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Effects of alpha-tocopherol associated with lovastatin on brain tissue and memory function in SHRSPs

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HIGHLIGHTS

- Lovastatin and alpha-tocopherol presented antioxidants effects.
- Alpha-tocopherol and lovastatin preserved memory and cognitive function.
- Alpha-tocopherol and lovastatin improved spatial memory of SHRSP rats.
- The hippocampus can be preserved on SHRSP rats with both treatments.

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ABSTRACT

Strokes are preceded by oxidative stress and inflammation, two processes linked to atherosclerosis and hypertension. Statins have been widely employed to control atherosclerosis; however, there could be neurological implications to its use—including cognitive impairment. Thus, we aimed to determine whether alpha-tocopherol is capable of reversing the neurological side effects of statins and enhancing its anti-inflammatory properties. To assess these effects, 15-week-old stroke-prone spontaneously hypertensive rats (SHRSPs) were divided into four groups (n = 6, each): alpha-tocopherol (AT), lovastatin (LoV), alpha-tocopherol + lovastatin (AT + LoV), and control (C). We administered 120 IU of alpha-tocopherol diluted in 0.1 ml of coconut oil, whereas the dose of lovastatin was administered at a ratio of 1 mg/kg of rat body weight. The control group received 0.1 ml coconut oil. All animals received the treatments via orogastric gavage. We assessed body weight, diuresis, food and water intake, oxidative stress (malondialdehyde levels), the total cellular injury marker (lactate dehydrogenase), short- and long-term memory, cognition, and histopathological changes in the hippocampus. The results demonstrated that lovastatin treatment did not negatively affect the memory of our animal model. In fact, the animals treated with AT and LoV showed improvement in memory and cognition. Additionally, both treatments decrease lactate dehydrogenase and oxidative stress levels. Furthermore, our study also demonstrated hippocampal tissue preservation in the treated groups.

1. Introduction

In the genesis of stroke there are two important events: inflammatory process and oxidative stress [1], present mainly in the atherosclerosis and hypertension pathophysiology [2]. It seems that to control these two events would be beneficial for both primary prevention and secondary prevention.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-Co-A) reductase, an enzyme responsible for inhibiting cholesterol biosynthesis, and is in wide use today [3]. Besides having a lipid-lowering effect, a statin is associated with a reduction in all causes of death from non-obstructive coronary artery disease [4]. Additionally, some authors have found it to have a positive effect on vascular inflammation [5], and linked it to a lower risk of developing venous thromboembolism [6].

However, the literature indicates that there are a number of side effects, with neurological implications that include cognitive impairment [7]. Recent studies report that statins may potentially cause such disease of the nervous system as intracerebral hemorrhage [8].

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In addition, other authors suggested that statin use may affect vitamin E levels. According to Jula et al. [9], a decrease in plasma vitamin E concentrations was noted upon treatment with statins.

Interestingly, Murad et al. [10] found improved cognitive capacity and preserved short- and long-term memory and neuronal tissue in stroke-prone spontaneously hypertensive rats treated with alpha-tocopherol. Furthermore, a different study reported an association between increases in serum vitamin E levels and lower risk of cognitive impairment [11].

Alpha-tocopherol, a powerful antioxidant, is the most potent compound in the antioxidant vitamin E family and the predominant form of vitamin E in vitamin supplements [12]. This vitamin exhibits several influences on physiologic parameters [13,14], that could preserve neuronal tissue and enhance cognitive functions.

Therefore, this study aimed to investigate whether alpha-tocopherol is able to reverse the statin neurological side effects and potentiates its anti-inflammatory action.

2. Material and methods

2.1. Animals

Twenty-four (24) male, 15-week-old SHRSPs (stroke-prone spontaneously hypertensive rats) weighing 180–230 g, housed in metabolic cages in a bioterium at the Federal University of the State of Rio de Janeiro (UNIRIO), were kept under controlled environmental conditions: temperature of 21 ± 2 °C, light cycle kept dark for 12 h with artificial light from 7:00 am–7:00 pm, humidity of 60 ± 10%, and air flow cycle of 15 min/h. All the animals were given ad libitum Nuvilab (from Nuvital Co.) rat-food pellets and water. All procedures were carried out in accordance with the protocol in the conventional guide for animal experimentation (Publication No.85-23, NIH, revised 1996) and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123, Strasbourg 1985). The Ethics Committee for Animal Experimentation at the Federal University of Rio de Janeiro State approved the laboratory trials used in this study.

