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ANTI-INFLAMMATORY, ANTI-APOPTOTIC AND HISTOARCHITECTURAL EFFECT OF VITAMIN C AND E ON ROHYPNOL-INDUCED HIPPOCAMPAL INJURIES ON WISTAR RATS

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ABSTRACT

Aim: This aimed at investigating the anti-inflammatory, anti-apoptotic and histoarchitectural effect of vitamin C and vitamin E on Rohypnol-induced hippocampal injuries on male wistar rats. **Methods:** This six-week experimental study, involved 25 adult male Wistar rats weighing between 200–220kg, divided into five groups (n=5). Group A served as the control, Group B received 1mg/kg body weight of Rohypnol, Group C received 1mg/kg body weight of Rohypnol + 100 mg/kg body weight of Vitamin C, Group D received 1mg/kg body weight of Rohypnol + 100 mg/kg body weight of Vitamin E, and Group E received 1mg/kg body weight of Rohypnol + 100 mg/kg body weight of Vitamin C and E for 28 days concurrently. Neurobehavioural studies were conducted; afterwards, the hippocampus and blood samples were collected for further analysis. **Results:** Groups C, D, and E exhibited increased TNF-α and decreases Bcl-2 levels compared to Group B, which displayed decreased and increase levels respectively.

Group B showed significantly reduced SOD, GPx, and GSH levels, whereas Groups C, D, and E demonstrated increased levels. Additionally, Group B exhibited elevated MDA levels, while Groups C, D, and E displayed decreased levels. The histopathological findings in our study revealed mild to severe damage on the cells and tissues of the hippocampus with focal area of hemorrhage, liquefactic necrosis, severe lymphocytic infiltration and vacuolations in the group B. Mild distortions in the hippocampal tissues were observed in other groups. **Conclusion:** Vitamin C and E effectively protected against Rohypnol-induced neurodegeneration, suggesting their potential as essential supplements.

KEYWORDS: Anti-inflammatory, Anti-apoptosis, Rohypnol, Hippocampus, Vitamin C and Vitamin E.

INTRODUCTION

Rohypnol is a central nervous system depressant which belongs to a drug class called benzodiazepines.[1] It slows down the function of CNS thereby inducing sedation, sleep and amnesia. Benzodiazepines are sedative-hypnotics that influences and enhances the neurotransmitters called GABA in the brain thereby reducing the brain activity. They are used in the short term treatment of insomnia, anxiety, sleep and seizure disorders; they are also used as skeletal-muscle relaxants.[2] Due to its additive nature, individuals use Rohypnol for purposes order than that prescribed by physician. They are commonly abused, especially by young people to get the knocked out and anti-anxiety effects as relieve to the side effects such as irritability and agitation, associated with cocaine binges, therefore it is associated with multiple-substance abuse. It has been investigated that students of high school, colleges, street gang members, rave party attendees, heroin and cocaine abusers, equally abuse Rohypnol to produce profound intoxication, boost the high of heroin, and modulate the effects of cocaine. Frequent intake of Rohypnol degenerate the brain cells and causes a number of cognitive impairments.

The hippocampus is region of the brain where neurogenesis continues even in adult life[3], it is also part of the brain mostly affected in neurodegenerative diseases.[4] Meanwhile the function of the hippocampus to human cannot be overemphasized.

Vitamin C (ascorbic acid), a well know water-soluble vitamin and an anti-oxidant that protects the human cells against the effect of free radicals; is rated amongst the most available and affordable non-enzymatic antioxidant molecule used to lessen the effects of

oxidative damages in the body.[5] [6] Amidst a couple of functions, it enhances sperms DNA and integrity[7] [8], ameliorates organophosphate pesticide-induced hematological and bio-chemical alterations in humans and animals.[9] [10] [11] Deficiencies in this vitamin lead to cognitive impairment.[12] This readily available, cheap and relatively non-toxic antioxidant possesses great benefit in the amelioration of toxic effects by most xenobiotics[13], such as Rohypnol.

