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Research Article

Keywords: Alzheimer's Disease, Curcumin, Neurodegeneration, Cognitive Dysfunction, Resveratrol, Streptozotocin

Posted Date: September 9th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-6363905/v2

Additional Declarations: The authors declare no competing interests.

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Abstract

Bacground: Alzheimer's disease (AD) is a degenerative neurological disorder characterized by progressive impairment of memory, cognition, and behavior. Since current treatment approaches have been unable to significantly slow the progression of the disease, there is an increasing need for new neuroprotective strategies. This study aims to investigate the protective effects of curcumin and resveratrol on neurotoxicity and the resulting cognitive impairments.

Methods: A total of 60 male Wistar albino rats were included in the study. On day 1, an experimental Alzheimer's model was induced by a single intracerebroventricular injection of streptozotocin (STZ) at a dose of 3 mg/kg. Following model induction, the animals were randomly assigned into six groups: Alzheimer's disease (ALZ) group, ALZ+curcumin (ALZ+CUR) group, ALZ+resveratrol (ALZ+RES) group, ALZ+curcumin+resveratrol (ALZ+CUR+RES) group, SHAM group, and control group. Morris water maze test, open field test and passive avoidance test were used for behavioral tests; GFAP and caspase-3 tests for immunohistochemical analysis; TAS/TOS values for biochemical analysis and Cavalieri method for stereological examination.

Results: Behavioral tests demonstrated that curcumin and resveratrol significantly improved neurobehavioral outcomes. Histological analyses revealed that curcumin and resveratrol reduced the density of cells stained with Caspase 3 (+) and glial fibrillary acidic protein (GFAP) (+), thereby alleviating neuronal cell death and astrocyte activation. Stereological analyses demonstrated that curcumin and resveratrol improved the STZ-induced hippocampal volume reduction. Serum levels of Total antioxidative status (TAS) and Total oxidative status (TOS) measured in serum samples indicated that oxidative stress induced by STZ was reduced by curcumin and resveratrol. While these two compounds exhibited neuroprotective effects when administered alone, no such effect was observed when administered together, suggesting that combination therapy did not produce the expected synergistic effect.

Conclusion: This study demonstrated that curcumin and resveratrol, when administered alone, reduce oxidative stress and neuroinflammation caused by STZ, prevent neuronal apoptosis, and exhibit significant neuroprotective effects in ALZ, thereby improving learning and memory functions. These findings suggest that curcumin and resveratrol may serve as effective therapeutic agents in the treatment of Alzheimer's disease.

Keywords: Alzheimer's Disease; Curcumin; Neurodegeneration; Cognitive Dysfunction; Resveratrol; Streptozotocin

Introduction

Alzheimer's disease (AD) is a type of neurodegenerative disease that is one of the most common causes of dementia worldwide. Approximately 44 million people worldwide are reported to be affected by AD, and this number is estimated to reach 115 million by 2050 [1]. Alzheimer's is a chronic progressive disease characterized by behavioral and cognitive impairments. The pathology of AD is composed of amyloid plaques, neuroinflammation, loss of neurons and synapses and neurofibrillary tangles. One of the most important basic factors involved in the formation and progression of these pathologies is oxidative stress [2]. As the underlying pathologies progress, the connections between the limbic system and the temporal and frontal lobes of the brain are disrupted. The pathological process that occurs due to these disruptions involves neuronal atrophy and loss, with an inflammatory response characterized by amyloid plaques and neurofibrillary tangles that affect the temporoparietal cortex intensively and the frontal lobe. Associated with this pathology, cerebral atrophy [3–5]

In recent years, the effects of oxidative stress, which occurs as a result of damage to neurons especially in AD, on the progression of the disease have been investigated. Rats with Icv-Stz-induced cognitive impairments have been proposed as a research model for understanding AD. ICV administration of Stz leads to oxidative stress and neuroinflammation. There is currently no definitive treatment for AD. Current treatment procedures include symptomatic therapies to slow the progression of the disease [6]. The importance of antioxidants, vitamins and the Mediterranean diet have been emphasized among the protective effects of AD. Vitamin C, α-tocopherol, β-carotene, curcumin, α-lipoic acid, resveratrol, ginkgo biloba, selegiline and melatonin are the most frequently studied antioxidants. More studies are needed on the possible effects of these antioxidants on AD [7]. Various factors such as individual differences and environmental factors may cause contradictory results. Therefore, studies need to be diversified [8].

Curcumin is an important active diphenolic compound extracted from the root of Curcuma longa (turmeric) and is used as a food additive as well as in medicines. It has few side effects and easily crosses the blood-brain barrier [9]. Many studies have shown that curcumin has antioxidant, antiallergic, antitumor, anti-inflammatory, anticarcinogenic, antidementia effects and free radical scavenging activity [10]. Curcumin provides a certain level of neuron protective effect in neuronal cell death induced by oxidative stress [11]. Curcumin has been shown to have a beneficial role in neurodegenerative disorders with various neuroprotective properties [12, 13]

Resveratrol is a natural compound of the polyphenol group found in a wide variety of plant species, including red grapes, mulberries and peanuts. It is an antioxidant with anti-inflammatory and anti-apoptotic effects. Recent studies have shown favorable effects of resveratrol in animal models of AD; yet, the relevance of these results to the clinic is still unclear [14, 15]. Resveratrol has been shown to reduce platelet aggregation, promote vasorelaxation, prevent atherosclerosis, reduce lipid peroxidation and improve serum cholesterol and triglyceride concentrations. In addition, against the doubts about the bioavailability of resveratrol, many in vivo studies have indicated its protective effects in animal models of stress and disease [16]

Due to the multifactorial pathophysiology of Alzheimer's disease, combination therapies targeting multiple mechanisms are gaining attention. Curcumin and resveratrol are natural compounds that act through different biochemical pathways. While curcumin inhibits NF-κB and affects β-amyloid aggregation, resveratrol activates SIRT1 and influences mitochondrial functions [17, 18]. In consideration of these discrete mechanisms, a hypothesis is proposed that posits the potential for synergistic or complementary effects to be produced by the combined use of these two compounds. The present study was conducted with the objective of investigating whether the combined use of curcumin and resveratrol provides the expected neuroprotective effect.

In this study, it was aimed to investigate the effects of curcumin and resveratrol, known for their potent antioxidant properties, on the hippocampus as the brain region first affected by Alzheimer's disease, using histomorphometric, behavioral and biochemical approaches in rats. It is believed that the results of this study will contribute to the studies on Alzheimer's disease, a neurodegenerative disease whose incidence has been increasing in world in recent years.

Materials and Methods

Animals and Experimental Design

In this study, 60 male Wistar albino rats (weighing 210-250 g) were obtained from Ondokuz Mayıs University Experimental Animal Application and Research Center. During the experiment, rats were kept in a controlled environment with a temperature of 22°C and 55-60% relative humidity. Rats were kept on a 12-hour light/dark cycle and had free access to standard rat chow and tap water. The study protocol was approved by Ondokuz Mayıs University Experimental Animal Ethics Committee and all experimental procedures were performed in strict adherence to the ethical rules (Ethics Code: 2020/67).

A single intracerebroventricular (ICV) injection of STZ (3 mg/kg in 5 µL saline) was administered on Day 1 under stereotaxic guidance to induce Alzheimer's-like pathology, as described in previous studies [19, 20]. For intracerebroventricular (icv) injection, animals were anesthetized by intraperitoneal administration of ketamine (Merck, Cat. No: K2753),-xylazine (Merck, Cat. No: X1251 mixture (100 mg/kg-10 mg/kg, respectively) and fixed in a stereotaxic apparatus. 5 µl of STZ (3 mg/kg) (Santa Cruz Biotechnology, Cat. No: U-9889) dissolved in citrate buffer was drawn into the Hamilton micro-injector. A single slow (1µl/min) STZ injection was administered into the left lateral ventricle (0.8 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture and 3.6 mm below the brain surface) via icv with a Hamilton micro syringe. Following the injection, the Hamilton syringe was left in for 2 minutes without being pulled out immediately. On the first day of the experiment, the injection procedure was performed only once. Following a 7-day recovery period, rats were randomly assigned to six groups: Control (C), Alzheimer (ALZ), Alzheimer+Curcumin (ALZ+CUR), Alzheimer+Resrevatrol (ALZ+RES), Alzheimer+Curcumin+Resveratrol (ALZ+CUR+RES) and SHAM. This randomization post-induction ensured unbiased group allocation. Rats in the ALZ+CUR group received curcumin (100 mg/kg) (Sigma-Aldrich, Cat. No: C1386) by gavage once daily for 21 days following icv-STZ injection. Rats in the ALZ+RES group received resveratrol (25 mg/kg) (Merck, Cat. No: R5010) by gavage once daily for 21 days following icv-STZ injection. Rats in ALZ+CUR+RES group received curcumin (100 mg/kg) and resveratrol (25 mg/kg) by gavage for 21 days following icv-STZ injection. In the combination group, the doses of curcumin and resveratrol were administered at the same levels used in their individual applications (100 mg/kg curcumin, 25 mg/kg resveratrol). This decision was made to mitigate potential toxicity risks and to align with the doses that have been demonstrated to

be effective in preceding animal studies. Rats in the SHAM group received icv injection of serum citrate buffer which is the solvent of STZ. No other treatment was given to the animals during the experiment. No treatment was applied to the control group.