2.2. Reagents

For the laboratory assays we used DL-alpha-tocopherol acetate (Sigma-Aldrich, T-3376, St. Louis, MO), lovastatin (Sigma-Aldrich, M-2147, St. Louis, MO), coconut oil (Sigma® C-1758, St. Louis, MO), thiopental sodium (Abbott Laboratories, Abbott Park, IL), and 2-thiobarbituric acid (Sigma-Aldrich, T-5500, St. Louis, MO).

2.3. Treatment

The animals were kept under basal conditions for ten days. Thereafter, the animals were divided into four groups (n = 6, each): alpha-tocopherol (AT), treated with 120 IU alpha-tocopherol diluted in 0.1 ml of coconut oil; lovastatin (LoV), treated with 1 mg/kg body weight of lovastatin; alpha-tocopherol + lovastatin (AT + LoV), treated with 120 IU alpha-tocopherol diluted in 0.1 ml of coconut oil + 1 mg/kg body weight of lovastatin; and control group that only received 0.1 ml of coconut oil (C). The treatment was administered by orogastric gavage, daily, with a polyethylene catheter PE 190 for a period of four weeks.

2.4. Physiological parameters

Both groups were observed daily to note food and water intake, diuresis, body weight and physical aspects (skin, mucosa and hair), posture and behavior [15].

2.5. Memory and cognition tests

2.5.1. Maze test

The maze test was applied to assess neurological cognition and the ability to store memories using a cognitive map [16]. For this test, the animals were placed in a maze measuring 30 × 55 × 55cm, and the time it took them to complete the entire course was noted.

2.5.2. Morris Water Maze

The Morris Water Maze measured 1.8 m in diameter and was 60 cm deep. Before filling it with water we added an escape platform affixed in a permanent location in the maze. Next we filled the apparatus halfway with water plus milk powder, to a depth of 30 cm. Thus, the hidden platform remained a few millimeters below the surface of the water. SHRSPs were placed in the Morris Water Maze, and when released, the animals swam around the pool in search of an escape (hidden platform). The time it took the animals to find the platform was measured in seconds. The test was conducted twice daily for each rat.

2.5.3. Novel Object Recognition Test

Initially, each SHRSP was placed in a plastic box and introduced to two differently-shaped objects to explore freely for 5 min. This procedure was repeated after 180 min. Additionally, 180 min after the last exercise, one of the objects was replaced by a new object with a different shape. We thus studied the time that the rat spent with the new object and with the familiar object. Each test lasted 10 min. In this step we studied the preservation of short-term memory.

To assess the preservation of long-term memory, 24 h after the last test, the novel object from the previous day was replaced by another. The time spent with this novel object and the familiar object also was recorded. These tests lasted 10 min.

For the short- and long-term-memory procedures, exploration was defined as sniffing or touching the object with the nose or front leg. When an animal finished the test and another was to be introduced, the objects were cleaned with alcohol and dried with paper towels, to prevent a bias in the results due to olfactory perception. These tests were applied during the basal period and twice a week over the course of the entire experiment.

2.6. Sacrifice and blood collection

Once the experiment was complete, deep coma was induced in all the rats by administering barbiturates (thiopental sodium) intraperitoneally (25 mg/kg). Then, we used the cervical-sterno-laparotomy surgical approach to open wide the thoracic and abdominal cavities. We then collected blood via cardiac puncture and placed it in tubes for centrifugation at 2000 ×g for 10 min in order to obtain the serum.

2.7. Brain resection

After taking the blood, we injected 0.9% NaCl saline solution into the left ventricle of the heart to wash the brain. Subsequently, extravasation was performed by flushing saline solution through a slice in the abdominal aorta.

The euthanized animals were pinned in prone position and a deep horizontal incision was made into the dorso-cervical region. A vertical incision was made in order to expose the skullcap. The skullcap was then fragmented to access the brain. Once accessed, we used a smooth pinch and leveraged the brain. The attached nerves were sectioned to complete the removal. The removed brains were then placed in a 10% formaldehyde solution for impregnation and subsequent storage.

