Research in Exercise Nutrition
ISSN: 2980-8960

RESEARCH ARTICLE

Research in Exercise Nutrition 4(4), 9-20

Interactive Effect of Nanoliposomal Ursolic Acid Supplementation and Enriched Environment on Oxidative Stress, Cerebellar Purkinje Neuron Survival, and Balance Function in Aged Rats

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Open Access

REN

Submitted:
02 September 2025
Revised:
24 December 2025
Accepted:
01 January 2026
Published:
01 January 2026

Research in Exercise Nutrition:

2025, Volume 4 (4), Pages 9-20

Publisher:
University of Kurdistan

ISSN: 2980-8960

Abstract

Introduction: The aim of this study was to investigate the simultaneous effect of nanoliposomal ursolic acid (UA) supplementation and physical activity in an enriched environment (EE) on changes in malondialdehyde (MDA) concentration, superoxide dismutase (SOD) activity, neuronal population based on changes in Purkinje cells in cerebellar tissue, and behavioral balance performance in aged rats.

Methods: In this experimental study, twenty rats aged 16-20 months were randomly divided into four equal groups: control, aged + solvent, aged + supplement, aged + exercise, and aged + supplement + exercise. Physical activity was performed for 4 weeks, 5 sessions per week, and each session was 30 minutes, including voluntary exercise in an enriched environment consisting of a larger than standard cage, a running wheel, and a set of 14 different objects. Nanoliposomal UA supplement gavage 250 mg/kg body weight was performed in the fasting state and in accordance with the training days. MDA concentration and SOD activity in cerebellar tissue were measured by ELISA, and the number of Purkinje neurons was measured by cresyl violet tissue staining. Balance performance was also measured by Beam behavioral test. Data were analyzed by two-way ANOVA and Bonferroni post hoc tests at $P \leq 0.05$ level.

Results: UA supplementation and EE physical activity each separately led to a decrease in MDA and an increase in SOD, neuronal population and improved balance ($p=0.001$). The interactive effect of UA and EE was significant in increasing SOD ($p=0.001$), neuronal population ($p=0.001$) and improving balance ($p=0.039$).

Conclusions: It seems that the interaction of regular physical activity in an enriched environment and UA administration in aged mice may have an adjunctive therapeutic role in maintaining cerebellar neuronal survival, improving balance function, and enhancing antioxidant defense through increasing SOD.

Key Words: Exercise Training, Tri Trepnoids, Ageing, Brain

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Citation:#
Zare B.A, Rahimi A, Feizollahi F. Interactive effect of nanoliposomal ursolic acid supplementation and enriched environment on oxidative stress, cerebellar purkinje neuron survival, and balance function in aged rats**. Research in Exercise Nutrition.** 2025;4(4):9_20**.** Doi: https://doi.org/10.22034/ren.2026.144440.1125

1. Introduction

Aging is a major risk factor for neurodegeneration. Among various age-related diseases, neurodegeneration and the associated decline in cognitive function are particularly important due to their significant impact on lifespan and quality of life (1). Age-related neurodegeneration also leads to the inability to maintain balance in the elderly, which is a major cause of falls in these individuals and, in some cases, is even a major cause of death. The cerebellum, a critical region for motor control and cognitive functions, plays a pivotal role in the timing of movements, especially during complex activities, to promote integrated perceptual coordination and interaction with the environment. Understanding the impact of aging on this brain region, which serves as a major center of neuronal assembly and houses more than 60% of the brain's neurons, is crucial given its significant vulnerability during aging (2).

Oxidative damage is closely associated with neuronal degeneration in the cerebellum during the aging process. The cerebellum exhibits particular vulnerabilities to oxidative stress with age, and this type of stress sensitizes neurons to damage. Studies have shown that oxidative DNA damage, characterized by increased levels of markers such as malondialdehyde (MDA), is enhanced with age in the cerebellum and increases the risk of developing neurodegenerative diseases (3). High levels of MDA in the brain are associated with age-related decline in cellular function and neuronal degeneration. In models of aging, such as Ercc1-deficient mice, increased MDA levels have been observed with synaptic degeneration and morphological changes in neurons, particularly in the cerebellum, suggesting that MDA may play a role in the cascade of events leading to neuronal death (4). On the other hand, with advancing aging and weakening of endogenous antioxidant defenses, brain levels of superoxide dismutase (SOD) can decrease, leading to the stimulation of oxidative stress and neurodegeneration. Studies have shown that SOD deficiency in animal models leads to increased age-related oxidative damage, emphasizing the importance of SOD in maintaining neuronal health during aging (5). SOD is involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's, and its activity is crucial for preventing the accumulation of superoxide radicals, which can lead to cellular damage and neuroinflammation. Increasing SOD activity or mimicking its function could provide a strategy to combat oxidative stress and its associated neuronal damage (6).

