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Research Article

Melatonin and Vitamin C modulate cholinergic neurotransmission and oxidative stress in scopolamine-induced rat model of memory impairment

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Dementia, Melatonin, Vitamin C, Memory, Scopolamine.

ABSTRACT

Background: Cognitive dysfunction which characterizes dementia is reportedly caused by multiple factors including oxidant-antioxidant imbalance, inflammation, alteration in synaptic neurotransmission. Despite the arrays of drugs available in managing dementia, it appears no single drug can effectively treat dementia. Since it is multifactorial, combining potential drugs may provide neuroprotective impact. As such, this study investigated the neuroprotective effects of melatonin and vitamin C on scopolamine model of cognitive impairment in rats and the possible mechanism of action. **Methods**: Thirty male Wistar rats were divided to receive either normal saline (5 ml/kg, p.o), scopolamine (1 mg/kg, i.p.), donepezil (2 mg/kg, p.o), melatonin (10 mg/kg, p.o), vitamin C (100 mg/kg. p.o) or melatonin *plus* vitamin C. Cognitive impairment was induced by daily injection of scopolamine (1 mg/kg, i.p.), after which different treatment regimen were administered for 15 days. Spatial memory was assessed using Morris Water Maze and modified light and dark box. The brain was processed for malondialdehyde (MDA), reduced glutathione (GSH) and acetylcholinesterase (AchE) activity. **Results**: Scopolamine-treated rats with no intervention showed impaired learning and memory as depicted by a significant ($p<0.05$) increase in escape latency, reduction in the frequency of visit to the escape aperture, increased MDA, decreased GSH and elevated acetylcholinesterase activity when compared to other groups. Interventions with melatonin or/and vitamin C reversed these responses respectively. The melatonin *plus* vitamin C treated group compared favorably with donepezil (reference group). **Conclusion**: Melatonin and vitamin C show neuroprotective effect in attenuating cognitive impairment in scopolamine-induced model by modulating oxidative stress pathway and enhancing cholinergic neurotransmission.

INTRODUCTION

Dementia is a progressive disorder characterized by gradual decline of mental and intellectual abilities. It interferes with social or occupational functioning such that as the disease progresses, the ability to function independently of an affected individual deteriorates due to memory loss and impaired cognitive ability (Tewari *et al.*, 2018). Globally, World Health Organization (WHO) estimated that about 5-8% of the general population aged 60 and above suffer from memory impairment, with approximately 60% living in low and middle-income countries. Annually, there about 10 million new cases (WHO, 2019). Therefore, it is imperative to curb the progression of cognitive decline before it crosses the threshold to dementia.

Involvement of oxidative stress in the pathogenesis of cognitive impairment has been reported. The role of oxidative stress in many neurodegenerative diseases is not shocking because the brain is rich in fatty acids, consumes lot of oxygen and deficient of endogenous antioxidants. These make it highly susceptible to reactive oxygen species (ROS) (Uttara *et al.*, 2009). Multiple lines of evidence indicate that oxidative stress

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does not only participate in cognitive impairment but plays an important role in inducing and activating multiple cell signaling pathways that contribute to the formation of toxic substances which promotes the development of dementia. Due to the increasing evidence that cognitive impairment is mediated by oxidative stress (Gil-Yong *et al.,* 2017), cumulative lines of evidence have demonstrated that consumption of antioxidant-rich foods can enhance cognitive performance (Ataie *et al.,* 2010; Craggs and Kalaria, 2011; Valls-Pedret *et al.,* 2013).

