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Research report

Protective effects of different exercise modalities in an Alzheimer's disease-like model

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HIGHLIGHTS

- Aerobic exercise improves anxiety-like behaviour in an Alzheimer's disease-like model.
- Aerobic, resistance and combined exercises protect from oxidative stress and memory decline.
- Exercise has neurotrophic effects.
- Exercise decreases Aβ burden in developmental stage of Alzheimer's disease-like conditions.

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ABSTRACT

Our aim was to investigate the probable protective effects of aerobic, resistance and combined exercise methods on ovariectomy and D-galactose induced Alzheimer's Disease (AD)-like model. D-galactose (100 mg/kg) or saline were administered intraperitoneally for 6 weeks to ovariectomized or sham-operated rats (n = 8/group). Aerobic (AE), resistance (RE) and combined exercises (CE) (aerobic + resistance) were performed for 3 times a week for 6 weeks. Anxiety level and cognitive functions were evaluated via hole-board and object recognition tests. Brain myeloperoxidase, malondialdehyde, nitric oxide activity, lucigenin-enhanced chemiluminescence, glutathione and serum insulin like growth factor-I (IGF-I) assays were done. Hippocampal mRNA levels of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and amyloid precursor protein 695 (APP695) were measured. Amyloid Beta (Aβ), NGF, BDNF, IGF-I immunoreactive neurons were evaluated. Freezing time were increased in AD-like model and decreased back with AE ($p < 0.05$). Deteriorated working memory in AD-like model was improved with all exercise types ($p < 0.05$-0.001). Reduced glutathione levels in AD-like model were increased and increased malondialdehyde levels were reduced and serum IGF-I levels were increased by all exercises ($p < 0.05$–0.001). Increased APP mRNA levels in AD-like model were decreased via CE ($p < 0.05$). Elevated Aβ scores in AD-like model were decreased by RE and CE ($p < 0.01$) in hippocampus and by all exercise types in cortex ($p < 0.05$–0.01). Decreased cortical NGF immunocytochemical scores of AD-like model were increased by CE ($p < 0.05$). Different exercise models may have protective effects in development stage of AD via reducing oxidative stress and Aβ scores, and by improving antioxidant system and brain plasticity.

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Abbreviations: AD, Alzheimer's disease; AE, aerobic exercise; RE, resistance exercise; CE, combined exercise; NO, nitric oxide; IGF-I, insulin like growth factor-I; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; Aβ, amyloid Beta; APP695, amyloid precursor protein 695.

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1. Introduction

Alzheimer's disease (AD) has been reported as a multi-factorial disorder [1] since many evidence have shown that nitro-oxidative and oxidative stress are early events and associated with the pathogenesis of AD [2,3]. The other important factor is neuroplasticity failure and all risk factors associated with AD have potentials to alter neuroplasticity [4]. Estrogen deprivation is also one of the risk factors which is related to the pathogenesis of AD thus the findings suggest that estrogen has a protective role in the AD and it is well known that estrogen promote neuronal plasticity in limbic system of males and females [4].

Long-term injection of D-galactose (D-GAL) in animal models can lead to decline in recognition memory [5], and an increase in anxiety levels, ROS production, and neuronal damage [6]. Addionally, long-term intraperitoneal administration of D-GAL into the ovariectomized (OVX) rats have been reported to deprivate estrogen levels and to increase oxidative stress which have synergistic effects on facilitating the improvement of AD [7,8]. Also it is suggested that low estrogen levels can increase the physiological inconvenience of neuroplasticity [4].

Although it has been widely reported that regular exercise may promote neuroprotection, the underlying mechanisms are not clear. In literature, it has been discussed that exercise eliminates the oxidative stress [9], on the other hand increases the antioxidant capacity, neurotrophins and growth factors that improve the cerebral neuroplasticity [10]. Plasticity is a well-defined adaptive response of the nervous system to the alterations in the external environment moreover the cognitive plasticity may be strenghtened via aerobic exercise [11]. Furthermore, voluntary running exercise has been shown to decrease Aβ load in a transgenic mouse model of AD [12]. Regular swimming exercise which was performed before $A\beta_{1-40}$ infusion has been revealed to be able to protect against $A\beta_{1-40}$-induced neurotoxicity [13]. Besides, aerobic exercise leads to increase in neutrophic factors and related molecules such as insulin like growth factor-I (IGF-I) and brain-derived neurotrophic factor (BDNF) in hippocampus and resistance exercise also increases perihperal and hippocampal IGF-I levels in rats [14]. In a recent study, combined exercise increases cell regeneration and decreases apoptosis in the hippocampus and enhances cognitive function in ovariectomized rats [15]. While the best exercise regimen for AD has not been defined yet, evidents suggest that multi component exercise regimen may be useful [10] that's why the possible protective effect of aerobic, resistance or combined exercise types on AD attracts attention.

Based on the aforementioned evidence, the aim of the present study was to investigate the probable preventative effects of aerobic, resistance and combined exercise methods on oxidative system, cognitive functions and neuroplasticity by using cognitive tests, bio- and immunocyto-chemical analyses in an experimental AD-like model.

2. Material and methods

2.1. Experimental procedures

The study protocol was approved by the Marmara University (MU) Animal Care and Use Committee (30. 2014. Mar). Female Wistar rats (n = 64), aged six to seven months, weighing 260–280 g were used. Rats were supplied by the MU Experimental Animals Research and Implementation Centre (DEHAMER). Animals were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature (22 ± 2 °C) and relative humidity (65–70%) were kept constant. Rats were fed with standard rat pellets and tap water ad libitum. All animal experiments carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Experimental design

Rats were weighed and randomly divided mainly into 2 groups and basal anxiety level and cognitive function measurements were performed via hole-board and object recognition tests. The first group was sham-operated, and the second group was ovariectomized and 2 weeks after operations treated with saline or D-GAL, respectively. Both of the groups had subgroups such as sedentary, aerobic (swimming), resistance (climbing with weight on vertical ladder) and combined (aerobic + resistance) exercise. Exercise adaptation sessions were started just after the first week of recovery period followed by surgical procedure. D-GAL (Sigma-Aldrich, St. Louis, MO, USA; 100 g/kg/day) was dissolved in 0.9% saline solution (vehicle) and was administered by intraperitoneally (i.p.) for six weeks. Physiological saline solution was administered by the same route and in the same dosage to the sham-operated groups in the same six weeks period. At the end of experimental protocol, hole-board and object recognition tests were repeated and blood samples were taken via cardiac puncture under thiopenthal sodium anesthesia (Fig. 1). Brain tissue samples were removed for measurements of myeloperoxidase (MPO) activity, malondialdehyde (MDA), and gluthatione (GSH) levels, nitric oxide (NO) release and lucigenin-enhanced chemiluminescence determinations. Additional samples were taken for immunocytochemical analysis of Amyloid $\beta_{1\text{-}42,}$, NGF, BDNF, IGF-I, and gene expression analysis of APP695, NGF, BDNF, IGF-I. The experiments were performed between 9:00 a.m. and 6:00 p.m.

2.3. Ovariectomy procedure

Surgical techniques were performed under anesthesia (ketamine, 100 mg/kg and xylazine, 10 mg/kg) and a small abdominal incision was made under aseptic conditions. The ovaries were then located and a silk thread was tightly tied around the oviduct, including the ovarian blood vessels. The oviducts were cut and the ovaries removed. The skin and muscle wall were then sutured with silk thread. After surgery, the rats received an injection of antibiotics (cefazolin, 40 mg/kg, intramuscular). Sham-operated rats were exposure to mimic ovariectomy according to the identical ovariectomy procedures [7]. The rats were remained in their cages for recovery period.

2.4. Exercise protocol

2.4.1. Aerobic exercise protocol

Swimming was performed 1 h/day, 3 days/week for 6 weeks. The swimming exercise protocol was adapted from de Lemos et al. [16]. Swimming was carried out in a cylindrical tank, diameters of 148 cm × 50 cm, containing temperature-controlled water (30–32 °C). The protocol was performed at the same time in every exercise session. The rats initially swam 15 min/day (5 days/week) until a regimen of 60 min/day was achieved. After 1 week of recovery period following the surgery, 1 week of exercise adaptation period was started. After the adaptations, the rats swam for 1 h, 3 days/week for 6 weeks. At the end of each exercise period the animals were dried with a towel carefully and kept in warm conditions. On the other hand, the sedentary rats were handled and dipped into water 3 times a week to mimic the same stress of touching the water that the exercised group had. All rats were weighed weekly and followed closely.

2.4.2. Resistance exercise protocol

Resistance exercise model had 8 climbing series (RE; climbing with weight on a vertical ladder) and were performed 3 times per

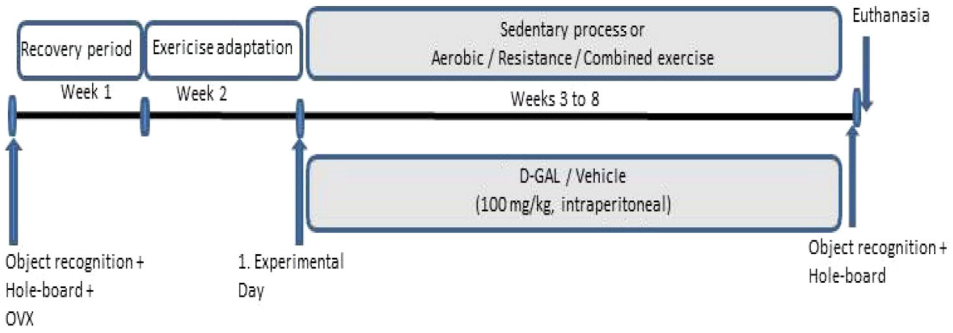

Fig. 1. Experimental protocol of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose.

week for six weeks. The resistance training protocol was modified from Rodrigues et al. [17]. Initially, the rats were adapted to climb a vertical ladder (1.1 m × 0.18 m, 2-cm grid, 80° incline) with weights attached to their tails. On the initial adaptation day, rats climbed the ladder without load and on the following exercise days the load was increased to 50% than to 75% and to 100% of the body weight, respectively. The load was adjusted weekly according to the body weight of the animal. The load (lead mass) was attached to the tail by wrapping the proximal portion of the tail with a self-adhesive foam strip (Vega adhesive tape). With the load attached to the tail, each rat was placed at the bottom of the ladder and familiarized with climbing. If necessary, a stimulus was applied to the animal's back with a ruler to initiate the movement and rat pellets were used to motivate the rats to climb. At the top of the ladder, the rats reached a housing chamber (21.6 cm × 24.1 cm × 13.97 cm), where they were let to recess for 120 sec intervals between each repetition.

2.4.3. Combined exercise protocol

Combined exercise model was consist of aerobic and resistance exercise types, alternately. One week was scheduled as two days of swimming (aerobic) and one day of climbing to ladder (resistance), and the following week was planned as one day of swimming, and two days of climbing to ladder. Combined exercise protocol was designed 3 days per week to imitate the exercise sessions that the patients having AD can achieve.