Antioxidants have been investigated as therapeutic options to reduce oxidative damage and its consequences on neuronal health. One natural antioxidant substance that has recently been widely used with a wide range of pharmacological activities to treat neuronal damage is ursolic acid (UA), a triterpenoid compound naturally occurring in the skin of fruits and in many plants such as rosemary. Available evidence suggests that ursolic acid, with its anti-inflammatory properties, prevents brain damage and reduces cognitive impairment by suppressing NF-kB signaling pathways (7). Previous studies have also demonstrated antioxidant and neuroprotective activity of UA. In this regard, a study reported that ursolic acid supplementation in aged mice subjected to neurotoxicity improved memory and learning by reducing oxidative damage in these samples (8). Also, the results of Salao et al. (2021) indicate the neuroprotective effect of UA against oxidative damage in isolated mouse brain, which is demonstrated by their ability to reduce oxidative stress, purinergic and cholinergic disorders, along with the simultaneous suppression of proteolytic activity (9). However, the results of a recent study by Kazemi et al. (2023) showed that supplementation with ursolic acid alone or in combination with exercise training did not cause significant changes in plasma levels of MDA and SOD in aged diabetic mice (10). Given the limited and contradictory findings, it seems that further studies are needed to clarify the mechanism of the effect of ursolic acid on positive changes in neural tissue and related disorders.

The role of physical activity in reducing oxidative damage in aging has been well studied. Epidemiological studies have shown that exercise not only improves overall brain health by regulating oxidative stress, but also acts as a potent anti-aging treatment with minimal adverse effects (11). In this regard, a recent meta-analysis reported that regular traditional exercise training in middle-aged and elderly individuals reduced oxidative stress by improving SOD and MDA levels (12). Also, Bratti et al. (2021) showed that 8 weeks of continuous and intermittent aerobic training in aged mice was able to reduce MDA protein expression in the hippocampus of the brain (13). Another recent study in 2023 reported that 8 weeks of aerobic training in aged mice resulted in a significant decrease in serum MDA levels and an increase in SOD (14). It seems that exercise during old age can play an effective role in reducing oxidative damage during aging through various mechanisms, especially strengthening antioxidant defense by increasing the activity or expression of endogenous antioxidant enzymes, improving mitochondrial function, and reducing systemic inflammation (16, 15).

According to the aforementioned literature, regular exercise in old age, in addition to its antioxidant benefits in neural tissues such as the brain and cerebellum, can have a positive and potential role in neuronal repair and survival (17); also, in old age, the combination of regular physical activity and UA administration can probably be considered as a multifaceted strategy for neuroprotection, support of the cerebellar neuronal population, and improvement of balance function, especially in conditions where oxidative stress is the cause of neurodegeneration. Studies have shown that UA contributes to neuronal regeneration in periods of oxidative stress-related neurodegeneration by increasing oligodendrocyte proliferation, reducing inflammation, and oxidative stress. Also, the role of exercise in increasing antioxidant activity and reducing free radical production confirms that the combination of exercise with ursolic acid may have synergistic protective effects. Although the current evidence does not examine the interactive effect of the two interventions on cerebellar neuronal population and oxidative status in aging, this represents an important research gap in this field. On the other hand, given that the evidence on the positive effects of ursolic acid supplementation in improving age-related neurological damage is limited, it seems that the interaction of administering this antioxidant supplement with voluntary and regular exercise in aging may have positive effects on improving cognitive and motor function, reducing neurodegeneration, and oxidative damage. Although few studies have examined the interactive effects of exercise and ursolic acid on the neuronal degeneration of cerebellar tissue in aging, and the lack of detailed investigation of molecular mechanisms is considered a research gap in this field. This study can probably provide strong scientific evidence to improve the quality of life of the elderly, prevent and treat neurological and cognitive disorders in this age group.

2. Methods

2.1. Participants

Male Wistar rats (16 to 20 months old) with an average body weight of 430 ± 20 g were purchased from the Pasteur Institute of Tehran. The elderly mice were kept in standard cages made of transparent polycarbonate for 2 weeks with the aim of adapting to the environment, with 12 hours of light and 12 hours of darkness at a temperature of 20 to 24 degrees Celsius, humidity of 45-55%, and they had free access to water and standard laboratory food produced by Behparvor Animal Feed Company. It should be noted that all laboratory methods in this study were carried out in accordance with the 1964 Helsinki Guidelines for the Care and Use of Laboratory Animals and also in accordance with the guidelines of the Ethics Committee of Islamic Azad University. This study was conducted based on the research plan of the doctoral thesis with tracking code 162947740 registered at Islamic Azad University, Karaj Branch.

