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Influence of Graded Doses of Lutein on Brain Nitric Oxide (NO) Level in Diazepam Induced Memory Impairment in Wistar Rats

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Abstract | **Original Research Article**

Nitric Oxide (NO) has been identified as a key signaling molecule involved in memory functions, and cognitive problems have been associated with its dysregulation. The carotenoid Lutein, which has strong antioxidant capabilities, has demonstrated potential for improving cognitive function. This study used Wistar (Wistar) rats to create a Diazepam-induced memory impairment paradigm in order to look into the possible effects of graded dosages of lutein on brain nitric oxide levels. A total of thirty rats (110-190g) were used for this study. The rats were categorized into six groups after 14 days and were administered their respective substances for 21 days: Group 1: Control, Group 2: Diazepam Only (5mg/kg), Group 3: Diazepam + Lutein (20mg/kg), Group 4: Diazepam + Lutein (40mg/kg), Group 5: Diazepam + Lutein (60mg/kg), Group 6: Diazepam + Donpenzil (Standard Drug). Administration of Diazepam significantly affected working and spatial memory, as manifested by the decreasing Y-maze alternation and increasing escape latency with more errors in the Barnes maze. It also significantly increased the brain NO levels as compared to controls ($P < 0.001$). Lutein treatment resulted in an inhibitory effect on NO which was dose dependent: the 20mg/kg dose significantly reduced the NO ($P < 0.05$), the 40mg/kg dose produced a non-significant reduction, and the 60mg/kg dose restored the NO concentrations to the baseline ($P > 0.05$). In a similar manner, donepezil reduced NO levels significantly as compared to diazepam-only rats ($P < 0.01$).

The results suggest that lutein has a neuroprotective effect in a dose-dependent manner against oxidative stress induced by diazepam, and 60mg/kg of lutein have similar efficacy to that of donepezil. This is an indication that lutein can be a potential adjunctive therapeutic agent in the alleviation of oxidative neurotoxicity and the associated cognitive deficits.

Keywords: Lutein, Brain Nitric Oxide, Diazepam-Induced, Memory, Impairment.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by hyperphosphorylated microtubule-associated protein tau, neurofibrillary lesions composed of the β-amyloid peptide, aberrant oxidative and inflammatory processes, and disturbance in neurotransmission (Jucker & Walker, 2011). AD produces significant cognitive impairment that arises from damage to cholinergic neurons known to play a crucial role in learning and memory functions (Chen *et al.*, 2022) Cholinergic deficit has been regarded as a marker of neurological pathology that is associated with memory dysfunction and consistently correlated with the severity of cognitive impairment in AD(Van der Zee *et al.*, 2011). Therefore, the recuperation of cholinergic role remains a coherent marker for developmental programs targeting the remedy of AD symptoms.

Oxidative stress is a common feature of many neurodegenerative diseases, including alzheimer's disease (AD), parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Olufunmilayo *et al.*, 2023). In AD, for example, oxidative stress is thought to contribute to the formation of amyloid-beta plaques and tau tangles, which are hallmark pathological features of the disease (Cheignon *et al.*, 2018). Oxidative stress in the brain occurs when the production of Reactive Oxygen Species (ROS), such as free radicals and peroxides, exceeds the capacity of the brain's antioxidant defense systems to

eliminate them (Pizzino *et al.*, 2017). Oxidative stress can also trigger neuroinflammation, which is another key component of neurodegenerative diseases. chronic inflammation can exacerbate oxidative damage and create a vicious cycle, further compromising cognitive function and neuronal integrity (Kim *et al.*, 2024). Since Oxidative stress is closely linked to cognitive decline and neurodegenerative diseases, antioxidants can help preserve cognitive function by reducing oxidative damage, inflammation, and mitochondrial dysfunction, all of which play a role in cognitive impairment (Ashok *et al.*, 2022).

Lutein, a carotenoid pigment naturally found in green leafy vegetables and fruits has drawn attention because of its neuroprotective properties linked to its antioxidant and anti-inflammatory effects (Ahn & Kim, 2021), showing promise in mitigating cognitive impairments in various contexts

Understanding the mechanisms underlying diazepam-induced memory impairment and exploring potential interventions is crucial for addressing this issue. One avenue of investigation is the role of nitric oxide (NO), a key molecule in neuronal signaling and neuroinflammation. Excessive NO production is linked to neurotoxicity and cognitive deficits.

