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The beneficial effects of nettle supplementation and exercise on brain lesion and memory in rat

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Abstract

Regular swimming and phytotherapeutic supplementation are assumed to alleviate the severity of neurodegeneration leading to dementia. The effect of swimming training and that of enriched lab chow containing 1% (w/w) dried nettle (*Urtica dioica*) leaf on the prevention of severity of brain injury caused by *N*-methyl-D-aspartate (NMDA) lesion in Wistar rats were investigated. Nettle supplementation and regular swimming exercise seem to improve the adverse effect of brain injury caused by NMDA lesion assessed by passive avoidance test and open-field test. Nettle supplementation decreases the level of reactive oxygen species, measured by electron paramagnetic resonance, and the DNA-binding activity of NF-κB. The data reveal that nettle supplementation has an effective antioxidant role, down-regulates the inflammatory transcription factors and could also promote learning performance in the brain. Regular swimming increases the concentration of reactive species in the cerebellum and alters the activity of transcription factors toward inflammation. The additive effect of the two treatments was more profound in the down-regulation of inflammatory transcription processes in NMDA lesion.

Keywords: Neurodegeneration; NMDA lesion; Brain; Stinging nettle; Swimming exercise; Oxidative stress; Neurotrophins; Transcription factors

1. Introduction

It has been shown that environmental enrichment with voluntary exercise has a significant potential role in attenuating the age-associated decline in cognitive function in experimental animals [1–5]. It has been reported that voluntary running promotes the number of new hippocampal cells, long-term potentiation [6] and brain plasticity [7]. Exercise can stimulate neurogenesis [2,5] and improve learning and mental performance [8]. In addition, exercise has been shown to ameliorate the extent of oxidative stress and related consequences after artificial brain lesion, ischemia/reperfusion or stroke [9–11].

The mechanism behind these effects of exercise can include increased expression of vascular endothelial growth factor, angiogenesis [12], glucose uptake [13], increased generation of neurotrophins [2,5], increased activity of neprilysin, a β-amyloid degrading enzyme [14] and proteosome [15], as well as influence the signaling pathways in the brain.

In addition, exercise appeared to alter the antioxidant and redox state of the brain [16]. It is well known that increased level of reactive oxygen species (ROS) is involved in the aging process and the pathogenesis of a number of neurodegenerative diseases [17].

N-Methyl-D-aspartate (NMDA) injection-induced lesion has been used to imitate some of the characteristics of neurodegenerative diseases, especially age-associated deterioration, and, indeed, NMDA lesion has been shown to result in impaired brain function [18,19]. Besides, the destructive effects of NMDA lesion on brain function, it

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has also been found that lesion induces inflammation, generation of ROS and closely mimics dementia [20]. It has been shown that neurodegenerative diseases are associated with increased formation of ROS, oxidative protein damage, decreased level of degradation of damaged protein and increased inflammation mediated by nuclear factor kappa B (NF-κB) [9,17].

As part of a healthy way of living, diet can also have a significant role in brain function [3]. Stinging nettle (*Urtica dioica* L.) leaf has a long history as an herbal remedy and nutritious addition to the diet [21]. Nettle is rich in minerals and vitamins, such as pro-vitamin A and vitamin C, which could have an anti- or pro-oxidant role like iron, which is found in large concentrations in nettle leaf [22].

Epidemiological and laboratory studies indicated that carotenoids may have anti-carcinogenic [23], anti-ulcer [24] or anti-aging properties [25]. Nettle leaves are a good source of essential amino acids [26], ascorbic acid [27], available and unavailable carbohydrates, and several mineral elements [28]. It is also known that nettle has an antioxidant, anti-inflammatory, immune-suppressive and antirheumatoid role [29–31], but the possible effects of nettle supplementation in the brain remain to be tested. In central European countries, nettle leafs are traditionally used for tea with the aim to reduce the consequences of rheumatic arthritis and other inflammatory diseases. We were interested in testing whether the traditional belief can be supported by the effects of nettle on artificially induced inflammation, which mimics a neurodegenerative disease. Therefore, in addition to the antioxidant role of nettle, the main reason for our selection was its possible anti-inflammatory role, which could mean that it can be used more effectively than other antioxidants.