2.2. Study design

20 elderly male rats were randomly selected and after adaptation to the new environment, they were divided into 4 groups, including 5 rats in each group, by simple randomization: 1- Elderly + Solvent Control Group, including elderly rats that did not have access to the enriched environment for 4 weeks and received only solvent (distilled water). 2- Elderly + Supplement Group, including elderly rats that received ursolic acid supplementation for 4 weeks. 3- Elderly + Exercise Group, including elderly rats that engaged in physical activity in the enriched environment for 4 weeks. 4- Elderly + Supplement + Exercise group included elderly mice that received both the supplement and physical activity intervention for 4 weeks. Considering the new restrictions provided by the Ethics Committee for Working with Laboratory Animals and preventing the unnecessary killing of animals, and in order to obtain an ethics code, it was suggested that a minimum number of animals (5 mice in each research group) be considered.

2.3. Sample size calculation

In the present study, G Power software was used to determine the sample size. In this software, by selecting the F test and then the statistical method of analysis of variance (main and interactive effects), a large or strong effect size (equal to 0.6), an α value equal to 0.05, and a statistical power of 0.70 were considered, and finally 20 mice were determined for 4 groups.

2.4. Nutritional intervention / Supplementation protocol

In order to administer ursolic acid, to overcome its pharmacological limitations, including low water solubility and low bioavailability, this compound was formulated in a nanoliposomal form to provide more effective delivery and higher stability in the animal body. Liposomes are known as vesicles with a lipid bilayer composed of phospholipid and cholesterol,

 https://doi.org/10.22034/ren.2026.144440.1125

which can surround lipophilic compounds in their lipid layer and, due to their nanometric size and bilayer structure, facilitate stability in aqueous solutions, improved drug absorption, and targeted release. To prepare nanoliposomal ursolic acid, the conventional method involves using the thin-film hydration technique. In this method, ursolic acid and lipid components including lecithin and cholesterol were dissolved in an organic solvent, then the solvent evaporated and a thin film was formed on the container wall. After hydration, the film was homogenized with an appropriate buffer to achieve nanometer-sized liposome vesicles that would trap the drug in their lipid structure (10). According to the protocol of Kazemi et al. (2023), rats in the aged + supplement group and the aged + supplement + exercise group received nanoliposomal ursolic acid supplement at a dose of 250 mg/kg body weight (1 mg/ml), fasting and 5 days a week (corresponding to exercise days) by gavage (10).

2.5. Exercise protocol

To familiarize the samples with the enriched environment and the exercise protocol, before the start of the main study period, which consisted of 4 weeks, the samples of the elderly + exercise group and the elderly + supplement + exercise group were placed in this environment for one session for 30 minutes. Then, based on a model of the exercise protocol of Zhou and Grace (2021), these samples were placed in the enriched environment in the morning hours, 5 sessions per week, each session lasting 30 minutes, and voluntarily engaged in physical activity in this environment (18). The enriched environment consisted of a cage (122 cm × 61 cm × 46 cm) larger than a standard metal cage, with climbing structures (e.g., ramps), shelter opportunities (houses, PVC pipes, cardboard tunnels), chew materials (pieces of wood), a spinning wheel, and a collection of 14 non-chewable plastic toys and objects (balls, geometric shapes, Legos) (19). The collection of toys and objects was rotated and cleaned twice a week.

2.7. Laboratory examination methods

2.7.1 Balance performnace test

After the end of the study period and before tissue collection, the sensorimotor functions of the forelimbs and hindlimbs were assessed using a tapered edged board via the Beam Test to examine balance function. Mice were trained to cross the board for 3 days before the main test, and the Beam Test was performed one day before sacrifice. This test involves walking on a tapered beam with overhanging edges on each side to allow for foot misalignment without falling completely. The end of the beam is connected to a black box (20.5 × 25 × 25 cm) with a platform at the starting point. A bright light was placed above the starting point to stimulate the mice to start moving; at the other end of the board, there was a food reward. The performance of the mice was recorded and later analyzed by calculating the ratio of forelimb and hindlimb slips (number of slips/total number of steps). Steps on edges are counted as half-slips and full-slips if the limb is completely separated from the edge (20).

2.7.2 Tissue Sampling

In order to eliminate the acute effects of training, cerebellar tissue was collected in each group 48 hours after the last training session and supplemental gavage. The animals were anesthetized with an intraperitoneal injection of ketamine and xylazine (80 to 10 mg of ketamine and xylazine per kilogram of body weight) and cerebellar tissue was collected immediately. The tissue sample from each animal was immediately placed in a microtube and frozen at -80°C.