After the 14th day, open field test, passive avoidance and Morris water maze tests were performed and on the 23rd day of the experiment, the animals were weighed again and sacrificed under general anesthesia with a mixture of xylazine (10 mg/kg) and ketamine (50 mg/kg). Following the sacrifice of the rats, the appropriately removed brain tissues were weighed and recorded and then placed in 10% buffered formaldehyde fixative for histologic tissue follow-up. The obtained blood serum samples were stored at -80 °C for biochemical analysis.

Behavioral Tests

Open Field Test

The open field test is one of the tests used to detect changes in the emotional state of experimental animals caused by procedures applied to them. In addition, it is also used to examine the changing emotions associated with anxiety. In the open field test used in the study, a platform made of white wood, with a floor divided into 49 equal squares, open at the top and dimensions of 100x100x30 cm (width x length x height) was used. The rats were placed in the apparatus from a certain corner with their backs facing the open field and observed for 5 minutes (min) by camera recording. After each animal, the boxes were cleaned with 70% ethanol. In the recorded videos, the number of the animals' rearing and the number of frames traveled in the periphery were used to evaluate locomotor activity. The number of groomings, the number of squares visited in the central zone and the time spent in the central zone were also used to assess the anxiety states of the animals[21].

Passive Avoidance Test

In this test, a One-Trial Step-Through Passive Avoidance Set-up (Ugo-Basile model 7551, Italy) consisting of two 25x15x15 cm compartments, one light and one dark, divided by a guillotine door, was used. Before the acclimatization period as the first phase of the passive avoidance test, the rats were taken one by one to the light zone and given a 30 s waiting period to acclimatize to the conditions of the environment. Then, all of the rats in the light zone were allowed to move to the dark zone respectively. After the learning process during the

acclimatization period, the rats that were placed in the light zone in the first phase of the test were allowed to move to the dark compartment by automatically opening the guillotine door. Rats were exposed to a low dose of electric shock (0.5 mA, 3 s) after switching to the dark compartment. The latency time of the rat's transition from the light compartment to the dark compartment was recorded as “latency time to the dark zone”. The rats were taken into their cages after being given electric shocks. The same procedure was repeated 24 hours after the learning period and the “retention time” which is the time of entrance to the dark compartment, was measured and recorded, assuming that this new information, which was thought to have been learned and stored by the rats, would be remembered 24 hours later. Nevertheless, the retention time of rats that did not move to the dark area within 300 s on the second day of the experiment was considered 300 s. No electric shock was applied on the second day [21].

Morris Water Maze Test (MWM)

This test is widely used to assess spatial memory and learning. A circular swimming pool with a diameter of 150 cm and a height of 80 cm and a platform in the pool is used for the experiments. It lasts for five days in total, with the first four days being the trial phase in which the learning sessions are applied and the last day being the probe phase in which the memory test is conducted [22].

Before the experiments, various images were placed on the north, east and west sides of the platform in order to divide the pool imaginatively into four quadrants (southwest, southeast, northwest and northeast). The pool was filled with tap water ensuring that the platform was approximately 2 cm below the water level and the water temperature was set to 25±1°C with an automatic heater. The platform was placed in the southwest quadrant of the pool and was not moved until the end of the experiments.

During the first four days, subjects were dropped into the pool from different points to learn the location of the platform. On each test day, subjects were released from the other three quadrants or directions where the platform was not present and swam freely for 90 s. Subjects who found the platform at any time within 90 s were allowed to stay on the platform for 30 s to observe the visual cues. Subjects who could not find the platform within this period (90 s) were left on the platform to observe the visual cues for 30 s to recognize the platform. On the fifth day, during the probe phase planned to test the memory of the subjects, food coloring was added to the tap water in the pool so that it was the same color (white) as the pool and the platform, and

the platform was removed. Subjects were released into the water during the probe phase from any of the other three quadrants except the target quadrant. During this phase, the subjects were expected to swim directly to the platform and the time spent in the quadrant with the platform (maximum 60 s) was recorded.

Histopathological analysis

Brain tissues fixed in 10% buffered neutral formalin solution were subjected to routine histological procedures and blocked. From the paraffin blocks, 20 µm thick sections were taken for stereologic sampling and stained with Cresyl violet stain (ISOLAB, Cat. No: 912.D01. Additionally, 4µm thick sections were taken for immunohistochemical analysis.

Histopathological Damage Scoring (HDS)

A semi-quantitative scoring system ranging from 0 to 3 was utilized in the analysis of hippocampal sections that had been stained with Cresyl violet. [23]. Neurons were classified based on their morphology and staining characteristics in three randomly selected areas of each tissue section.

Score 0- Normal: Neurons are round or oval, have a light-colored nucleus, and distinct Nissl bodies. Cells appear healthy.

Score 1 - Mild Damage: Damage is observed in a small number of neurons. Cytoplasms appear pale or nuclei are slightly pyknotic.

Score 2 - Moderate Damage: Neurons show significant damage. Cytoplasms are reduced, nuclei are hyperchromatic, and cellular structures are disrupted.

Score 3 - Severe Damage: Neurons are largely lost. Cell structure is unrecognizable. Severe damage indicators such as gliosis and vacuolization are observed.

GFAP Immunocytochemistry

The sections were placed in an oven at 50 ºC overnight for deparaffinization, then clarified in xylene and rehydrated in alcohol series. To elicit antigen, sections were heated in 10 mM citrated buffer (pH: 6) in a 400-watt microwave oven for 20 minutes and then allowed to cool in the same buffer for 30 minutes at room temperature. For the elimination of endogenous peroxidase activity in the tissues, the sections were treated with a 3% hydrogen peroxide solution and left for 15 minutes. Then, all staining steps were performed using a fully automated immunohistochemistry instrument (VENTANA, Benchmark/I.T., Ventana Medical Systems, USA) under constant temperature and conditions. GFAP monoclonal antibody (Abcam, Cat.

No. ab33922, RRID:AB_732571) was used as the primary antibody to make the targeted protein visible in autostained sections. The incubation time with antibodies was set at 2 hours. After the staining process was completed, the sections were passed through alcohol series (70%, 80%, 96% and 99% ethyl alcohol) for 2 minutes each. The sections were dried at room temperature, kept in xylol for 15 minutes and covered with entellan. For the evaluation of immunohistochemical staining, all sections were examined under a light microscope.

The evaluation of GFAP staining results was conducted by means of a random selection of 10 different areas at 20x magnification for each animal. The analysis encompassed five sections and 50 images for each animal. A total of 200 cells were counted in each field, with the cells being classified as either $GFAP$ (+) or $GFAP$ (−). The density of $GFAP$ (+) cells in each animal was then calculated using the following formula [24].

$$GFAP\ (+)\ cell\ density = \frac{GFAP\ (+)\ stained\ cell\ number}{Total\ number\ of\ GFAP\ (+)\ and\ GFAP\ (-)\ stained\ cells} \text{ x } 100$$

Caspase-3 Immunocytochemistry

5 µm thick sections from brain tissues were automatically stained for caspase-3 immunohistochemical reactivity using the VENTANA BenchMark GX System (Ventana Medical Systems, Inc.). Antigenic marker regions for caspase-3 were unfolded with vapor in citrate buffer for 60 minutes. For caspase-3 staining, IgG grade Caspase-3 antibody (Santa Cruz, Cat. No: sc-65497, RRID: AB_2054782) and mouse monoclonal IgG2a (kappa light chain) were diluted 1:80. The sections were incubated in antibody solution for 32 minutes at 37°C, followed by the use of the ultraView Universal DAB imaging kit (Ventana Medical Systems, Inc.). DAB (Sigma-Aldrich, Cat. No: D8001) was used as chromogen and staining was counterstained with hematoxylin. The specificity of the staining was confirmed by processing negative control sections in which the primary antibody was not used.

