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Research Article

L-Arginine Administration Improves Cognition and Oxidative Stress Parameters in the Hippocampus and Frontal Lobe of 4-Vinylcyclohexene Diepoxide Perimenopausal Female Rats

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ABSTRACT

Neuropsychiatric symptoms like cognitive impairment and anxiety are prominent in the perimenopausal period and have been related to increased oxidative stress. Study evaluated effect of L-arginine on these neuropsychiatric symptoms and oxidative stress parameters. Immature female Sprague-Dawley rats were divided into 3 groups; Premenopausal injected with Corn-oil (2.5µl/g) for 15 days; VCD perimenopausal, injected with 4-vinylcyclohexene diepoxide (160mg/kg) diluted in Corn-oil for 15 days; and AGING perimenopausal group. Fourteen weeks after VCD/corn-oil administrations, and 180 days in AGING perimenopausal group, rats were further divided into 2 sub-groups that received L-Arginine (100mg/kg) and distilled water for 30 days. Thereafter, neurobehavioural assessments were carried out in animals at diestrus using Y-maze and elevated plus maze. Animals were humanely sacrificed, hippocampus and frontal lobe were isolated from the brain and homogenized for measurement of oxidative stress parameters. Percentage correct alternation was significantly higher ($P< 0.05$) in premenopausal compared to VCD and AGING perimenopausal groups. It was significantly lower ($P < 0.05$) in AGING perimenopausal group administered distilled water compared to AGING perimenopausal group administered L-Arginine with no change in other groups. Close Vs Open arm ratio was significantly lower ($P < 0.05$) in premenopausal compared to VCD and AGING perimenopausal groups. Similarly, L-Arginine significantly reduced ($P < 0.05$) Close Vs Open arm ratio in AGING PRM group while it significantly improved ($P < 0.05$) oxidative stress parameters in all groups. L-arginine improved cognition and anxiety in AGING perimenopausal with no change in premenopausal and VCD perimenopausal rats.

Keywords: *L-arginine, oxidative stress, anxiety, memory, perimenopause*

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INTRODUCTION

Cognitive impairment and anxiety related behaviours are commonly reported during the perimenopausal transitory period (Santoro *et al.,* 2015, Muslic and Jokic-Begic, 2016). The perimenopausal transitory period is characterized by changes in the levels and function of reproductive hormones such as FSH, LH, oestrogen, progesterone, testosterone and dihydrotestosterone (Hale *et al*., 2014, Reis *et al.*, 2014, Brinton *et al.,* 2015, Santoro, 2016).

These changes are associated with increased oxidative stress which occur secondary to a marked increase in the production of reactive oxygen species. Increased oxidative stress in the perimenopausal period may account for the neurobehavioural symptoms seen during this transitory period (Joshi *et al*., 2015). This is because oxidative stress has been implicated in both cognitive dysfunction and anxiety disorders in the general population and recent studies have demonstrated the beneficial role of antioxidants in the management of these conditions (de Oliveira *et al*., 2015, Zhao *et al.,* 2017, Li *et*

al., 2018). Furthermore, a recent study showed that the antioxidant; melatonin improved psychosomatic symptoms in post-menopausal women (Chojnacki *et al.*, 2018). These findings suggest that antioxidants may be beneficial in ameliorating the neuropsychiatric symptoms experienced in perimenopausal women.

L-arginine (L-ARG), is one of the commonly used antioxidant drugs. It is converted to nitric oxide (NO) by nitric oxide synthase (NOS) in the presence of heme and tetrahydrobiopterin as co-factors. Nitric oxide acts as both an antioxidant and a neurotransmitter and decreased NO transmission in the brain has been implicated in cognitive dysfunction (Zhu *et al*., 2017, Stephan *et al.*, 2017). It has been shown that drugs replacing NO levels are beneficial in disorders affecting memory and the ability of L-ARG to improve cognition has been attributed to its antioxidant potential as well as its neurotransmitter function (Fonar *et al.*, 2018).

A study demonstrated the protective effect of L-ARG on lipopolysaccharide induced memory deficit by improving oxidative stress parameters (Hosseini *et al.*, 2018). While cognitive impairment is associated with low levels of NO, increased NO levels has been implicated in anxiety related disorders (Zhou *et al.*, 2018). It has been shown that one of the mechanism of action of anxiolytic drugs is downregulating neuronal nitric oxide synthase (nNOS) and reducing NO levels (Zhou *et al*., 2018), although a few studies reported conflicting findings (Kalouda and Pitsikas, 2015, Trevlopoulou *et al*., 2016).