2.7.3 Crisyl violet tissue staining

Cresyl violet tissue staining was used to examine neuronal population and Purkinje cell number. Identical sections of frozen cerebellum were used to assess Purkinje cell number. For this purpose, coronal sections were stained with 0.1% Luxal Fast Blue (LFB) solution (British Drug House, London, UK) and stored at 60°C for 1 h. Cerebellar tissue sections were quickly immersed in 0.05% lithium carbonate followed by repeated immersion in alcohol reagent (70%). Sections were washed in distilled water and then incubated with 0.1% Cresyl Fast Violet (Merck, Germany) for 4 min. Then, sections were quickly washed with distilled water and quickly dried in a graded alcohol series and cleared in xylene. Using ImageJ software, the number of Purkinje cells in each section was estimated as a percentage of the total cerebellum (7). In Figure 1, the survival status of Purkinje cells in different groups can be seen as brightly colored spherical granules between the granular and molecular layers (the so-called Purkinje layer).

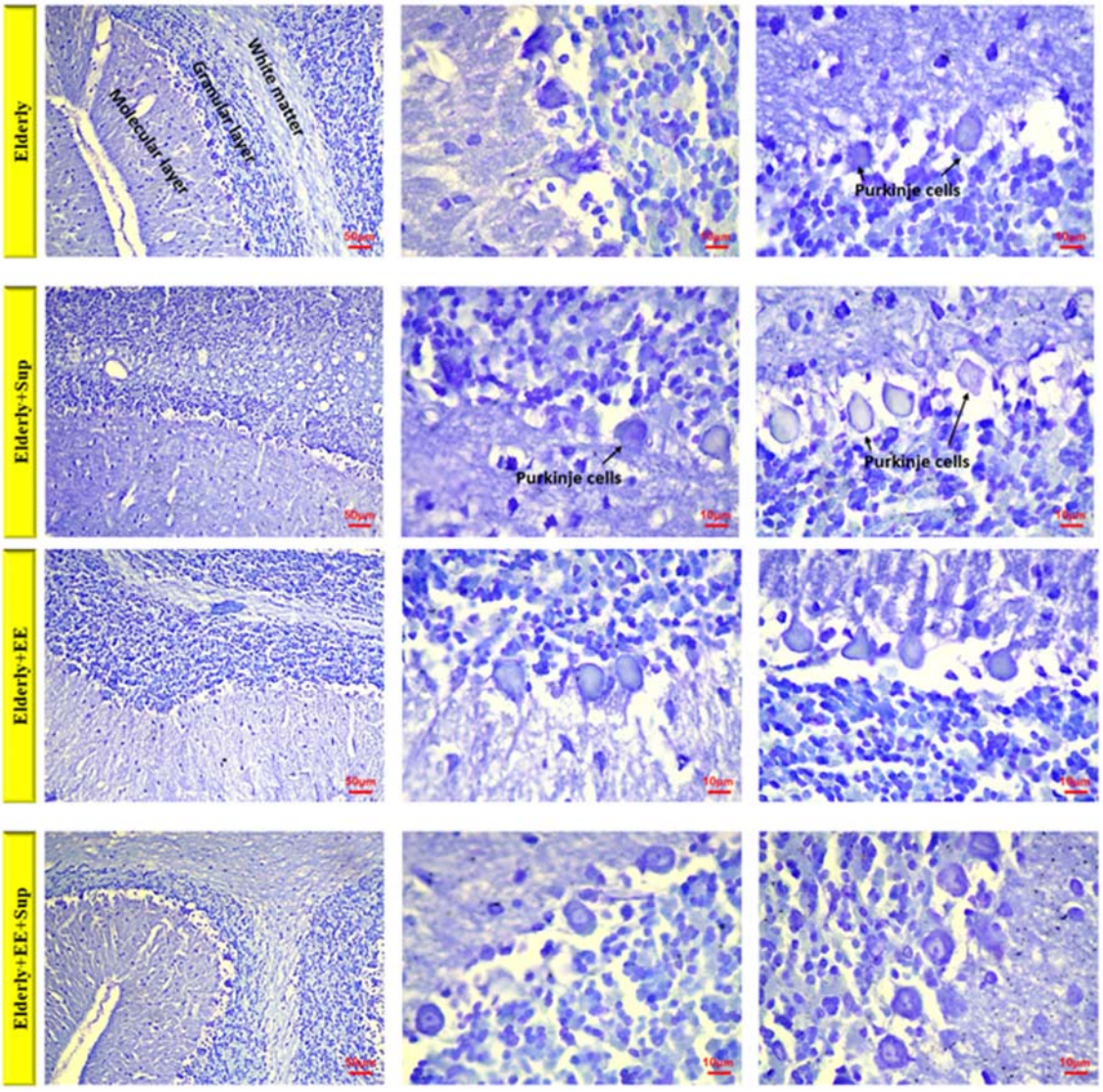

Figure 1 Pictures of Imaje J software to examine the number of Purkinje cells in each part of the cerebellum in the research groups; the images on the left were taken at a magnification of 50 microns and the rest of the images were taken at a magnification of 10 microns.