Vitamin E is a fat-soluble anti-oxidant that inhibits reactive oxygen species (ROS) during oxidation of fats and also destroys free radicals found in cellular membrane.[14] [15] Reported decrease alcohol-induced oxidative-stress and apoptosis in developing hippocampus and cerebellum via vitamin E. The deficiency symptoms of vitamin E include peripheral neuropathy, ataxia, skeletal myopathy and impairment of immune response.[16] Vitamin E is also considered as a treatment strategy in some neurodegenerative diseases because of its effects in neuroprotection and neurogenesis.[17] A combined therapeutic effect of these two vitamins can help mitigate the devastating effect of Rohypnol degenerative effects, hence this study aimed at investigating the anti-inflammatory and anti-apoptotic effect of Vitamin C and E on the Rohypnol-induced hippocampal injuries on wistar rats.

MATERIALS AND METHODS

This research was carried at the animal holding facility of College of Health Science, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria. It involved a total of 25 adult male wistar rats weighing between 200kg to 220kg. The rats were randomly divided into five groups of five rats each n=5; groups A to E.

Group A (control group) received only feed and water Ad Libitum, Group B received 1mg/kg body weight of Rohypnol only, Groups C and D received 1mg/kg body weight of Rohypnol and 100mg/kg body weight of vitamin C and vitamin E respectively, while Group E received 1mg/kg body weight of Rohypnol and 100mg/kg body weight of vitamin C and Vitamin E concurrently. All these substances were grounded into fine powder using electric blender, and 500g of each were obtained dissolved in 5ml of distilled water separately. A stock solution of 100mg/ml was obtained corresponding to 1000mg/kg dose. They were fed these extract for a period of four weeks concurrently. The experiment lasted for a total of six weeks, two weeks acclimatization inclusive.

LD50 of the extracts were obtained using Dietrich Lorke (1983) method.

During the experiment, neurobehavioural test using Morris water test, was carried out twice, before and during administration of the extracts to the animals.

At the end of the fourth week, the animals were sacrificed using chloroform inhalation, blood samples were collected and the brain excised. Blood samples were analysed using Elisa kit, while immunohistochemical tissue procedures were carried out and photomicrographs were obtained. Data obtained were statistically analysed using Analysis of variance (ANOVA) followed by post HOC Fisher LSD multiple comparison; data were considered significant at $P<0.05$ means significant and $P>0.05$ means not significant.

Ethical approval

Ethical approval was gotten from the ethical committee Nnamdi Azikiwe University, College of Health Sciences, Nnewi, Anambra State, Nigeria.

Table 1: Showing the Groups, doses of administration and duration.

Groups	No of Rats	Administration	Doses	Duration
Group A	5	Control		4 Weeks
Group B	5	Rohypnol	100mg/kg body weight	4 Weeks
Group C	5	Rohypnol + Vitamin C	100mg/kg body weight of Rohypnol + 100mg/kg body weight of Vitamin C	4 Weeks
Group D	5	Rohypnol + Vitamin E	100mg/kg body weight of Rohypnol + 100mg/kg body weight of Vitamin E	4 Weeks
Group E	5	Rohypnol + Vitamin C & E	100mg/kg body weight of Rohypnol + 100mg/kg body weight of Vitamin C & E	4 Weeks

RESULTS

Table 2: Hippocampus Expression Count of Immunohistochemical Markers.

	Groups	Mean ± SEM	p-value
TNF-α (µg/mL)	Group A	27.80 ± 4.24	6.253
	Group B	49.52 ± 2.00	0.018*
	Group C	46.95 ± 6.14	0.215
	Group D	40.75 ± 3.22	0.255
	Group E	28.22 ± 3.12	1.000
Bcl-2 (µg/mL)	Group A	39.12 ± 2.96	2.001
	Group B	29.14 ± 4.54	0.536
	Group C	51.70 ± 7.83	0.745
	Group D	43.84 ± 7.50	0.999
	Group E	42.96 ± 3.99	0.994

From Table 2 above, there is a significant increase ($p<0.05$) in the immunohistochemical expression of TNF-a in group B, indicating neuronal degeneration in the hippocampus as a

result of Rohypnol administration. Consequently, there is a decrease in the immunohistochemical expression of TNF-a in groups treated with Vitamin C, E or C and E, this decrease is though not significant (p>0.05) in comparison to the control group that given feed and water only.