2.5. Anxiety-like behaviour

Hole-board test was used to assess anxiety level. The duration of the test was 5 min. The hole-board apparatus consisted of a wooden board (40 × 40 cm) with 16 equally spaced holes (each 3.8 cm in diameter). The hole-board test was performed by placing the rat in the center of the wooden board, and the test was recorded by a video camera for 5 min. When no movement of the trunk, head or extremities was observed, it was evaluated as freezing behaviour and the 'freezing time' [18] was determined from the videotape. Increased freezing time indicated a reduction in the exploratory behaviour and increased anxiety levels.

2.6. Recognition memory

The object recognition tasks were used to examine recognition memory functions. The test has familiarizing, sample object and novel object steps [19]. The dimensions of the test plexiglass box were 50 × 50 × 30 cm. The floor of the box was cleaned after each rat, and 2 objects (2 old or 1 old and 1 new) were assigned to each box. The rats were familiarized with the researcher before the test.

2.6.1. Familiarizing

Each rat was put into the boxes one by one for 10 min, during which the rat was adapted to the environement.

2.6.2. Sample object (old object)

Two identical (sample) objects were put and to prevent coercion to explore the objects, rodents were released against the center of the opposite wall with its back to the objects. The sample object exposure time was recorded for 10 min by a video camera. It is important that no other stimulus was present in the environment that would distract the rat's attention. Before putting the next rat into the box, the apparatus and the objects were cleaned by 70% isopropyl alcohol and left to dry for 1 min.

2.6.3. Novel object (new object)

The rats, which completed the familiarizing step returned to a housing box. After an hour, one of the objects was changed. Delay time period of one hour allows evaluation of short term memory. One sample (old) and one novel (new) object were put in the box and the rats were released against the center of the opposite wall with its back to the objects. The test duration was 3 min and recorded by a video camera.

2.6.4. Evaluation

The results were assigned by comparing the time spent with the sample and the novel objects. Object recognition is reflected by spending more time in interacting with the novel than familiar object, and is given a positive difference score. The time difference spent with the novel object was calculated by the formula below and the results were expressed as a difference score [19].

Difference score (second): Time spent with the novel object−time spent with the sample object.

2.7. Euthanasia and removal of tissue samples

The animals were euthanized by thiopenthal sodium (50 mg/kg) 24 h after the last cognitive test session. The brain and the muscle (m. soleus, m. flexor digitorum brevis) tissues were removed, washed with cold saline solution and stored at −80 °C until analysis.

2.8. Measurement of serum cortisol, IGF-I and blood cholesterol levels

Serum used for cortisol and IGF-I analyses was prepared by collecting blood into a Microtainer® Brand Serum Seperator Tube (Beckton Dickinson) allowing it to clot for 10 min at room temperature. Then, it was centrifuged (3000 rpm) for 10 min. Serum

was collected and was stored at −80 °C until analyse. Commercially available Enzyme-Linked Immuno Sorbent Assay (ELISA) kits were used for serum cortisol and IGF-I (SRB/Shanghai) following the manufacturer's instructions. The cortisol and IGF-1 results were expressed as ng/mL. The blood cholesterol concentrations were determined by using a micro-autoanalyzer (Accutrend Plus®, Roche Diagnostics), according to the manufacturer's instructions. The results were presented in mg/dL.

2.9. Measurement of fiber cross-sectional area of flexor digitorum brevis and soleus muscles

To evaluate the efficiency of the exercises, the cross-sectional areas of the flexor digitorum brevis (FDB) and soleus muscles were measured. Transverse sections (40-μM thick) were cut from the flexor digitorum brevis muscle in a cryostat (Laica CM1950) at −20 °C and were stained with Meyer's hematoxylin eosin (H&E) dye. Digital images of stained sections were achieved with an Olympus bright field microscope BX51, fitted with a DP72 camera (Tokyo, Japan) with a 20X objective. Blind analysis of the cross-sectional area of 100 fibers per muscle sample was performed by using the software image J (NIH image, USA).

2.10. Determination of myeloperoxidase activity

Myeloperoxidase, a heme protein expressed by phagocytic cells [21]. MPO activities were measured in 0.2–0.3 g brain tissue samples. The brain cortex samples were homogenized in 10 vols of ice-cold potassium phosphate buffer (50 mM K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5% w/v). The homogenate was centrifuged at 12000*g* for 10 min at 4 °C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (w/v) HETAB and 10 mM ethylenediaminetetraacetic acid (Sigma). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianizidine·2 HCl. One unit of enzyme activity was defined as the amount of MPO present that causes a change in absorbance of 1.0 unit/min at 460 nm and 37 °C and was expressed in units per g tissue [22].

2.11. Measurement of MDA and GSH levels

MDA levels, as a marker of lipid peroxidation, were measured in brain tissues. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a pink colored complex, which has maximum absorbance at 532 nm. The brain cortex samples were homogenized in 10 vols of ice-cold 10% trichloracetic acid and centrifuged at 3000 rpm for 15 min at 4 °C. Supernatant was removed and recentrifuged at 15000 rpm at 4 °C for 8 min. Lipid peroxide levels were expressed in terms of MDA equivalents as nmol MDA/g tissue [23]. GSH as an antioxidant molecule was determined by a spectrophotometric method which is a modification of Ellman procedure [24]. Briefly, after centrifugation at 2000*g* for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/L $Na_2HPO_4{\cdot}2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (1% sodium citrate, 0.4 mg/mL) was added and the absorbance at 412 nm was measured immediately after mixing. The results are expressed in μmol GSH/g tissue.

2.12. Determination of superoxide and nitric oxide release in brain tissues

Superoxide and NO release from fresh brain tissues were evaluated by the chemiluminescence (CL) method. CL is a non-invasive method that is based on light emission as a result of chemical reaction. Brain cortex specimens (approx. 25–30 mg) were slightly cut and put into vials containing PBS-HEPES buffer (0.5 M phosphate buffered saline containing 5 mM HEPES, pH 7.8). For superoxide radical determination, lucigenin (bis-*N*-methylacridiumnitrate) were added as an enhancer into the tubes for a final concentration of 0.2 mmol/L. NO release were measured by addition of luminol (sodium salt)-H_2O_2 system [25]. All measurements were made at room temperature (Mini Lumat Junior LB 9509 luminometer, EG&G Berthold, Germany). Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min and were corrected for wet tissue weight (rlu/mg tissue).

2.13. Gene expression analysis with real-time PCR method

2.13.1. Total RNA isolation

Total RNA isolation of samples was performed with GenUP™ Total RNA Kit (Biotechrabbit GmbH, Germany). Four hundred fifty μl of lysis buffer was added into the 20 mg of fresh samples and homogenized via rotor-stator homogenizer (T 25 digital ULTRA-TURRAX®, IKA, Germany). After homogenizing the samples, RNA isolation steps were done according to supplier's instructions. RNA amount was measured via Qubit® RNA HS Assay Kit (Life Technologies, USA) with Qubit® 2.0 Flourometer (Life Technologies, USA).

2.13.2. cDNA synthesis

Total RNA from each sample was used to generate cDNA using a iScriptTM Select cDNA Synthesis Kit (BioRad, USA) according to manufacturer's manual. Briefly, 100 pg of total RNA was mixed with 2 μl of oligo (dT), 4 μl of 5 × iScript select reaction mix, 1 μl of iScript reverse transcriptase. RNA, oligo (dT), and enzyme were mixed first, then heated at 42 °C for 90 min and reaction was inactivated at 85 °C for 5 min.

2.13.3. Oligonucleotide primers

The primers used for expression analysis of BDNF, IGF, NGF and APP695 genes were custom designs and were as follows:

BDNF; forward: 5′-TGGCTGACACTTTTGAGCAC-3′, reverse: 5′-TTCCTCCAGCAGAAAGAGCA-3′, NGF; forward: 5′-CCTGAAGCCCACTGGACTAA-3′, reverse: 5′-ATTGTACCATGGGCCTGGAA-3′, IGF-1; forward: 5′-TCTCCTAGTCCCTGCCTCTT-3′, reverse: 5′-TCTGTGAAGGAAGCGGCTTA-3′, APP 695; forward: 5′-GACCAGCAGAACACTCCCTC-3′, reverse: 5′- AAAACTCATACTTGAAATACGCTT-3′, TAU; forward: 5′-TCTCTGGGGAGACCACTAGC-3′, reverse: 5′-GCAGGTTGCTTGTCAGTTGG-3′. The housekeeping gene was β-actin and the sequence; forward: 5′-CTCTCAGCTGTGGTCGTGAA-3′, reverse: 5′-AGCCATGTACGTAGCCATCC-3′.

2.13.4. Real-Time PCR

Real-time quantitative PCR amplification reactions were carried out in a Line Gene 9640 Real Time Thermal Cycler system (Biotools, Spain) in a 20 μl end volume. The reaction mixture consisted of 1 × PCR buffer containing SYBR-Green and 100 nm of each primer (QPCR Green Master Mix, Biotechrabbit GmhH, Germany). 100 ng of cDNA template was added to each reaction. All reactions were carried out in duplicate. The PCR conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, and 50–60 °C for 25 s and 72 °C for 20 s. The fluorescent spectra were recorded during the elongation phase of each PCR cycle. The amplification plots and CT-values were exported from the exponential phase of PCR directly into a Microsoft Excel worksheet for further analysis.

2.14. Histological analysis

Coronal sections from the brains of animals from each group were cut and then immersion fixed in 10% buffered formalin.

Immunohistochemical staining was performed using the peroxidase conjugated polymer based system. Briefly, sections of 3 μm were cut from the paraffin embedded tissue and mounted on positive charged slides. The sections were dried overnight at 37 °C and deparaffinized in xylene and hydrated in graded ethanols. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide diluted in methanol, and followed by washing under running tap water. To unmasking the antigens, for 20 min the slides were microwave-treated in 10 mM citrate buffer (pH 6.0). The tissue sections were rinsed in phosphate-buffered saline (PBS, pH 7.4) and to minimize background staining the sections were incubated with protein blocking solution (Expose Rabbit spesific HRP/DAB detection IHC kit, Abcam, Cambridge, UK) for 10 min. Sections were incubated overnight at 4 °C with rabbit polyclonal antibodies for beta Aβ immunoreactivity determinations (ab14220, Abcam, Cambridge, UK) at dilution 1:200, for NGF (ab6199, Abcam, Cambridge, UK) at dilution 1:2000, for IGF1 (ab40657, Abcam, Cambridge, UK) at dilution 1:1000 and for BDNF (ab6201, Abcam, Cambridge, UK) at dilution 1:5000. After washing with PBS, the bound primary antibody were detected by incubation with goat anti-rabbit HRP conjugate according to the manufacturer's instructions (Expose Rabbit spesific HRP/DAB detection IHC kit, Abcam, Cambridge, UK). Staining was visualized with 3.3′-diaminobenzidine tetrahydrochloride (DAB). The slides were counterstained with Mayer hematoxylin, dehydrated in ethanol and mounted. Immunohistochemistry was evaluated semi-quantitatively (Olympus BX51 Tokyo, Japan), using an additive immunoreactive score reflecting signal intensity, that is 0: negative, 1: weak, 2: inter-mediate, and 3: strong, and the number of immunopositive cells, that is, 0: less than 5% positive cells, 1: 5–25% positive cells, 2: 26–50% positive cells, 3: greater than 50% positive cells. 2 scores were added [26,27].