2.7.4 Enzyme assay

To measure lipid peroxidation indices and antioxidant defense, cerebellar tissue was washed and homogenized with cold PBS (pH=7.4) immediately after harvest (~100 mg tissue in 1 ml buffer), then centrifuged on ice (4000–6000 rpm, approximately 20 min), and the supernatant was stored for testing (avoid freeze/thaw cycles; store at −20 °C until measurement). MDA was measured using the colorimetric/microplate method of the ZellBio kit made in Germany (CAT No. ZB-MDA-96A/48A); Thus, after preparing the standards from 0 to 100 µM in serial dilutions, the sample/standard was reacted with the chromogenic reagent, incubated in a boiling bath for one hour, and after cooling on ice and centrifugation, 200 µL of the supernatant was transferred to the plate and the absorbance was read at 535 nm, and the final concentration was calculated according to the standard curve.

 https://doi.org/10.22034/ren.2026.144440.1125

According to the instructions of the same kit, the MDA measurement range is 0.78 to 50 µM and the sensitivity of the method is 0.1 µM.
SOD activity was measured with the ZellBio kit (CAT No. ZB-SOD-96A/48A) and the colorimetric method; the color production from the reaction of superoxide anion with chromogen was monitored at 420 nm at times 0 and 2 minutes, and based on the manufacturer's formula, the enzyme activity was calculated in IU/mL. The measurement range of SOD activity in this kit is 5 to 100 IU/mL and its sensitivity is reported to be 0.01 IU/mL.

2.8. Statistical analysis

To test the assumption of normality of the data, the Shapiro-Wilk test was used, and to test the statistical hypotheses, two-way analysis of variance and Bonferroni post hoc tests were used at the P≤0.05 level using SPSS version 26 software.

3. Results

3.1. Primary outcomes

Descriptive data from the research variables after the intervention is presented in Figure 2.

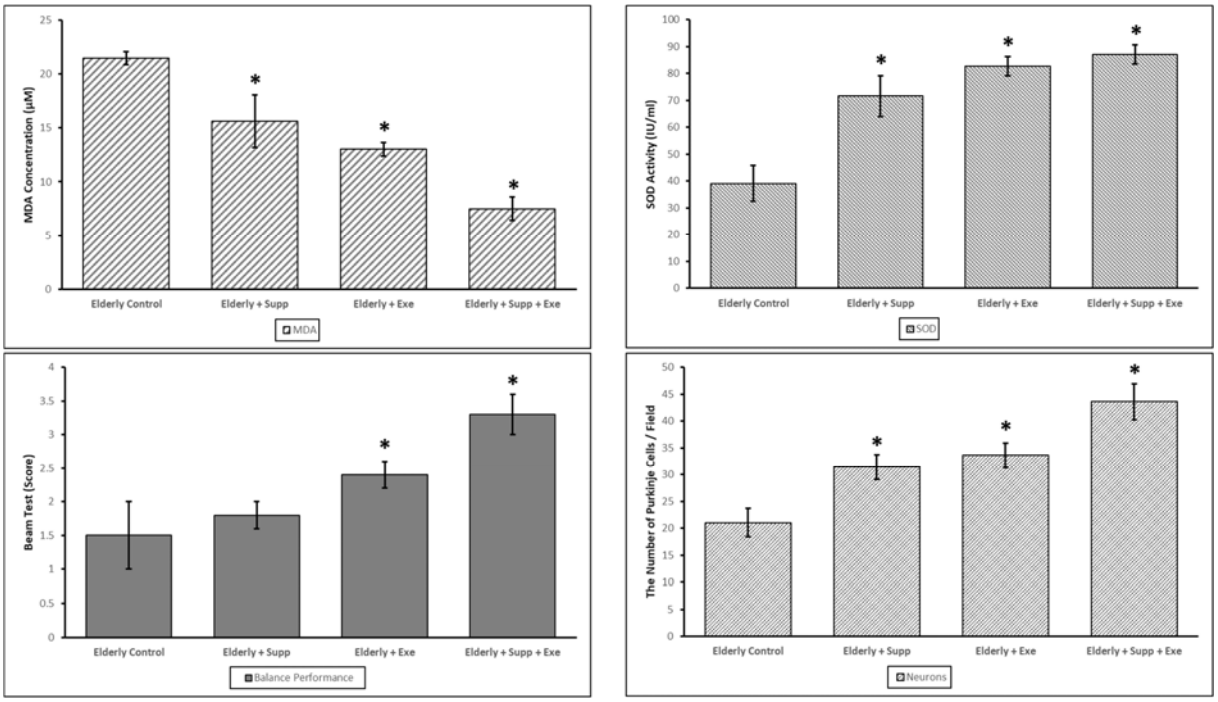

Figure 2 Descriptive data based on mean and standard deviation; * indicates a significant difference with the elderly control group (P≤0.05).