Melatonin is an amphiphilic tryptophan-derived indolamine which has a free radical scavenging, and antioxidant effects (Hardeland *et al.,* 1993). It scavenges reactive oxygen and nitrogen species and increases antioxidant defenses, thus it prevents tissue damage and blocks transcriptional factors of pro-inflammatory cytokines. Melatonin has beneficial effects including stimulation of antioxidant enzymes, inhibition of lipid peroxidation, and contributes to protection against oxidative damages (Manchester *et al.,* 2015).
Endogenous melatonin secretion is regulated by the circadian clock and by light/dark cycles. Through the indirect pathway, light influences sleep and secondarily influences mood and hippocampal-dependent cognition. Studies have shown that melatonin can positively influence cognition in adults with mild cognitive impairment (Furio *et al.,* 2007; Cardinali *et al.,* 2012)
In addition, exogenous melatonin has been found to be beneficial in improving certain aspects of cognitive function in elderly people (Peck *et al.,* 2004), in various animal models such as Alzheimer's disease (AD) (Xian *et al.,* 2002; Feng *et al.,* 2004; Olcese *et al.,* 2009).
Vitamin C, also known as ascorbic acid, is an essential nutrient involved in the repair of tissue and the enzymatic production of certain neurotransmitters. It is required for the functioning of several enzymes and is important for immune system function. It also functions as an antioxidant (Linus, 2019). The antioxidant effect of vitamin C has been well documented (Duarte and Lunec, 2005). It is an important water soluble antioxidant which is reported to neutralize ROS and reduce the oxidative stress (Verma *et al.,* 2007).
Antioxidants have been reported to prevent oxidative damage caused by the generation of free radicals. Oxidative stress is a culprit in the pathogenesis of various neurodegenerative disorders (Patockova *et al.,* 2003). Despite the availability of reports that support the memory enhancing effects of either melatonin or vitamin C and these effects have been strongly linked to their antioxidant capacities, yet there is no available evidence that combination of melatonin and vitamin C might be beneficial in cognitive impairment. As such, the present study investigated the effect of combination of melatonin and vitamin C on learning and memory in scopolamine-induced rat model of cognitive impairment.

MATERIALS AND METHODS

Drugs and reagents

All chemicals/drugs and reagents used were of analytical grade and drug solutions were freshly prepared before use. Scopolamine was a product of Hubei Tianyao pharmaceutical Co. Ltd. Hubei, China. Donepezil was produced by Torrent Pharma Ltd, United Kingdom. Ketamine and Melatonin were purchased from Sigma chemical company (St. Louis, MO, USA). Vitamin C (Biopharma Nigeria Ltd) was purchased from Aromokeye Pharmacy, Ilorin, Nigeria. The drugs were dissolved in normal saline and administered based on body weight.

Table 1: Animal grouping and administration

Groups		Dosage administered to each group (daily)
1	Control	Vehicle (5 ml/kg normal saline) orally
2	Scopolamine treated	Scopolamine (1mg/kg) ip.
3	Donepezil treated	Scopolamine (1mg/kg) ip. + Donepezil (2mg/kg) orally
4	Scopolamine/Melatonin	Scopolamine (1mg/kg) ip. + Melatonin (10mg/kg) orally.
5	Scopolamine/Vitamin C	Scopolamine (1mg/kg) ip. + Vitamin C (100mg/kg) orally.
6	Scopolamine/Melatonin + Vitamin C	Scopolamine (1mg/kg) ip. + Melatonin (10mg/kg) + Vitamin C (100mg/kg) orally.

i.p=Intraperitoneally

Experimental animal

Thirty male Wistar rats with an average weight of 125g were obtained from the breeding colony of Department of Biochemistry, University of Ilorin, Ilorin. They were housed in cages and fed with standard diet and water *ad libitum*, in the animal holding of the Faculty of Basic Medical Sciences, College of Health Sciences,

University of Ilorin, Ilorin. The rats were kept under standard laboratory conditions (12h light/dark cycle, temperature: 22 ± 3ºC) and acclimatized for two weeks before the commencement of the experiment. The experiment was performed in compliance with NIH guidelines for the humane use of laboratory animal.

The experiment was conducted in the morning (between the hours of 08:00 and 10:00). Administration of treatments regimen as shown above lasted for 15 consecutive days. Cognitive impairment was induced in all groups except control by a daily single injection of scopolamine (1 mg/kg, i.p). One-hour post scopolamine injection, rats were administered either donepezil, melatonin, vitamin C or a combination of melatonin and vitamin C. On the last day of administration, Morris water maze (MWM) and modified light and dark box were used to assess spatial memory function. Thereafter, each rat was anaesthetized, the brain was excised and then homogenized. The supernatant was processed for biochemical analysis of malondialdehyde (MDA), reduced glutathione (GSH) and Acetylcholinesterase.