2.8. Malondialdehyde (MDA) levels

To assess oxidative stress, we analyzed malondialdehyde levels, which is a lipid peroxidation marker. We performed this test using the

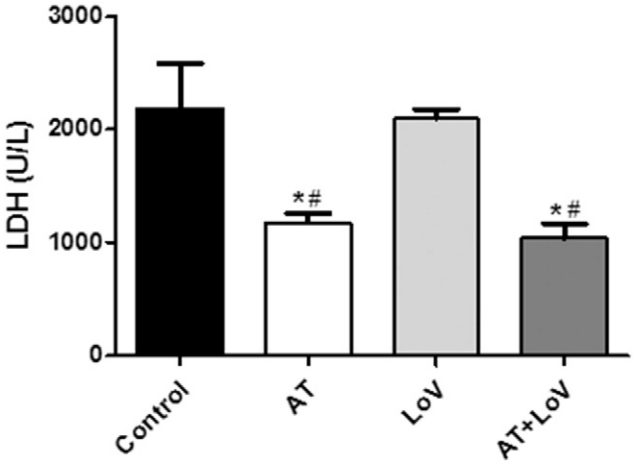

Fig. 1. Comparison of the MDA levels between groups (n = 24). *p < 0.05 considered significant when compared to the control group. #p < 0.05 considered significant when compared to the lovastatin group.

reaction of thiobarbituric acid and rat blood plasma. The spectrophotometer Micronal, model B442 (São Paulo, SP, Brazil) was used to analyze the colorimetry results and calculate the concentration of MDA from the absorption of light at the 532-nm wavelength. The values were expressed in nmol/ml.

2.9. Total cellular injury marker

Total lactate dehydrogenase was determined by using the enzymatic assay method described by Bergmeyer [17]. The values were expressed in U/l.

2.10. Brain histopathologic analysis

The brains conserved in 10% formaldehyde solution were cleaved and embedded in paraffin for processing for microscopic analysis. The paraffin block was cut to a thickness of 5 μm using a Gung RM 2025 microtome (Leica, Nussloch, Germany) and stained with hematoxylin and eosin (H&E). Signs of injury to the hippocampal region were identified using an optical microscope (Olympus Optical BX-40, Tokyo, Japan) with a magnification range of 40× to 400×. The total numbers of cells in the hippocampus and in the CA1, CA2 and CA3 subregions were counted. The slides were prepared in cross-sections, and quantification

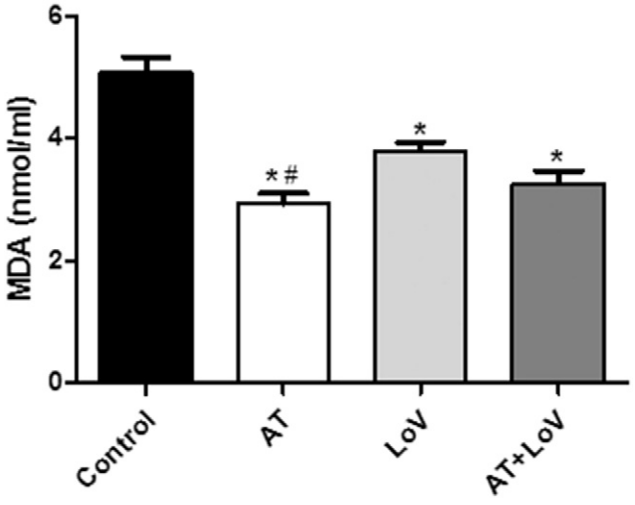

Fig. 2. Comparison of the LDH levels between groups (n = 24). *p < 0.05 considered significant when compared to the control group.

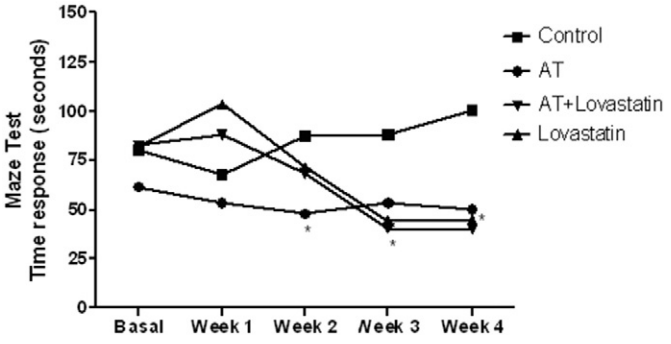

Fig. 3. Results of time response (s) on maze test (n = 24). *p < 0.05 considered significant when compared to the control group.

was performed via microscopic observation of 40 fields per slide at high magnification (200×).