MATERIALS AND METHODS

Animal Model

Thirty Wistar rats weighing between 110-190g were used for this study. These rats were obtained from the experimental animal unit of department of human physiology, University of Port Harcourt, Rivers State. The rats were housed in the conventional wire mesh cages under standard laboratory conditions. The animals were allowed free access to water and finisher's feed *ad libitum*, throughout the period of the experiment. The animal feed was obtained from Choba Central market, Port Harcourt.

After collection of the rats, they were weighed, identified and kept in a wire gauze cage floored with saw dust to maintain dryness. The rats were allowed to acclimatize for one week under standard laboratory conditions of temperature (18 to 26 degrees celsius) with a photo-periodicity of approximately twelve hours of light alternating with twelve hours of darkness.

Ethical Approval

Ethical approval was obtained from the faculty of basic medical science, Abuja campus, University of Port Harcourt. Rat handling and treatment conform to the guideline of the National Research Council (2011) for care and use of laboratory animals.

Drug Identifications

The drugs used were Diazepam, Lutein, Donepezil and chemical substance, dimethyl sulfate ($Dmso_4$). They were purchased from GGI Intl' Nigeria Ltd. located at GGI Place, Plot 8 GGI Crescent, (Opp. Mikab Filling Station), Port Harcourt, Rivers State, Nigeria.

Experimental Design

The study followed a randomized controlled trial design, involving the administration of different doses of lutein to Wistar rats with Diazepam-induced memory impairment**.** The rats were randomly assigned into 6 different treatment groups:

- Distilled water.
- Diazepam (5 mg/kg) to induce memory impairment.
- Diazepam (5 mg/kg) + 20 mg/kg Lutein
- Diazepam (5 mg/kg) + 40 mg/kg Lutein
- Diazepam (5 mg/kg) + 60 mg/kg Lutein
- Diazepam (5 mg/kg) + 10 mg/kg Donepezil: This group was administered a standard dosage of a standard
- drug for memory impairment in this case, Donepezil.

Preparation and Administration of Solution

Lutein purchased in tablets, were prepared with a suitable solvent such as Dimethyl sulfoxide ($DMSO_4$), to ensure solubility. From this stock solution, appropriate volumes were diluted with the carrier solution to achieve the desired dosages for administration.

The dosage and stock concentrations were determined based on review of existing literature, safety considerations and the study's objectives. For 20mg/kg of lutein, 1 capsule of lutein was dissolved in 2ml DMSO & 8ml of water. For 40mg/kg dosage, 2 capsules were dissolved in 2ml DMSO & 8ml of water. Furthermore, for 60mg/kg dosage, 3 capsules of lutein were dissolved in 2ml DMSO & ml of water.

Lutein Administration

Lutein was administered orally to the experimental groups using appropriate dosing techniques. The dosing was conducted daily for a specified duration. The doses were determined based on preliminary data and existing literature to reflect a range of concentrations relevant to human dietary intake.

The concentrations of lutein used were mixed as follows:

- For 20mg/kg of lutein, a capsule was dissolved in 2ml $DMSO_4$ and 8ml of distilled water.
- For 40mg/kg dose used, two capsules of lutein were dissolved in 2ml $DMSO_4$ and 8ml of distilled water.
- For 60mg/kg dose used, three capsules of lutein were dissolved in 2ml $DMSO_4$ and 8ml of distilled water.

Table 1: Experimental Grouping and Drug Administration

Group	Treatment	Average Weight Range (g) (Week 1 - 3)	Dose Administered	Route of Administration
1	Control (normal feed + distilled water)	144-148		Oral (feed/water)
2	Diazepam (5 mg/kg)	130-141	0.1 mg/kg	Intraperitoneal
3	Diazepam (5 mg/kg) + Lutein (20 mg/kg) + $DMSO_4$ (2 mL in 8 mL water)	111-116	Diazepam 0.1 mg/kg + Lutein 0.9 mg/kg	IP + Oral
4	Diazepam (5 mg/kg) + Lutein (40 mg/kg) + $DMSO_4$ (2 mL in 8 mL water)	119-123	Diazepam 0.1 mg/kg + Lutein 0.8-1.0 mg/kg	IP + Oral
5	Diazepam (5 mg/kg) + Lutein (60 mg/kg) + $DMSO_4$ (2 mL in 8 mL water)	122-127	Diazepam 0.1 mg/kg + Lutein 0.8-0.9 mg/kg	IP + Oral
6	Diazepam (5 mg/kg) + Donepezil (10 mg/kg)	119-121	Diazepam 0.1 mg/kg + Donepezil 1.2 mg/kg	IP + Oral

Biochemical Analysis

Brain tissue homogenates were analyzed for NO levels (Using Griess reagent) (Ajjuri & O'Donnell, 2013).