2.15. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). Groups of data were compared with analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The diference between two groups were analysed by Student *t*-test. p value of less than 0.05 was considered to be significant. Statistical analysis was carried out using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. OVX + D-GAL application alter body weight

Our results indicated that the exercise protocols applied for 6 weeks caused muscle hypertrophy which was specific to the execise type (data not shown). Additionally, compared to the initial body weight, all of other groups did not have a noticeable weight change except, OVX + D-GAL sedentary group, which had an increase in final body weight ($p < 0.05$) (Table 1).

Table 1
Body weight measurements (g).

Groups	Initial body weight (g)	Final body weight (g)
Sham sedentary	266.9 ± 4.202	283.6 ± 4.531
Sham + aerobic exercise	279.9 ± 7.034	287.9 ± 6.818
Sham + resistance exercise	280.6 ± 8.369	286.0 ± 7.860
Sham + combined exercise	279.4 ± 6.225	283.3 ± 4.208
OVX + D-GAL sedentary	281.6 ± 8.287	314.8 ± 4.913*
OVX + D-GAL aerobic exercise	273.8 ± 3.063	301.9 ± 4.498
OVX + D-GAL resistance exercise	275.5 ± 6.100	306.8 ± 8.194
OVX + D-GAL combined exercise	273.9 ± 5.076	304.8 ± 7.622

*$p < 0.05$, compared to initial body weight. Each group consists of 8 animals.

3.2. Behavioural analysis

3.2.1. Anxiety-like behaviour

Compared to sham sedentary group a significant increase was observed in the freezing time of sedentary OVX + D-GAL group ($p < 0.01$), which indicates increased anxiety-like behaviour, while aerobic exercise decreased this increase ($p < 0.05$) (Fig. 2A). Basal freezing time levels were not different than each other among the groups (data not shown).

3.2.2. Cortisol levels

The cortisol levels, as an indicator of stress response, were increased in OVX + D-GAL sedentary and sham resistance exercise groups compared to sham sedentary group ($p < 0.05$–0.01) (B).

3.2.3. Recognition memory

The difference score that shows the time difference spent with the novel and sample objects, was decreased significantly in OVX + D-GAL sedentary rats compared to sham sedentary group ($p < 0.01$), showing defective cognitive abilities. The time spent with the novel object in OVX + D-GAL groups were prolonged by all exercise modalities in different levels when compared to their own sedentary group ($p < 0.05$–0.001) (Fig. 2C).

3.2.4. Cholesterol levels

There was a significant increase in cholesterol values of OVX + D-GAL sedentary group (164.5 ± 2.964 mg/dL) compared to sham sedentary group (99.80 ± 5.314 mg/dL) ($p < 0.001$), while this elevated cholesterol levels were reduced with aerobic exercise (130.8 ± 11.81 mg/dL) ($p < 0.01$).

3.2.5. Serum and brain IGF-I levels

All exercise procedures elevated serum IGF-I levels in both of the sham-operated and OVX + D-GAL groups when compared with their own sedentary groups ($p < 0.05$–0.001) (Fig. 3). Moreover, OVX + D-GAL resistance exercise further increased IGF-I levels in serum and aerobic exercise also improved IGF-I score in hippocampus compared to their own sedentary groups ($p < 0.05$) (data not shown).

3.2.6. Oxidative stress markers and antioxidant levels

The MDA levels of the brain tissue were risen in OVX + D-GAL sedentary rats ($p < 0.01$), while all exercise modalities significantly reduced this OVX + D-GAL induced elevation ($p < 0.05$) (Fig. 4A). Additionally, resistance and combined exercise applications decreased the MDA levels in also sham-operated groups ($p < 0.05$). Moreover, brain MPO activity in the OVX + D-GAL sedentary and resistance exercised groups were higher compared to sham sedentary group ($p < 0.05$), while MPO activity in aerobic and combined exercised groups were not significantly altered with OVX + D-GAL model (Fig. 4B). When compared with the sham sedentary group, there was an elevation in lucigenin levels of OVX + D-GAL sedentary and aerobic exercised groups ($p < 0.05$–0.01), and also aerobic and combined exercised sham-operated rats ($p < 0.05$), which indirectly shows elevated superoxide radical content (Fig. 4C). Not only the endogenous antioxidant GSH levels which was significantly depleted in the OVX + D-GAL sedentary group ($p < 0.01$) compared to sham sedentary group were improved in all exercise groups ($p < 0.05$), but also the GSH levels of sham operated groups were enhanced with all exercise models ($p < 0.05$) (Fig. 4D). NO release of brain tissue,

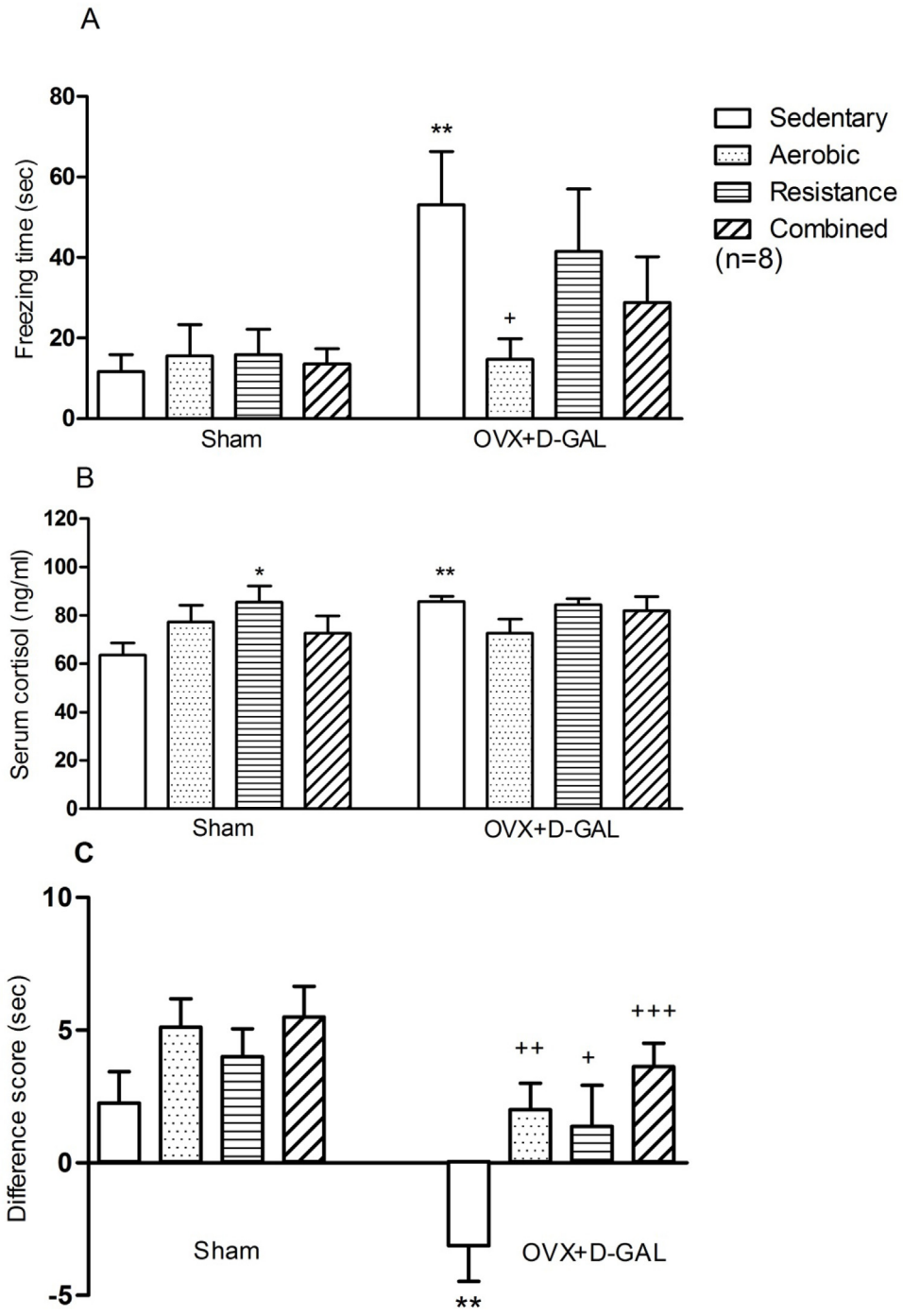

Fig. 2. Analysis of hole-board (A) serum cortisol levels (B) and object recognition tests (C) of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. Difference score (sec): Time spent with the novel object−time spent with the sample object. OVX: ovariectomized, D-GAL: D-galactose, sec: second. *p < 0.05, **p < 0.01, compared to sham sedentary group; +p < 0.05, ++p < 0.01, +++p < 0.001, compared to OVX + D-GAL sedentary group. Each group consists of 8 animals.

that shows the nitro-oxidative stress, was found to be significantly amplified in all OVX + D-GAL groups compared to sham sedentary group (p < 0.01–0.001) (Fig. 5).

3.2.7. Gene expression levels of APP695, NGF and BDNF

APP695 and NGF gene expression levels were higher in OVX + D-GAL sedentary and aerobic exercised groups (p < 0.05–0.01) compared to sham sedentary group, while these increaments were reduced by combined exercise (p < 0.05) (Fig. 6A-B). There was no significant difference in hippocampal BDNF gene expression levels among groups (C).

3.2.8. Immunoreactivity scores of $A\beta_{1-42}$, NGF and BDNF in cortex and hippocampus

All OVX + D-GAL groups have shown elevated Aβ immunoreactivity scores in hippocampus and cortex (p < 0.05–0.001) compared to sham sedentary group and these increased scores were declined via resistance and combined exercise applications in hippocampus (p < 0.01) (Figs. 7 .1A; 8), while all exercise types decreased

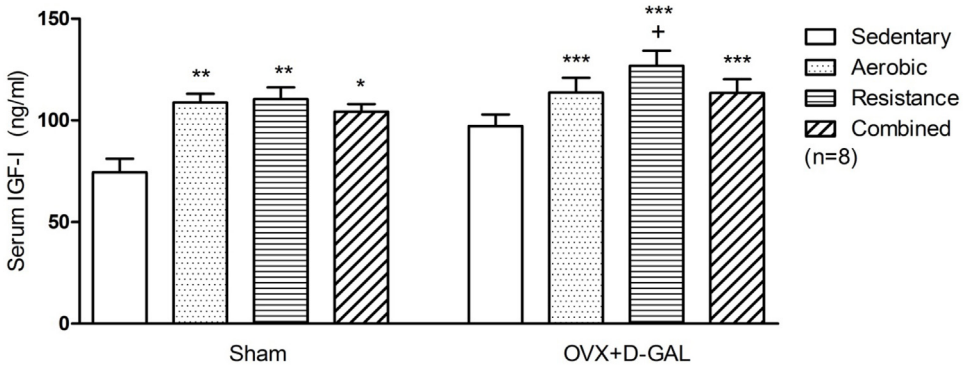

Fig. 3. Serum IGF-I levels of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose. *p < 0.05, **p < 0.01, ***p < 0.001; compared to sham sedentary group, +p < 0.05, compared to OVX + D-GAL sedentary group. Each group consists of 8 animals.