Behavioral Tests

Morris Water-Maze Test

Spatial memory was evaluated using the Morris water maze (Morris, 2008). The maze is made up of an open circular pool of about 200 cm in diameter and 70cm deep filled with water up to about 60cm of the pool. A hidden platform with a top surface of about 15cm, maintained at the same position throughout the experiment was submerged at about 1.5cm below the water surface. The platform was made hidden by adding milk to make the water opaque thereby creating a nearly invisible platform-to-background. First, animals were trained to locate the platform. The maximum cut off duration for swimming was set at 60 seconds. When the rat locates the invisible platform the timer was stopped and the rat was removed but if the rat did not find the platform during the allotted time, the rat was guided on to the platform. At the end of the trial, the rats were removed from water, dried with towel and placed back in their home cages to keep warm. During acquisition trial, escape latency time (ELT), time taken to locate the hidden platform, was noted as an index of learning which was recorded with the aid of a video system. Each animal was subjected to the four acquisition trials per day for 5 consecutive days before the administration. On the last day of administration (15th day), the animals were re-exposed to the maze (to test for their spatial and long-term memory functions), a video camera was placed above the center of the pool to capture images of the swimming animal, for measures of the escape latency. The time spent by the animal in locating the hidden platform (escape latency) was noted as an index of learning. At the end of the test, the rats were removed from water, dried with towel and placed back in their home cages to keep warm.

Light and dark box

The light and dark box, an apparatus normally use for mood assessment was modified to evaluate spatial memory (Ayinla *et al.,* 2019). The tool consists of two compartments, the light and dark section. The light and dark box used was 50cm x 30cm x 100cm in size and the two sections were linked with an opening of $8cm^2$. Before administration, the rats were trained three trials a day (5 minutes per trial) for three consecutive days. At the end of training, only animals that developed memory of an opening route between the light and dark compartments were used for the experiment. To establish formation of memory in rats, the opening route was blocked from the dark compartment using black plank. Animals were then reintroduced and allowed to move freely, increased probing of the blocked-exit showed memory formation. One hour after the last treatments on day 15, the rats were re-introduced to the modified light and dark box and could explore the maze for 5 minutes. This was recorded with an overhead camera (Logtech Webcam, 5MP) and later analyzed by a blinded investigator. The number of time (frequency) each rat probed the blocked aperture was documented as the index for intact/enhanced spatial memory.

Sample Collection

On the 15th day of administration, after behavioral assessments, the rats were anaesthetized with intraperitoneal injection of ketamine (100 mg/kg). The brain tissues were isolated weighed and homogenized in 0.1 M phosphate buffer solution (pH 7.4). The homogenate was centrifuged at 3000 rpm for 10 minutes and the supernatant were separated and kept at -20°C prior to biochemical analysis.

Biochemical Analysis

Estimation of Acetylcholine esterase (AchE) level

The cholinergic marker, acetylcholinesterase, was estimated using Acetylcholinesterase Activity Assay kit (Elabscience, China). The assay kit is an optimized version of Ellman's method (Ellman, 1961) in which thiocholine, produced by AChE, reacts with 5, 5-dithiobis (2-nitrobenzoic acid). This homogenate was incubated for 5 min with 2.7mL of phosphate buffer and 0.1ml of Ellman's reagent (5, 5-dithiobis 2-nitrobenzoate, DTNB). Then, 0.1ml of freshly prepared acetylthiocholine iodide (pH 8) was added and the absorbance was read at 412nm.