In the Bcl-2 test, there was a non-significant (p>0.05) increase in Groups C, D and E compared to Group B that showed non-significant decrease (p>0.05).

Table 3: Hippocampus Expression Count of Oxidative Markers.

	Malondialdehyde level (um/L)	Superoxide Dismutase (U/ml)	Glutathione Peroxidase (Um/ml)	Reduced glutathione (Um/ml)
	MEAN±SEM	**MEAN±SEM**	**MEAN±SEM**	**MEAN±SEM**
Group A (control)	0.71±0.11	23.39±0.38	6.05±0.79	10.17±0.28
Group B (1mg/kg of Rophynol)	2.87±0.20*	9.01±0.04*	3.40±0.79	5.20±0.06*
Group C (1mg/kg of Rophynol +100mg/kg of Vitamin C)	0.69±0.01*	20.87±0.81*	6.06±0.00*	10.12±0.22*
Group D (1mg/kg of Rophynol +100mg/kg of Vitamin E)	0.58±0.01*	11.14±0.93*	8.15±0.26*	10.17±0.08*
Group E (1mg/kg of Rophynol +100mg/kg of Vitamin C&E)	0.74±0.00*	13.34±0.04*	8.68±2.89*	10.34±0.11*
F-value	**5.34**	**14.04**	**17.61**	**6.24**

Result revealed a significant increase in MDA level in group B compared to A (p=0.021). Groups C, D and E (p=0.011, p=0.010, p=0.002) had a significantly decreased MDA level compared to group B. The SOD level result showed a significant decrease in group B compared to A (p=0.009), while groups C, D, and E (p=0.028, p=0.00, p= 0.00) had a significant increase compared to group B. The GPx level showed a significant decrease in group B as compared to A (p=0.024), while groups C, D and E (p=0.003, p=0.033) had significant increase compared to group B. GSH level significantly decreased in group B compared to A (p=0.010), while groups C, D and E (p=0.038, p=.0001, P=0.025) had a significant increase in comparison to group B.