Regular exercise depending on certain conditions such as age, tissue and timing could increase or decrease the activity of NF-κB, which is one of the main transcriptional regulators of inflammation [29]. In our earlier study, we tested the effects of exercise and nettle supplementation on rats without NMDA-induced lesion [32]. We have setup our experimental design to be able to see the effect of exercise with and without nettle supplementation on NMDA excitoxic lesion-caused neurodegenerative processes like oxidative status, inflammatory mechanisms and the behavioural and learning performance of the brain. Accordingly in our hypothesis, the design of the study would allow to test whether lifestyle-related changes (nettle consumption and/or exercise training) would be beneficial to reduce NMDA lesion-mimicked neurodegenerative disorders.

2. Methods

2.1. Animals, diet and exercise

In the present study, 68 four-month-old male Wistar rats were divided into eight experimental groups: sham control (SH), NMDA lesioned (NM), swimmer and sham-operated (SWSH), and swimmer and NMDA lesioned (SWNM) groups fed with standard or with nettle-enriched lab chow. In the exercise protocol, rats were swimming for 1.5 h/day, five times a week, for a total of 7–9 weeks. Dried stinging nettle leaf was purchased from Herbaria (Budapest, Hungary) and its dose in the chow was set at 1% w/w to reach a daily dose of 30 mg/kg. Dried chopped nettle was mixed into the lab chow by the [redacted] company that supplied the standard food (Bioplan, Budapest, Hungary). Rats had free access to normal or nettle-enriched (1% w/w in standard rat chow for 8 weeks) diet. The protocol of the study was evaluated and approved by the local ethics committee of the university.

One day after ending the behavioral tests, the rats were sacrificed and their brains were removed and immediately frozen in liquid nitrogen and stored at −70°C until analyses.

2.2. Surgery and NMDA lesion

Half of the rats from each experimental group were subjected to excitotoxic brain lesion of NMDA. The other half was sham operated and served as controls. The region of cholinergic neurons in the nucleus basalis magnocellularis (NBM) was injected with the NMDA solution unilaterally in the right hemisphere, at the intermediate level of the nucleus projecting to the ipsilateral neocortex using an injection procedure described earlier [18,19]. Surgery was performed under pentobarbital (60 mg/kg) anesthesia. The rats were positioned in a stereotaxic frame, and 0.5 μl of phosphate-buffered saline (pH 7.4) containing 30 nmol of a racemic mixture of ~~*N*-methyl-D,L-aspartate~~ (NMDA, Sigma, St. Louis) was slowly injected in steps of 0.1 μl into two dorso-ventral positions within the NBM (0.6 mm apart). Thus, a total amount of 60 nmol was injected into the NBM region in a total volume of 1.0 μl during a 20-min infusion period. After each injection, the needle was left *in situ* for 5 min to allow proper drug diffusion and to avoid the spread of the toxin solution during withdrawal of the needle. For sham surgery, the needle was placed at the appropriate site, but no infusion was made. Food and water were available *ad libitum* for 6 days following surgery and then the rats were returned to the normal daily schedule.

2.3. Behavioral tests

2.3.1. Orientation response to novelty

The open-field test is widely used to study the reaction to novelty and it also provides some insight into the state of anxiety in rodents. The test was performed on the fifth postoperative day. Rats were positioned into the center of an open-field box consisting of a cylindrical arena of 80 cm in diameter, divided into 20 sectors by concentric and radial lines, and surrounded by a 35-cm-high wall [33]. During a 3-1min recording period, the number of lines crossed between sectors and the number and duration of rearings were scored. Normal exploratory behaviour in this test is in favour of the outer zone (thigmotaxis or wall hugging) and thus greater exploration in the central zones is indicative of less anxiety. The intensity of rearing activity was expressed

by a combined score, which summed the number and duration of rearings, representing increased aspects of motor and exploratory activity.

2.3.2. Retention of passive avoidance learning

The retention of passive avoidance learning behavior and memory retrieval was investigated in a one-trial step-through paradigm [34] from the sixth to the eighth postoperative day. The apparatus consisted of two equally sized compartments, a dark one and a well-lit white compartment (20×25×25 cm each), separated by a small sliding door. On Day 1 of training, a 3-min adaptation was allowed in the dark compartment, which was followed by a single trial by placing the rat into the illuminated white compartment and allowing it to enter the dark chamber. On Day 2 after the third entrance, a mild electric foot shock (0.8 mA, 3 s) was delivered in the dark box through the stainless steel bars on the floor. On Day 3, the latency of the entrance into the dark compartment was recorded, whose measure was used to differentiate the individuals for statistical analysis (ANOVA) and which served to express the retention of the learned avoidance response and memory retrieval.