Estimation of Malondialdehyde (MDA) level

Malondialdehyde (MDA), marker of oxidative stress was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) based on the method of Mihara and Uchiyama, (1978). Briefly, 3 ml of 1% H3PO4 and 1 ml of 0.6% TBA aqueous solution were added to 0.5 ml of 10% homogenate of the tissue sample. It was stirred and the mixture was heated on a boiling water bath for 45 minutes and allowed to cool. After which 4 ml of n-butanol was added, shook and the butanol layer was separated by centrifugation. The optical density was read at 535 and 520 nm

Estimation of reduced glutathione (GSH) level

Reduced glutathione was assayed according to the method of Ellman (1959). The colorimetric assay involves carefully optimized enzymatic recycling method using glutathione reductase and Ellman's reagent; DTNB. Glutathione reductase reduces GSSG to GSH. DTNB (5-5-dithiobis (2-nitrobenzoic acid) reacts with GSH to form yellow colour chromophore, 5 – thionitrobenzoic acid (TNB) and GS- TNB. GS – TNB was further reduced to GSH and TNB by glutathione reductase. The absorbance was read at 415nm and compared with standard curve for GSSG.

Statistical Analysis

The results were expressed as mean ± standard error of mean (SEM). Statistical significance was done using one-way analysis of variance (ANOVA) and then subjected to post-hoc Newman-Keul test using Graph pad prism version 5. Values were considered statistically significant at $p<0.05$.

RESULTS

Effects of melatonin and vitamin C on escape latency in Morris water maze test:

In Fig 1, the scopolamine-treated group showed an increase Escape Latency (EL) compared with control group which is an indicator of cognitive impairment (as this group of rats took a longer time to identify the escape platform).

Treatment with donepezil (reference drug), melatonin or/and vitamin C enhanced memory function as depicted by a significant ($p<0.05$) decrease in EL when compared with the group that received scopolamine only. Furthermore, there was no significant difference in EL in rats treated with melatonin or/and vitamin C when compared with donepezil treated group. Also, there was no significant difference in between the intervention groups as they show similar pattern.

Effects of melatonin and vitamin C on probing frequency in modified light and dark box:

As shown in Fig 2, there was a significant ($p<0.05$) reduction in the probing frequency (number of times

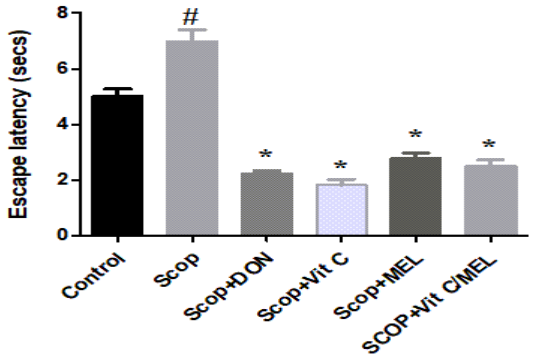

Fig. 1. Effects of melatonin and vitamin C on escape latency in Morris water maze test. Values are expressed as mean ± SEM of 5 rats per group. F (5, 29) = 48.10; #p<0.05 vs Control group, *p<0.05 vs scopolamine treated group Scop = scopolamine, DON= donepezil, Vit.C = vitamin C, MEL= melatonin.

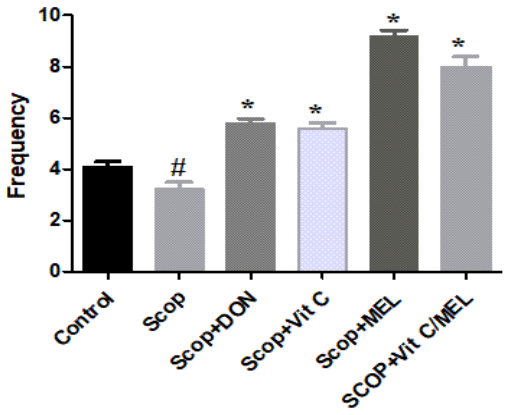

Fig 2. Effects of melatonin and vitamin C on probing frequency in light and dark box. Values are expressed as mean ± SEM of 5 rats per group. F (5, 29) = 78.33. #p<0.05 vs Control, *p<0.05 vs Scopolamine treated group. Scop = scopolamine, DON= donepezil, Vit.C = vitamin C, MEL= melatonin

the rat visited the escape route/aperture) in the scopolamine-treated rats when compared with control rats. Intervention with melatonin or/and vitamin C caused a significant increase in the probing frequency compared with scopolamine group. However, there was no significant difference in the probing frequency between the rats treated with donepezil (reference drug) and the three intervention groups. Also, there was no significant difference in between the intervention groups