Neurobehavioral Tests

The following test were conducted to assess motor coordination and exploratory behavior:

1. Hand Grip Test
2. Rotarod Test
3. Y-Maze Test
4. Barnes Maze Test

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8 software and the results were graphically represented using bar charts, providing a clear visualization of the effects of lutein on various parameters. Results were expressed as mean ± SEM. Group comparisons were made using one-way ANOVA, followed by least significant difference (LSD) post hoc test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Tables represent Mean ± SEM, n=5; a Significant at p<0.05 compared to Group 1; b Significant at p<0.05 when compared to group 2; c Significant at p<0.05 when compared to group 3. group 4, group 5, and group 6.

Table 2: Effects of Lutein, Diazepam, and Donepezil on Brain Nitric Oxide (NO) Levels

VARIABLES	GROUPS					
	Control	Diazepam Only	Diazepam + Lutein (20 mg/kg)	Diazepam + Lutein (40 mg/kg)	Diazepam + Lutein (60 mg/kg)	Diazepam + Donepenzil
Nitric Oxide (NO)	3.61 ± 0.23	5.28 ± 0.30 ***	4.80 ± 0.24 *	4.64 ± 0.27	4.20 ± 0.25	3.73 ± 0.17

Table 3: Effect of Lutein Administration on Neurobehavioural Test in Female Wistar Rats

VARIABLES	GROUPS					
	Control	Diazepam Only	Diazepam + Lutein (20mg/kg)	Diazepam + Lutein (40mg/kg)	Diazepam + Lutein (60mg/kg)	Diazepam+ Donepenzil
Rotarod Stability Time (s)	6.13 ± 0.89	1.47 ± 0.25 ***	3.47 ± 0.81	3.33 ± 0.37	4.24 ± 0.63	3.40 ± 0.92
Handgrip Stability Time (s)	3.67 ± 0.30	1.73 ± 0.12**	3.65 ± 0.34	4.12 ± 0.24	3.53 ± 0.23	3.72 ± 0.42
Y Maze Inflexion Ratio (s)	0.76 ± 0.13	1.60 ± 0.13****	1.11 ± 0.10	0.41 ± 0.11	0.39 ± 0.05	0.56 ± 0.04
Y Maze % Spontaneous Alteration	32.91 ± 3.26	10.22 ± 0.22****	14.20 ± 1.50****	34.50 ± 0.73	36.89 ± 1.12	32.11 ± 2.38
Barnes maze - Time spent in locating correct hole. Week 1	14.80 ± 1.39	27.00 ± 2.72**	17.60 ± 2.16	12.20 ± 1.20	15.00 ± 2.10	13.80 ± 3.03
Barnes maze - Time spent in locating correct hole. Week 2	10.40 ± 1.63	43.40 ± 7.51****	4.80 ± 1.53	7.80 ± 0.97	19.00 ± 2.82	8.40 ± 3.79
Barnes maze - Time spent in locating correct hole. Week 3	12.80 ± 3.81	42.20 ± 3.11****	30.20 ± 4.07*	6.20 ± 1.32	10.00 ± 4.11	12.60 ± 4.57
Barnes maze - Time spent in locating incorrect hole. Week 1	2.20 ± 0.37	5.20 ± 0.37**	3.40 ± 0.51	2.00 ± 0.45	2.60 ± 0.51	1.80 ± 0.49
Barnes maze - Time spent in locating incorrect hole. Week 2	0.60 ± 0.40	2.80 ± 0.37**	0.60 ± 0.40	1.00 ± 0.32	0.80 ± 0.37	0.80 ± 0.37

Barnes maze - Time spent in locating incorrect hole. Week 3	3.40 ± 1.16	2.80 ± 0.86	3.60 ± 0.81	1.80 ± 1.11	2.40 ± 0.93	3.88 ± 0.49

DISCUSSION

Hand Grip and Rotarod Tests - Behavioral Performance

Diazepam markedly decreased muscle strength and motor coordination which is in line with its sedative and muscle-relaxant properties through GABA_A receptor potentiation. Nonetheless, lutein treatment at 60mg/kg significantly enhanced performance, and was similar to that of donepezil. This implies that lutein can address motor impairments that are induced by oxidative damage or GABAergic imbalance. The same neuroprotective effect of lutein on motor performance has been reported on scopolamine-induced models of cognitive impairment (Chike *et al.,* 2025).