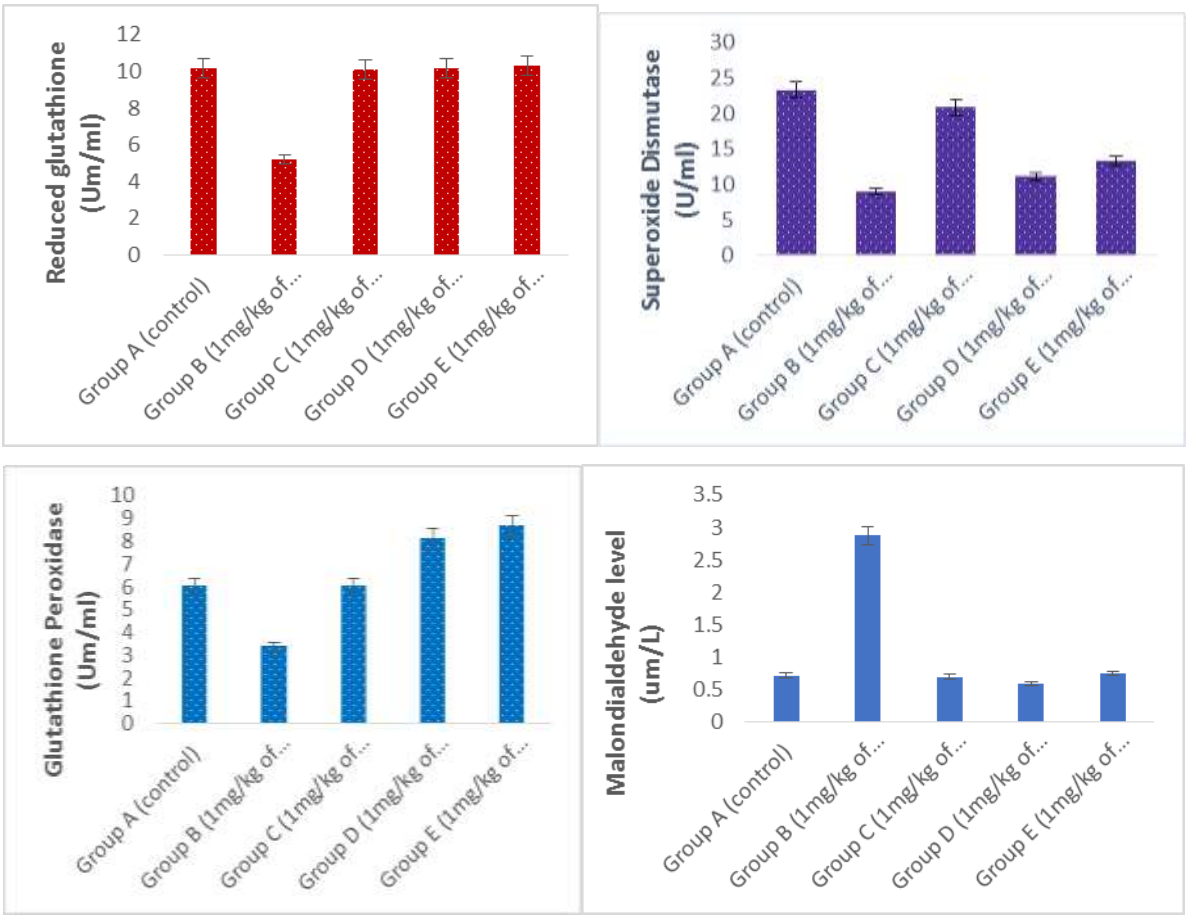

Fig. 1: Chats showing results of the different Oxidative markers.

Figure 1. Showing chat representation of the different Oxidative markers; the level of MDA was greatly increased in the group B, which received Rohypnol only with any form of treatment, while the groups C,D and E which received Rohypnol and vitamin C or E had normal MDA serum level as the control group. The other antioxidant markers had a higher level in the groups treated with either vitamin C, E or C and E. This result confirms the anti-oxidative property of these vitamins.

Table 4: Effect of Vitamin C and E on cognition using moriss water maze test following Rophynol toxicity.

		Mean	±SEM	P-value	T-value
Group A (control)	**Pre**	3.00	±1.00	0.021*	-1.28
	Post	7.50	±2.50		
Group B (1mg/kg of Rophynol)	**Pre**	5.50	±1.50	0.050*	0.60
	Post	2.00	±1.00		
Group C (1mg/kg of Rophynol +100mg/kg of Vitamin C)	**Pre**	2.00	±1.00	0.012*	-5.00
	Post	4.50	±0.50		
Group D (1mg/kg of	**Pre**	5.00	±1.00	0.009*	-7.00

Rophynol +100mg/kg of Vitamin E)	**Post**	8.50	±0.50		
Group E (1mg/kg of Rophynol +100mg/kg of Vitamin C&E)	**Pre**	6.00	±1.00	0.025*	-2.33
	Post	9.50	±0.50		

Result revealed a significant increase the spatial memory and learning in groups C, D, and E comparing the pre-time and post time; while group B showed a significant decrease in the post-time compared to pre-time. This indicates a significant decline in cognition in group B, administered Rohypnol, while the improvement noticed in groups C, D and especially E, shows cognitive recovery from the toxic effect of Rohypnol.