Effects of melatonin and vitamin C on acetylcholine esterase (AchE) activity:

In Fig 3, the activity of AchE (an enzyme which breaks down Ach neurotransmitter) was significantly increased

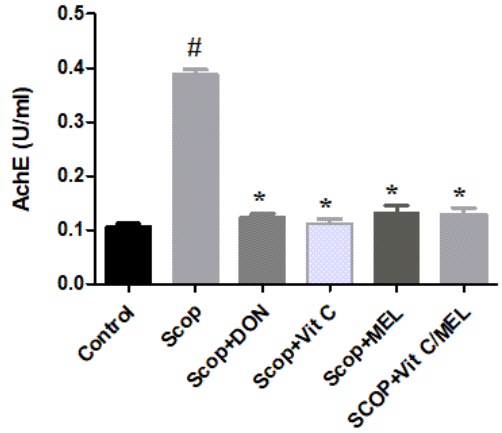

Fig 3. Effects of melatonin and vitamin C on acetylcholine esterase (AchE) activity. Values are expressed as mean ± SEM of 5 rats per group. F (5, 29) = 224.6; #p<0.05 vs control group, *p<0.05 vs Scopolamine treated group. Scop = scopolamine, DON= donepezil, Vit.C = vitamin C, MEL= melatonin

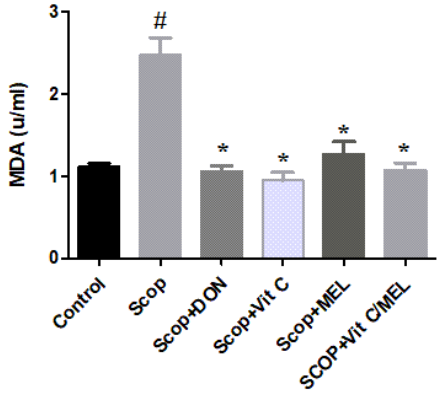

Fig. 4. Effects of melatonin and vitamin C on malondialdehyde (MDA) level. Values are expressed as mean ± SEM of 5 rats per group. F (5, 29) = 21.84; #p<0.05 vs control group, *p<0.05 vs Scopolamine treated group. Scop = scopolamine, DON= donepezil, Vit.C = vitamin C, MEL= melatonin

in the scopolamine group when compared with control group. Conversely, administration of melatonin or/and vitamin C reduced its activity when compared with the scopolamine group. The reduction in the activity of AchE in the combined treatment group follow a similar pattern with the donepezil treated group. However, there was no significant difference in between the intervention groups.

Effects of melatonin and vitamin C on malondialdehyde (MDA) level:

Fig 4 shows that the level of MDA, an index of lipid peroxidation, was significantly increased in scopolamine group when compared with control. Treatment with melatonin or/and Vitamin C significantly ($p<0.05$) attenuated the level of MDA compared with the untreated scopolamine group. There was no significant difference in MDA level across the three intervention groups when compared with the reference (donepezil) group.

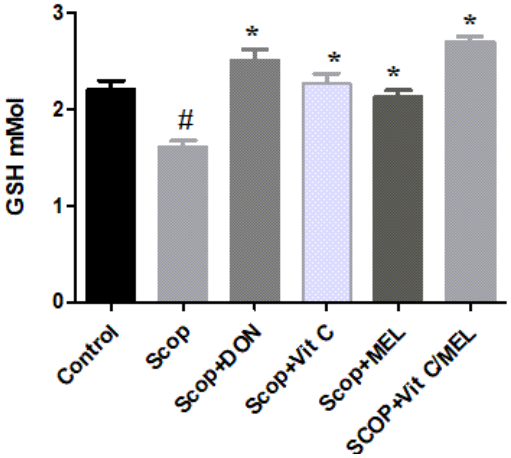

Fig. 5: Effects of melatonin and vitamin C on reduced glutathione (GSH) level. Values are expressed as mean ± SEM of 5 rats per group. F (5, 29) = 11.73; #p<0.05 vs control group, *p<0.05 vs Scopolamine treated group. Scop = scopolamine, DON= donepezil, Vit.C = vitamin C, MEL= melatonin