Low levels of NO and L-ARG has been reported in the perimenopausal period. A relatively lower plasma levels of L-ARG in perimenopausal compared to premenopausal women has been reported (Klawitter *et al*., 2017). Studies in animal models of perimenopause has also demonstrated decreased levels of NOS activity and reduced NO production in the ovaries of perimenopausal rats (Zhao *et al*., 2012). Therefore, it appears that reduced levels of L-ARG may account for the changes associated with perimenopause and its replacement may be beneficial in the management of cognitive impairment. Thus, based on the antioxidant and NO increasing properties of L-ARG, we hypothesise that L-arginine supplementation will improve cognition with a variable effect on anxiety in perimenopausal animal models.

MATERIALS AND METHODS

Chemicals: 4-vinylcyclohexene diepoxide (VCD) was purchased from Sigma-Aldrich (St. Louis, MO, USA). L-arginine was purchased from Nowfoods (USA).

Experimental animals and diet: Thirty-six female Sprague-Dawley rats were obtained from the animal laboratory center, College of Medicine, University of Lagos. All the animals were housed in groups of 6 rats per clear polypropylene cage lined with wood shavings. Rats were kept under normal light conditions (12 hours light/dark cycle) and normal room temperature (23 ± 1 °C). Pelletized normal rat chow and water was made available *ad libitum*, and all rats were weighed weekly. All experimental procedures were carried out in compliance with the international principles for laboratory animals as obtained in the Helsinki's declaration (NIH 1985) guide for care and use of laboratory animals. The research protocol was also in line with the guidelines of the College of Medicine, University of Lagos, Health Research Ethics Committee.

Experimental design and animal groupings: Twenty-four post-weaned female Sprague-Dawley rats at 28 days of age and twelve matured female Sprague-Dawley rats at 70 days of age were allowed to acclimatize for a week and the post-weaned rats were randomly divided into 2 equal groups i.e. Premenopausal (PREMP) and VCD perimenopausal (VCD PRM) groups, while the matured rats served as the AGING perimenopausal (AGING PRM) group. The PREMP group received daily subcutaneous injection of corn oil (2.5 µl/g BW) for 15 consecutive days and allowed to grow till the 14th week. The VCD PRM group received daily subcutaneous injection of VCD (160 mg/Kg) (Mayer *et al.,* 2004; Lohff *et al.,* 2005) diluted in Corn oil (2.5 µl/g BW) for 15 consecutive days and also allowed to grow till the 14th week. The AGING PRM group was allowed to grow till 180 days of age.

Then all animals in the three groups were further subdivided into Distilled water (DW) and L-arginine (L-ARG) groups. L-arginine was administered via oral cannula at a dose of 100mg/kg in distilled water to rats in the L-ARG sub-groups for additional duration of 4 weeks (Arikawe *et al.,* 2019), while distilled water only in equal volume as dissolved L-ARG was orally administered daily via oral cannula to rats in the DW sub-groups for additional duration of 4 weeks. The average age of rats in the PREMP and VCD PRM groups was 160 days, while in the AGING PRM group was 210 days and 14 weeks as stated above signified the number of weeks after either corn oil or VCD administration. We have previously observed that female rats injected with VCD subcutaneously usually transit towards reproductive senescence from 80 to 100 days of age (yet to be published data).

At 150 – 165 days in PREMP and VCD PRM groups, and 200 – 215 days in AGING PRM, vaginal smears were analyzed following the methods of Marcondes, Bianchi, and Tanno (Marcondes *et al*., 2002). Estrous cycle was monitored daily for 15 days consecutively and only rats showing regular estrous cyclicity were used. A regular estrous cycle was defined as a 4-day sequence of metestrus, diestrus, proestrus, and estrus.

Neurobehavioural assessments were carried out in the animals on diestrus morning using the Y maze and elevated plus maze (EPM). All the female rats were pre- exposed to the test battery a day before the tests and diestrus was determined by the predominance of small circular leukocytes, nucleated epithelial cells and non-nucleated cornified cells in vaginal smear under the microscope (Marcondes *et al*., 2002).