3.2. Secondary outcomes

The results of the two-way analysis of variance statistical method at a significance level of P≤0.05 to determine the intergroup changes of the studied variables can be seen in Table 1, as well as the results of the Bonferroni post hoc test for intergroup comparisons in Figure 2 (above):

Table 1 Results of two-factor analysis of variance for changes in the studied variables

Variables	Source	SS	df	MS	F	p	η²
MDA concentration (µM)	Training	344.03	1	344.03	175.01	0.001*	0.92
	Supplement	161.97	1	161.97	82.39	0.001**	0.84
	Training × Supplement	0.15	1	0.15	0.07	0.784	0.00
	Error	31.45	16	1.96			
SOD activity (IU/ml)	Training	4395.07	1	4395.07	136.39	0.001*	0.90
	Supplement	1698.03	1	1698.03	52.69	0.001**	0.77
	Training × Supplement	991.45	1	991.45	30.77	0.001***	0.66
	Error	515.55	16	32.22			
Balance (Beam test score)	Training	7.44	1	7.442	73.86	0.001*	0.82
	Supplement	1.68	1	1.68	16.70	0.001**	0.51
	Training × Supplement	0.51	1	0.51	5.08	0.039***	0.24
	Error	1.61	16	0.10			
Neuronal population (Purkinje cells/section)	Training	768.80	1	768.80	106.41	0.001*	0.87
	Supplement	520.20	1	520.20	72.00	0.001**	0.82
	Training × Supplement	250.00	1	250.00	34.60	0.001***	0.68
	Error	115.60	16	7.22			

* Indicates a significant effect of physical activity in an enriched environment on changes in research variables (P≤0.05)

** Indicates a significant effect of ursolic acid supplementation on changes in research variables (P≤0.05)

*** Indicates a significant interaction effect of physical activity in an enriched environment with ursolic acid supplementation on changes in research variables (P≤0.05)

According to Table 1, the results of the two-way ANOVA statistical test in determining the presence of a significant effect of physical activity in an enriched environment among the groups showed that the main and independent effect of exercise on the levels of MDA (p<0.001), SOD (p<0.001), balance (p<0.001) and neuronal population (p<0.001) was significant. So that physical activity in an enriched environment alone led to a significant increase in SOD activity, neuronal population and improved balance function, as well as a significant decrease in MDA concentration in the cerebellar tissue of aged rats. Also, the results of this test in determining the presence of a significant effect of ursolic acid supplementation among the groups showed that the main and independent effect of supplementation on the levels of MDA (p<0.001), SOD (p<0.001), balance (p=0.001) and neuronal population (p<0.001) was significant. So that ursolic acid supplementation alone led to a significant increase in SOD activity, neuronal population and improved balance function and also a significant decrease in MDA concentration in the cerebellar tissue of aged rats. In addition, the results of two-factor ANOVA in determining the presence of a significant interaction effect of physical activity in an enriched environment with ursolic acid supplementation among the groups showed that the interactive and simultaneous effect of exercise and supplementation on SOD levels (p<0.001), balance (p=0.039) and neuronal population (p=0.001) was significant. So that physical activity in an enriched environment with ursolic acid supplementation simultaneously led to a significant increase in SOD activity, neuronal population and improved balance function in the cerebellar tissue of aged rats. However, the interactive effect of exercise and supplementation on MDA levels was not significant (p=0.784). However, according to Figure 2, the results of the Bonferroni post hoc test in the between-group comparison of MDA levels showed that there was a significant difference between the mean MDA in the elderly + supplement + exercise group and the elderly control group (p=0.001). So that physical activity in an enriched environment along with supplementation with ursolic acid reduced the MDA concentration compared to the elderly control group. However, this difference

 https://doi.org/10.22034/ren.2026.144440.1125

indicates the combined or cumulative effect of exercise and supplementation.

4. Discussion

The results of the present study showed that physical activity in an enriched environment along with ursolic acid supplementation led to increased SOD activity, the number of Purkinje neurons in cerebellar tissue, and improved balance function in aged rats. Although physical activity alone and ursolic acid intake separately reduced MDA concentration and increased SOD activity, the number of Purkinje neurons, and balance function. However, the interaction of supplementation and physical activity had a greater effect on increasing SOD, neuronal population, and balance function compared to exercise or supplementation alone.