Effects of melatonin and vitamin C on reduced glutathione (GSH) level:

In Fig 5, the scopolamine group showed a significant decrease in the level of GSH compared with control rats. Conversely, administration of melatonin and Vitamin C led to a significant ($P<0.05$) increase in GSH when compared with scopolamine group. Also, the level of GSH in the three intervention groups compare favorably with donepezil treated rats and control group. However, there was no significant difference in between the intervention groups.

DISCUSSION

Antioxidant used as a potential treatment for cognitive impairment has been of interest for many years. Studies report that oxidative stress is positively associated with impaired cognitive function. Multiple lines of evidence demonstrate beneficial effects of antioxidants on cognition and dementia (Farah *et al.,* 2016).

Several drug induced dementia models have been used to evaluate the effects and therapeutic potentials of novel drugs in dementia; these include colchicine, scopolamine, okadaic acid and trimethyltin (Malekzadeh

et al., 2017). Scopolamine model used in this study has been reported to antagonize acetylcholine activities, increase brain oxidative stress status and impair learning and memory functions similar to memory deficit observed in dementia (Bubser *et al.,* 2012; Falsafi *et al.,* 2012; Gil-Yong *et al.,* 2017). Furthermore, the use of acetycholine esterase (AchE) inhibitors such as Tacrine, donepezil, and rivastigmine has been approved for the treatment of dementia symptoms (Amenta and Tayebati, 2008). Donepezil used in the present study as a reference drug is a known acetycholine esterase inhibitor which increases acetylcholine concentration and up-regulate brain cholinergic receptors (Cacabelos, 2007). Ketamine was used as an anesthetic agent in this study. Chronic administration of ketamine in animal model of schizophreina has been shown to increase AChE level in brain tissues which leads to cognitive deficit (Zugno *et al.,* 2014). This might not affect the present result as it was administered at a sub-chronic dose once and across all the groups.

In this study, the significant decrease in the escape latency recorded in the group treated with melatonin and vitamin C shows the memory-enhancing effect of the drugs. This confirms the reports of Xia *et al*., (2016). Although these authors used isoflurane to induce impairment of spatial memory. Also our results are in conformity with the findings of Olcese *et al.,* (2009). Similarly, the increase in the frequency of visiting the blocked escape route (modified light and dark box test) by rats treated with melatonin, vitamin C and combination of melatonin and vitamin C is suggestive of improvement in cognitive impairment. Furthermore, the observed improvement in the cognitive decline is consistent with the report of Tongjaroenbuangam *et al.* wherein pre-treatment with melatonin prior to dexamethasone-induced memory deficit resulted in shorter escape latencies and a longer time spent in the target quadrant which implies an improvement in memory function (Tongjaroenbuangam *et al.,* 2013). However, in contrast to our own finding, a study by Yilmaz *et al.,* (2015) in which melatonin was given together with vitamin C, showed no reversal of memory deficit in a dexamethasone-induced model of cognitive impairment in rats. Also, earlier studies using the AD model showed improvement in cognitive function (Xian *et al.,* 2002). However, a study by Eslamizade *et al.* (2016) showed no effect of melatonin on the cognitive function despite its protective effects against Aβ-induced increase in NF-κB and shrinkage of the CA1 pyramidal neurons. The disparity between these reports and ours may be attributed to differences in the melatonin dose, route and duration of administration, age of animal, and method of inducting cognitive impairment.