2.4. Biochemical assays

DNA-binding activities of NF-κB and activated protein-1 (AP-1) were measured by electrophoretic mobility shift assay (EMSA) as described by Kim et al. [35] from pooled brain (cerebellum) samples. The preparation of nuclear extracts was based on a method described previously [36]. The oligonucleotides with the sequence of 5′-GAGAGG-CAAGGGATTCCCTTAGTTAGGA-3′ for NF-κB, and 5′-GAG GTG AGG GCC TTC CCT TAG-3′ and 3′-AC TCC CGG AAG GGA ATC AATC-5′ for AP-1 were terminally labeled with ^{32}P using [γ^{32}P]-ATP and T4 polynucleotide kinase. For binding assay, 10 μg of nuclear proteins was mixed with the labeled probe in a buffer containing 1.0% Nonidet P40. The mixtures were incubated at room temperature for 20 min, and the [^{32}P]-labeled oligonucleotide–protein complex was separated from the free oligonucleotide by electrophoresis through a 5% native gel in a running buffer containing 50 mM Tris-HCl (pH 8.0), 45 mM sodium borate and 0.5 mM EDTA. After separation, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film for 1 day at −80°C. To determine the specificity of the nuclear protein binding, competition with the corresponding unlabeled oligonucleotide was carried out under the same conditions.

Electron paramagnetic resonance (EPR) measurements were carried out as described by Stadler et al. [37] previously. In brief, measurements with an X-Band computer-controlled EPR spectrometer constructed by Magnettech (Berlin, Germany) were carried out. Approximately 100 mg of tissue samples from the forebrain and the cerebellum was frozen into a rod-shaped form, and spectra of the samples were recorded at 77 K using a quartz finger Dewar filled with liquid nitrogen. Instrument settings were 100 kHz modulation frequency, 0.7050 mT modulation amplitude, 18 mW microwave power, 1 min scan time and 20.63 mT field sweep. For evaluation, a method of double integration of the EPR signals with Mn/MnO as an internal standard was used.

The carbonyl measurements were done according to the description of Radak et al. [38]. In brief, each sample was incubated for 1 h in 500 μl of 10 mM dinitrophenylhydrazine or 2N HCl as a blank. Later, 500 μl 20 w/w% trichloroacetic acid was added to the samples. After centrifuging for 10 min at 20,000×*g*, the supernatants were discarded. Samples were washed in ethanol two times and once in acetone. The remaining pellets were dissolved in 8N urea. The pellet-urea solution was incubated for half an hour at 37°C. The absorbance of the samples was detected by spectrophotometry at 360 nm.

2.5. Statistical analysis

Statistical significance was assessed using parametric ANOVA, followed by Duncan's post hoc tests. Fisher's and Student's *t*-test were performed for analysis of data variance and normal distribution statistics; one-way ANOVA test was used for the behavioral data. Pearson's correlation of the variables was also calculated. The significance level was set at $P<.05$ and $P<.01$.

3. Results

3.1. Behavioral findings

The activity and exploration rate of the rats were assessed by open-field activity test. The most profound changes were detected in total rearing scores, which strongly correlated with the animals NMDA lesion-caused anxiety and behavioral disturbances. NMDA lesion-suffered rats showed

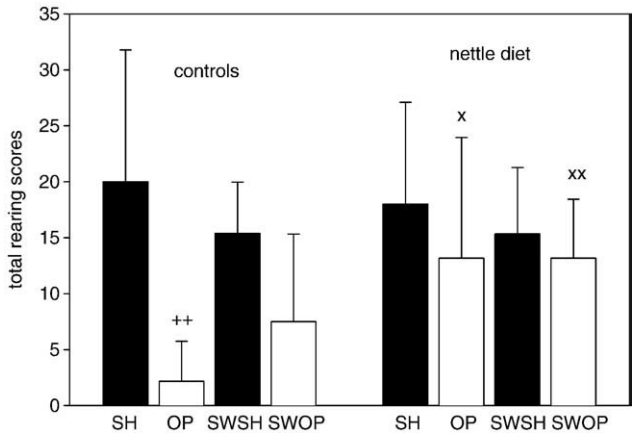

Fig. 1. Bars show the open-field behavior results in total rearing scores. Results are means±S.D. for five to seven animals per groups. ($^{++}P<.01$ vs. control SH; $^{xx}P<.01$ vs. control OP; $^{x}P<.05$ vs. control OP.) Abbreviations: SH — sham control, OP — NMDA lesioned, SWSH — swimmer and sham lesioned, SWOP — swimmer and NMDA lesioned animals. The black columns represent sham operation and the white columns NMDA lesion.

