eosin for 30 seconds - 2 minutes was followed by dehydrating in 95% alcohol and 100% alcohol, 3 changes each for 2 minutes. Clearing was done in 3 changes of xylene for 2 minutes each. Specimen were mounted.

2.3. Cresyl Fast Violet for Nissl Substance [9]

Cresyl Violet Acetate solution stains Nissl substance in neurons, hence, useful in identifying neuronal structure especially in the CNS. Sectioned were de-waxed and rehydrated followed by staining in 0.1% Cresyl Violet 4-15min. Dehydrating through 2x3min changes of absolute ethanol was followed by clearing in xylene x2 and mount in DePeX.

2.4. Immunohistochemistry: GFAP - Astrocyte Marker [10]

Subjects were transcardially perfused with heparanized saline (0.9% w/v NaCl) followed by 4% w/v paraformaldehyde and 15% saturated picric acid in 0.1 M. phosphate buffer pH 7.4. Brains incubated in 20%w/v sucrose in 0.1 M PB (24 hours at 4°C) followed by freezing in isopentane (-40°C). 30 micron coronal sections cut on freezing microtome/cryostat for free floating IF.

2.5. Cytochrome C Oxidase Assay [11] [12]

Measurement of cytochrome c oxidase activity: The absorption of cytochrome C at 550 nm would change with its oxidation state. Cytochrome C was reduced with dithiothreitol and then reoxidized by the cytochrome C oxidase. The difference in extinction coefficients (De mM) between reduced and oxidized cytochrome C was 21.84 at 550 nm.

2.6. Glucose-6-Phosphate Dehydrogenase Assay Protocol [13]

G6PDH is unique for its dual coenzyme specificity. When assayed under conditions that are optimal for the particular coenzyme, the ratio of observed catalytic activity is $\text{NAD}/\text{NADP} = 1.8$. The reaction velocity is determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD or NADP. One unit reduces one micromole of pyridine nucleotide per minute at 30°C and pH 7.8 under the specified conditions.

3. Results

Rohypnol and Alcohol Effects General Hippocampal Formation, Dentate Gyrus and CA

Rohypnol and alcohol use did not cause extensive alterations and distortions in the histo architectural outline and organisation of the hippocampus [Figure 1-4]. High dose Rohypnol caused reduction in the morphological definitions of the dentate gyrus neurons that stained relatively less intense under the same conditions as other groups dentate gyrus. The combination of both substances also altered the morphologies of the CA4 regions neurons and resulted in morphological aberrations of the cells relative to the control [Group A] and other treated groups.



Figure 1. Photomicrographs of the Hippocampal Formation of the Groups A-E Experimental Animals Showing the Dentate Gyrus and the CA Areas [H&E X640]. Dentate Gyrus Formation and CAs are Properly demonstrated. Cells of the Dentate Gyrus and are Relatively Normally Defined Across the Groups [HP- hippocampus].

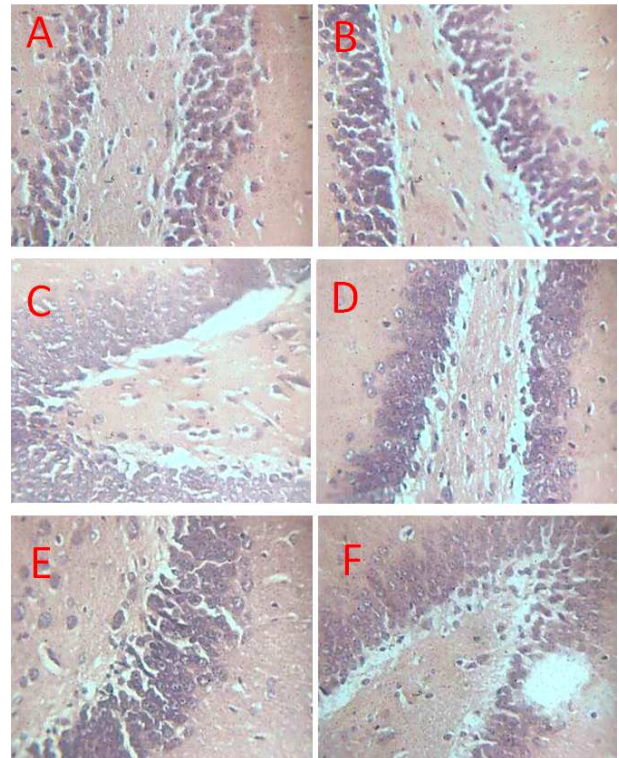


Figure 2. Photomicrographs of the Hippocampus Dentate Gyrus of the Groups A-E Experimental Animals [H&E X640]. Dentate Gyrus Formation is Demonstrated. Cells of the Dentate Gyrus and are Relatively Normally Defined Across the Groups. Dentate Gyrus Neurons are Less Clearly Demonstrated in the Group C Administered the Higher dose of Rohypnol.