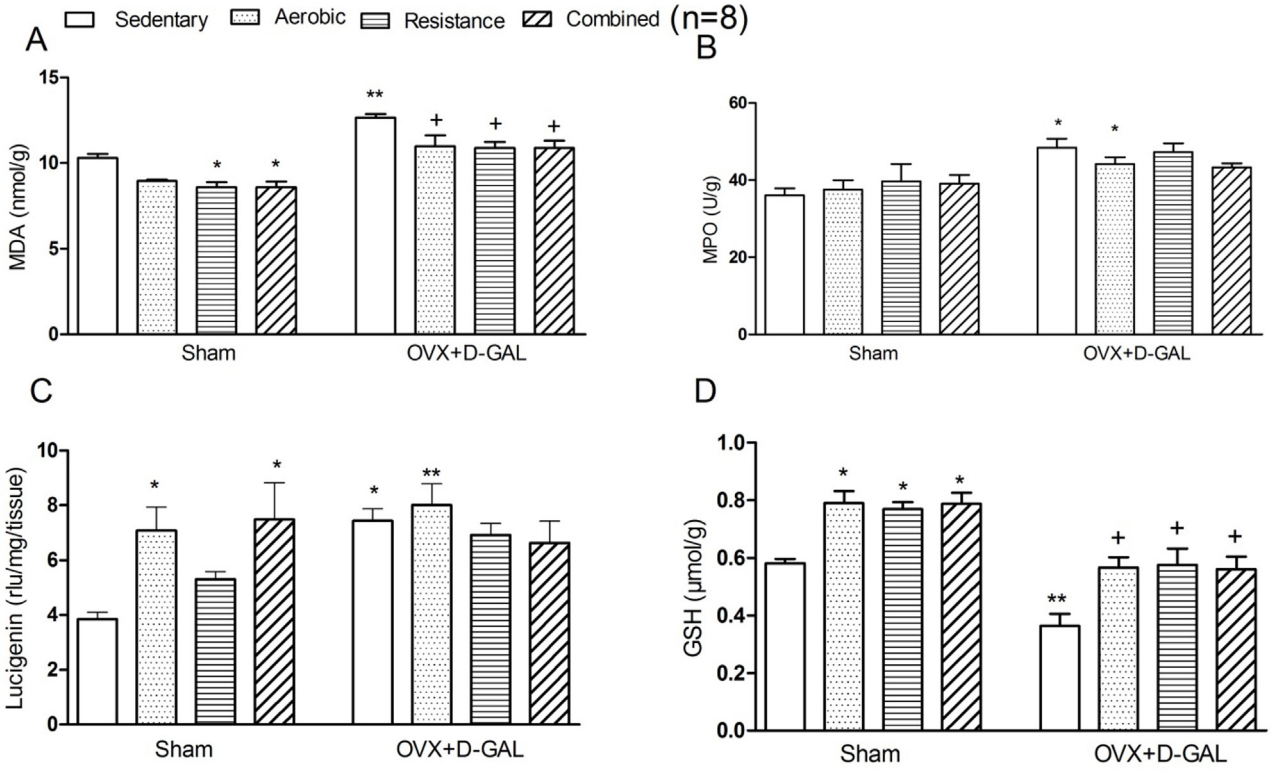

Fig. 4. Malondialdehyde (MDA) level (A), myeloperoxidase (MPO) activity (B), lucigenin-induced chemiluminescence levels (C), and glutathione (GSH) measurements (D) in the brain tissue of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose. *p < 0.05, **p < 0.01, compared to sham sedentary group, +p < 0.05, compared to OVX + D-GAL sedentary group. Each group consists of 8 animals.

this elevated Aβ scores in cortex (p < 0.05–0.01) (Figs. 7.1B; 9). While aerobic exercise group had improved hippocampal NGF and BDNF immunoreactivity scores (p < 0.05–0.01), resistance exercise had elevated NGF score (p < 0.05) and combined exercise group had increased hippocampal BDNF score (p < 0.05) in sham groups compared to sham sedentary group, there was no significant alterations in hippocampal and cortical BDNF scores of OVX + D-GAL groups (Figs. 7.2A, 3A-B; 10 , 12, 13). On the other hand, in cortical NGF immunoreactivity scores, OVX + D-GAL sedentary and aerobic exercise groups have shown significant decreases when compared with sham sedentary group (p < 0.01), while combined exercise group has shown increased scores compared to OVX + D-GAL sedentary group (Figs. Fig. 6.2B; 11) (p < 0.05).

4. Discussion

In the present study, we used the OVX + D-GAL induced experimental AD-like model to investigate the probable protective effect of chronic swimming, resistance and combined exercise methods on oxidative system, cognitive functions and neuroplasticity. We aimed to perform all exercise types on the devolepmental stage of AD. The results demonstrate that all exercise types improved short term working memory function in OVX + D-GAL model and aerobic

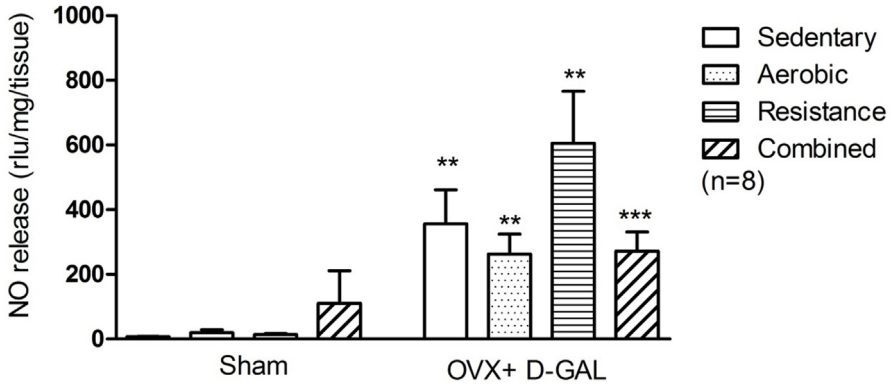

Fig. 5. NO release in the brain tissue of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to sham sedentary group. Each group consists of 8 animals.

exercise was also ameliorative on anxiety-like behaviour. All exercise type reduced oxidative stress via decreasing lipid peroxidation and increasing antioxidant content in brain tissue. Combined exercise had rising effect on NGF levels in cortical part of the brain tissue that shows the improvement of neuroplasticity. Besides, the histopathological changes observed with AD-like model were alleviated with all exercise models.

In the current study, while the resistance exercise groups exhibited FDB muscle fiber hypertrophy as expected; the aerobic exercise groups presented hypertrophy in soleus muscle fibers. These results confirmed the effectiveness of the exercise protocols and support previous studies [14].

Obesity and/or increase in adiposity are significant risk factors for AD [28]. This study is the first one indicating that body weights were increased by combined OVX with D-GAL model in sedentary rats. Taken together in our study exercise models were protective from weight gain as expected.

Most of the studies have reported that cholesterol has important role in production and metabolism of Aβ protein such as increasing cholesterol levels augments Aβ amount in animal experiments [29]. Moreover different exercise models have shown to affect the cholesterol levels such as swimming exercise is one of them [30]. As expected in the current study, blood cholesterol levels were increased with OVX + D-GAL model, and this increase was inhibited via aerobic exercise suggesting the protective effect of swimming exercise from hyperlipidemia.

Elevated cortisol level, which is a stress marker, in AD patients, may contribute to the development and/or maintenance of AD. Many AD patient hypersecrete glucocorticoids and this elevated level correlate with cognitive deterioration and neuronal atrophy [31]. Brureau et al. have suggested that amyloid toxicity affects on adaptive response of the HPA axis to stress in an acute model of AD and elevated glucocorticoids observed in AD may be the first consequence of amyloid toxicity [32]. In accordance with the previous results, in our study we have shown increased serum cortisol levels in OVX + D-GAL model.

Moreover, the increased freezing time that shows the elevated anxiety level in AD was ameliorated by aerobic exercise suggesting the protective effect of swimming from anxiogenic factors in AD. Previous studies indicate that aerobic exercise training has anxiolytic effects and protects the animals from stress [33] and reduces anxiety-related behaviour [34]. In accordance with our results, by our group swimming exercise previously has been reported to have the anxiolytic effect in a cat odor stres model in rats [20]. The novel object recognition task is a non-spatial memory test which shows working memory [19]. Although there is no data about the effects of OVX + D-GAL model on object recognition test or working memory, in another Alzheimer model, impairment in working memory was shown [35]. In the present study, declined working memory in AD-like model was improved with all exercise modalities, thus suggested the possible protective effect of all exercise types. In accordance with our results, treadmill and swimming exercises were shown to improve short-term memory in rats with Aβ-induced AD [36,13], however, in literature, the effect of resistance and combined exercise models on cognitive functions of AD is not known. In recent studies, resistance exercise was reported to prevent age-related cognitive decline of working memory among elderly people [37] and combined exercise was reported to have beneficial effect on cognition in patients with dementia [38]. These studies were focused on the changes that occur in cognitive functions with ageing or dementia but for the first time we reported the beneficial effects of these exercise types in AD-like conditions.

IGF-I as a neurotrophic and neuroprotective hormone, decreases in serum during ageing and neurodegenerative diseases [39,40]. IGF-I crosses the blood brain barrier and reflects the brain tissue levels [40]. By ageing, Aβ burden increases with decreasing serum IGF-I levels. This relationship reflects the ability of IGF-I to induce clearance of brain Aβ load [40]. Although increased $A\beta_{42}$ and lowered serum IGF-I was shown to be associated with cognitive decline [41], in contrast, it has been shown that serum IGF-I level was risen in AD patients [42]. In our study, we observed increased Aβ immunoreactivity, however no significant difference in serum IGF-I levels in AD-like model. In a human study, Duron et al. [43] have reported a significant association between low IGF-I serum levels with AD occurrence in men, however no significant difference in women, thus our result correlates with this outcome. Previous studies have shown increased IGF-I levels in hippocampus and in serum samples via treadmill exercise and a positive correlation between the cognitive functions and IGF-I levels of adolescent rats [44], and also increased serum IGF-I levels in a moderate swimming exercise model [45]. In accordance with previous data, in our study, increased serum IGF-I level was observed with all exercise types. Moreover, resistance exercise was beneficial by increasing serum IGF-I levels in case of AD-like conditions.