The number of Caspase-3 (+) cells was counted and divided by the overall cell count for each field. Each section was evaluated on average across six fields. The percentage of stained cells was assessed: 0 for no stained cells; 1 for 25%-50% stained cells; 2 for 25%-50% stained cells;

3 for >50% stained cells. Staining intensity was scored as follows :none (0); low (1); moderate (2); severe (3). The Immunostaining Intensity Distribution Index (IIDI) was calculated using the formula: IIDI = stained cells x staining density. The IIDI represents the average of all six fields [25]

Stereological analysis

Considering previous studies, the sampling interval of the hippocampal volume in the brain was accepted as 1/6 (Systematic). According to this sampling interval, 20 µm-thick sections were taken from the brain blocks and 1/6 systematic and uniform random sampling was performed. The Cavalieri method was used to measure the area of the sections and to calculate the total volume of the hippocampus. A Stereo-Investigator (Microbrightfield, Colchester, VT) was used in the study. This device had special software that included a dotted area ruler for area calculation with the Cavalieri method. The total volume was automatically calculated by the software according to the Cavalieri principle [25].

Biochemical Analysis

Collection of Serum Samples

The blood obtained from the heart under anesthesia was transferred into serum tubes (BD SST™ II Advance Serum Separator Tube). After the blood was collected in serum tubes, it was gently turned upside down 5-6 times and allowed to clot for 30 minutes. Following the clotting, the serum tubes were centrifuged at 1300-2000 x g for 10 minutes at room temperature (25 °C). After centrifugation, the sera were collected in 0.5 ml microcentrifuge tubes. They were then placed in a deep freezer at -80 ˚C and stored until the day of measurement. Total Oxidant Status (TOS) and Total Antioxidant Status (TAS) levels were measured from the sera obtained.

Measurement of TOS and TAS

Rat serum TAS and TOS levels were determined by double antibody sandwich-ELISA kits were analyzed (using Rat Serum TAS Bioassay Technology Laboratory, Shanghai, China, catalog no: E1710Ra and Rat Serum TOS Bioassay Technology Laboratory, Shanghai, China, catalog number: E1512Ra). The samples were added to wells pre-coated with TAS/TOS monoclonal antibodies. After a certain period of incubation, the monoclonal antibodies were combined with TAS/TOS in the samples. Following the incubation and washing, biotinylated rat TAS/TOS antibody was added. This antibody was bound to TAS/TOS in the immune

complex. Streptavidin-HRP was then added and completed the reaction by binding to biotinylated TAS/TOS antibody. After incubation and washing, substrate solution was added. Color developed in proportion to rat TAS/TOS levels. The reaction was stopped by adding acidic stop solution. Absorbance values were measured at 450 nm. The hue of the solution showed a positive correlation with rat TAS/TOS levels at 450 nm.

Statistical Analysis

The numerical data of the groups obtained in the study were statistically evaluated using the SPSS program (SPSS version 21.0; SPSS Inc., Chicago, IL, USA). The data used were expressed as mean±standard deviation. As a result of the normality and homogeneity tests of the data of the groups, the differences between the groups with normal distribution were evaluated through One-Way ANOVA and Tukey tests, while the groups without normal distribution were analyzed through Kruskal-Wallis and Tamhane's T2 tests. A value of $p < 0.05$ was considered statistically significant in statistical evaluation. (*) indicates that $p < 0.05$, (**) indicates that $p < 0.01$, (***) indicates that $p < 0.001$.

RESULTS

Behavioral Test Results

Open Field Test

No statistically significant difference was found between the groups in the comparisons made for the number of grooming among the open field test parameters ($p > 0.05$). When the number of grooming movements was evaluated, the number of grooming movements observed in the control group was significantly higher than ALZ and ALZ+CUR+RES groups ($p < 0.01$). When the number of line crossings in the periphery was evaluated in intergroup comparisons, a significant difference was found only between ALZ and ALZ+CUR+RES groups ($p < 0.01$). The number of line transitions in the 24 squares adjacent to the walls around the perimeter of the open field platform was significantly higher in the ALZ group than in the ALZ+CUR+RES group. No significant difference was found between the groups in the central line crossing number and center duration parameters ($p > 0.05$) (Figure 1).

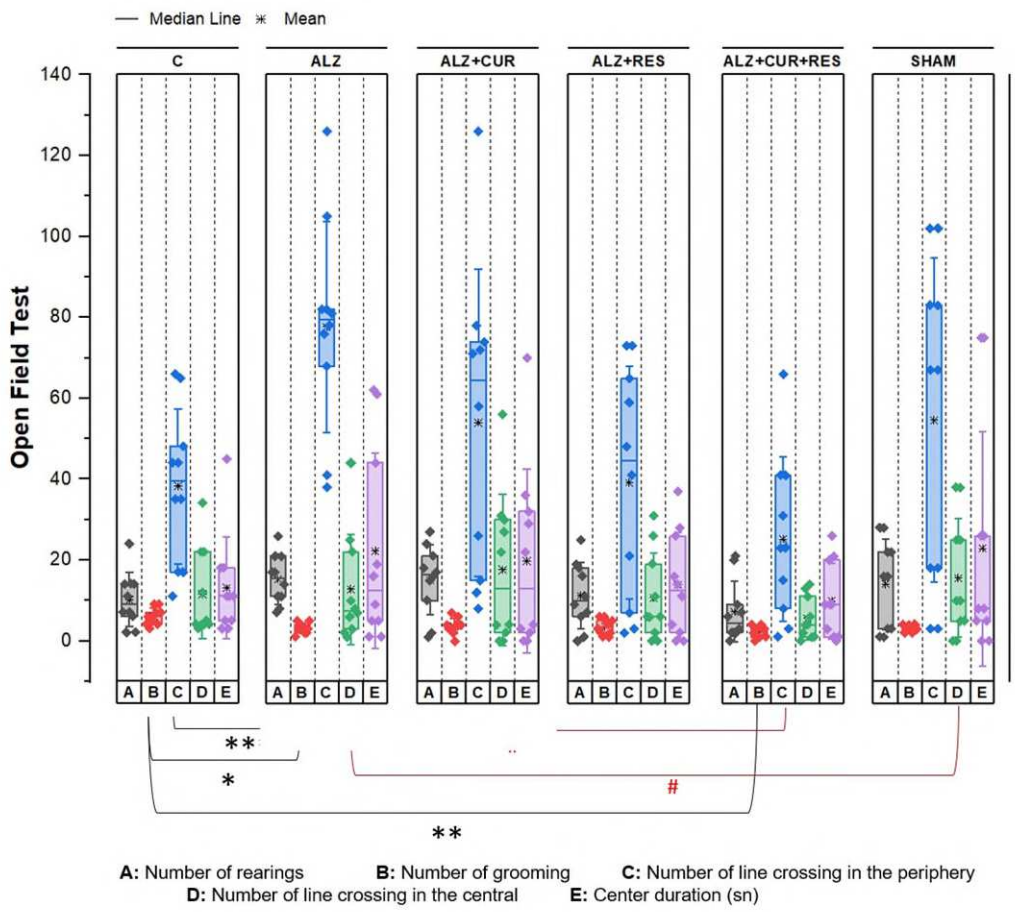

Figure 1 Effects of curcumin and resveratrol on anxiety-related behaviors of Alzheimer model rats in the open field test. *Indicates statistical difference compared to control group, $p < 0.05$, ** Indicates statistical difference compared to control group $p < 0.01$, *** Indicates statistical difference compared to control group, $p < 0.001$, # Indicates statistical difference compared to ALZ group, $p < 0.05$.

Passive Avoidance Test

In the passive avoidance test, which is widely used in the evaluation of short and long-term memory, the mean data and standard errors of the time (s) required for the groups to move to the dark zone are presented in Figure 2. According to statistical analysis, the mean dark zone transition time of the ALZ group was 45.08±28.49 s, which was significantly lower compared to all groups ($p < 0.001$). When the transition times to the dark zone were compared between the groups, no significant difference was found between C, SHAM and ALZ+RES groups. In ALZ+CUR+RES and ALZ+CUR groups, the time to transition to the dark zone was significantly lower than in the C, SHAM and ALZ+RES groups and higher than in the ALZ group ($p < 0.001$).