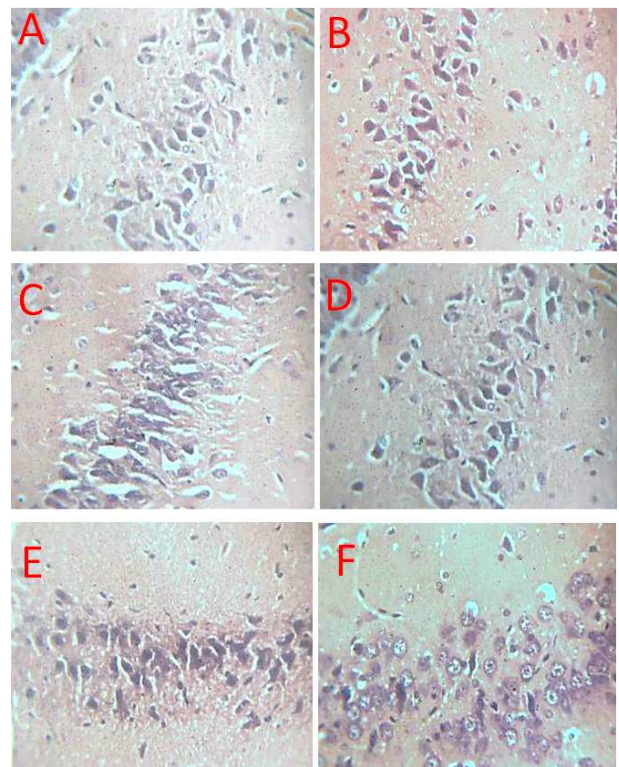


Figure 3. Photomicrographs of the Hippocampus CA4 of the Groups A-E Experimental Animals [H&E X640]. CA4 Formation is Demonstrated. Cells of the CA4 and are Relatively Normally Defined Across the Groups. Neurons are Morphologically Aberrant in the Group E Administered Both Rohypnol and Alcohol.

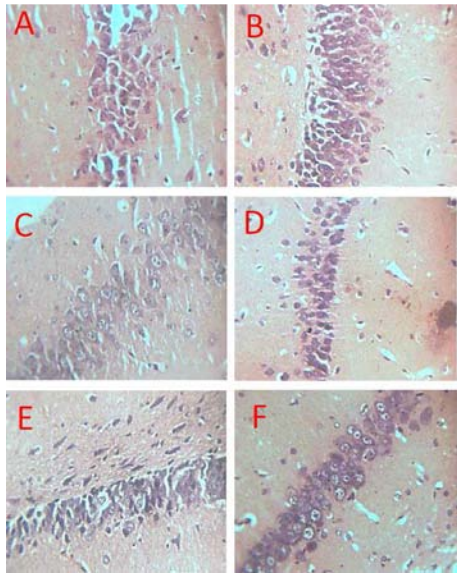


Figure 4. Photomicrographs of the Hippocampus CA2 of the Groups A-E Experimental Animals [H&E X640]. CA2 Formation is demonstrated. Cells of the CA4 and are Relatively Normally Defined Across the Groups. However, Cells Stain Pale Relatively in Group C Administered with High dose Rohypnol and Appear Mildly Morphologically Aberrant in Group E Administered Both Rohypnol and Alcohol.

Rohypnol and Alcohol Effects, Dentate Gyrus Nissl Bodies Expressions

Rohypnol at high dose could be observed to slightly reduce the intensity of Nissl bodies expression in the Group C. This could be an indication of a relative reduction, albeit, mild in the activities of ribosome bound rough endoplasmic reticulum in the dentate gyrus. Generally, there is no specific evidence of extensive compromise in the expression, and consequently, the demonstration of Nissl bodies in the dentate gyrus of the experimental animal.