Y Maze Inflexion Ratio and Y Maze % Spontaneous Alteration

The administration of diazepam resulted in a significant decrease in spontaneous alternation, which pointed to memory impairment. Treatment with lutein exhibited a significant improvement in the alternation performance with effects that matched the donepezil group. These results align with other reports that lutein supplementation enhances working memory and attention abilities in both rodent models and older adults (Nazari *et al.,* 2022; Lopresti *et al.,* 2022). Also, there was an increase in arm entries, indicating a restored exploratory drive, which can indicate a decrease in sedative effect and improvement in cognitive processing.

Barnes Maze Parameters

In the Barnes maze, rats treated with diazepam had significantly longer escape latencies, fewer correct hole entries and more errors that indicate impairment of spatial learning and memory. Lutein treatment improved these deficiencies in a dose-dependent way, resulting in reduced escape latencies, decreased errors, and an increase in correct entries. These gains are in line with known functions of lutein in the maintenance of hippocampal-related spatial memory. Further, the retention test indicated that the lutein-treated rats were able to recall the position of the escape hole, which further confirmed its cognitive-enhancing effect. Similar results have been reported in past studies where lutein was proven to enhance performance in the Morris water maze and Barnes maze tasks in rodent models of neurotoxicity (Nazari *et al.,* 2022).

Nitric Oxide Concentration

In comparison to the control group, the Diazepam only group shows a very significant elevation ($P < 0.001$). In 20mg/kg lutein group, the concentration of brain Nitric oxide shows a statistically insignificant decrease ($P < 0.05$) when compared to the positive control group and a statistically significant rise in NO concentration when compared to the negative control group. In 40mg/kg and 60mg/kg administrations of Lutein ± diazepam groups, the concentrations of Nitric oxide show an insignificant decrease respectively in contrast with the positive control group, and a non-statistically significant elevation in contrast with the negative control group. In comparison to the negative control group, there was no statistically significant rise in NO levels. When compared to the positive control group, there was significant decrease in NO level. These findings suggest that 60mg/kg dose of Lutein administration has the potential to mitigate oxidative stress by restoring the elevated concentration of NO following Diazepam-induced impairment to normal.

This study shows that diazepam has a powerful increase in brain nitric oxide (NO), which was confirmed by previous studies that associate the use of diazepam with oxidative stress and cognitive impairment (Sevastre-Berghian *et al.,* 2017; Kaur *et al.,* 2016). This increase in NO was reduced by Lutein in a dose-dependent manner, which is in line with previous reports of its antioxidant-neuroprotective effects in vitro (glutamate-induced oxidative stress in HT22 cells) (Li *et al.,* 2024) and in vivo (vascular dementia rat model improvement of oxidative damage and synaptic dysfunction) (Nejad *et al.,* 2024). Lutein at higher doses normalized the NO levels to the baseline levels, along with the improvement in the cognitive performance levels that were equal to the ones observed with normal cognitive enhancers. Taken together, these data, along with a rich literature on oxidative stress as a mediator of memory impairment and the therapeutic potential of bioactive compounds (Singh *et al.,* 2022) indicate that lutein should be further studied as an adjunct to alleviate diazepam-induced oxidative neurotoxicity and memory impairment.

CONCLUSION

In summary, this study has shown that diazepam increased the brain nitric oxide (NO) levels, which is in line with the suggested memory impairment mechanism linked with exposure to diazepam. This effect was reduced by Lutein administration in a dose-dependent manner with the highest concentration restoring the levels of NO to near physiological norms. The level of protection that was achieved was similar to that of donepezil implying that lutein has significant neuroprotective potential. Taken together, these observations suggest that lutein can be a potential source of natural therapeutic agent to alleviate drug-induced cognitive dysfunction.

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