These findings have also been confirmed in recent studies; in line with these results, a study in 2020 reported that 6 weeks of aerobic exercise in aged mice resulted in a decrease in serum MDA levels and an increase in SOD (21). Yi et al. (2021) also reported in a systematic study that regular aerobic exercise in elderly subjects reduced MDA levels and increased SOD (22). In this regard, the findings of another new study in 2023 showed that a period of aerobic exercise in aged mice was effective in significantly reducing serum MDA levels and increasing SOD (14). The role of exercise in neural growth and survival has also been studied. Consistent with the findings of the present study, Nakanishi et al. (2021) reported that a period of balance and coordination exercise training in aging mice significantly improved cognitive function, reduced neuronal inflammation, age-related neuronal loss, and reduced oxidative stress (23). Chen et al. (2020) also examined the effects of lifelong exercise (14 months) on cognitive function and memory, oligodendrocyte number, and myelination in aged mice. The results showed that long-term running exercise effectively delayed age-related decline in spatial learning ability and white matter atrophy by protecting against age-related changes in myelinated fibers and oligodendrocytes in white matter (24). Regarding the effects of ursolic acid on oxidative status, consistent with the results of the present study, Zhao et al. (2023) reported in a systematic and meta-analytic study that findings from animal and laboratory studies show that ursolic acid reduces the levels of inflammatory cytokines, increases antioxidant enzymes (increases in SOD and GSH), and reduces the level of oxidative stress (decreases in MDA), which provides convincing evidence for the anti-inflammatory and antioxidant properties of this supplement (25). Also, Rudy Soleimani et al. (2025) reported in a review study that supplementation with ursolic acid reduces MDA and ROS levels and increases SOD and GPx (26). The results of a recent study by Salao et al. (2021) also demonstrate the neuroprotective effect of ursolic acid against oxidative damage in isolated mouse brains, as demonstrated by its ability to reduce oxidative stress, purinergic and cholinergic disorders, along with the simultaneous suppression of proteolytic activity. Supplementation with ursolic acid in this study was able to increase SOD, catalase and glutathione activities and reduce MDA levels (9). Also, considering the neuroprotective properties of ursolic acid, the results of a study by Liu et al. (2023) showed that ursolic acid intake in animal models of epilepsy reduced neuronal damage and cognitive impairment by suppressing neuroinflammation and oxidative stress (27). Despite numerous evidences about the beneficial effects of physical activity and reports of the antioxidant and neuroprotective properties of ursolic acid, the literature on the simultaneous and interactive effect of these two interventions in aging is very small and is mainly limited to separate studies of these two interventions. The research gap in identifying interactive mechanisms and their possible synergy on neuronal population and neuronal survival during the aging process highlights the necessity of conducting this study.

The main mechanism of the positive effects of ursolic acid in reducing brain damage and cognitive deficits and improving neuroprotection in the elderly seems to be related to its antioxidant properties. In fact, the significant antioxidant effects of ursolic acid contribute to neurogenesis and axonal myelination. It acts by modulating oxidative stress and inflammatory pathways, especially the NF-kB pathway, which are crucial in neuroprotection and regeneration. Ursolic acid has been shown to increase the activity of endogenous antioxidant enzymes such as SOD, catalase, and glutathione peroxidase, thereby reducing oxidative damage in neural tissues. In addition, the ability of ursolic acid to scavenge free radicals and inhibit lipid peroxidation and its markers, including MDA, further supports its neuroprotective role (28).

On the other hand, the mechanism of the effect of exercise on increasing neuronal population and improving neuronal damage is probably related to increasing antioxidant enzyme activity and reducing free radical formation. Regular exercise leads to improved enzymatic activities, enhanced antioxidant capacity, and mitochondrial biogenesis in neuronal tissue

(29). Previous studies have shown a strong correlation between PGC-1α as the most important indicator of mitochondrial biogenesis and neuronal health, and this relationship can be strengthened by exercise. Regular aerobic exercise can play an important role in increasing SOD, reducing MDA, and generally reducing oxidative stress, and subsequently improving the neuronal population of the brain (30).