There are controversial data about the effects of exercise on the expression levels of brain IGF-I in literature. While it has been revealed that IGF-I mRNA levels of hippocampus were not changed by treadmill exercise which was applied 3 times per week [46], elevated IGF-I levels in hippocampus of aerobic and resistance exercised rats [14] and in hippocampus and blood samples of treadmill exercised rats were reported [44] when the exercise frequency is increased to 5–6 days per week. Our findings suggest that aerobic

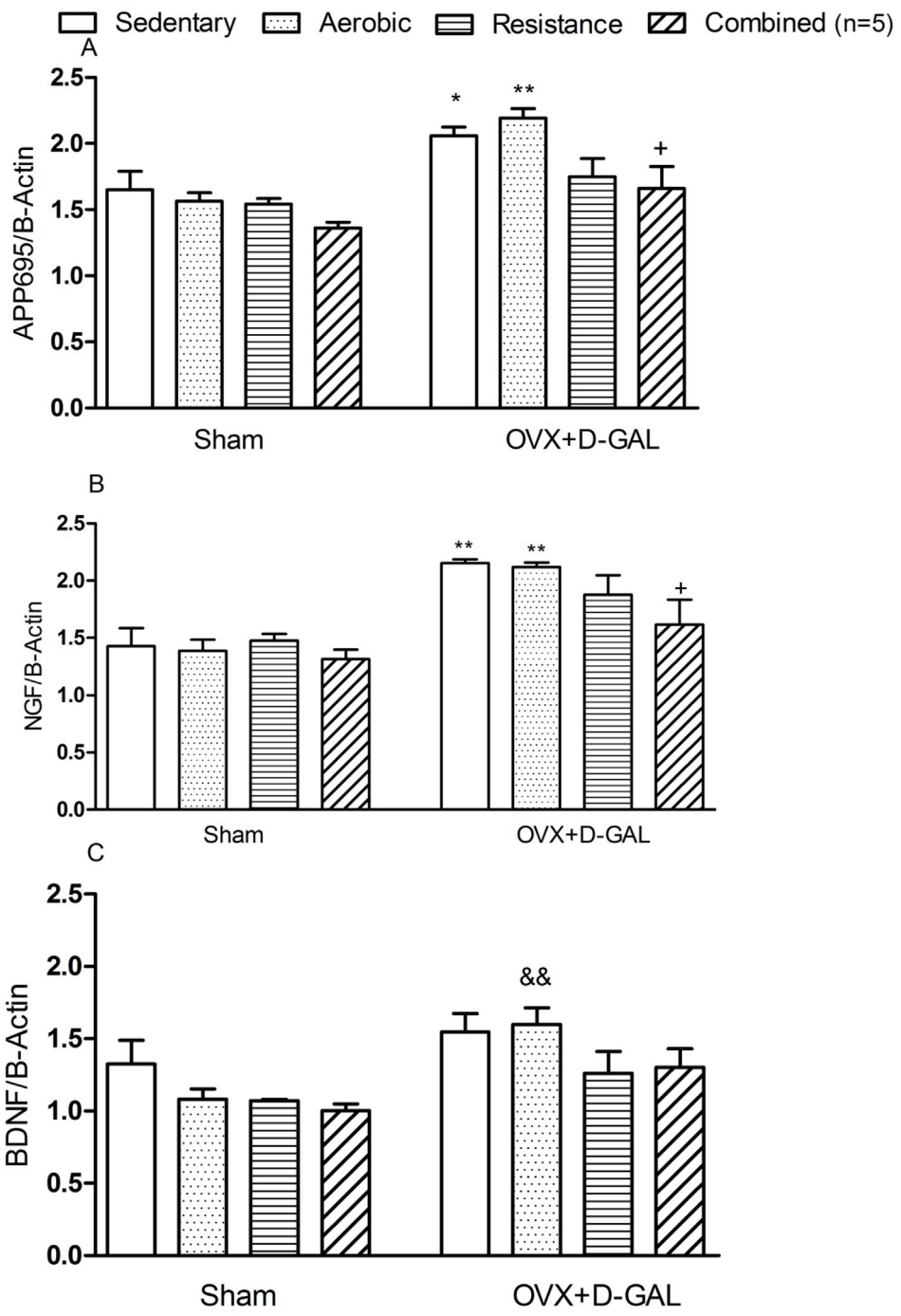

Fig. 6. Gene expression levels of hippocampal amyloid precursor protein (APP) 695 (A), neuronal growth factor (NGF) (B) and brain derived neurotrophic factor (BDNF) (C), of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose. *$p < 0.05$, **$p < 0.01$, compared to sham sedentary group, +$p < 0.05$, compared to OVX + D-GAL sedentary group. Each group consists of 5 animals.

exercise which was performed 3 times per week for 6 weeks even increased hippocampal IGF-I protein immunoreactivity that may be a sign of increased neurogenesis in sham aerobic exercise group.

Lipid peroxidation directly damages neuronal membranes and have neurotoxic activity thus decreasing the brain lipid peroxidation level is a potential therapeutic target in AD [47]. Furthermore, increased brain oxidative damage was reported to contribute in AD pathogenesis before Aβ accumulation [48]. Numerous studies have demonstrated increased lipid peroxidation in brain tissue of patients with AD [3], and a previous study has shown elevated MDA levels in our AD model [49]. There is no data about the effect of exercise on lipid peroxidation levels observed in AD, besides there are conflicting effects of exercise in rat brain such as increased MDA and decreased antioxidants levels and unchanged TBARS levels were observed with chronic swimming exercise [50]; [51], and increased MDA levels and MPO activity in brain of the stressed rats with a concomitant reduction in the GSH levels were turned back via swimming exercise [52]. In the current study, MDA load of brain tissue has been shown to decrease via all exercise models indicat-

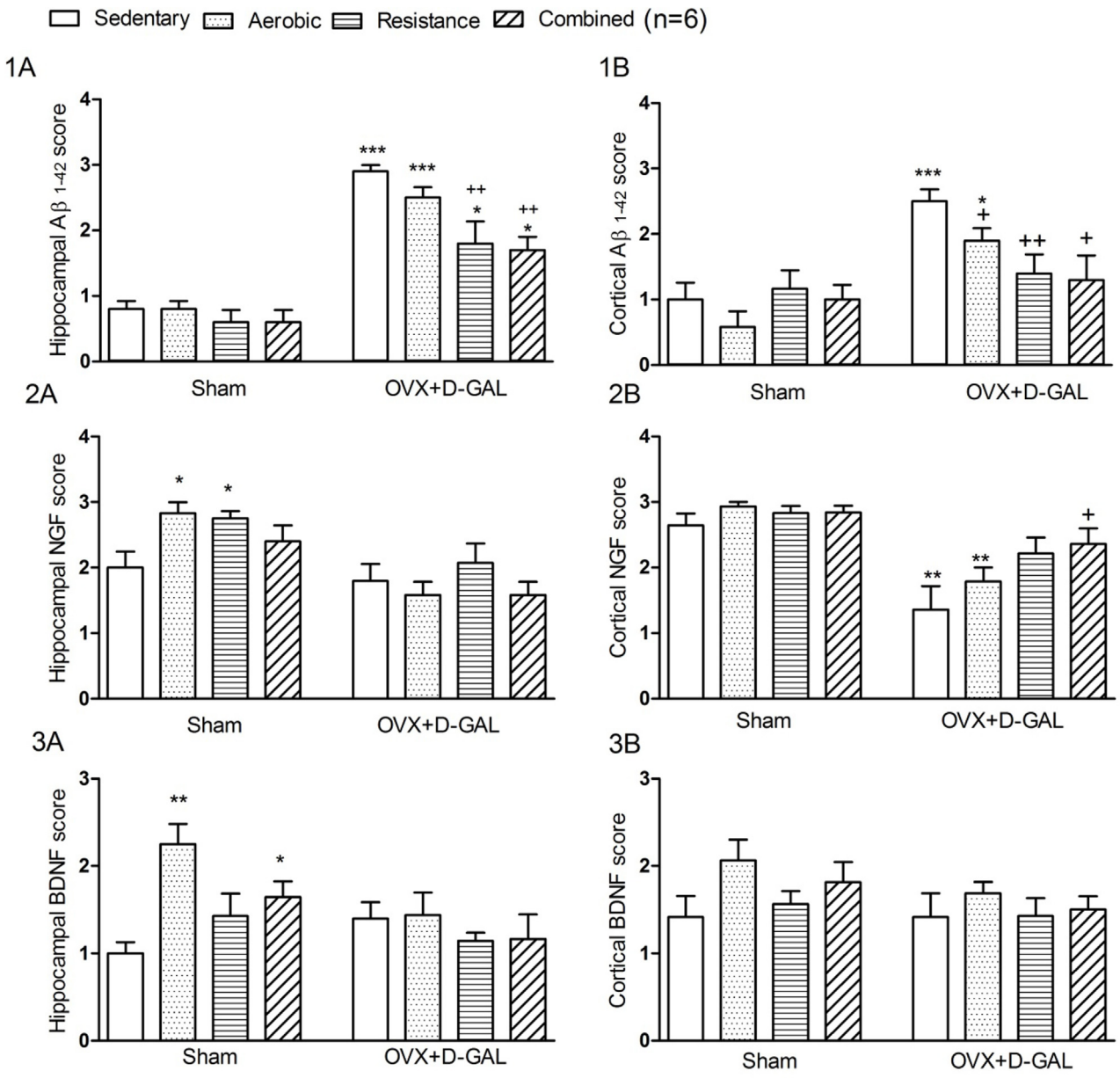

Fig. 7. Immunohistochemistry evaluation of hippocampal (1) and cortical (2) scores of amyloid beta (Aβ) (A), nerve growth factor (NGF) (B), brain derived neurotrophic factor (BDNF) (C), of rats, which were either sham-operated + saline-treated or ovariectomized + D-GAL-treated, and which were kept sedentary or performed aerobic, resistance or combined exercise procedures for 6 weeks. OVX: ovariectomized, D-GAL: D-galactose. *p < 0.05, **p < 0.01, ***p < 0.001, compared to sham sedentary group, +p < 0.05, ++p < 0.01, compared to OVX + D-GAL sedentary group. Each group consists of 6 animals.

ing a possible beneficial effect of chronic variant exercise types in development of AD.

MPO, a heme protein expressed by microglia in brain, generates oxidants which are asserted to subscribe to tissue destruction during inflammation [21]. There is a limited data about MPO activity in AD, such as increase in neuronal MPO activity levels were observed in a human and also in an experimental AD model [21,53]. Our results have shown increased MPO activity in this AD-like model for the first time and support indirectly increased microglial activation occurred in AD [54]. In AD, it is not known whether the exercise protects the brain tissue from microglial activation or not. According to our results, although none of the exercise types ameliorated elevated MPO levels significantly aerobic and combined exercises seem to protect the rats from increasing MPO activity. Previously we have shown unchanged MPO levels in the brain tissue of chronic swimming execised rats [52], in accordance with this outcome, in present study the MPO levels were not altered with aerobic exercise. On the other hand, reduced MPO levels in the brain tissue of chronic swimming exercised rats under acute stressed conditions were detected [20]. These dissimilar results may be related to oxidative injury model or perfomed exercise regimen.