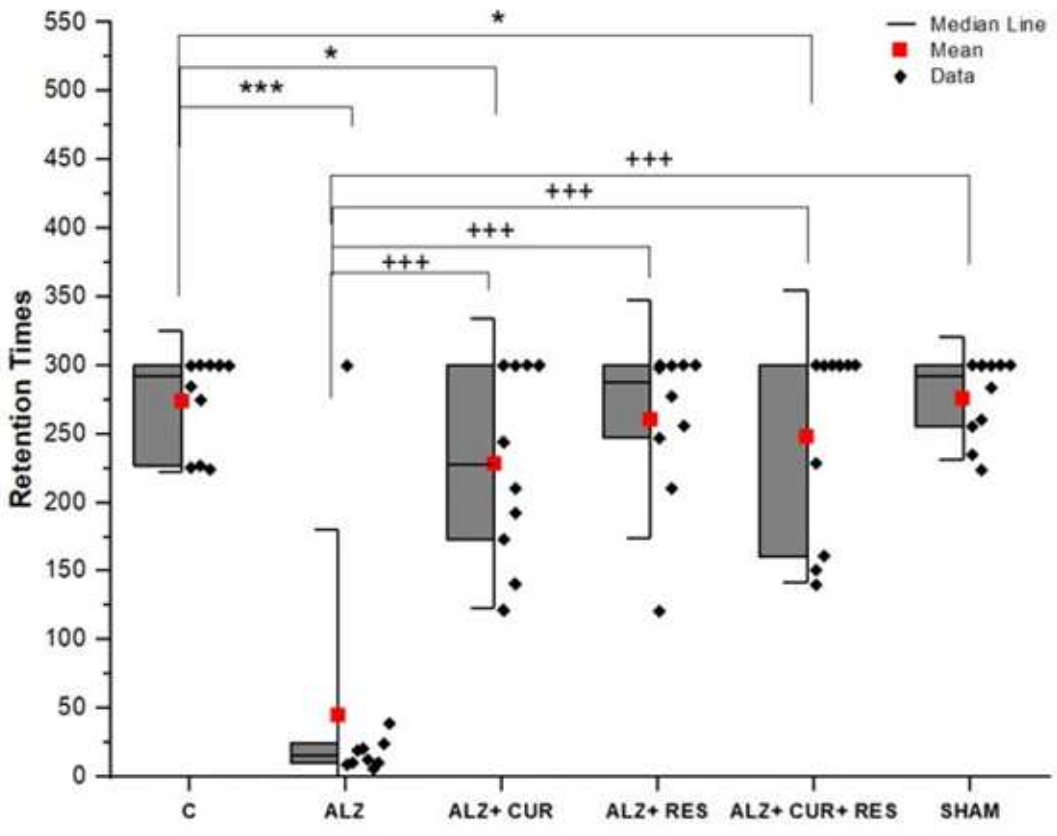

Figure 2 Retention times of rats in all groups in the passive avoidance test

*Indicates statistical difference compared to control group, $p < 0.05$, *** Indicates statistical difference compared to control group, $p < 0.001$, ### Indicates statistical difference compared to ALZ group, $p < 0.001$. Rats that failed to enter the dark compartment within 300 seconds were assigned a retention time of 300 seconds, in accordance with the experimental protocol. Consequently, the presence of overlapping data points at the upper limit indicates subjects who exhibited complete avoidance behaviour.

Morris Water Maze Test

The average values of the time to find the platform and the time to stay in the quadrant where the platform is located on the day of the experiment are presented in Figure 3. In intergroup comparisons, no significant difference was found between the groups on the first day of the trial phase ($p > 0.05$). When the probe phase durations on the 5th day of the Morris water maze test were analyzed, it was found that the control group spent more time in the target quadrant compared to ALZ, ALZ+CUR and ALZ+CUR+RES groups and a statistically significant difference was observed ($p < 0.001$). In addition to the control group, the time spent in the target quadrant in the ALZ group was significantly lower than in the ALZ+CUR, ALZ+RES and

SHAM groups ($p < 0.05$, $p < 0.001$). The time spent in the target quadrant by rats in the ALZ+RES group was similar to the control and Sham groups ($p > 0.05$), but higher than the other treatment groups. ($p < 0.001$). The ALZ group spent the least time in the target quadrant with an average of 10.10±0.86 seconds.

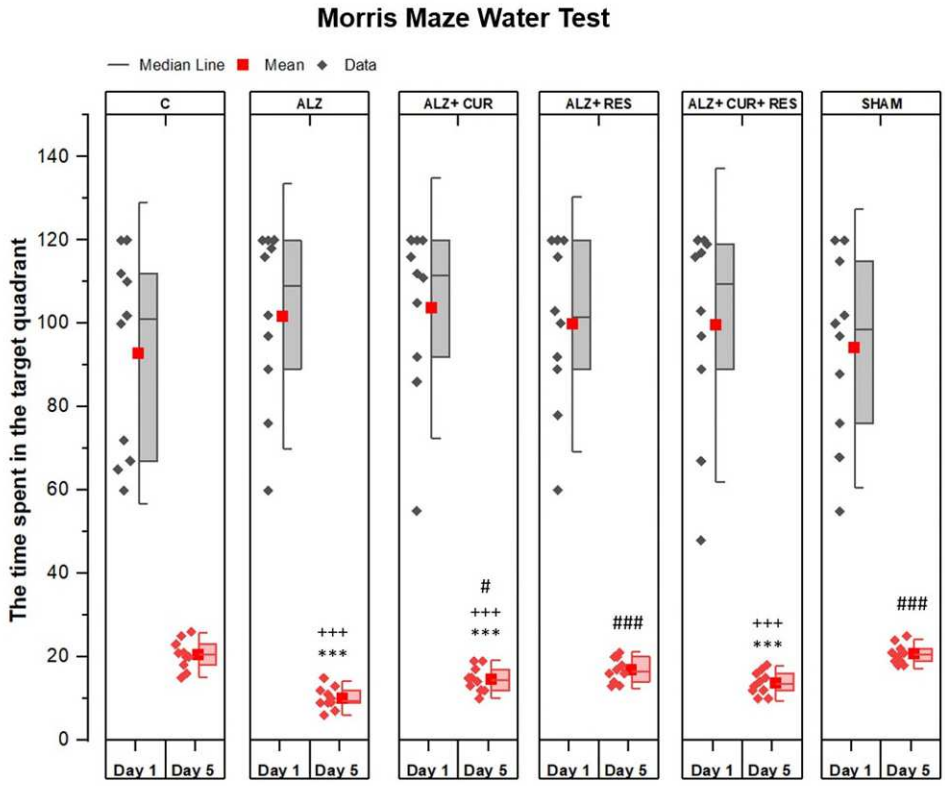

Figure 3 Effects of curcumin and resveratrol on spatial learning and memory of Alzheimer's model rats in the Morris water maze (MWM)

*** Indicates statistical difference compared to control group, $p < 0.001$, # Indicates statistical difference compared to ALZ group, $p < 0.01$, ### Indicates statistical difference compared to ALZ group, $p < 0.001$, +++ Indicates statistical difference compared to ALZ+RES group, $p < 0.001$.

Stereological results

Total Hippocampal Volume

Hippocampal volumes of rats of all groups were calculated by the previously mentioned stereological methods (Figure 4). When the data obtained were evaluated statistically, the lowest area volume was observed in the ALZ group. The total hippocampal volume of ALZ group was significantly lower than Control, ALZ+CUR, ALZ+RES and SHAM groups ($p < 0.001$). The hippocampal volumes of ALZ+CUR and ALZ+RES groups were statistically significantly lower than the Control group. Total hippocampal volume of ALZ+CUR+RES group was significantly lower than Control, ALZ+CUR, ALZ+RES and SHAM groups ($p < 0.001$). On the other hand, there was no statistically significant difference between the mean hippocampal volumes of ALZ and ALZ+CUR+RES groups ($p > 0.05$).