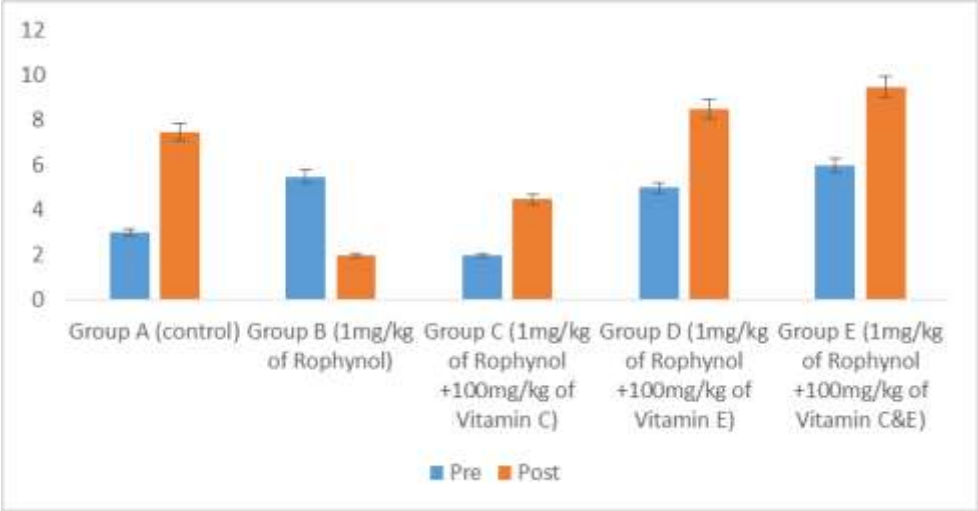

Fig. 2: Neurobehavioural Results.

Chat representation of the neurobehavioural test carried out using Morris Water Test.

The neurobehavioural test showing the effects of rohypnol, Vitamin C and E on spatial learning. The control group (Group A), groups that received Vitamin C,E or C and E (Groups C, D and E) had increased spatial learning, which is the reverse in the group B treated with Rophynol only.