2.11. Statistical analysis

The results were expressed in mean ± standard deviation and subsequently analyzed using the one-way ANOVA test with Tukey's post hoc test. The statistical software package used was GraphPad Prism® 5.0 for Windows® (Graph Pad Software, San Diego, CA, USA).

To assess the ability to recognize novel objects in the Novel Object Recognition Test, a discrimination index (DI) provided the new-object-exploration-to-recognition time ratio. This index was determined using the following formula: Mean novel object-exploration time/(sum of mean total exploration time [mean novel object-exploration time + mean familiar object-exploration time]) × 100. For these data, we also applied the one-way ANOVA test and Tukey's post hoc test. The value p < 0.05 was considered statistically significant.

3. Results

3.1. Lovastatin associated with alpha-tocopherol significantly reduces LDH levels

Despite evidence suggesting that it has a protective effect on cells, the lovastatin group did not show a significant decrease in lactate dehydrogenase levels. On the other hand, the association of lovastatin + alpha-tocopherol did lead to a significant decrease in the marker. Furthermore, the alpha-tocopherol group also had decreased the LDH levels. These results show that alpha-tocopherol may be capable of modulating LDH levels. The control group presented increased values of lactate dehydrogenase (Fig. 1).

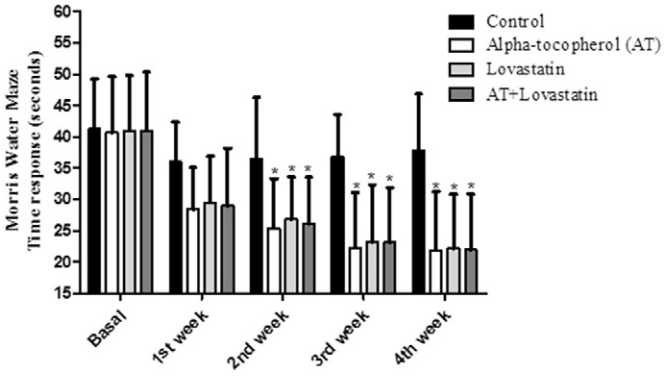

Fig. 4. Results of time response (s) on Morris Water Maze test (n = 24). *p < 0.05 considered significant when compared to the control group.

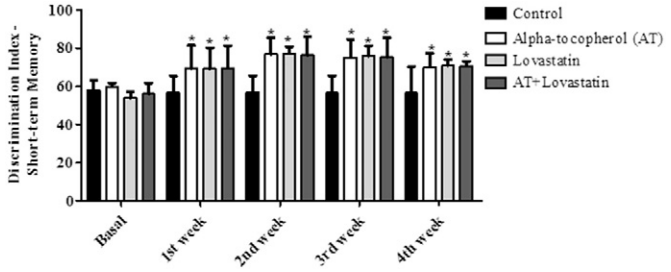

Fig. 5. Discrimination index of Novel Object Recognition Test: Short-term memory (n = 24).

3.2. Lovastatin presented an antioxidant effect, but alpha-tocopherol administered alone reduces more efficiently thiobarbituric acid-reactive substances

Surprisingly, lovastatin treatment was shown to have a significant antioxidant effect and promote a significant decrease in malondialdehyde (MDA) levels. However, this product of lipid peroxidation was significantly lower in the alpha-tocopherol group ($p < 0.05$). The alpha-tocopherol + lovastatin group also showed a significant decrease in MDA, though this data was not significant when compared to the lovastatin group ($p > 0.05$) (Fig. 2).