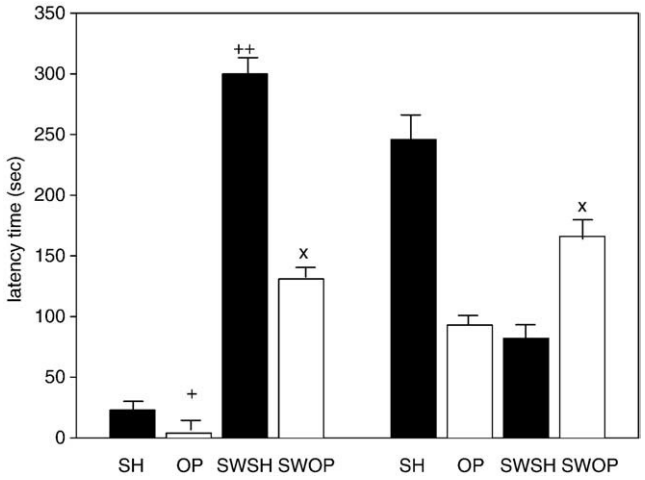

Fig. 2. Bars show the passive-avoidance learning test latency times results in medians. ($^{++}P<.01$, $^{+}P<.05$ vs. control SH; $^{\times}P<.05$ vs. control OP.) Results are medians for five to seven animals. (See Fig. 1 for the abbreviations used here.)

significant brain deterioration compared to their sham controls (Fig. 1). NM animals, kept on control diet and subjected to NMDA brain injection, showed a much lower level of rearing activity than their controls (vs. SH, $P<.005$). The difference was nearly 10-fold, suggesting that NMDA lesion massively reduced the exploration activity of the animals and increased anxiety. Exercise training and nettle supplementation, on the other hand, resulted in the attenuation of the lesion-associated impairment, since the rearing activities of these groups (SWNM kept on control diet, the NM and SWNM groups kept on nettle diet) did not differ from that of SH controls kept on control diet. In addition, the NM and SWNM groups kept on nettle diet showed a significantly higher rearing activity as compared to the NM group kept on control diet ($P<.05$ and $P<.005$, respectively). Consequently, only the lesioned rats' behavior

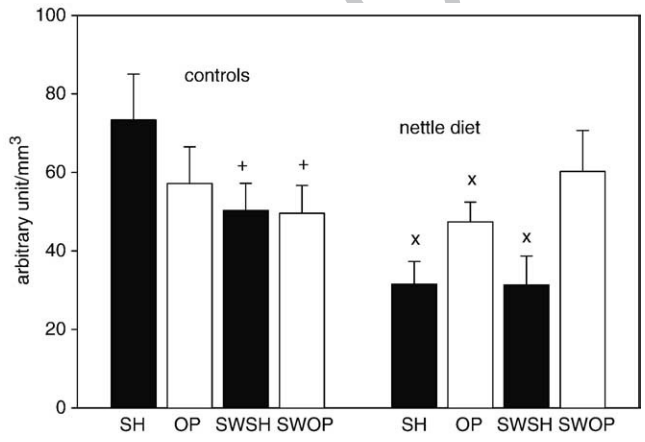

Fig. 3. The free electron accumulation in the frontal lobe is shown as obtained by EPR measurements. Results are means±S.D. for five to seven animals. ($^{\times}P<.05$ vs. control SH; $^{+}P<.05$ vs. control SH). (See Fig. 1 for the abbreviations used here.)