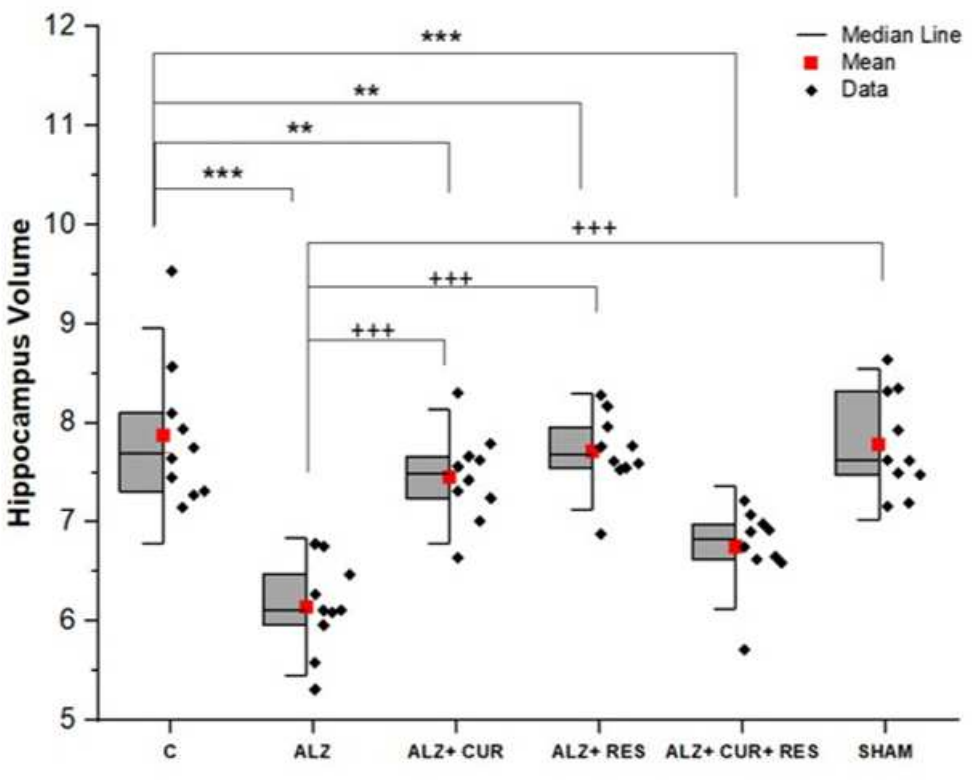

Figure 4 The graph shows the hippocampus volumes of rats in all groups

** Indicates statistical difference compared to control group $p < 0.01$, ***Indicates statistical difference compared to control group, $p < 0.001$, ### Indicates statistical difference compared to ALZ group, $p < 0.001$.

Histopathologic Results

Cresyl Violet Stain

The histopathological damage scores in the hippocampal sections of rats from all groups are presented in the right graph of Figure 5. Following comprehensive analysis that it t was determined that the general structure of the hippocampus and neurons in the control and SHAM groups exhibited no significant abnormalities, with cell borders remaining clearly delineated. When the general structure of the hippocampal region was examined in the brain sections of the ALZ group, neurons with euchromatic nuclei, narrow and darkly stained cytoplasm were found. In most of the neurons in this group, cell boundaries were not clear and significant cell loss was observed. Specifically, the boundaries of neurons that were about to degenerate could not be distinguished. Neurons that showed structural integrity were also reduced in size. Additionally, degenerated structures with possible cell debris were frequently observed. In the ALZ+CUR and ALZ+RES groups, it was evident that the degeneration and cell reduction observed intensely in the ALZ group was less, whereas the number of neurons with normal structure was higher. In the ALZ+CUR+RES group, the histologic appearance of the hippocampus was quite similar to the ALZ group. In this group, the density of degenerated cells with reduced nucleus size, narrow and darkly stained cytoplasm was significantly higher than in the control and SHAM groups. Additionally, when compared to the sections in ALZ+CUR and ALZ+RES groups, the density of healthy neurons was lower and the density of degenerated neurons was higher (Left image of Figure 5).

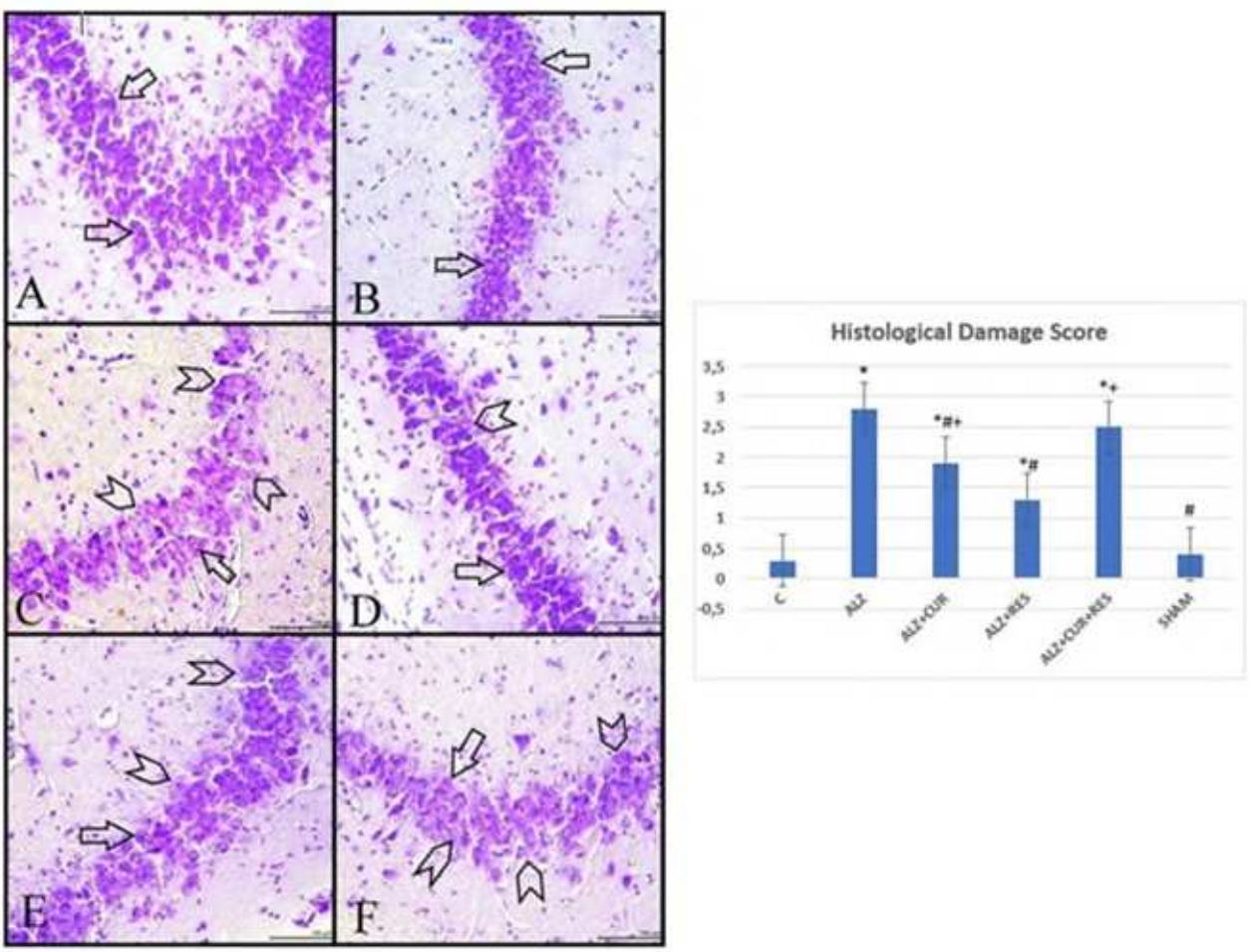

Figure 5 Left image: Light microscopic images showing histological changes in hippocampal sections of rats from each group. Arrows and arrowheads indicate healthy and degenerated neurons, respectively (A: Control; B: SHAM; C: ALZ; D: ALZ+RES; E: ALZ+CUR; F: ALZ+CUR+RES). Staining: Cresyl violet; magnification ×400.

Right graph: Semi-quantitative histological damage scores (HDS) in the groups. *Indicates significant difference compared to the Control group (p<0.05); # compared to the ALZ group (p<0.05); + compared to the ALZ+RES group (p<0.05).

Immunohistochemical Results

GFAP Immunocytochemistry

The intensity of GFAP (+) stained cells was evaluated throughout the hippocampus in brain sections of rats in all groups (Figure 6). In the sections examined, the increase in the intensity of GFAP (+) stained cells in the hippocampus of rats in the ALZ group was remarkable

compared to all other groups ($p < 0.05$). It was also observed that the cytoplasmic extensions of these cells increased and branched into numerous branches. While no significant difference was observed between the control and SHAM groups ($p > 0.05$), the ALZ+CUR group had slightly more GFAP (+) stained cells than the control group. The ALZ+CUR+RES group showed a similar density of GFAP (+) stained cells as the ALZ group ($p > 0.05$). Although the ALZ+CUR and ALZ+RES groups showed an increase in GFAP (+) stained cells compared to the control group ($p < 0.05$), this increase was much less evident than in the ALZ group. Besides, the cytoplasmic extensions of GFAP (+) cells in ALZ+CUR and ALZ+RES groups were less branched and elongated compared to ALZ group. In particular, the density of GFAP (+) stained cells and cytoplasmic extensions in the ALZ+RES group were more similar to those in the control group.