3.3. Alpha-tocopherol and lovastatin preserve memory and cognitive function

The results from the maze test showed a significant difference in the runtimes of the lovastatin and alpha-tocopherol groups when compared to the control group. Firstly, on week two the alpha-tocopherol group showed a significant decrease in the time that it took to complete the task. From the third week, the lovastatin and alpha-tocopherol + lovastatin groups lowered the time that it took them to complete the task. There was no significant difference between the alpha-tocopherol, alpha-tocopherol + lovastatin and lovastatin groups (Fig. 3).

3.4. Alpha-tocopherol and lovastatin improve spatial memory in SHRSPs

The results from the Morris Water Maze test were similar to those from the maze test. The data indicates that there was a significant decrease in escape latency in the groups treated with lovastatin and alpha-tocopherol. These results may suggest that spatial memory formation is preserved and improved in the SHRSPs treated with lovastatin and alpha-tocopherol (Fig. 4).

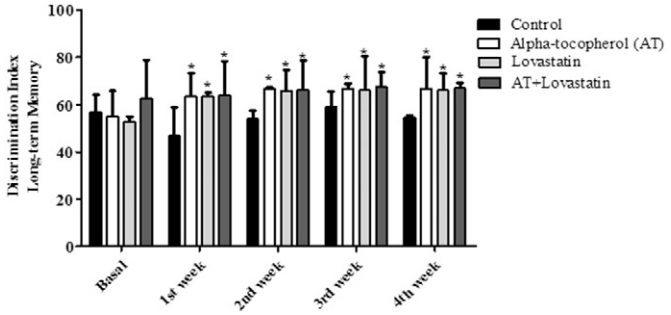

Fig. 6. Discrimination index of Novel Object Recognition Test: Long-term memory (n = 24).

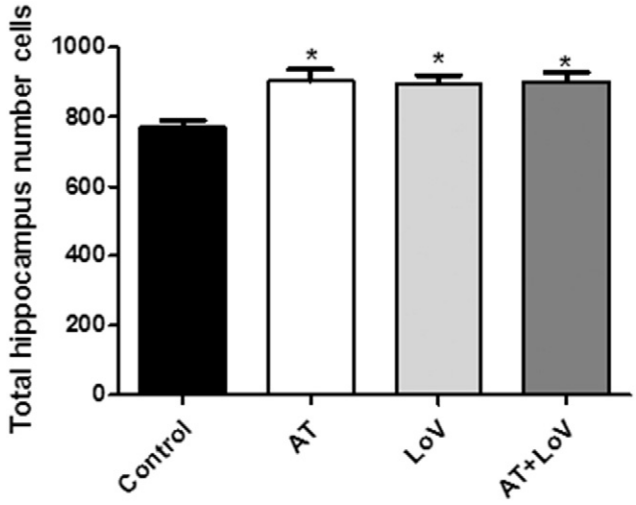

Fig. 7. Counting of the total cell number in the hippocampus (n = 24). *p < 0.05 considered significant when compared to the control group.

3.5. Treatment with alpha-tocopherol and lovastatin enhances the discrimination index in the Novel Object Recognition Test

The control group showed a reduced discrimination index, which could indicate cognitive impairment. Treatment with lovastatin or alpha-tocopherol prompted an improvement in the discrimination index when compared to the control group. These results seem to indicate that treatment with either alpha-tocopherol or lovastatin, or with a combination of these two substances, makes a significant contribution to the preservation of memory (Fig. 5 and Fig. 6).

3.6. The hippocampus can be preserved in SHRSPs treated with lovastatin and alpha-tocopherol

Regarding histopathological evaluation, examination of the hippocampus revealed that the total number of cells in the hippocampus was significantly higher in the rats treated with lovastatin or alpha-tocopherol (Fig. 7). The CA1, CA2 and CA3 subregions of the hippocampus were also examined, and the cell counts confirm these findings (Fig. 8). These data do indeed suggest preservation of the neuronal tissue and consequently possible enhancement of memory and cognition.