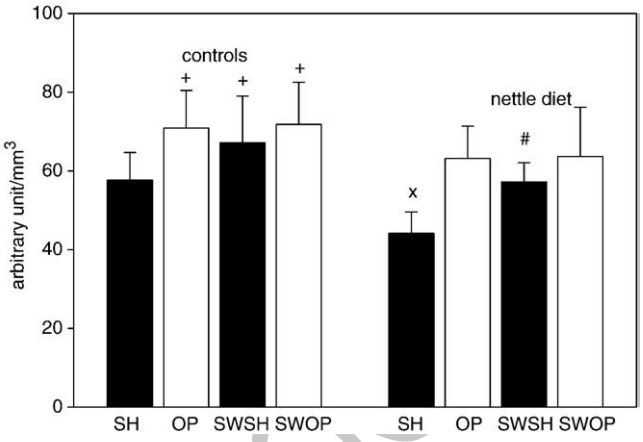

Fig. 4. Free electron accumulation in the cerebellum is represented in bars. Results are means±S.D. for five to seven animals. ($^{+}P<.05$ vs. control SH; $^{\times}P<.05$ vs. control SH; $^{\#}P<.05$ vs. control SWSH.) (See Fig. 1 for the abbreviations used here.)

was influenced by the interventions, i.e., regular swimming and nettle supplementation and by both in a positive way.

The learning performance and memory retrieval of SH rats, assessed by passive avoidance test, were significantly impaired by NMDA lesion (Fig. 2, $P<.05$). On the other hand, regular swimming attenuated the lesion-induced decline in memory retrieval (NM vs. SWNM: $P<.05$) and increased the performance in sham-operated rats (SWSH vs. SH: $P<.05$). Nettle supplementation only in combination with regular exercise could exceed significance in both sham and NM groups compared to control SWNM group ($P<.05$, respectively).

3.2. Neurochemical findings

With the help of EPR, we could detect the level of free radicals, which play a role not just in oxidative stress but also in the activation of redox-sensitive transcription factors like

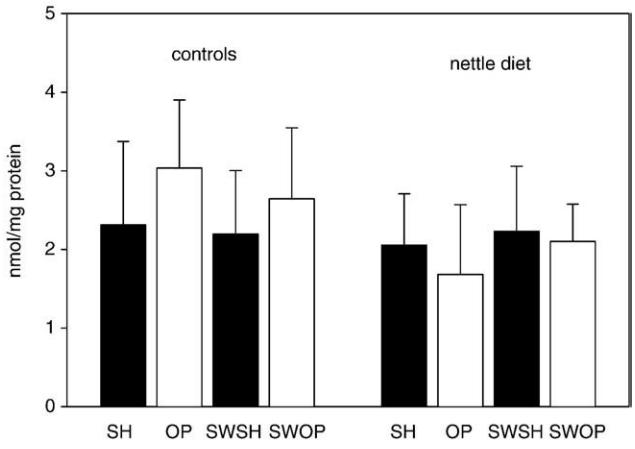

Fig. 5. The bars show the quantitative measurement of reactive carbonyl derivative content in the brain. No significant differences were found. Results are means±S.D. for five to seven animals. (See Fig. 1 for the abbreviations used here.)

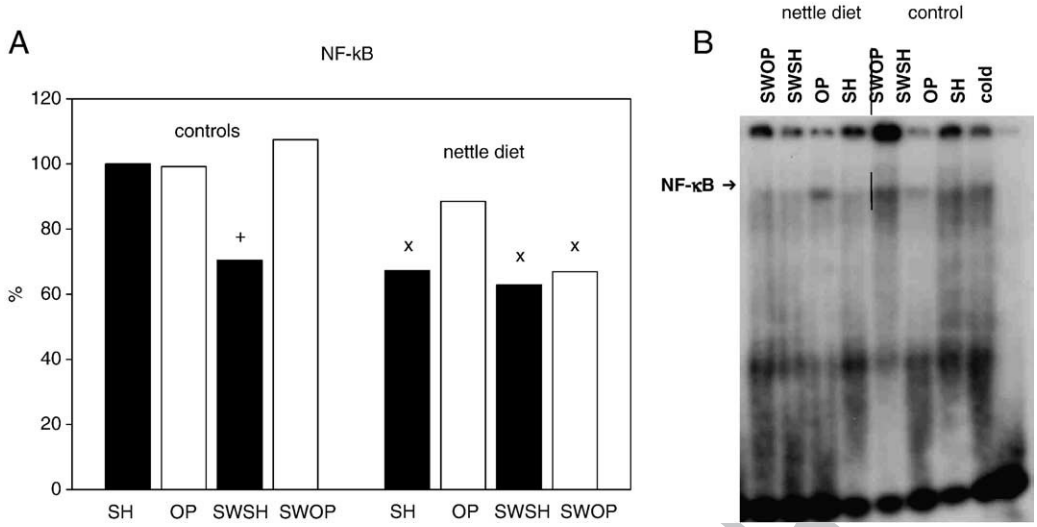

Fig. 6. The NF-kB binding activity to DNA was measured by EMSA from pooled brain (cerebellum) samples (Panel A). Each band demonstrates the pooled sample for six animals for the cerebellum. Panel B shows the densitometric results of EMSA assay. The difference exceeded $^{+,\times}\leq$20%. (See Fig. 1 for the abbreviations used here.)