Y-Maze test: The Y maze is used to measure locomotion activity and percentage correct alternation. The locomotion activity measures the complex neuronal circuit involved in movement and emotionality of the rats (Martin, 2003, Ramos, 2008), while the proportion of correct alternation is a function of working/spatial memory (Onaolapo and Onaolapo, 2013). The apparatus is a Y-shaped maze with three wooden arms

labelled A, B and C at a 120° angle from each other. The animals were allowed to freely explore the three arms for 4 minutes, after introduction to the centre of the maze. An entry occurred when all four limbs are within the same arm. Correct alternation occurred when the rats entered the three arms sequentially. The number of arm entries and the number of correct alternation were recorded in order to calculate the percentage of alternation (Onaolapo and Onaolapo, 2013).

Elevated Plus Maze : The elevated plus maze (EPM) is a validated model for testing anxiety related behaviour, preference for the closed arms suggests anxiety (Carobrez and Bertoglio, 2005). The EPM is a plus shaped maze with a central area and four arms; 2 open and 2 enclosed arms with a height of 50cm. The central area is 10cm by 10cm in size while each arm is 50cm long and 10cm wide. The wall of the closed arms is 40 cm high. The animals were placed in the centre of the maze facing an open arm and were allowed to explore the maze for 4 min. Arm entry was recorded starting when two paws had crossed the line into the arm. The ratio of the time spent in the closed arm to open arm was then calculated.

Brain tissue for oxidative stress assessment: The rats were humanely sacrificed by using a standard cervical dislocation procedure. The brains were quickly isolated with hippocampus and frontal lobe dissected from the whole brain, weighed, washed in ice-cold normal saline and homogenized in 10% ice-cold 0.1 M potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 RPM for 15 minutes in a cold centrifuge with supernatants separated and stored at -70º C for the measurement of oxidative stress markers superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), and catalase as described in a previous study (Arora *et al.*, 2010).

Glutathione level and glutathione peroxidase activity (GPx) were estimated using the method described by Ellman (Ellman, 1959). Catalase activity was determined by the colorimetric method as previously described (Clairborne, 1985). Malondialdehyde was estimated using the method described by Ohkawa and co-workers (Ohkawa *et al.,* 1979), and Superoxide dismutase activity was determined by the pyrogallol auto-oxidation method described by Marklund (Marklund, 1985).

Statistical analysis: All results were expressed as mean ± S.E.M of rats 10 to 12 in each group. The data were statistically compared by carrying out One-way Analysis of Variance (ANOVA) using GraphPad Prism 8 followed by post-hoc Tukey's test for inter-group comparisons. A value of $p < 0.05$ was considered statistically significant for comparison

RESULTS

Percentage correct alternation: The percentage correct alternation in female rats administered DW was significantly higher ($P < 0.05$) in the PREMP group compared to the VCD PRM and AGING PRM groups (Figure 1). Likewise, in the female Sprague-Dawley rats administered L-ARG, percentage correct alternation was significantly higher ($P < 0.05$) in the PREMP group compared to the VCD PRM and AGING PRM groups (Figure 1). However, in perimenopausal rats administered oral L-ARG, percentage correct alternation was significantly lower ($P < 0.05$) in the VCD PRM group compared to AGING PRM group (Figure 1). Also, comparing the intra-groups, percentage correct alternation was significantly lower ($P < 0.05$) in the AGING PRM group administered DW compared to AGING PRM group administered L-ARG (Figure 1). There was no significant difference in percentage correct alternation in VCD PRM DW group compared to VCD PRM L-ARG group

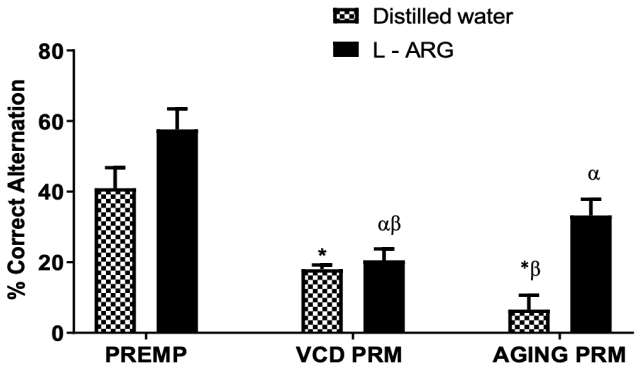

Figure 1
Percentage Correct Alternation in PREMP, VCD PRM, and AGING PRM female rats
*P < 0.05 Vs. PREMP Distilled water; αP < 0.05 PREMP L-ARG; βP < 0.05 AGING PRM L-ARG