Additionally, some neuronal injuries were found in the control group. In the hippocampus of this group, glial nodules and vessel proliferation were detected. In contrast, the hippocampus tissue of the SHRSPs treated with alpha-tocopherol, lovastatin or alpha-tocopherol + lovastatin did not show any such changes (Fig. 9). These findings may corroborate the impairment of cognitive and memory functions found

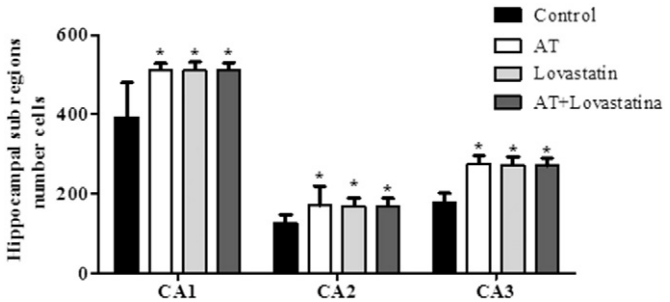

Fig. 8. Counting of the hippocampus CA1, CA2 and CA3 subregions. *p < 0.05 considered significant when compared to the control group.

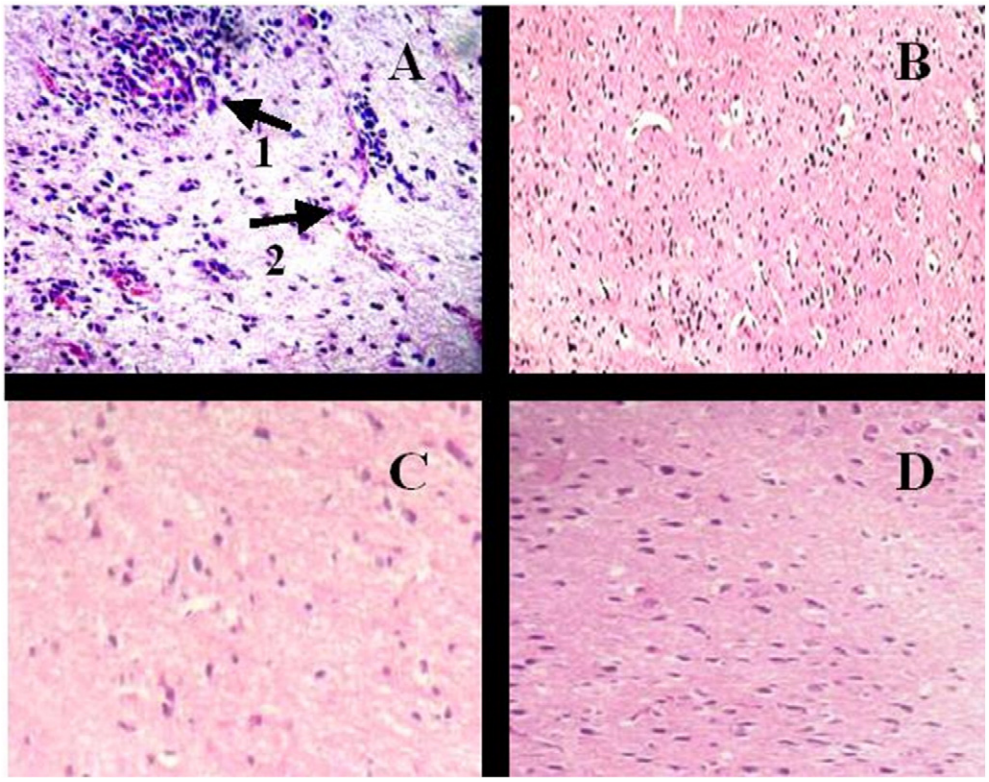

Fig. 9. Histopathological injuries observed in cross sections of the hippocampus. (A) Control group. Presence of glial nodules (1) and proliferative vessels (2) (H&E, ×40). (B) Lovastatin group (H&E, ×40). (C) Alpha-tocopherol group (H&E, ×400). (D) Alpha-tocopherol + lovastatin (H&E, ×400).

in the control group and the preservation of these functions in the groups receiving treatment.

3.7. Alpha-tocopherol and lovastatin did not change physiological parameters

All the SHRSPs showed fluctuations in body weight during the four weeks of treatment, however, with no statistical significance. Neither did the assessment of diuresis and water and food intake identify significant changes between the groups at any point during the four-week experiment (Table 1). Physical examination of the animals did not reveal any changes that would indicate any adverse effect resulting from the treatment.