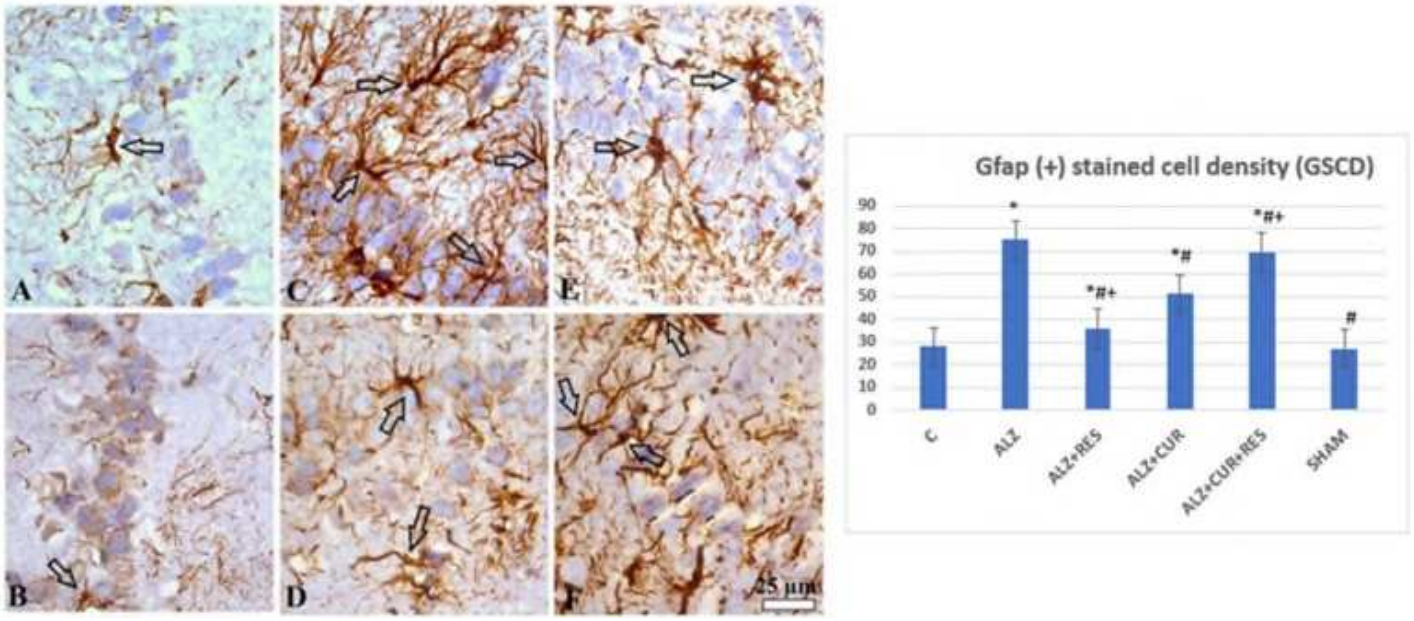

Figure 6. Left image: Distribution of GFAP (+) stained cells in the CA region of the hippocampus. Arrow: GFAP (+) stained cells. **(A):** Control Group, **(B):** SHAM Group, **(C):** ALZ Group, **(D):** ALZ+RES Group, **(E):** ALZ+CUR Group, **(F):** ALZ+CUR+RES Group. × 1000

Right graph: Quantitative analysis of GFAP (+) stained cell density (GSCD). *Indicates significant difference compared to the Control group ($p < 0.05$); # compared to the ALZ group ($p < 0.05$); + compared to the ALZ+RES group ($p < 0.05$).

Caspase-3 Immunocytochemistry

In the immunohistochemical analysis using caspase-3, brain sections from the groups were examined and the Immunostaining Intensity Distribution Index (IIDI) was calculated (Figure 7). The staining intensity in the cytoplasm of cells in the hippocampus exhibited variability from cell to cell. The presence of high levels of apoptosis was indicated by a dark brown staining, while no staining was observed in areas exhibiting weak activity. The evaluation of the groups was based on the extent of dark staining. In this context, no significant difference was observed between the control and SHAM groups ($p > 0.05$). The IIDI value was found to be considerably elevated in the ALZ group in comparison to the Control group ($p < 0.05$). The study revealed no statistically significant differences between the ALZ+CUR+RES and ALZ groups ($p > 0.05$). While the IIDI value of the ALZ+CUR and ALZ+RES groups was higher than that of the Control group, it was significantly lower than that of the ALZ group ($p < 0.05$).

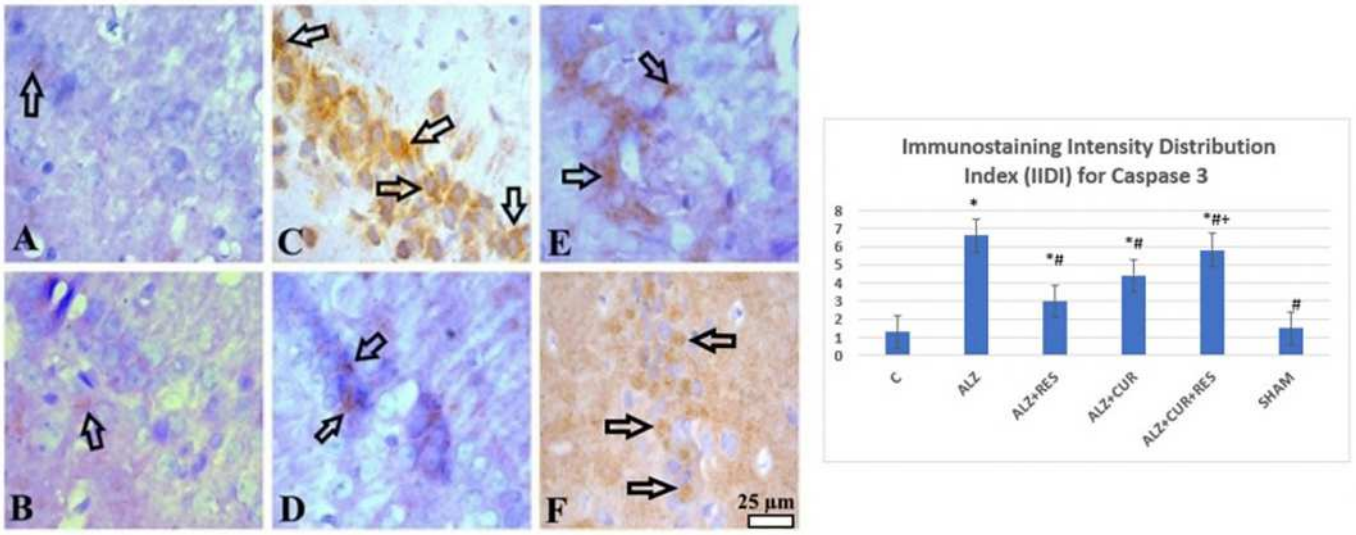

Figure 7. Left image Light microscopic findings of caspase 3 immunoreactivity in hippocampus sections of rats in all groups. Arrow: Caspase 3 stained cells. **(A):** Control Group, **(B):** SHAM Group, **(C):** ALZ Group, **(D):** ALZ+RES Group, **(E):** ALZ+CUR Group, **(F):** ALZ+CUR+RES Group. × 1000

Right graph: Immunostaining intensity distribution index (IIDI) values for caspase-3 immunoreactivity in all groups. *Indicates significant difference compared to the Control group ($p < 0.05$); # compared to the ALZ group ($p < 0.05$); + compared to the ALZ+RES group ($p < 0.05$).

Biochemical Results

The mean values and standard errors of TAS and TOS values, which are among the oxidative stress parameters used in the study, are presented in Figure 8. As a result of the statistical analysis, it was determined that there was a significant difference between the ALZ group and the Control and ALZ+RES groups in terms of TAS level (p<0.05). According to this result, the total antioxidant level of the ALZ group was significantly lower than the other groups. No significant difference was found between the Control group which had the highest TAS level and the other groups.