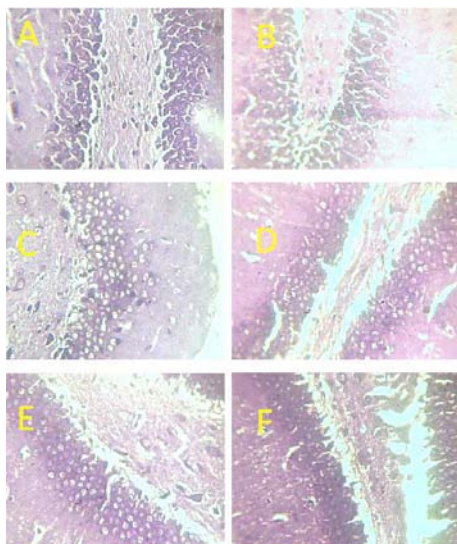


Figure 5. Photomicrographs of the Hippocampal Dentate Gyrus of the Experimental Animals in Groups A-F [CFV X640] to Demonstrate Nissl Bodies with Respect to Cytoplasmic Protein Synthesis. There are Observable Significant Aberrations Across the Treated Groups B-F. Group C, Administered High dose Rohypnol had Relatively Reduced Intensity for Nissl Expression.

Rohypnol and Alcohol Effects, Dentate Gyrus and CA 2 Nissl Bodies Expressions

Nissl bodies are demonstrated across the Rohypnol and alcohol treated animals groups relatively normal when compromised with the control Group A. There is no evidence of extensive or significant alterations in the expression of Nissl bodies in the treated groups relative to the control and this suggested that the administered agents would not significantly compromise Nissl bodies in the established cells of the CA regions.

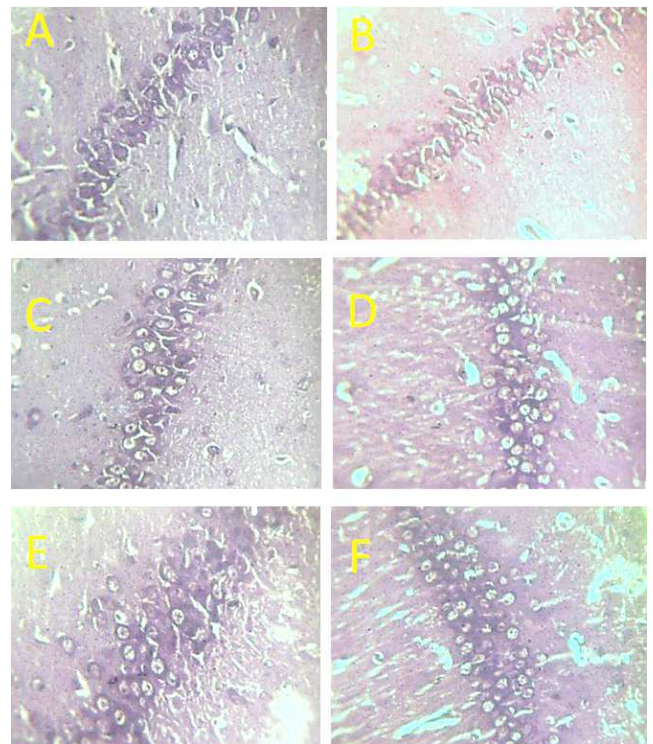


Figure 6. Photomicrographs of the Hippocampal Cornu Ammonis of the Experimental Animals in Groups A-F [CFV X640] to Demonstrate Nissl Bodies with Respect to Cytoplasmic Protein Synthesis. There are Observable Significant Aberrations Across the Treated Groups B-F.

GFAP Demonstration of Astrocytic Expressions.

The Glial Fibrillary Acidic Protein method was used to dentate reactive astrocytes as a possible indication neuroinflammations in the hippocampal formation. Results as shown in figure 7 showed that the administered agents either independently or combined did not result in significant inflammatory reactions within the hippocampal formation. This further showed that the agents did not produce neuroinflammation to which the astrocytes could have reacted.

Effects of Rohypnol and Alcohol Administration on Brain Enzymes Cytochrome C Oxidase and Glucose-6-Phosphate Dehydrogenase

Rohypnol increased the levels of the activities of Cytochrome C Oxidase significantly in the brain. The effect increased with dosage; so also alcohol, which effects increased the activities of the same enzyme even greater than rohypnol. Both agents combined altered the enzyme's

activities significantly and higher than each agent when administered independently.

Also, G-6-PDH activities levels were raised in the brains in all treated groups significantly. The higher dose of rohypnol caused higher increase than the lower dose of rohypnol or either doses of alcohol; however the combination of both alcohol and rohypnol; increased the enzyme's activities level higher than aver other it is in very other group.