Given the results of the present study on the interactive effect of physical activity and ursolic acid on enhancing antioxidant activity and improving neural function and neuronal survival, it seems likely that this synergistic effect of exercise and ursolic acid is related to the common mechanism of these two interventions, namely the activation of the SIRT1 and PGC-1α pathways; the activation of the SIRT1 and PGC-1α pathways following exercise and also the consumption of ursolic acid by increasing mitochondrial function can improve the balance between reactive oxygen species and antioxidant enzymes, which seems beneficial in protecting neural precursor cells against the damaging effects of ROS (31). Recent scientific findings indicate that ursolic acid affects neurogenesis and neuronal maintenance and survival through the interaction and activation of the PGC-1α/SIRT1 pathway. Overall, the interaction between ursolic acid, PGC-1α, and SIRT1 suggests a potential mechanism by which ursolic acid can promote neurogenesis and myelin regeneration and protect against neuronal injury through increased mitochondrial biogenesis and function (32).

However, since the interaction of physical activity and ursolic acid did not significantly reduce MDA concentrations in this study, it is important to note that both exercise and ursolic acid independently activate the oxidation-sensitive Keap1–Nrf2–ARE pathway, which results in the induction of different SOD isoforms, especially MnSOD/SOD2, in mitochondria and improves mitochondrial quality. SOD displaces the initial oxidative load by degrading superoxide ($O_2^{\bullet-}$) to H_2O_2; the resulting H_2O_2, in addition to partially neutralizing the damage, plays a signaling role for mitochondrial biogenesis and neuronal survival (34, 33). Therefore, a significant increase in SOD and neuronal/functional outcomes without requiring a significant reduction in MDA is quite expected. In contrast, MDA is the terminal marker of lipid peroxidation, which proceeds mainly with the hydroxyl radical derived from the Fenton–Haber–Weiss reaction (from H_2O_2); sustained control of MDA usually requires simultaneous enhancement of downstream H_2O_2 detoxification factors (i.e., catalase, glutathione peroxidases, especially GPx4, which detoxifies phospholipid hydroperoxides) and membrane antioxidants (35). If a combined intervention primarily increases SOD but CAT/GPx/GPx4 are not stimulated to the same extent, a shift of active species occurs, such that superoxide is reduced but H_2O_2 flux allows the final lipid peroxidation output, MDA, to decrease only slightly (36) and not reach a significant threshold. Overall, exercise with ursolic acid can create a favorable mitohormesis: increased SOD2 and regulatory H_2O_2 activate mitochondrial adaptation signaling pathways (especially Nrf2, PGC-1α/AMPK pathways), resulting in the maintenance or enhancement of neuronal survival with a modest reduction in MDA levels.

Given the current financial constraints and the lack of measurement of downstream indicators of H_2O_2 detoxification (i.e., catalase and glutathione peroxidases and membrane antioxidants) in the present study, it is suggested that future studies measure these indicators in a more comprehensive and precise manner to investigate these mechanisms. Given the sensitivity of cerebellar tissue in laboratory methods, other possible limitations of this study include the stress caused by supplemental gavage in aged mice and its possible impact on functional brain damage.

5. Conclusion

Based on the findings, voluntary physical activity and ursolic acid supplementation, each separately, may have a potential role in reducing neuronal damage, maintaining neuronal survival, and balance function by improving oxidative stress in aged mice. However, it seems that the interaction of ursolic acid with regular voluntary physical activity in aging may have an adjunctive role in improving neuronal population, neuronal function, and enhancing antioxidant defense.

6. Highlights

A combined approach of behavioral therapy (regular voluntary exercise) and phytochemical therapy (ursolic acid) could potentially be considered as an adjunctive therapy strategy to maintain neuronal survival and neurological function in old age, although the pattern of biological response of oxidative markers may be heterogeneous and requires optimization of timing and evaluation of lipid peroxidation indices in future studies.

https://doi.org/10.22034/ren.2026.144440.1125

Statements

Funding
The costs of the study were borne by the researcher.

Acknowledgments
The author thanks the Physiology Laboratory of Karaj Azad University for their sincere cooperation.

Conflicts of Interest
The authors declare that there are no conflicts of interest regarding this article.

Authors' Contributions
Conceptualization, [Rahimi.A.] and [Feizollahi.F.]; methodology, [Feizollahi.F.]; formal analysis, [Rahimi.A.]; investigation, [Zare.A.] and [Feizollahi.F.]; resources, [Zare.A.]; data curation, [Zare.A.]; writing—original draft preparation, [Zare.A.]; writing—review and editing, [Rahimi.A.] and [Feizollahi.F.]; supervision, [Rahimi.A.]; project administration, [Zare.A.]. All authors have read and agreed to the published version of the manuscript.#

Supplementary Materials
Not applicable

Data Availability Statement
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Institutional Review Board Statement
The animal study protocol was approved by the Institutional Animal Care and Use Ethics Committee of Islamic Azad University. All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. This study was conducted based on the research plan of the Ph.D. thesis with tracking code 162947740 registered at Islamic Azad University, Karaj Branch.

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