4. Discussion

Despite studies reporting neurological impairment due to the use of statin, the present article demonstrates that treatment with lovastatin promotes the preservation of memory and cognition. These results corroborate the findings of Mans et al. [18], who demonstrated that acute treatment using simvastatin for 2 to 4 h in C57BL/6 mice significantly increases the magnitude of long-term potentiation (LTP) of synapses in the hippocampal CA1 and CA3 fields. The authors also showed that phosphorylation of protein kinase B increased significantly in the CA1 region after treatment and that inhibition of this protein suppresses the increase in LTP induced by simvastatin. These results suggest that activating protein kinase B can promote an increase in LTP in the hippocampus through statin treatment.

Table 1
Results of biological parameter measurement (n = 24).

Parameters	Groups	Basal period	1st week	2nd week	3rd week	4th week
Body weight (g)	Control	197.33 ± 13.74	201.23 ± 16.83	210.87 ± 7.84	218 ± 10.55	216.97 ± 9.86
	AT	200 ± 9.74	203 ± 14.56	215.69 ± 13.56	220.76 ± 11.27	215.67 ± 11.56
	Lovastatin	196.69 ± 10.13	205.61 ± 12.23	211.23 ± 14.50	213.45 ± 11.67	212.35 ± 12.48
	AT + lovastatin	198.81 ± 11.71	202.21 ± 11.33	210.76 ± 12.06	211.79 ± 12.96	211.88 ± 13.73
Diuresis (ml)	Control	6.87 ± 3.43	5.63 ± 2.03	6.54 ± 2.33	6.45 ± 2.33	7.83 ± 1.23
	AT	7.56 ± 2.03	5.26 ± 3.03	5.38 ± 2.03	6.23 ± 1.55	6.87 ± 0.92
	Lovastatin	6.43 ± 0.63	5.71 ± 1.47	6.81 ± 0.91	7.12 ± 3.11	6.98 ± 2.56
	AT + lovastatin	6.19 ± 2.65	6.63 ± 1.75	6.52 ± 1.73	6.73 ± 0.69	6.41 ± 0.81
Food intake (g)	Control	14.56 ± 2.32	15.47 ± 1.34	15.99 ± 0.87	13.57 ± 2.07	14.56 ± 3.36
	AT	14 ± 1.46	17.45 ± 1.21	15.88 ± 1.60	16.95 ± 3.05	13.48 ± 3.81
	Lovastatin	14.85 ± 2.45	16.29 ± 2.84	12.41 ± 2.98	12.19 ± 3.33	10.92 ± 1.26
	AT + lovastatin	15.17 ± 2.52	17.82 ± 2.15	14.82 ± 1.45	13.44 ± 1.88	12.31 ± 2.24
Water intake (ml)	Control	45.98 ± 3.46	49.69 ± 4.76	37 ± 6.68	32.68 ± 3.23	38. 67 ± 5.78
	AT	47.74 ± 8.87	46.95 ± 6.78	40.31 ± 7.43	37.69 ± 5.41	34.19 ± 6.12
	Lovastatin	41.78 ± 4.22	35.15 ± 2.19	39.51 ± 1.25	33.32 ± 2.73	35.64 ± 6.97
	AT + lovastatin	41.97 ± 5.19	32.32 ± 6.31	31.39 ± 7.97	32.36 ± 2.94	28.93 ± 9.44

The values represent the mean ± SD of 24 animals.
AT: alpha-tocopherol.

Our work also indicates that alpha-tocopherol was capable of modulating memory formation and preserving cognition. According to Wu et al. [19], alpha-tocopherol can increase molecular factors involved in memory consolidation, such as BDNF (brain-derived neurotrophic factor), CREB (cAMP response element-binding) and synapsin I. These factors may be affected by oxidative stress. Previous studies by our group demonstrated that alpha-tocopherol can be considered a modulator for stroke-risk factors and a neuroprotective agent, promoting the preservation of neural tissue and function [10].

Additionally, vitamin E-deficiency may lead to an impaired induction of LTP, which could have an influence on the memory formation [20]. Moreover, vitamin E can modulate protein kinase B, possibly increasing survival of neuronal cells [21].

Furthermore, the authors report that in the process of neuronal injury, the levels of thiobarbituric acid, hydroperoxides and isoprostane increased in the brain of the rats affected by oxidative stress [22].