Histological Findings

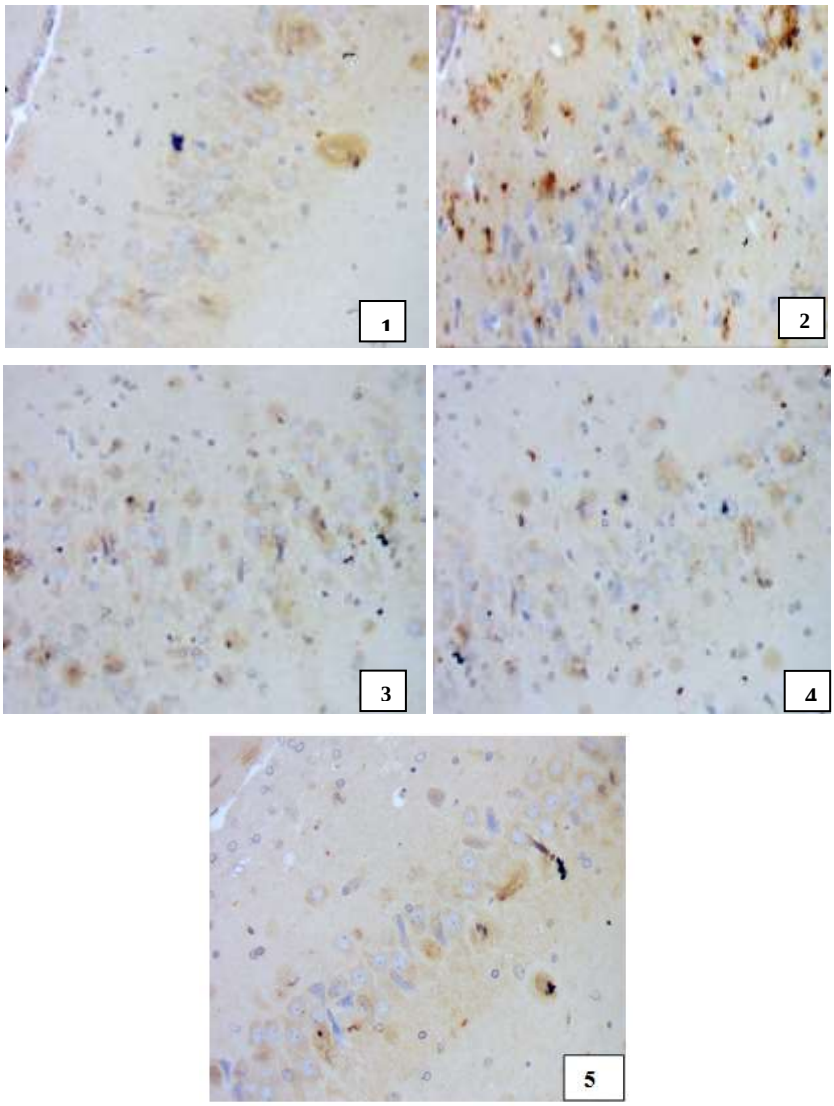

Fig. 3: Hippocampus Tumor Necrosis Factor Alpha (TNF-α) Expression.

PLATE 1: Photomicrograph of group A Section of hippocampus shows a normal expression of Tumor Necrosis Factor Alpha (TNF-α) on the hippocampal region of the brain. **PLATE 2:** Photomicrograph of group B Section showing increased expression of Tumor Necrosis Factor Alpha (TNFα) on the hippocampal region of the brain, indicative of neuro-toxicity. **PLATE 3**: Photomicrograph of group C Section showing increase in the expression of Tumor Necrosis Factor Alpha (TNF-α) on the hippocampus, although this expression is minimal in comparison to group B, this show a measure of recovery from the toxic effects of Rohypnol administration. **PLATE 4**: Photomicrograph group D Section of hippocampus shows an increase in the expression of Tumor Necrosis Factor Alpha (TNF-α) on the hippocampal region of the brain. **PLATE 5**: Photomicrograph Group E Section of hippocampus shows an

increase in the expression of Tumor Necrosis Factor Alpha (TNF-α) on the hippocampal region of the brain.