Elevated plus maze - Closed versus open arm entry ratio: The closed versus open arm ratio in female Sprague-Dawley rats administered DW was significantly higher ($P < 0.05$) in the perimenopausal (VCD PRM and AGING PRM) groups compared to the premenopausal (PREMP) group (Figure 2). Likewise, in perimenopausal rats administered L-ARG, closed versus open arm ratio was significantly higher ($P < 0.05$) in the VCD PRM group compared to the AGING PRM group (Figure 2). Also closed versus open arm ratio was significantly higher ($P < 0.05$) in the AGING PRM group administered DW compared to AGING PRM group administered L-ARG (Figure 2).

The closed versus open arm ratio in female Sprague-Dawley rats administered DW was significantly higher ($P < 0.05$) in the perimenopausal (VCD PRM and AGING PRM) groups compared to the premenopausal (PREMP) group (Figure 2). Likewise, in perimenopausal rats administered L-ARG, closed versus open arm ratio was significantly higher ($P < 0.05$) in the VCD PRM group compared to the AGING PRM group (Figure 2). Also closed versus open arm ratio was significantly higher ($P < 0.05$) in the AGING PRM group administered DW compared to AGING PRM group administered L-ARG (Figure 2).

Oxidative stress parameters assessment in distilled water groups

Frontal lobe: In the frontal lobe in all animals that had distilled water, MDA level was significantly lower ($p<0.05$) in the AGING PRM DW group compared to the PREM DW group (Table 1). On the other hand, there was no significant change in GSH levels, the activity of CAT and SOD in the VCD PRM DW and AGING PRM DW groups compared to the PREM DW group (Table 1).

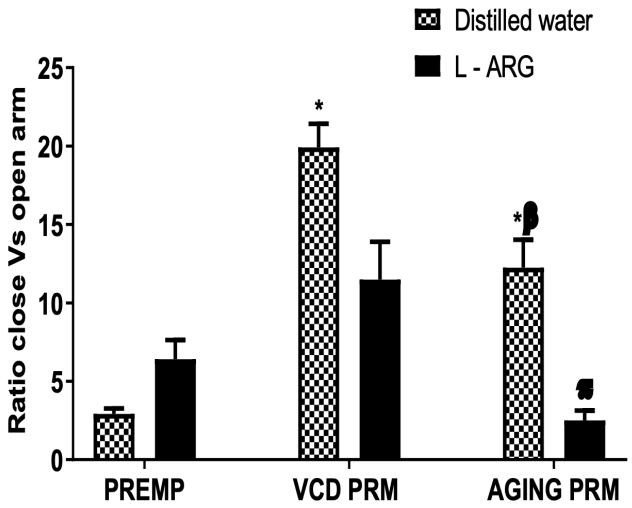

Figure 2
Ratio close Vs open arm in PREMP, VCD PRM, and AGING PRM female rats
*P < 0.05 Vs. PREMP Distilled water; πP < 0.05 Vs VCD PRM L-ARG;
βP < 0.05 Vs AGING PRM L-ARG

Hippocampus: In the hippocampus in all animals that had distilled water, there was no significant change in the activity of SOD. However, CAT activity was significantly higher ($p<0.05$) in the VCD PRM DW group compared to the PREM DW group (Table 2). On the other hand, there was no significant difference in the levels of GSH and MDA amongst the groups (Table 2).

Effects of L-Arginine supplementation on oxidative stress parameters assessment

Frontal Lobe: In the frontal lobe in all animals that had L-ARG supplementation, there was a significant decrease ($p<0.05$) in the activity of SOD in the PREMP L-ARG group compared to the PREM DW group (Table 1). MDA level was also significantly lower ($p<0.05$) in all the L-ARG supplemented groups compared to the distilled water groups (Table 1).