Brain is in particular sensitive to oxidative stress by ROS production [55] since the excess of ROS can damage cellular components such as lipids, proteins, or DNA [56]. Superoxide anion (O_2^-) is the first free radical of ROS and it initiate the oxidative detriment in AD brain [57]. In the present study, we have shown elevated lucigenin-induced chemiluminesance levels in AD-like model and in sham operated aerobic and combined exercised ones. A recent study supports our results and shows increased hippocampal production of reactive oxygene/nitrogen species after swimming exercise in rats [58]. As the brain blood flow and oxygen consumption were shown to be increased during exercise and both of these changes may be

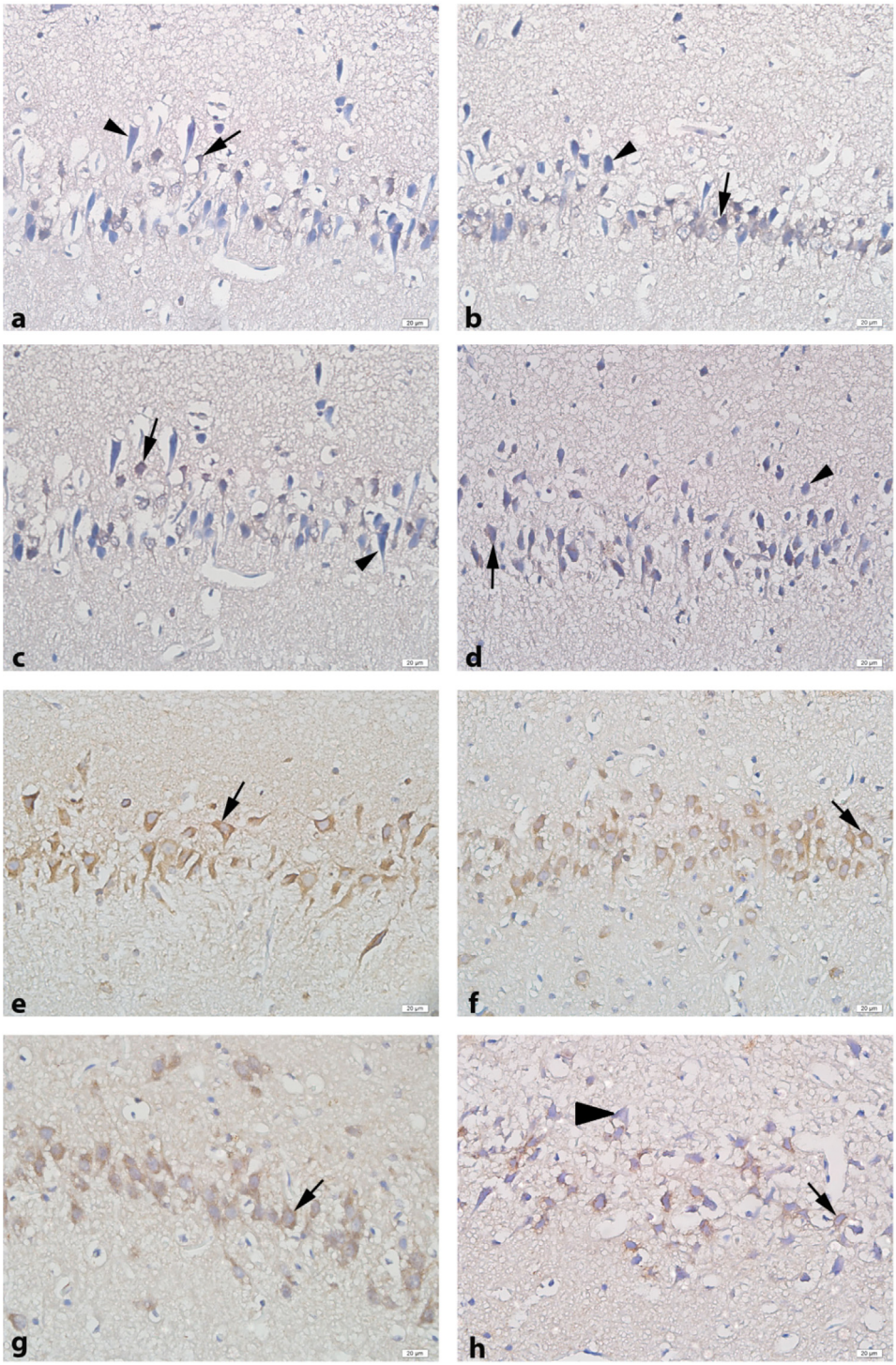

Fig. 8. Representative images of immunohistochemical staining of Aβ1-42 protein in the rat hippocampus 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. Strong expression of Aβ immunoreactive neurons was located at the CA region of the hippocampus in the OVX + D-GAL groups. Slightly Aβ expression was detected in sham experimental groups. Arrow: Aβ1-42 immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: x40.

result of an increase in brain metabolism and may increase ROS production [59].

GSH is the most important endogenous antioxidant defence molecule, and the detoxification of ROS is crucial in the brain and astroglial glutathione is essential for this process [60]. In the current study, decreased GSH levels in AD-like model demonstrate decline in protective antioxidant status. Lower antioxidant content in AD is suggested to indicate increased oxidative stress and to be an important factor in the deterioration of cognitive function [61]. A previous study has shown astrocyctic degeneration and decrease in GSH level via OVX + D-GAL model [62]. On the other hand, exercise has been relevant to antioxidant adaptations [63]. In previous studies, the levels of hippocampal GSH were increased after chronic exercises, this increament was associated with improvement in hippocampal antioxidant status probably for dealing with the increased levels of ROS [58,63]. In the present study, we sug-

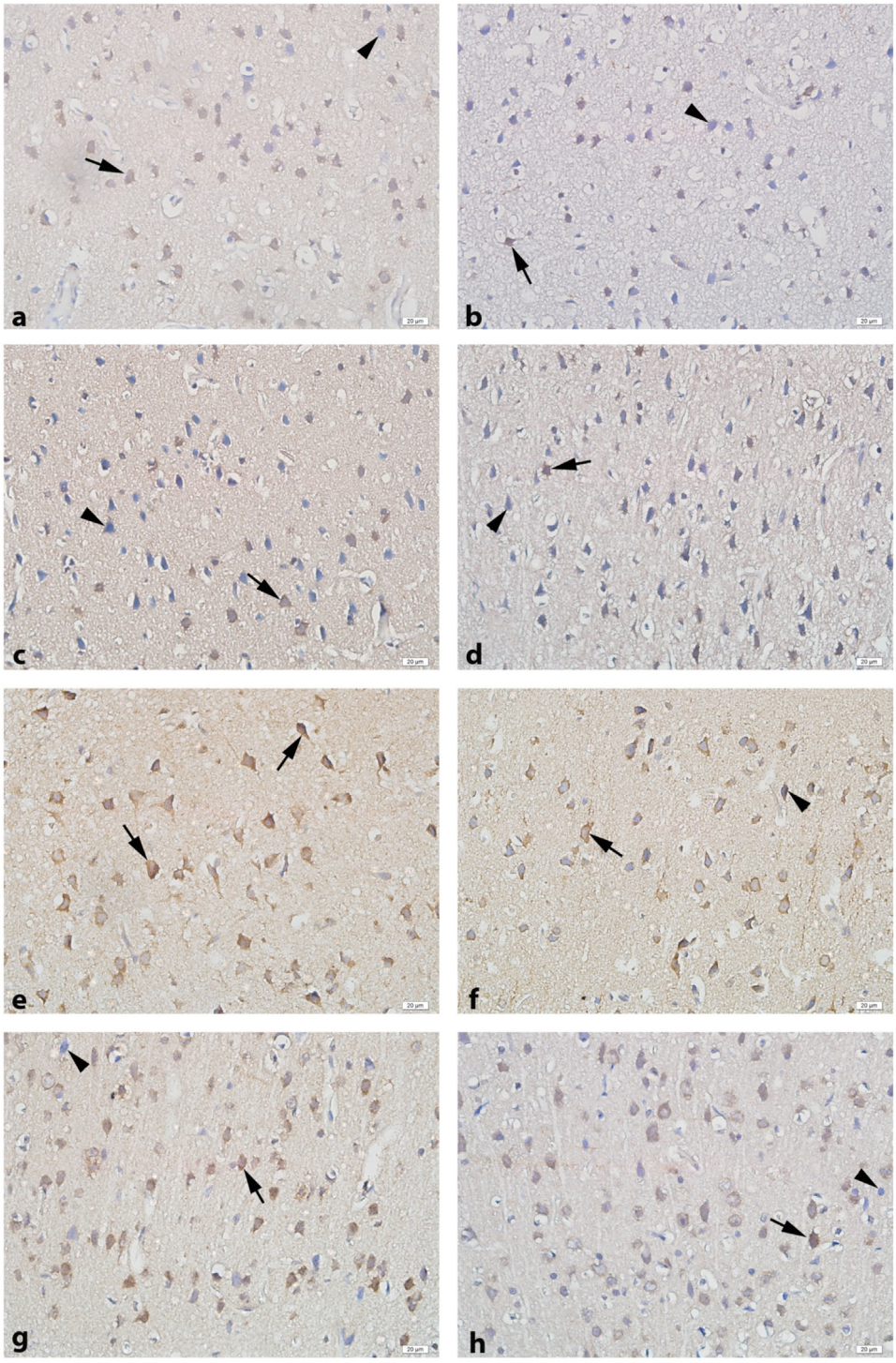

Fig. 9. Representative images of immunohistochemical staining of Aβ1-42 protein in the rat cortex 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. Strong expression of Aβ immunoreactive neurons was located at cortex in the OVX + D-GAL groups. Slightly Aβ expression was detected in sham experimental groups. Arrow: Aβ1-42 immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: ×40.

gest that moderate regular exercise attenuates oxidative stress by improving antioxidant status via up-regulating antioxidant GSH levels, together with reports of other investigators [58,63].