When TOS levels were compared between groups, a significant difference was found between ALZ group and Control, ALZ+RES and SHAM groups (p<0.05). TOS level in the ALZ group was significantly higher compared to these groups (p<0.05). Accordingly, the mean TOS level of the ALZ+CUR group was significantly higher than the Control and SHAM groups, and the mean TOS level of the ALZ+RES group was significantly higher than the Control and SHAM groups. The TOS level of ALZ+CUR+RES group was also significantly higher than the Control and SHAM groups (p<0.05).

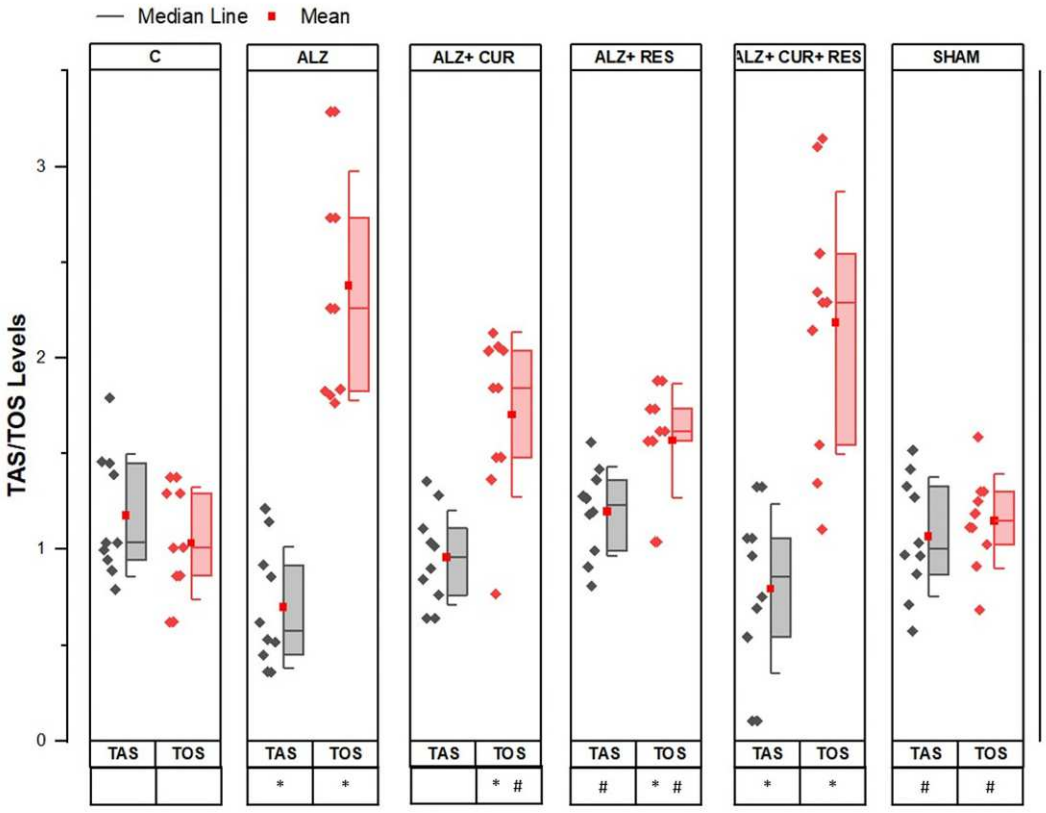

Figure 8 Mean TAS and TOS values in serum samples from rats in all groups

*Indicates statistical difference compared to control group, $p < 0.05$.

# Indicates statistical difference compared to ALZ group, $p < 0.05$.

Discussion

Alzheimer's disease, a growing global health concern, is a critical issue with no known cure. The objective of this study was to determine whether different antioxidants can protect against the damage caused by oxidative stress, which plays an important role in the pathogenesis of Alzheimer's disease, in the hippocampus. This was accomplished through the implementation of social behavior tests, biochemical analyses, stereological analyses, and histopathological examinations.

In the open field test, a widely utilized method for evaluating anxiety-like behaviors and locomotor activity in animals, it has been documented that a reduction in the time spent in the central area is indicative of anxiety [26]. In this test, it was also noted that the increase in locomotor activity was parallel to the increase in anxiety, and that the increase in grooming behavior could provide indirect information about anxiety [27, 28]. This study revealed two notable findings during the open field test. The initial observation pertained to a conspicuously elevated grooming frequency in the control group in comparison to the other groups. The subsequent observation revealed an augmented line crossing frequency in the ALZ group. The observed outcomes can be ascribed to elevated anxiety levels, which are concomitant with augmented locomotor activity and thigmotaxis

The Passive Avoidance and Morris Water Maze tests administered in this study indicated a substantial decline in learning and memory performance in rats treated with STZ. The results of the Passive Avoidance Test demonstrated a significant decrease in retention time in the ALZ group, while curcumin and resveratrol treatment effectively prevented this deterioration. On the fifth day of the Morris Water Maze test, rats in the ALZ group exhibited a reduced duration of time spent in the target quadrant. It is noteworthy that the performance of the group treated with resveratrol was nearly equivalent to that of the control group and yielded more favorable results compared to the other treatment groups. These findings suggest that resveratrol may exert a more pronounced effect on cognitive functions than curcumin.

STZ-induced cognitive impairments have been linked to elevated oxidative stress and inflammation within the brain. This phenomenon has been demonstrated to result in neuronal

damage, impaired synaptic connections, and disrupted memory processes. Furthermore, STZ has been shown to cause damage to pancreatic beta cells, resulting in a reduction in insulin production and subsequent development of insulin resistance. These disruptions in glucose metabolism also have a detrimental effect on brain function [29, 30].

Curcumin and resveratrol have been demonstrated to reduce oxidative stress by neutralizing free radicals, thanks to their antioxidant properties. Furthermore, these compounds have been shown to suppress the production of inflammatory cytokines and to inhibit inflammation-related signaling pathways [31]. These compounds have been demonstrated to support synaptic plasticity by increasing BDNF levels, improve neuronal energy metabolism by protecting mitochondrial function, and thus enhance learning and memory processes [32–34]. In addition, in vitro studies have demonstrated that these polyphenols may impede the formation of toxic Aβ oligomers and protofibril intermediates [35].

A salient finding of the study was the elevated total oxidant level (TOS) and diminished total antioxidant level (TAS) in the STZ-treated group. Although no significant increase in TAS was observed in the groups treated with curcumin and resveratrol, a significant decrease in TOS was observed. This finding indicates that both compounds exert an effect on oxidative stress, particularly through the suppression of TOS levels. Numerous studies in the extant literature have reported that curcumin and resveratrol play a protective role against apoptotic cell damage and cell death by increasing antioxidant capacity [31, 36].

In this study, TAS and TOS measurements were performed to assess systemic oxidative stress. These parameters are considered reliable biochemical indicators, reflecting the overall oxidative balance in the body. Furthermore, cellular-level inflammation and apoptosis processes were evaluated using immunohistochemical markers such as GFAP and caspase-3, thereby indirectly demonstrating neuroinflammation and oxidative stress-induced neuronal damage. However, the evaluation of cytokines such as TNF-α, IL-1β, IL-6, and oxidative stress-related signaling pathways (e.g., Nrf2, HO-1) could enhance the scope of the study by providing a more detailed understanding of inflammatory processes. In this context, the inclusion of molecular markers is considered an important area for future development that should be considered in future studies.

In this study, GFAP immunohistochemistry was utilized to evaluate astrocytes that become active in the formation of neuronal damage and synthesize GFAP. In the examined sections, it was observed that the density of GFAP-positive cells was significantly increased in rats from

the ALZ group compared to rats from the other groups. A substantial body of research employing icv-STZ as an AD model has evidenced a notable augmentation in GFAP-positive staining within the brains of icv-STZ rats. The most pronounced staining was observed in the hippocampal and ventricular regions, suggesting the presence of more severe neuroinflammation in the hippocampus[35, 37]. A reduction in oxidative stress in astrocytes is regarded as a prospective therapeutic approach for the prevention of inflammation and apoptosis [38]. In the ALZ+CUR and ALZ+RES groups, this increase was less pronounced but still higher than in the control group. This result suggests that both compounds have the potential to reduce astrocytic activation. As demonstrated in previous animal and cell studies, resveratrol and curcumin have been shown to suppress astrocyte activation by reducing oxidative stress and inflammation [38, 39].