NF-κB and AP-1. Data obtained by EPR measurements revealed that free radical accumulation in the cerebellum was significantly reduced by nettle diet (Fig. 3, $P<.05$), especially in sham-operated animals. Exercise training increased the accumulation of free radicals in the cerebellum ($P<.05$), but nettle was able to reduce the swimming-caused elevation ($P<05$). NMDA lesion itself showed significant increase in oxidative stress, but this increase was reduced in nettle and combined NM groups.

The oxidative damage of whole brain samples was evaluated by the content of reactive carbonyl derivatives, but no significant change was found among the groups (Fig. 4). The marker of oxidative protein damage, accumulation of carbonyl groups, was not significantly altered by the experimental protocols used, indicating that the oxidative stress was not massive (Fig. 5).

From the pooled cerebellum samples, it can be concluded that the NMDA lesion did not change the DNA-binding activity of NF-κB in control animals (Fig. 6). However, both regular exercise and nettle supplementation on their own and in combination significantly reduced the NF-κB activation compared to sham-operated control animals. The combined effect of these treatments was additive in decreasing the NF-κB binding activity to DNA both in sham and, more importantly, in NM animals, suggesting a strong anti-inflammatory effect.

The AP-1 DNA-binding activity was quite different from that of NF-κB, since nettle administration did not change the

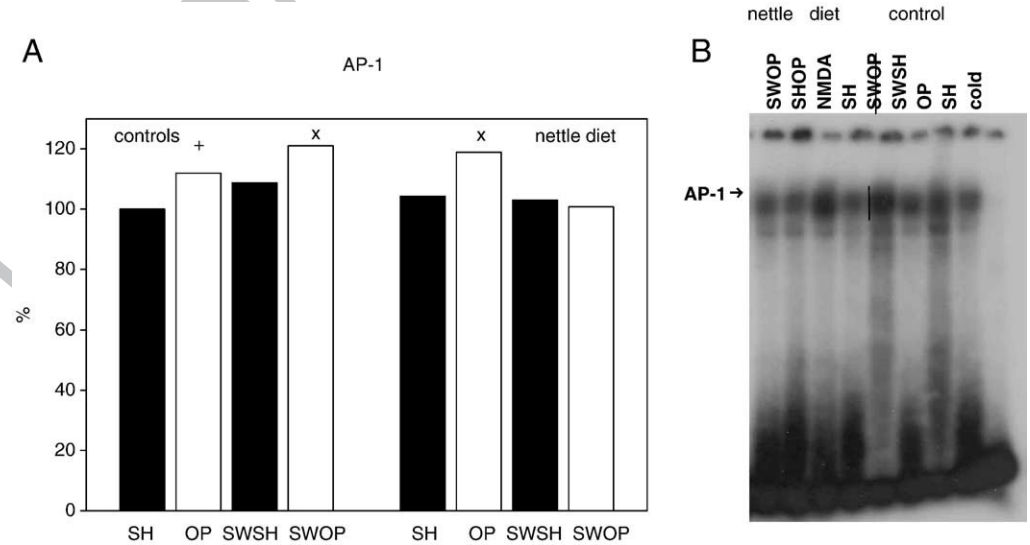

Fig. 7. Panel A shows the AP-1 DNA-binding activity. Each band demonstrates the pooled sample of six animals for the cerebellum. Panel B shows the densitometric result of EMSA assay. The difference exceeded $^{+}\leq$10% and $^{\times}\leq$20%. (See Fig. 1 for the abbreviations used here.)

association of AP-1 to DNA (Fig. 7). The AP-1 activity in the NMDA-lesioned brain, on the other hand, was increased except in the combined NM group, which did not change compared to the sham-operated one. Swimming alone also elevated the level of AP-1 activity but to a lesser extent than NMDA lesion.

Statistical calculation of the obtained data revealed a positive correlation between the cerebellum EPR signals and open-field data of all animals (R=−0.318, P=.027), indicating that higher level of free radicals can be associated with or may be a causative factor of poor behavioral performance. Moreover, the level of oxidative protein modification, the reactive carbonyl derivatives, also positively correlated with the activity of redox-sensitive transcription factors NF-κB and AP-1 (R=0.717, P=.045; R=0.68, P=.06, respectively).