Scopolamine has been reported to elevate acetylcholinesterase (AChE) levels in the cortex and hippocampus (Ahmed and Gilani, 2009). The data obtained from this study showed that the acetylcholinesterase activity was reduced in melatonin and vitamin C treated groups similar to donepezil which show that melatonin and vitamin C inhibited the action of acetylcholinesterase. This action could enhance cholinergic transmission and thus availability of acetylcholine at the synapse. Meanwhile, acetylcholine is an important neurotransmitter which is involved in memory consolidation (Feng *et al.,* 2004).

Elevated level of malondialdehyde recorded in the scopolamine rats agree with the study which showed that impairment of memory in scopolamine induced animal model is associated with altered status of brain oxidative stress (Roja *et al.,* 2017). As such, the significant decrease in the MDA level of groups treated with melatonin and vitamin C may be attributed to their antioxidant properties. These antioxidants protect brain cells from oxidative stress; thereby reducing brain damage and improved neuronal function. Epidemiological and laboratory findings reveal that antioxidants delay progress of neurodegenerative disease such as Alzheimer's disease probably due to prevention or neutralization of detrimental effects of free radicals (Small and Mayeu, 2002). Similarly, the low level of reduced glutathione recorded in scopolamine-treated rats confirms the findings of Reiter *et al.* (2007) wherein high oxidative stress was accompanied by reduction in total glutathione levels. Therefore, the significant increase in GSH level of melatonin and vitamin C treated groups as compared to the scopolamine group may be due to their ability to promote the activity of antioxidant enzymes (Kurutas, 2016; Najafi *et al.,* 2017).

The central nervous system is vulnerable to free radical damage because of the brain's high oxygen consumption, its abundant lipid content, and the relative paucity of antioxidant enzymes as compared to other tissues. The brain is deficient in oxidative defense mechanisms and hence is at greater risk of damage mediated by reactive oxygen species (ROS), resulting in cellular dysfunction (Gupta *et al.,* 2003). Oxidative stress has been demonstrated to be related to the pathophysiologic mechanisms involved in brain injury in various common neurodegenerative disorders, including Parkinson's, Alzheimer's and Huntington's

diseases. Oxidative stress decreases the antioxidant defense status in the brain, which may form the basis for impaired memory (Onodera *et al.,* 2003). Thus, in the present study, the memory deficits observed in scopolamine-treated rats might have resulted from increased free radical formation and subsequent oxidative injury to neurons. Oxygen free radicals and other products of oxidative metabolism have been shown to be neurotoxic. Studies have shown that dehydroascorbic acid, the oxidized form of ascorbic acid, enters the brain by means of facilitated transport (Agus *et al.,* 1997). GLUT-1 transporter present on the endothelial cells of the blood-brain barrier helps in the transport of glucose and dehydroascorbic acid into the brain (Huang *et al.,* 2001). Oral administration of ascorbic acid might have resulted in the entry of high levels of dehydroascorbic acid into the rat's brain, which could protect neurons from the deleterious effects of free radicals. Administration of Melatonin or/and vitamin C improved learning and memory deficits by inhibiting oxidative stress via reduction of lipid peroxidation as depicted by low level of MDA, elevating oxidized glutathione (GSH) levels, and by modulating cholinergic neurotransmission. Similarly, the improvement in the cognitive behavioral decline can be attributed to enhanced neural plasticity mediated by the preservation of Ach, a key neurotransmitter involved in the process of learning and memory (Feng *et al.,* 2004). The reduction in the activities of acetylcholinesterase is thought to have increased the concentration of Ach, hence its availability for neurotransmission. Melatonin reportedly improved cognitive functions by increasing the number or activity of ChAT in the brain (Xian *et al.,* 2002; Eltablawy and Tork, 2014). It is worthy of mention that intervention with melatonin + vitamin C did not improve cognitive impairment better than the groups that received either melatonin or vitamin c alone. The melatonin + vitamin c group compares favorably with other treatment groups.

CONCLUSION

Co-administration of melatonin and vitamin C improved cognitive impairment by modulation of oxidative stress pathway and enhancement of cholinergic neurotransmission. As such, a combination of melatonin and vitamin C could provide ameliorative effect in neurological disorders where cognition is impaired.

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