Oxidative stress can trigger the processes of necrosis and apoptosis [23]. These events can be determined by injury markers like LDH enzyme. Donnan et al. [24] reported an association between LDH levels and stroke. Some authors also report that alpha-tocopherol has an anti-inflammatory property, which could suggest a decrease in serum LDH levels [25].

Confirming this data, Tsai et al. [26] found that LDH increased after subjecting experimental animals to medium artery occlusion. However, in the same study when the authors treated the animals with resveratrol, a powerful antioxidant, there was a significant decrease in this injury marker. This could be associated with the antioxidant effect of resveratrol.

Reactive oxygen species can react with biological structures (e.g., plasmatic membranes), and generate measurable products. In the control group, LDH and malondialdehyde levels were higher than in the other groups, and only the control group was found to have hippocampal injury. This tissue injury may have been responsible for the impaired cognition and memory. Indeed, Watson et al. have reported that lipid peroxidation may be involved in the progressive decline in memory and learning performance [27].

On the other hand, rats treated with lovastatin or alpha-tocopherol showed improvement in neurological functions and no hippocampal injury was noted. LDH levels were significantly lower in the animals of the alpha-tocopherol group. However, lovastatin did not seem to alter injury-marker levels. Surprisingly, we found an interesting association between alpha-tocopherol and lovastatin, which together promoted a major decrease in LDH levels.

We also found that lovastatin promoted a significant decrease in malondialdehyde levels. Other authors also have described the antioxidant properties of lovastatin. Chang et al. [28] reported a decrease in malondialdehyde levels and oxidative DNA injury in rabbits treated with lovastatin. These data can be confirmed by Kumar et al. [29], that reported an increase in antioxidant-enzyme activity and a decrease in thiobarbituric acid-reactive substances in liver and heart tissue from rats treated with lovastatin.

The antioxidant activities of statins can be explained by the increase in catalase, paraoxonase, vitamin E and eNOS levels. These events could trigger a decrease in reactive oxygen species production and lipid peroxidation [30]. Additionally, Guasti et al. [31] linked the antioxidant effect of statins with the decrease in angiotensin II type 1 receptor expression, which is responsible for endothelial dysfunction.

Nevertheless, alpha-tocopherol was the most effective treatment for reducing the oxidative stress marker. The combination of alpha-tocopherol and lovastatin showed a slight decrease in lipid peroxidation levels, compared to the lovastatin group.

Recent findings demonstrate that lovastatin could prevent an increase in LDH levels and have a neuroprotective effect [32]. Clinical trials have shown improvement in the cognition of children with neurofibromatosis treated with lovastatin [33].

The antioxidant and anti-inflammatory effects of lovastatin and alpha-tocopherol could be what preserved the hippocampal tissue and increased the number of cells in the hippocampus (CA1, CA2 and CA3) in the test groups. In turn, the control group showed a decrease in the number of hippocampal cells, and histopathologically glial nodules and vessel proliferation were detected. Glial nodules may be associated with an inflammatory process and responsible for provoking learning and memory impairment [34,35].

Both histological alterations found in our work could be a signal of cerebral ischemia [36], explained by high blood pressure, which is a characteristic of the strain of animal used [37,38]. Additionally, the angiogenesis (proliferation vessel) here observed has been described by other authors. Studies report upregulation of angiogenesis gene expression a few minutes after ischemic stroke in rodents, as well as an increase in angiogenic proteins in the ischemic area weeks thereafter [39,40].

Furthermore, SHRSPs have a condition that exacerbates reactive oxygen species production, causing injury to cellular integrity and playing a part in processes that lead to the death of brain tissue [37,38].

Some studies suggest that reduced redox potential in hippocampal neurons provokes impairment of long-term potentiation and deficient cognitive functions [41]. However, according to Emmrich et al. [41], modulation of oxidative reactions by alpha-tocopherol could preserve brain cell metabolism and restore cognitive functions.

5. Conclusion

In sum, the evidence from this study demonstrates that treatment using lovastatin did not have a negative effect on cognition and memory nor produce any deleterious effects on our model. In fact, there was hippocampal tissue preservation and we observed an improvement of the cognition and memory functions in rats treated with lovastatin and alpha-tocopherol.

Disclosure

The authors declare no conflicts of interest.

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