NO plays an essential messenger role both in physiological and pathological processes such as enormous NO has neurotoxic effect and can be implicated in the pathogenesis of AD [2]. The reaction of NO with superoxide produces the peroxynitrite which is a powerful oxidant [64]. In the present study, we found elevated NO and superoxide levels in this AD-like model for the first time that suggests increased nitro-oxidative stress. In addition, none of the exercise methods have effected on the elevated NO levels in our study. Exercise may lead to an increase in NO production and this effect may be linked to the nature of exercise itself such as the increased production of NO via the endothelium surges the blood

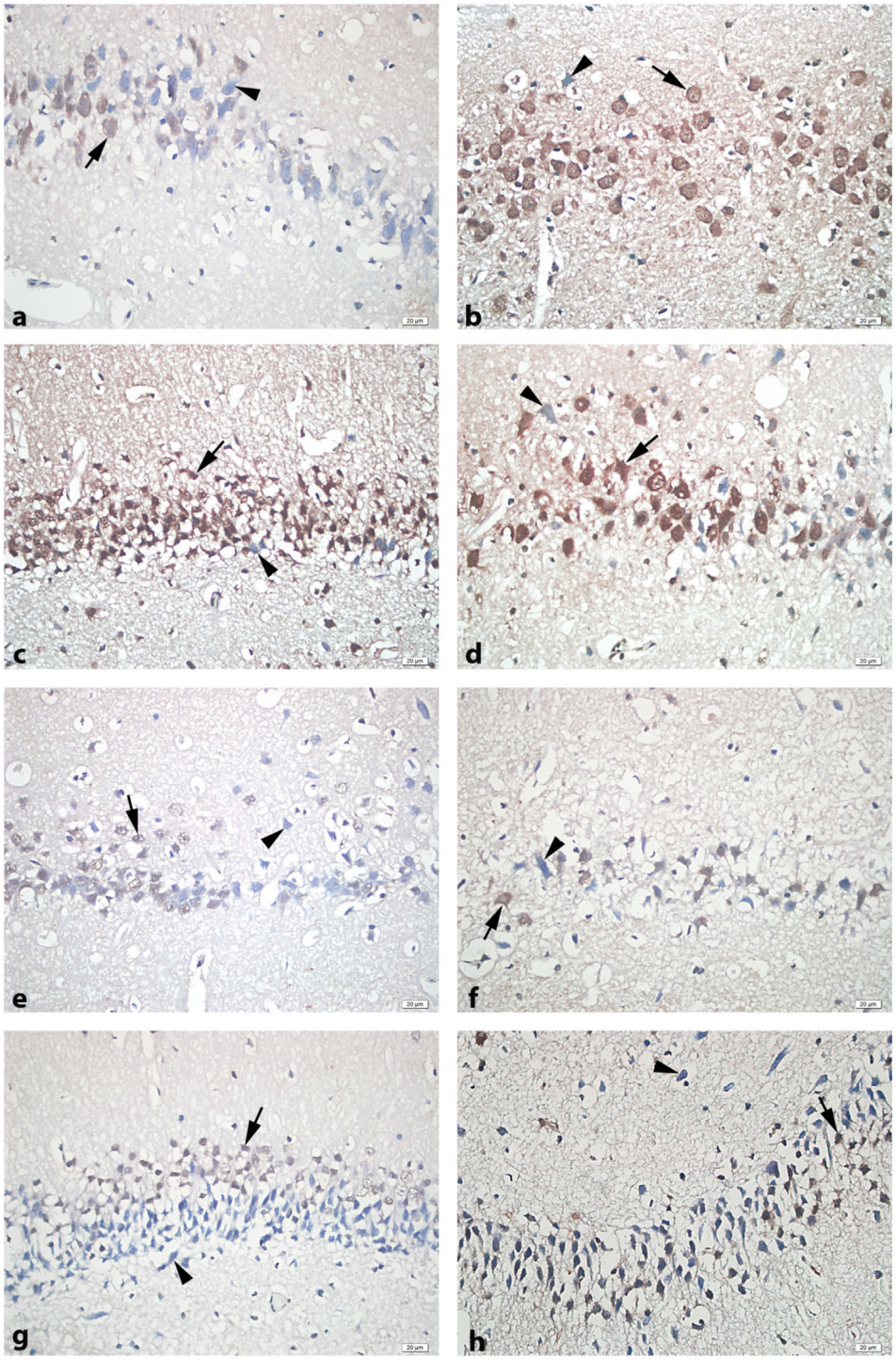

Fig. 10. Representative images of Immunohistochemical staining of NGF protein in the rat hippocampus 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. Strong expression of NGF immunoreactive neurons was located at hippocampal CA region in the sham aerobic and resistance groups compared to sham sedentary. There was no significant difference among OVX + D-GAL groups. Arrow: NGF immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: ×40.

flow, and results with a hyperemic response which is well known during exercise [65]. The uneffectiveness of exercise to decrease the increased NO release in disease conditions may be related to the nature of exericise.

In accordance with previous studies, in the present study, we observed strongly Aβ immunoreactive neurons in the hippocampus and cortex of AD [7,8], furthermore, gene expression of APP695 supported these histopathological changes in hippocampus. A previous study suggests that increased accumulation of APP may cause caspase-3-mediated cell death and microglial activation [66]. Moreover, overexpressing a mutated human APP is an AD model [67]. In the present study, increased APP695 gene expression and increased Aβ load in AD-like model were reduced by combined exercise. Additionally, swimming and resistance exercises were

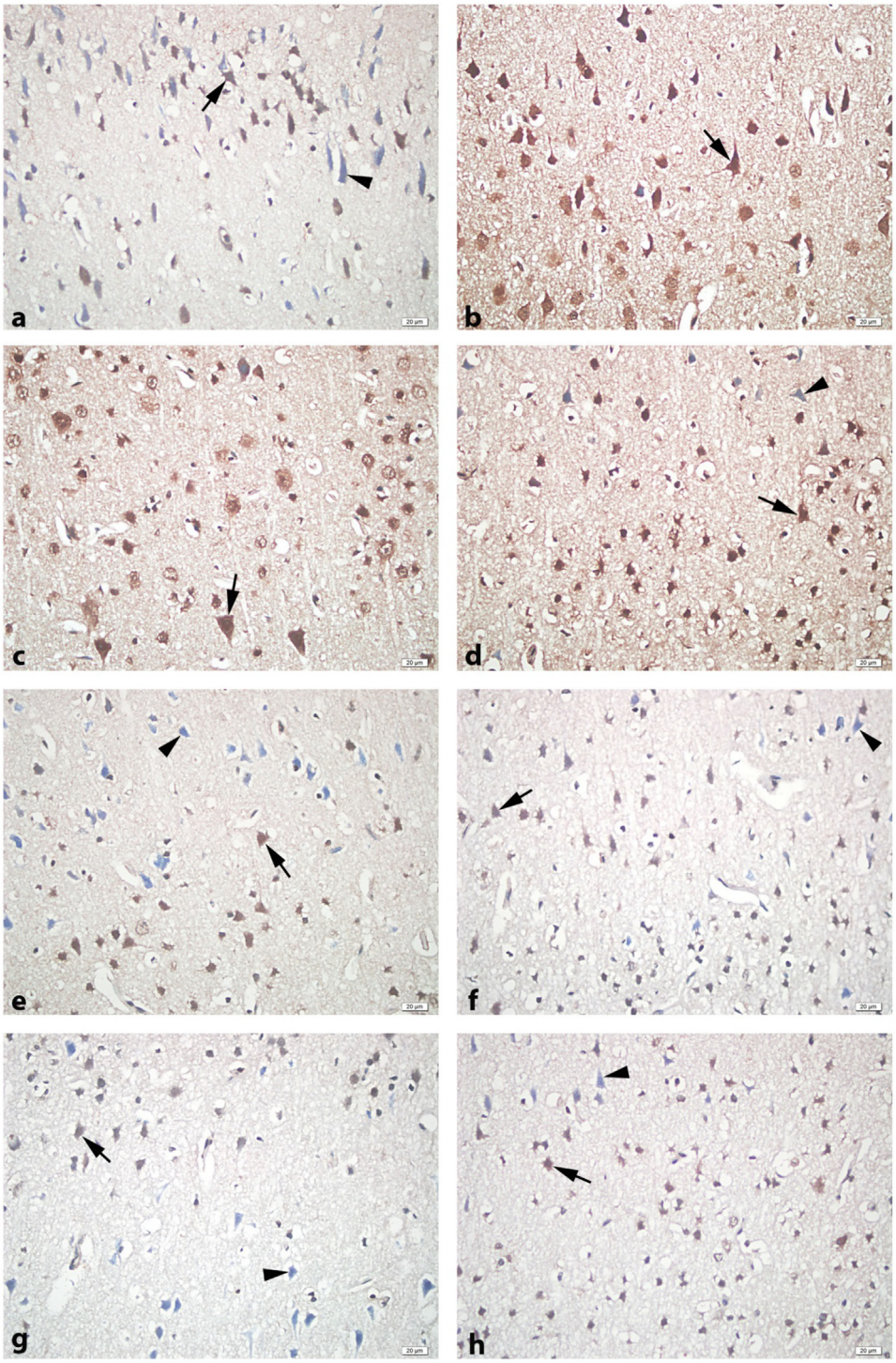

Fig. 11. Representative images of immunohistochemical staining of NGF in the rat cortex 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. OVX + D-GAL sedentary and aerobic exercise groups have shown decreased NGF immunoreactivity compared sham sedentary group. OVX + D-GAL combined group have incrased immunoreactivity their own sedentary group. Arrow: NGF immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: ×40.

also protective from increasing Aβ load in AD-like model. Although the effect of resistance and combined exercise types on Aβ levels in AD is not known, treadmill exercise was reported to reduce hippocampal $A\beta_{42}$ protein levels by modulating secretase enzymes in ageing rats [68], and was shown to elevate levels of Aβ clearance proteins in AD [69]. As the above studies show the effect of treadmill exercise, in our study we have reported the protective role of not only swimming exercise, but also resistance and combined exercise models for the first time in literature.

NGF has stimulatory effect on differentiation, maintenance, and growth of neurons in peripheral and central nervous system [70]. In the present study, increased gene expression of NGF but no significant difference of NGF immunoreactive neurons in the hippocampus of rats with AD-like model was detected. Intrahip-