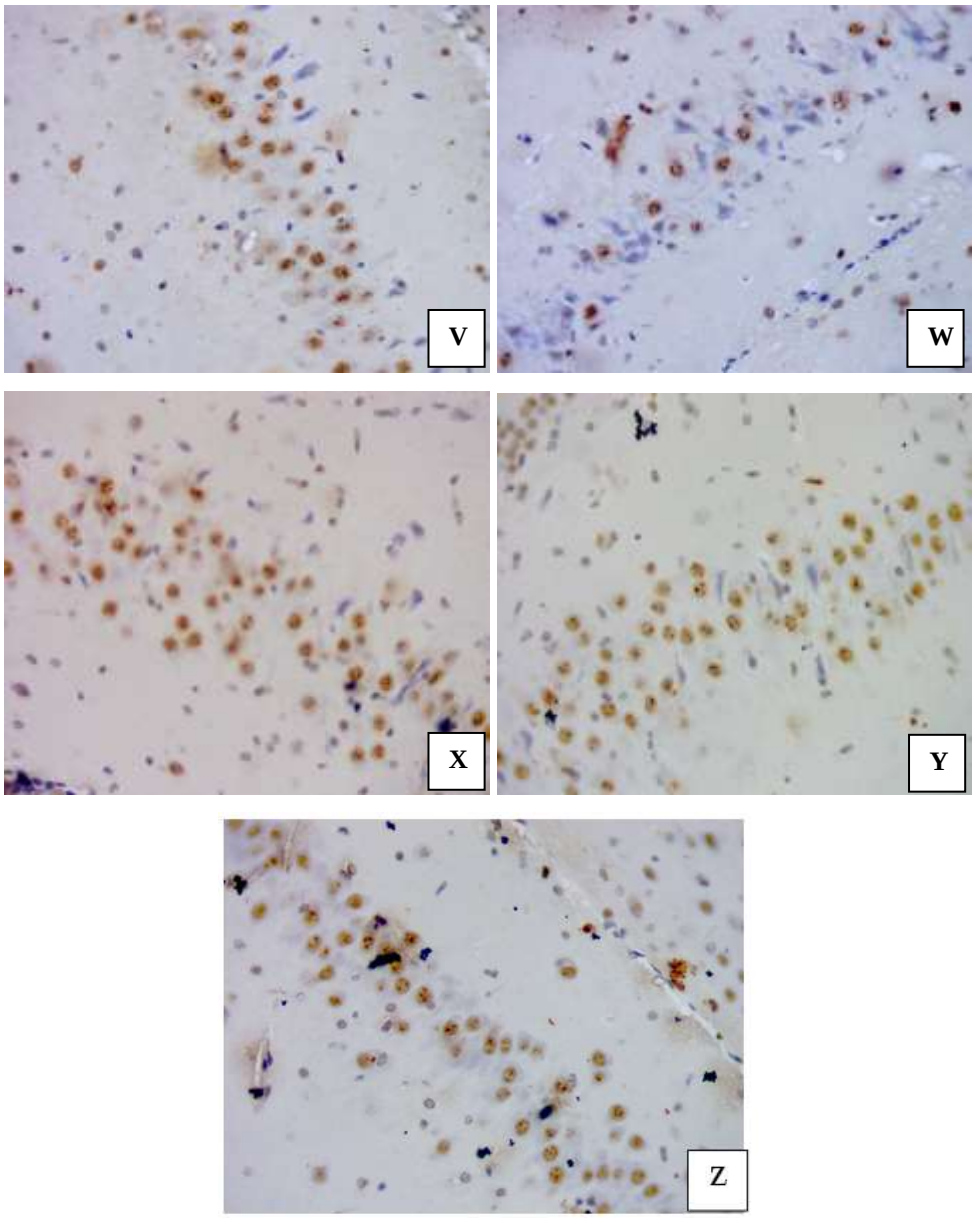

Fig. 4: Hippocampus Tissue Expression of B-cell Lymphoma 2 (BCL-2).

PLATE V: Photomicrograph Group A Section of hippocampus showing an increase in tissue expression of B-cell lymphoma 2 (BCL-2) on the hippocampal region of the brain. **PLATE W**: Photomicrograph Group B Section of hippocampus showing a decrease in the tissue expression of B-cell lymphoma 2 (BCL-2) on the hippocampal region of the brain. **PLATE X**: Photomicrograph Group C Section of hippocampus showing an increase in the tissue expression of B-cell lymphoma 2 (BCL-2) on the hippocampal region of the brain. **PLATE Y**: Photomicrograph Group D Section of hippocampus showing an inverse increase in

expression of B-cell lymphoma 2 (BCL-2) on the hippocampal region of the brain. **PLATE Z**: Photomicrograph Group E Section of hippocampus showing an inverse increase in the tissue expression of B-cell lymphoma 2 (BCL-2) on the hippocampal region of the brain.

DISCUSSION

[1]Stated the discovered date and purpose of Rohypnol. Its medical use includes sedative hypnotics for anxiety, insomnia and sleep disorders[2], although this drug has been abused, the level of damages it can cause to the brain tissue cannot be overemphasized.

The effect of Rohypnol, Vitamin C, E and C&E on the hippocampus oxidative stress markers in adult male wistar rats

Result showed a significant increase in MDA level in Rohypnol group compared to control group[19], while the other groups treated with vitamin C or E or both had a significantly decreased MDA level compared to group B.[20] In the same way, there is a significant decrease in group B SOD level signifying insult in the normal body physiology; while groups that received the vitamins, had significantly increased SOD level. These findings correspond with the findings of.[20]

Glutathione (GSH), a tripeptide (glutamate, cysteine and glycine), overtime has shown to have multiple functions including antioxidant effects.[21] The glutathione system detoxifies reactive oxygen and nitrogen species (ROS/RNS), and electrophiles generated in the body.[22] The GSH and GPx levels were significantly decreased in Rophenol only group, while groups C, D and E had significant increase levels, signifying a measure of recovery from the neuronal assault, caused due to Rophynol ingestion; these also concur with the work.[21]

The effect of Rohypnol, Vitamin C, E and C&E on the hippocampus immunological marker (TNF Alpha) in adult male wistar rats