The second immunohistochemical analysis applied in this study to evaluate neuronal apoptosis targeted the caspase-3 marker. Caspase-3 is widely regarded as one of the most significant effectors of apoptosis [40]. The study's findings indicate that the density of caspase-3-positive cells in the hippocampus exhibited a significant increase in the ALZ group compared to the control group. However, in the group treated with the combination of curcumin and resveratrol, this density remained at a level comparable to that of the ALZ group. Conversely, in groups treated with either compound alone, the number of caspase-3-positive cells was significantly reduced compared to the ALZ group, yet remained higher than the control group.

The STZ application has been demonstrated to induce DNA damage in neurons, which subsequently results in elevated activation of poly (ADP-ribose) polymerase (PARP). Excessive activation of PARP has been shown to lead to depletion of NAD+ and ATP, resulting in an energy crisis. This, in turn, has been observed to cause increased mitochondrial membrane permeability and cytochrome c release. The release of cytocrom c into the cytoplasm has been demonstrated to trigger the activation of caspase-9 and, consequently, caspase-3. Caspase-3 is the initiator of apoptosis, a process that involves the degradation of intracellular proteins [29]. STZ has also been demonstrated to increase the production of reactive oxygen species (ROS), which has been linked to oxidative stress. This, in turn, has been shown to contribute to mitochondrial dysfunction and the progression of apoptosis [41]. Furthermore, evidence has emerged that STZ triggers caspase-3-mediated apoptotic signals by augmenting the production of pro-inflammatory cytokines, such as TNF-α and IL-1β [42].

The progression of apoptosis through caspase-mediated pathways has been associated with neuronal dysfunction and cell death in chronic neurodegenerative diseases [43]. In Alzheimer's

disease, elevated levels of oxidative stress, particularly in the neurons of the hippocampus, have been documented to induce increased expression of the enzyme caspase-3 [44].

The anti-apoptotic properties of curcumin have been demonstrated in studies of STZ-induced diabetic rats. These studies have shown that curcumin suppresses apoptosis by reducing inflammation and oxidative damage, increasing the levels of anti-apoptotic proteins such as Bcl-2, and decreasing caspase-3 expression [45, 46]. In a similar manner, resveratrol has been documented to safeguard mitochondrial function by diminishing oxidative stress and inflammation and by reducing the expression of pro-apoptotic proteins, particularly caspase-3 [47–49]

The biochemical and histopathological findings are, in general, consistent with each other. The increase in TOS, elevated GFAP density, and increased caspase-3 activity observed in the ALZ group suggest that STZ administration may enhance neuronal damage through oxidative stress and contribute to neuroinflammatory processes by triggering astrocyte activation.

In the monitoring of cognitive decline in Alzheimer's disease (AD), determining the extent of tissue loss in the brain is an important parameter [50]. In this study, the Cavalieri principle, a stereological method, was employed to assess the volume of the hippocampus, with the objective of determining whether there was a loss of volume in the STZ-induced model [51]. The findings indicated a significant reduction in hippocampal volume in the ALZ group compared to other groups. Conversely, the volume of the hippocampus was maintained in subjects who received curcumin or resveratrol in isolation. However, in the group treated with both compounds, a decrease in hippocampal volume was observed, similar to the decrease seen in the ALZ group.

In the literature, widespread tissue loss in the limbic system and related cortical regions, particularly in the hippocampus, has been reported in AD [52–55]. Furthermore, studies conducted in various toxic models have demonstrated that both curcumin and resveratrol have protective effects against volume reduction in brain regions such as the hippocampus and prefrontal cortex [14, 56, 57].

The results of this study suggest that STZ may cause a loss of hippocampal neurons through oxidative stress and inflammation, contributing to a reduction in volume. Conversely, curcumin and resveratrol have been postulated to counteract these processes, thereby curtailing neuronal loss and, consequently, preserving the volume of the hippocampus.

The study indicated that when curcumin and resveratrol were administered concurrently, they did not provide the same level of protection as when administered individually. The precise rationale for this phenomenon remains to be elucidated; however, several potential explanations can be postulated. One possibility is that when administered concurrently, the medications may adversely affect each other's bioavailability, consequently diminishing their effectiveness. Another potential explanation is that the administered doses may have exceeded the optimal range or been insufficient. Additionally, the medications may influence disparate molecular pathways, thereby exerting a counteractive effect on each other. Finally, the medications may modulate immune responses in a contradictory manner, resulting in a reduction in the anticipated therapeutic effect. This distinctive finding from the present study gives rise to novel inquiries regarding the concomitant utilization of curcumin and resveratrol. It is imperative that future research systematically evaluate diverse dose combinations.

This study is one of the few that has thoroughly examined the effects of natural antioxidants, such as curcumin and resveratrol, on behavioral, biochemical, and histopathological parameters in an experimental model of Alzheimer's disease. The stereological assessment of hippocampal volume, in conjunction with the co-analysis of molecular markers such as GFAP and caspase-3, serves to enhance the study's uniqueness. From a clinical perspective, the neuroprotective effects of curcumin and resveratrol may be considered as supportive treatment options for progressive neurodegenerative diseases such as Alzheimer's disease. The low toxicity profile of both compounds enhances their safety for long-term use and provides insights for future human studies. Furthermore, the observed loss of efficacy when these compounds are used in combination necessitates further investigation of pharmacodynamic and pharmacokinetic interactions. This study offers pioneering data on the integration of antioxidant-based approaches into clinical protocols, particularly for patients with early-stage Alzheimer's disease.

Strengths and limitations

This study makes significant contributions to the multifaceted evaluation of the individual and combined use of curcumin and resveratrol, natural antioxidants, in an experimental Alzheimer's model induced by streptozotocin (STZ), at behavioural, biochemical, histopathological, and immunohistochemical levels. The study's methodological strengths include the integration of behavioural tests (the open field test, passive avoidance test, and Morris water maze) to assess different cognitive domains; the quantitative evaluation of hippocampal volume using stereological methods; and the immunohistochemical analysis of molecular markers (GFAP

and Caspase-3). Furthermore, the low toxicity profile of both compounds suggests their potential as complementary treatments for Alzheimer's disease. The scientific explanations provided for the lack of synergistic effects observed in combination therapy, based on pharmacodynamic and pharmacokinetic interactions, constitute another original aspect of the study.

However, it is important to note that the system is not without its limitations. The absence of a positive control group in the study limits the comparison of the findings with a reference treatment. The evaluation of oxidative stress was based exclusively on TAS and TOS levels, with molecular markers associated with inflammation and apoptosis (TNF-α, IL-1β, SOD, CAT, etc.) not being considered. The comments regarding the ineffectiveness of the curcumin and resveratrol combination, although theoretical, were not supported by mechanistic data, necessitating further studies.

Conclusion

The findings of the study demonstrated that STZ destroys neurons in vivo and antioxidants positively affect cell survival as expected. In addition, when the efficacy of curcumin and resveratrol from the same family group were compared, it was observed that resveratrol provided a higher neuroprotective effect than curcumin. It was found that if the two antioxidants were used together, they showed neurotoxic effects and their efficacy decreased. Further studies with different methods are necessary to elucidate the efficacy of resveratrol and curcumin on the STZ model. Besides, the study can be enriched by varying the doses in the experimental protocol, using different antioxidant and immunohistochemical methods.

The biochemical, histopathological, immunohistochemical, stereological and behavioral results in this study show that curcumin and resveratrol reduce the oxidative stress induced by STZ, reduce STZ-induced neurodegeneration, activate astrocytes, reduce the reduction in hippocampal volume, and protect learning and memory functions. The findings of this study reveal the potential therapeutic effects of curcumin and resveratrol against ADand provide important clues for future research.

Declarations

Ethics approval

The study protocol was approved by Ondokuz Mayıs University Experimental Animal Ethics Committee and all experimental procedures were performed in strict adherence to the ethical rules (Ethics Code: 2020/67).

Consent for publication

All authors have consented to the publication of the manuscript.

Data availability

Raw data can be obtained from the authors upon request. All other data generated or analyzed during this study are provided in this published article and in the supplementary information files.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This study was supported by Erciyes University Scientific Research Projects Unit with project number TDK-2021-11068.

Acknowledgments

The authors thank Dr. Süleyman Emre Kocacan for his valuable collaboration in creating a STZ-induced Alzheimer's model in rats.

Authors contributions

D.Ö.S: Visualization, Methodology, Investigation, Conceptualization.
E.U: Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation.
D.S: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Clinical trial number

Not applicable.

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