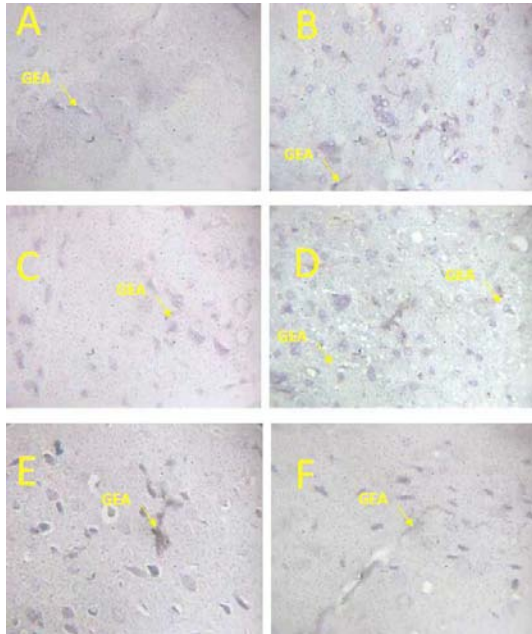


Figure 7. Photomicrographs of the Hippocampal Formation of the Experimental Animals in Groups A-F [GFAP X640] to Demonstrate GFAP Astrocytic Expressions. GFAP Expression is Relatively Normal in the Treated Groups B-F Relative to the Control Group A.

Legend: GEA- GFAP Expressing Astrocyte GFAP- Glial Fibrillary Acidic Protein

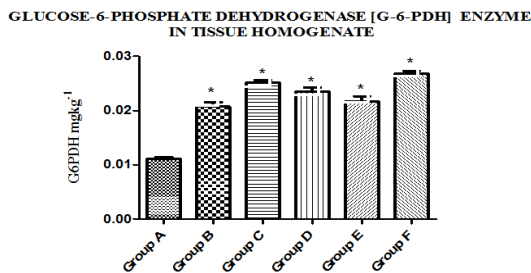
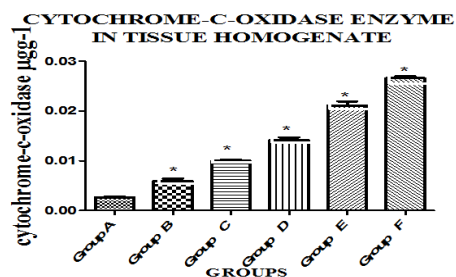


Figure 8. Charts of the Levels of Cytochrome C Oxidase and G6PDH Enzymes Activities in the Brain Tissues of the Experimental Animals. Treatments with Rohypnol, Alcohol and the Combination of Both Increased the Levels of These Enzymes Significantly in the Brain.

4. Discussion

4.1. Histology and Histochemistry Evidences for Structural and Functional Integrity

Dentate gyrus formation and CAs are properly demonstrated. Cells of the dentate gyrus and are relatively normally defined across the groups. Dentate gyrus formation is demonstrated. Cells of the dentate gyrus and are relatively normally defined across the groups. Dentate gyrus neurons are less clearly demonstrated in the Group C administered the higher dose of Rohypnol. CA4 formation is demonstrated. Cells of the CA4 and are relatively normally defined across the groups. Neurons are morphologically aberrant in the Group E administered both Rohypnol and alcohol. CA2 formation is demonstrated. Cells of the CA4 and are relatively normally defined across the groups. However, stains stain pale relatively in Group C administered with high dose Rohypnol and appear mildly morphologically aberrant in Group E administered both Rohypnol and alcohol. There are observable significant aberrations across the treated groups B-F. Group C, administered high dose Rohypnol had relatively reduced intensity for Nissl expression. There are observable significant aberrations across the treated groups B-F in Nissl bodies demonstration with respect to cytoplasmic protein synthesis. GFAP expression is relatively normal in the treated Groups B-F relative to the control Group A.

While the negative effects of Flunitrazepam on memory has been studied and reported [14]; [15]; what is not clearly known is the effects on the structural integrity of the primary part of the brain that is involved in the consolidation of memory- the hippocampus. To this end, the current results show that the effects of Flunitrazepam might not have included extensive disruption of the hippocampal formation, but effects significant to alter the biochemistry of the tissue enzymes negatively and that could produce effects capable of altering the normal morphologies of these cells.