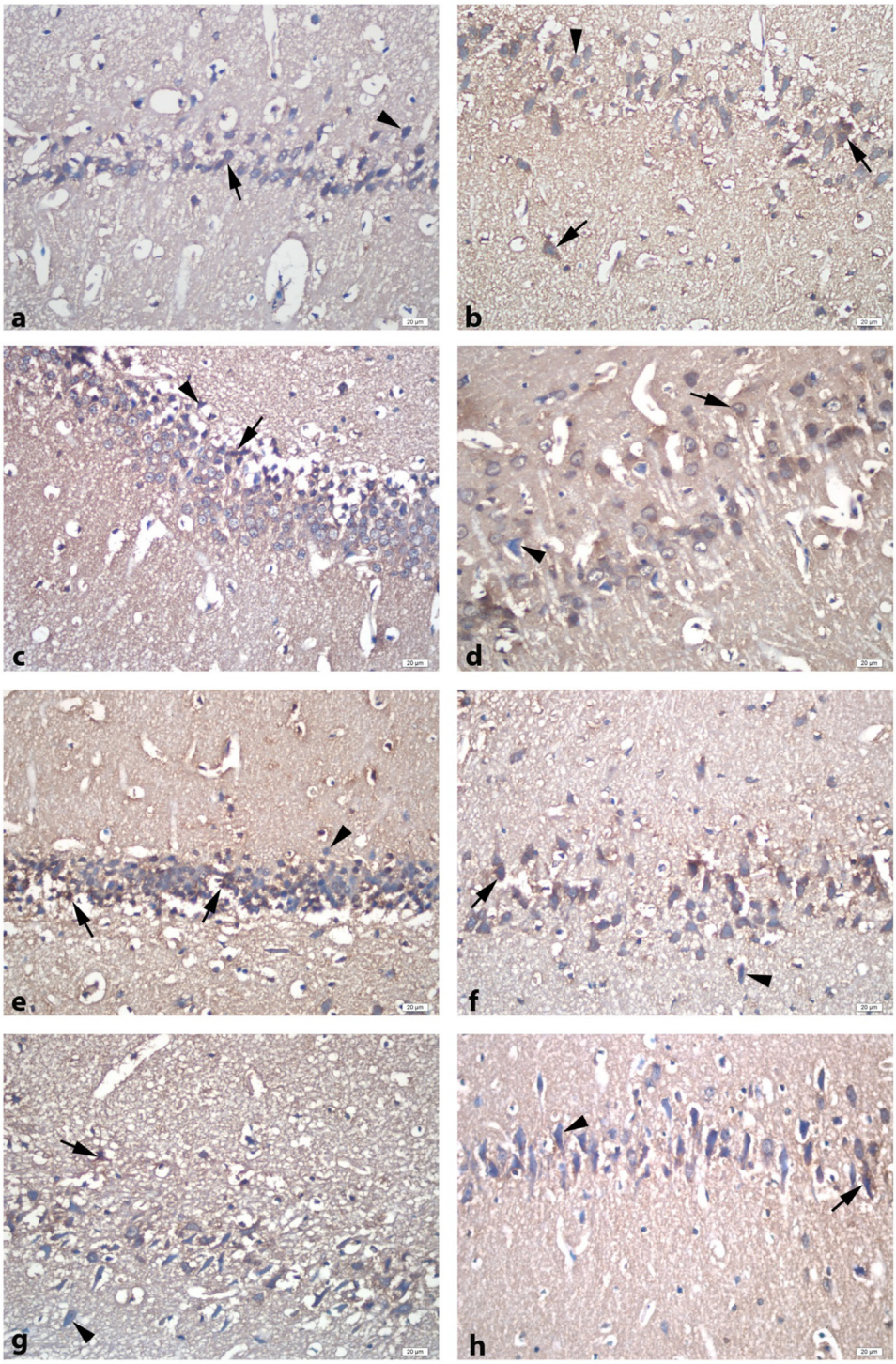

Fig. 12. Representative images of immunohistochemical staining of BDNF in the rat hippocampus 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. Strong expression of BDNF immunoreactive neurons was located at hippocampal CA region in the sham aerobic and combined group compared to sham sedentary. There was no significant difference among OVX + D-GAL groups. Arrow: BDNF immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: x40.

pocampal injection of Aβ oligomers provoked upregulation of pro-NGF in rats, moreover decreased conversion of pro-NGF to its mature form, and augmented degradation of mature NGF [71,72]. On the other hand, the level of mature NGF in hippocampus was not altered in case of AD [73]. The reason of unreflectiveness of increased gene expression of hippocampal NGF levels to NGF immunoreactivity may be related to suppression in conversion of pro-NGF to mature NGF, and this process is known to increase the severity of AD [74]. On the other hand, animal studies reported reduced expression levels of cortical NGF in AD [75]. In the current study, although we found unchanged NGF immunoreactive neurons in hippocampus, the cortical neurons were declined via AD-like model suggesting that the cortical neurons may be more sensitive to AD than the other parts of the brain.

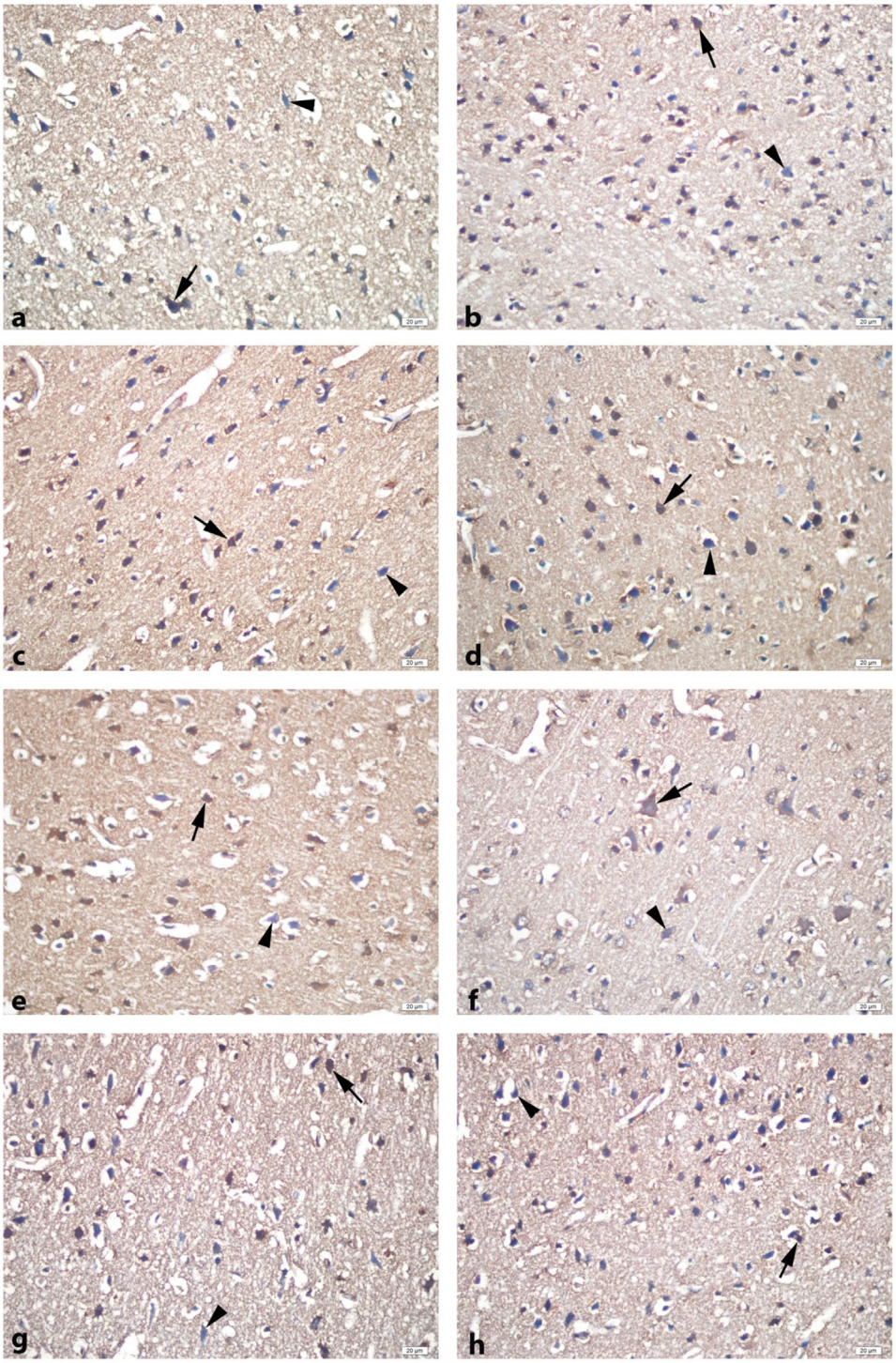

Fig. 13. Representative images of immunohistochemical staining of BDNF in the rat cortex 6 weeks after D-GAL injection. Sham-operated; sedentary (a), aerobic (b), resistance (c), combined (d) exercised groups and OVX + D-GAL; sedentary (e), aerobic (f), resistance (g), combined (h) exercised groups. There was no significant difference among groups. Arrow: BDNF immunoreactive cells, arrowhead: non-immunoreactive cells. Magnification: ×40.

In present study, hippocampal NGF immunoreactive neurons were increased by aerobic and resistance exercise under normal conditions and cortical NGF immunoreactivity was improved by combined exercise under disease conditions. Although the swimming exercise which was performed 6 days per week for 4 weeks had no effect on NGF gene expression [76], previous studies has found increases in NGF levels in several areas of the brain in response to aerobic exercise [77]. Our data is the first to indicate the beneficial effects of all exercise types that appear to be mediated by increasing hippocampal or cortical NGF immunoreactive neurons and this increase may lead to stimulate neuroplasticity.

BDNF contributes to the proliferation and survival of central nervous system neurons, and its expression is elevated significantly with exercise in general [78,79]. Although reduced expression levels of brain-derived neurotrophic factor were reported in AD [35], there was no data about our model. We found unaltered gene

expression levels of BDNF in hippocampus and accordingly there was no change in BDNF immunoreactive neurons with AD-like model. A previous study has reported unchanged serum BDNF levels in humans with AD [80]. In our study, the reason of unchanged BDNF immunoreactivity may be due to the AD model. The effect of exercise on brain BDNF gene expression and protein levels may change due to the sampling time following exercise. Rasmussen et al., [78] have reported significant increases in hippocampal and cortical BDNF mRNA levels, with levels peaking at 2 h post- treadmill exercise period and returning to baseline levels at 24 h after exercise, besides Oliff et al., [81] has reported increased BDNF gene expression and very quickly decreases after voluntary whell running exercise. In the present study, although hippocampal BDNF immunoreactivity was found to rise specifically in aerobic and combined exercise groups, mRNA expression was not significantly different than control. The reason may be the time past following exercise, such as we removed brain tissue samples 1 day after exercise sessions. Briefly, our results suggest that swimming and combined exercise may contribute to enhance neuroplasticity via increasing BDNF in hippocampus.

In conclusion, the decreased cognitive functions in AD-like model were improved via all exercise type, additionally the increased anxiety-like behaviour in AD-like conditions was inhibited with aerobic exercise. Our results display increased oxidative damage via elevated microglial activation, worsened oxidative and nitro-oxidative stress parameters and risen neuronal Aβ load in the brain tissue with AD-like model and show reduction in increased oxidative damage parameters and improvement in antioxidant defence system and alleviation of Aβ content of the brain tissue with all types of exercise. Moreover, well known neurotrophic factors that have role in brain neurogenesis and plasticity were improved with different exercise types in AD-like conditions. Eventually, the present study is the first one that indicates the protective role of different exercise models in AD-like conditions and suggests resistance and combined exercise models nearby aerobic exercise to be included in therepautic approach of AD.

Competing interests

None declared.

Author contributions

The experiments were performed at the Department of Physiology and Experimental Animals Research and Implementation Centre at the University of Marmara. D.Ö. and Ö.K.Ç. are responsible for the design of the work and interpretation of the data. G.S. analysed the gene expression data, N.Ö. analysed immonucytochemistry evaluations, B.K. analysed and interpreted the data related to gene expression, M.Y. performed the chemiluminesence measurements, Ö.T.Ç.K interpreted immonucytochemistry data. All authors were involved in drafting the work and critically revising the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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