TNF-α is a proinflammatory cytokine implicated in the pathogenesis of systemic inflammatory and neurodegenerative diseases.[23] Our findings, revealed significant increase in the immunohistochemical expression level of TNF-α in group B, indicating neuronal degeneration in the hippocampus which most likely is as a result of Rohypnol administration, which can produce pharmacological effects that include; memory impairment, sedation, and behavioural disinhibition.[19] TNF-α is involved in many systemic chronic inflammatory and degenerative conditions and are amongst the key mediators of neuroinflammation.[12], [24]

Decreases in the immunohistochemical expression level of TNF-α in groups treated with Vitamin C, E or C and E, were observed in this work, although these were not significant in comparison to the control group that received Normal Saline only. It has been established that when astrocytes are stimulated by pro-inflammatory cytokines such as IL-1 and IL-6, they become reactive astrocytes, and promote inflammation when activated, through the secretion of cytokines such as TNF-α and IL-6.[25] The observed recovery could have resulted due to the administration of the vitamin C and E and these accurately corresponds to the findings of[5] [6] [15] on administration of vitamin C and or E.

So it could be inferred that the increase and decrease in the activities of TNF-α is due to the toxicity caused by Rohypnol and the curative potential of vitamins C and E.

The effect of Rohypnol, Vitamin C, E and C&E on the hippocampus Apoptotic marker (BCL-2) in adult male wistar rats

Apoptosis, or programmed cell death, is a cell-suicide program, distinct from necrosis, which is activated in physiological processes such as tissue development and differentiation as well as in pathophysiological conditions.[26]

The Bcl-2 expression count shows a non-significant decrease in the Group treated with Rophynol, which may have been induced following inflammation. An increase in Bcl-2 expression level occurs in prevention of cell death.[26] The term apoptosis (Gk: falling off, like a tree leaf) had been coined some years earlier to refer to deliberate cell death, and thus is applied to the type of cell death blocked by BCL-2.[26] It could be inferred from our findings, that Rophenol is capable of inducing apoptosis.[1] Non-significant increases in Bcl-2 expression levels were observed in the groups C, D and E; consequently, these increases may be due to the healing potential of vitamin C and E.[1] The increase in Bcl-2 levels in the groups administered vitamin C and E, were observed to be higher than in the control group. This goes a long way to portray the effectiveness of these vitamins in the prevention of cell death.

The effect of Rohypnol, Vitamin C, E and C&E on the hippocampus histoarchitecture of adult male wistar rats

The rationale behind the extensive damage of the hippocampus could be said to be the resultant 7-Aminoflunitrazepam which is the toxic metabolite of Rohypnol having the ability to cross the blood brain barrier and alter the cellular integrity of brain.[27] The

histopathological findings in our study revealed mild to severe damage on the cells and tissues of the cerebral cortex with focal area of hemorrhage, liquefactic necrosis, severe lymphocytic infiltration and vacuolations in the group treated with Rophyno only, which is synonymous with the findings of.[27] [19] Meanwhile, measures of recovery were noted in the groups administered vitamin C and E, buttressing the impact of vitamin C and E in addressing the debilitating effect of Rophynol on the brain.[5][6]

The effect of Rohypnol, Vitamin C, E and C&E on neuro-bahavioural parameter (spatial memory and learning) in adult male wistar rats

Result revealed a significant increase the spatial memory and cognition in groups C, D, and E comparing the pre-time and post time; while group B showed a significant decrease in the post-time compared to pre-time. This is indicative of a decline in cognition following Rohypnol administration[19], while the improvement noticed in groups C, D and especially E shows a measure of cognitive recovery from the toxic effect of Rohypnol, as a result of vitamin C and E administration.[6]

CONCLUSION

From our findings, we discovered that vitamin C and E are effective antioxidants; anti-apoptotic and anti-inflammatory substance capable of ameliorating the toxic effect of Rohypnol on brain cells, hence can be suggested as daily supplement for users of certain drugs in the family of Rohypnol.

Conflicts of interest

There is no conflict of interest during the decision making or the review process of this manuscript.

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