4. Discussion

In this study, the effect of regular exercise and nettle supplementation was investigated in rats with excitotoxic NMDA-induced brain lesion, which resulted in deterioration of behavioral and certain learning abilities, assessed by open-field activity and passive avoidance learning tests. One of the most important findings was that both regular exercise and nettle, moreover, the combined effects of these two natural treatments, significantly attenuated lesion-associated decrease in brain function. The molecular mechanisms behind these beneficial effects, based on the results of the study, could be the following.

NMDA lesion resulted in increased formation of free radicals, as was shown by EPR measurements. Our data suggest that the extent of NMDA injection-induced oxidative stress was not just a local one, since the increased ROS production was measurable at the cerebellum. Indeed, the site propagation of NMDA lesion-induced oxidative stress was observed in an earlier study [39], which suggests that our finding on increased ROS level distant from the lesion is not a unique one.

The extent of the increased ROS level, observed in the cerebellum, could not be the only factor that resulted in deterioration of brain function, since the induced MMDA not only increases the monoamines release by reverse transport but also decreases extracellular GABA levels in rat striatum, as well as the glutamate efflux in nucleus accumbens, which independently can result in functional deficit [40].

Studies which applied the same or similar artificial brain damage reported enhanced inflammation [9,10]. However, the DNA-binding activity of NF-κB alone does not strongly support the occurrence of inflammation in our study as a result of NMDA lesion. On the other hand, the NF-κB activity was reduced by nettle supplementation as demonstrated in the study by Riehemann et al. [41], where nettle supplementation decreased the extent of inflammation via suppressing the activation of NF-κB. Besides being one of the key regulators of inflammation, NF-κB is involved in the transcription of Mn-SOD, DNA repair and apoptosis, which are associated with the level of ROS and significantly affect the fate of the cell [42]. Hence, down-regulation of NF-κB activity has more widespread effect on cell, which naturally could attenuate inflammation [42]. The activity of AP-1 could also indicate enhanced inflammation, since the AP-1 transcriptor protein plays an important role in inflammatory responses. Hence, numerous subsequent studies have provided further evidence regarding the essential role of JNK and c-Jun activation, which are constituent dimers of AP-1, on neural cell death induced by diverse stimuli (withdrawal of trophic support, DNA damage, oxidative stress, β-amyloid exposure and excitotoxic stress) [2,32,39,40,43]. The fact that AP-1 content is significantly increased by NMDA lesion shows that the lesion may have inflammatory and stress-related consequences in the tissue; however, we did not measure inflammatory markers but rather the activity of transcription factors. Again, regular exercise and nettle diet together proved to be a very powerful down-regulator of AP-1 activity in NMDA lesion similarly to that seen with NF-κB results. Therefore, it can be suggested that the combined effect of regular exercise and nettle supplementation results in decreased transcription of inflammation-associated proteins and might have an impact on apoptosis as well, since these transcription factors are the modulators of programmed cell death [2].

Regular exercise and nettle supplementation also altered the oxidation process of the brain tissue. Regular swimming elevated the concentration of free radicals, while it was decreased by nettle administration. This outcome is in accordance with the observation where nettle leaf extract, as an antioxidant agent, reduced the free electron accumulation in several brain areas [44,45]. Nettle was even an effective agent for reducing the NMDA lesion-caused free electron accumulation. Although the changes in carbonyl derivatives were not significant in this study, which could be due to the increased activity of proteasome complex, previous investigations have demonstrated a causative relationship between the accumulation of carbonyl groups and impaired brain function [46–48]. This relationship occurred in an indirect manner in our study as well, since the concentration changes of free radicals were associated with carbonyl content as well as with the impairment of brain function.

In conclusion, our results suggest that nettle supplementation has a potential to decrease the level of reactive species and the DNA-binding activity of NF-κB. Nettle was found to be an effective antioxidant supplement, to be a down-regulator of inflammatory transcription and could also promote learning performance in the brain. Regular exercise increases the concentration of reactive species in the cerebellum and alters the activity of transcription factors. The additive effect of the two treatments was more profound in the down-regulation of inflammatory transcriptor processes in NMDA lesion. The present study revealed that natural, physiological factors such as nutrition and regular exercise could play an important role in brain health.

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