Hippocampus: In the hippocampus, L-Arginine supplementation significantly reduced ($p<0.05$) CAT activity in the VCD PRM L-ARG group compared to the VCD PRM DW group (Table 2). GSH level was significantly increased ($p<0.05$) in the PREMP L-ARG group compared to the PREM DW group. Likewise, GSH level was significantly increased ($p<0.05$) in the VCD PRM L-ARG group compared to the VCD PRM DW group (Table 2).

DISCUSSION

This study demonstrated the presence of cognitive impairment and anxiety related behaviour in animal models of perimenopause and is in consonance with previous studies (Reis et al., 2014, Santoro et al., 2015, Muslic and Jokic-Begic, 2016). The perimenopausal (VCD PRM and AGING PRM) rats had impaired working memory, and this was demonstrated by a reduction in the proportion of correct alternation compared to premenopausal rats (PREMP group) as seen in Figure 1.

Table 1:
Level and activity of oxidative stress parameters in the frontal lobe

Frontal Lobe	PREMP		VCD PRM		AGING PRM	
	DW	L-ARG	DW	L-ARG	DW	L-ARG
GSH Level (µmol/ml)	11.17 ± 0.61	9.24 ± 0.63	11.30 ± 0.64	9.66 ± 0.61	9.75 ± 0.87	9.19 ± 0.98
MDA Level (µmol/ml)	0.62 ± 0.06	0.34 ± 0.07*	0.78 ± 0.11	0.15 ± 0.01δ	0.25 ± 0.02*	0.14 ± 0.02κ
CAT Activity (µmol/ml/min/mg pro)	17.27 ± 1.77	14.35 ± 1.72	20.32 ± 2.49	24.07 ± 2.42	18.42 ± 1.21	20.72 ± 1.21
SOD Activity (µmol/ml/min/mg pro)	4.00 ± 0.55	2.46 ± 0.14*	3.70 ± 0.54	4.33 ± 0.45	4.66 ± 0.25	4.97 ± 0.51

Level and activity of oxidative parameters in the frontal lobe in PREMP, VCD PRM, and AGING PRM female rats.
*P < 0.05 Vs. PREMP DW; δP < 0.05 Vs VCD PRM DW; κP < 0.05 Vs AGING PRM DW

Table 2:
Level and activity of oxidative stress parameters in the hippocampus

Hippocampus	PREMP		VCD PRM		AGING PRM	
	DW	L-ARG	DW	L-ARG	DW	L-ARG
GSH Level (µmol/ml)	10.70 ± 0.52	13.66 ± 0.53*	9.28 ± 0.29	11.97 ± 0.31δ	12.19 ± 0.95	10.98 ± 0.94
MDA Level (µmol/ml)	0.34 ± 0.071	0.10 ± 0.070	0.59 ± 0.095	0.58 ± 0.083	0.29 ± 0.080	0.41 ± 0.087
CAT Activity (µmol/ml/min/mg pro)	16.10 ± 2.59	14.49 ± 2.65	31.11 ± 2.81*	15.24 ± 2.78δ	19.25 ± 0.80	18.24 ± 0.71
SOD Activity (µmol/ml/min/mg pro)	5.54 ± 0.92	3.57 ± 1.00	4.38 ± 0.29	3.98 ± 0.38	4.17 ± 0.55	5.47 ± 0.56

Level and activity of oxidative parameters in the hippocampus in PREMP, VCD PRM, and AGING PRM female rats.
*P < 0.05 Vs. PREMP DW; δP < 0.05 Vs VCD PRM DW

This is in line with previous documentations in perimenopausal women (Greendale et al., 2011). Other studies on menopausal animal models have reported impaired cognition though there is paucity of data on animal perimenopausal models on cognition (Patki et al., 2013, Djiogue et al., 2018). Likewise, the perimenopausal (VCD PRM and AGING PRM) rats showed a preference for the closed arm of the EPM rather than the open arm which supports increased anxiety (Carobrez and Bertoglio, 2005). Reis and colleagues had earlier reported a decrease in the rate of open arm exploration in VCD induced perimenopause in rats (Reis et al., 2014) and our results support this (Figure 2).

Our results also showed that the L-Arginine supplementation had variable effects on cognitive function and anxiety in the three groups. In the PREMP group, there was no significant difference in the measures of anxiety and memory between the DW and L-ARG groups. We have earlier reported the beneficial role of a similar dose of L-Arginine in reducing drug-induced cognitive impairment with no significant change in normal rats (Olusanya et al., 2018). Likewise, a study reported that the administration of L-homoarginine, (a structural analogue of L-Arginine and NO precursor) to healthy humans without cognitive dysfunction showed no improvement in cognition (Schonhoff et al., 2018). These suggests that L-arginine may be more beneficial in cognitively impaired models rather than models without impaired cognition.