Alcohol has long been reported and reputed to interfere with the formation of new memories, consolidated by the hippocampus. More specifically, it has been reported to interfere with the activities of cells such as pyramidal cells in CA1 area of the hippocampus to communicate effectively with other regions of the brain [16]; [17]. Both short and long term memories could be affected [18]. This investigation also shows the biochemical alterations that might accompany these anomalies especially, the abnormally high and significant changes in the levels of cytochrome C oxidase and G6PDH in the brains of the treated animals. Though, extensive changes might not be reported of the structural integrity of the tissues, variations are observed especially in the morphologies of certain cells in localised or specific parts of the hippocampus.

Alcohol is often consumed in combination with certain other drugs [19]. An interesting observation is the morphological alteration of the pyramidal cells resulting in their heterogeneity as observed in the group administered the combination of Flunitrazepam and alcohol. Noting that this

was aimed at modelling the habitual or typical use of these agents [20]; there are reasons to suggest that the combine defects of these substances may alter certain cells morphologically and expectedly compromise their communications patterns. While this combination has also been associated with violence and memory compromise [20] and performance reduction, current reports suggest that the long term effects on brain structural integrity and functions should be investigated more thoroughly to find possible complications and potential spectrum of disorders.

4.2. Immunohistochemistry for Neuroinflammation

There has not been clearly evidences or reports on neuro inflammatory effects of Flunitrazepam in humans or experimental models. In this research, there was no observable neuro inflammatory reaction as marked by astrocytic reactions. This implied that Flunitrazepam did not produce typical toxic effects that could have results in astrocytic reactions. Also, alcohol routine consumption as modelled in this study also did not typically produce neuroinflammation. These facts show that the agents could alter brain chemistry and influence cells morphologies, but might not cause acute toxicities that might lead to death.

4.3. Biochemicals Functional Integrity

The two vital enzymes- cytochrome C oxidase and G-6-PDH, activities in the brain tissues of the experimental animals were significantly elevated across the groups. Treatments, either with each of Rohypnol or alcohol independently, or the combination of both increased the levels of these enzymes significantly in the brain. This points to the fact that the use of these substances altered the chemistry of the brain significantly; furthermore these observations suggest that both agents can influence the functional integrity of the brain. Interestingly, both are often combined together to facilitate the desired effects by the users, implying that both agents when used as such would later the chemistry of the brain furthermore.

Flunitrazepam influences mental functions through its selective effect on GABA-mediated synaptic transmission [21]. It is also reported to cause retrograde amnesia [22]. It is expected that its influence on GABA functions would slow down neuronal communication in certain regions of the brain. These could have effects on the chemistry of the cytological domains especially, enzymes that are involved in carbohydrate and energy metabolism. Interestingly, the affected cells had increase in the levels of the activities of these neurons.

The enzyme Cytochrome C oxidase is important for its role in the mitochondrial electron transport chain; it oxidises Cytochrome C molecules, transfers the electrons to oxygen to produce water; binds four protons and consequently creates a transmembrane proton electrochemical potential required for ATP synthase to synthesise ATP [23]; [24]. To this end the implications of the alterations and potential consequences on brain function should be noted. Glucose 6 phosphatase is an

enzyme that plays vital roles in managing oxidative stress within cells [25]. Also, high levels of G-6-PDH activities is associated with relatively high rate of cell growth or development [26]. Noting that the brain is continually and largely dependent on metabolic processes for the purpose of deriving energy from glucose; this enzyme by virtue of its importance also has vital implications and important consequences when its activities in tissues are altered or compromised. Relatively high doses of benzodiazepines and alcohol would induce sedation and sleep; however, in low to moderate doses the drugs can increase aggressive behaviour. An experiment on amnesic action of intravenous flunitrazepam showed that it caused amnesia without effect on the level of consciousness. Also, effects of flunitrazepam were harmful to coordination [4].

Results from the current investigation provide information on the use of rohypnol which is one of the popularly abused drugs but with limited experimental information on effects and consequences of use especially on mental and social health [27]. Further investigations into the use and circumstances surrounding such instances; as well as the possible neurological consequences of rohypnol will be very useful in managing its effects and controlling its abuse. Rohypnol and alcohol abuse deserve adequate research attention due to its influence on social life and mental health, sexuality, crime and driving behaviours among other factors. Its abilities to influence the human memory is also a major consideration [28]; [29]; [30].

5. Conclusion

Flunitrazepam and alcohol effects were observed in specific regions of the hippocampus to include morphological aberrations, resulting in mild heterogeneity. The biochemistry of the hippocampus cytochrome C oxidase and G6PDH enzymes was altered.- there were changes that resulted in significant increases. However, flunitrazepam and alcohol did not produce extensive structural disruption of the hippocampus.

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