L-arginine also appears to follow this similar pattern of response as demonstrated above on cognition in non-anxious states. A study investigating the effect of administration of a combination of L-Arginine and dehydroepiandrostenedione (a neurosteroid) on anxiety, found a significant reduction in anxiety scores whereas there was no difference between the anxiety measurement in non-anxious rats (Chakraborti et al., 2011).

In the AGING PRM rats, L-arginine administration significantly improved memory and reduced anxiety but had no effect on these parameters in the VCD PRM group. The beneficial effect of L-arginine and NO donors on cognition has been well documented (Fonar et al., 2018, Polis et al., 2018) whereas the effect on anxiety is controversial (Gulati et al., 2017). The reason for the beneficial effect may be related to the low dose given. The dose given was probably enough to increase the deficient levels of NO in the brain but not sufficient to induce anxiety since reduced NOS activity and NO levels have been documented in the perimenopausal period in women (Klawitter et al., 2017), and perimenopausal animal model (Zhao et al., 2012).

L-Arginine supplementation had differential effects on cognition and anxiety in both animal models of perimenopause in this study. The difference may be related to the reproductive age of the animals and more importantly mechanism underlying perimenopausal induction since it has been reported that perimenopause as a transitory period is characterized by fluctuating levels of reproductive hormones to a more sustained reduction in the late phases and distinct biochemical changes from beginning to the end of the spectrum (Allshouse et al., 2018). Plausibly, the VCD PRM group represents the later phase of perimenopause compared to the AGING PRM group as shown from our results (Tables 1 and 2). These findings support the view that severe oxidative changes develop as reproductive senescence progresses (Kolesnikova et al., 2015, Taleb-Belkadi et al., 2016), albeit because oxidative stress could be the primary cause of a disorder or occur secondary to the effect of a disorder (Sies, 2015).

The increased oxidative stress reported in the VCD PRM group was more pronounced in the hippocampus compared to the frontal lobe (Tables 1 and 2). This could be linked to changes in neurotransmission in the hippocampus which have been implicated in both cognitive changes and anxiety disorders (Rio-Alamos et al., 2017). Disruption in neurotransmission in the limbic structure made up of hippocampus, amygdala, perirhinal, entorhinal cortex, and basal forebrain results in anxiety disorder. Coincidentally, nNOS is highly concentrated in similar regions of the brain mediating anxiety (Zhou et al., 2018). Thus, our results support a link between NO pathway and the development of anxiety and a pathological basis for the development of anxiety in the perimenopausal period.

The Y-maze measures working and spatial memory and can be employed as a measure of interaction between the prefrontal cortex and the hippocampus. Thus, disruption in working memory suggests involvement of the prefrontal cortex (Roberson et al., 2012). L-arginine supplementation improved oxidative stress parameters in both perimenopausal groups and this supports the antioxidant potentials of L-arginine. However, the improved oxidative state did not translate into improved cognition or reduced anxiety in the VCD PRM group and this suggests an alternative mechanism of action of L-arginine since it has a pleotropic effect in the central nervous system. For example, apart from its role as an antioxidant, it is involved in neurotransmission, which is an essential component of memory formation, it also increases cerebral blood flow and maintains sleep-wake cycle (Kajitani et al., 2010, Paul and Ekambaram, 2011, Virarkar et al., 2013).

Memory impairment and anxiety disorders as common comorbidities in perimenopause are both associated with oxidative stress but seems to be on opposite end of the spectrum when it comes to nitric oxide replacement (Wultsch et al., 2007). Thus, drugs targeting nitric oxide replacement during perimenopause need to be carefully titrated to avoid excessively high levels of NO. Likewise, the L-arginine supplementation may be a useful approach in the management of perimenopausal neuropsychiatric disorders as suggested in this study. This is one of the few studies that have addressed the neuropsychiatric symptoms which are predominant during perimenopause. L-arginine supplementation improved oxidative stress, but it's effect on memory and anxiety during this transitory period seems to be independent of its antioxidant capacities.

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