

The effect of exercise and nettle supplementation on oxidative stress markers in the rat brain

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Abstract

Chronic swimming training and phytotherapeutic supplementation are assumed to alleviate oxidative damage, and support cell survival in the brain. The effect of forced, chronic swimming training, and enriched lab chow containing 1% (w/w) dried nettle (*Urtica dioica*) leaf were investigated for oxidative stress, inflammation and neurotrophic markers in Wistar rat brains.

The rats were divided into groups subjected to swimming training (6 weeks) or to nettle supplementation (8 weeks) or to a combination of these two treatments. The level of oxidative stress was measured by electron spin resonance (EPR), and by the concentration of carbonylated proteins. Nettle supplementation resulted in a decreased concentration of free radicals in both cerebellum and frontal lobe. Swimming, however, did not influence significantly the oxidative damage nor was it reflected in the carbonyl content. The protein content of nerve growth factor (NGF), and brain-derived neurotrophic factors (BDNF) was evaluated by E-Max ImmunoAssay in the cerebellum. No changes occurred either with exercise or nettle diet treatments. On the other hand, nuclear factor kappa B (NF- κ B) binding activity to DNA increased with the combined effect of swimming training and nettle diet, while the activator protein1 (AP-1) DNA binding activity showed a more profound elevation in the nettle treated animals. The amount of c-Jun decreased by swimming training.

In conclusion, the results suggest that both exercise and nettle influenced physiological brain functions. Nettle supplementation reduces the free radical concentration and increases the DNA binding of AP-1 in the brain. Nettle was found to be an effective antioxidant and possible antiapoptotic supplement promoting cell survival in the brain. Exercise, as a downregulator of c-Jun and in combined group as an upregulator of NF- κ B, may play also a role in antiapoptotic processes, which is important after brain injury.

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1. Introduction

The generation of reactive oxygen species (ROS) is a necessary and unavoidable consequence of aerobic metabolism. In response to acute or single bout of exercise, the body cannot adapt to the oxidative challenge because of the shortness of exercise duration and the physiological demands of in-

tensity. Physical exercise under these conditions generates increased levels of ROS, and results in oxidative damage to macromolecules [10,33].

Regular exercise, however, through its continuous radical generating effect, can also significantly contribute to the oxidative status [5,18]. The most marked effect of regular exercise is that it causes adaptation to the exercise-induced oxidative stress. Regular exercise has been shown to increase the activity of antioxidant enzymes in the brain, indicating that the oxidative stress-related adaptation takes place also in

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the brain [39]. In addition it may lead to muscle hypertrophy, better cardiac function, lower resting heart rate, improved glucose uptake, and better endurance [2].

Accumulating evidence suggests that physical exercise has the capability to beneficially effect certain brain functions [13]. It has also been shown that voluntary running increases the number of new hippocampal cells [30], brain plasticity [7], i.e. exercise can stimulate neurogenesis, increase resistance to brain insult and improve learning and mental performance [8]. It appears that voluntary physical exercise increases the production of trophic factors in the brain, which is associated with improved memory and learning [7,12], and long-term potentiation [31].

Neurotrophins elicit structural and physiological changes, and regulate gene expression in the neurons. There is an immediate answer in neurotrophin transcription to a number of changes occurring in brain functions. Neurotrophins themselves are involved in regulating their own expression [27,28]. According to our knowledge, at least two major neurotrophins play crucial roles in brain function related to exercise: the brain derived neurotrophin (BDNF), and the nerve growth factor (NGF).

Besides exercise, diet also has a significant effect on brain function [26]. Stinging nettle (*Urtica dioica* L.) leaf has a long history as a herbal remedy, and nutritious addition to the diet [35]. Nettle is rich in minerals (especially iron), vitamin C and pro-vitamin A [14]. Previous studies show that nettle leaves are a good source of essential amino acids [23], ascorbic acid [25], rare carbohydrates [24], and several mineral elements. It is also known that nettle has an antioxidant, anti-inflammatory, immune-suppressive, and anti-rheumatoid effect [3,6,42]. Epidemiological and laboratory studies indicate that carotenoids (pro-vitamin A) may have anti-carcinogenic [38], anti-ulcer [17] or anti-ageing properties [9].

The aim of the present investigation was to study the effects of regular exercise, nettle supplementation and their additive influence on brain reactions to oxidative stress and on several markers of brain function, such as the transcription of inflammatory factors and neurotrophin production.

2. Methods

2.1. Animals, diet and exercise

The protocol of the study was reviewed and approved by the local ethics committee. Twenty-eight, 4-month-old healthy Wistar rats were divided into four groups, which were subjected to swimming training (sw) (1.5 h swimming per day, 5 times/week, for 6 weeks) or to nettle supplementation (n) (1% w/w for 8 weeks) or to a combination of these two treatments (cbd) or left undisturbed as controls (c). Dried stinging nettle (*Urtica dioica*) leaf was bought from Herbaria (Budapest, Hungary). The component of nettle in normal lab chow was 1% w/w due to 30 mg/kg. This amount is equal to 1

tea bag consumption per day for humans. The dried, chopped nettle was mixed with normal lab chow. It was free excess to food. One day after the last exercise session rats were sacrificed and organs were separated and frozen in liquid nitrogen and stored at -80°C until analyses.

2.2. Biochemical assays

DNA binding activity of NF- κ B and activated protein-1 (AP-1) were measured by electrophoretic mobility shift assay (EMSA) as described by Kim et al. [20] from pooled brain (cerebellum) samples. The preparation of nuclear extracts was based on the method of Hattori et al. [16]. The oligonucleotide, with the sequence of 5'-GAGAGGCAAGGGATTCCCTTAGTTAGGA-3' for NF- κ B and 5'-GAG GTG AGG GCC TTC CCT TAG-3'; 3'-AC TCC CGG AAG GGA ATC AATC-5' for AP-1 was terminally labeled with ^{32}P using [γ ^{32}P]-ATP and T4 polynucleotide kinase. For binding assay, 10 μg of nuclear proteins were mixed with the labelled probe in a buffer containing 1.0% Nonidet P40 [19]. The mixtures were incubated at room temperature for 20 min, and the [^{32}P]-labelled 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 unlabelled oligonucleotide was carried out under the same conditions.

The c-Jun and phosphorylated c-Jun levels were measured by Western blot and the antibodies were obtained from Santa Cruz (CA, USA).

The concentrations of BDNF and NGF were determined using the E-Max ImmunoAssay System (Promega, Madison, WI). Standard 96-well flat-bottom Corning ELISA plates were incubated with carbonate coating buffer containing either polyclonal anti-NGF or monoclonal anti-BDNF overnight at 4°C . The next day, the plates were blocked with $1 \times$ B&S buffer for 1 h at room temperature. Serial dilutions of known amounts of NGF and BDNF, ranging from 500 to 0 pg, were performed in duplicate for the standard curve for each set of mouse tissue. For both the standards and the samples, 100 μl was added to each well in duplicate, and incubated for 6 h (NGF) or 2 h (BDNF) at room temperature. The wells were then incubated with a secondary monoclonal anti-NGF (overnight at 4°C) or antihuman BDNF polyclonal antibody (1 h at room temperature). Then, the wells were incubated with antirat IgG (NGF) or anti-IgY (BDNF) conjugated to HRP for 2.5 h (NGF) or 1 h (BDNF) at room temperature. A TMB solution was used to develop color in the wells for 10 min at room temperature. The reaction was stopped with the addition of 1N HCl to the wells. The absorbance was read at A45 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1 software, Sunnyvale, CA).

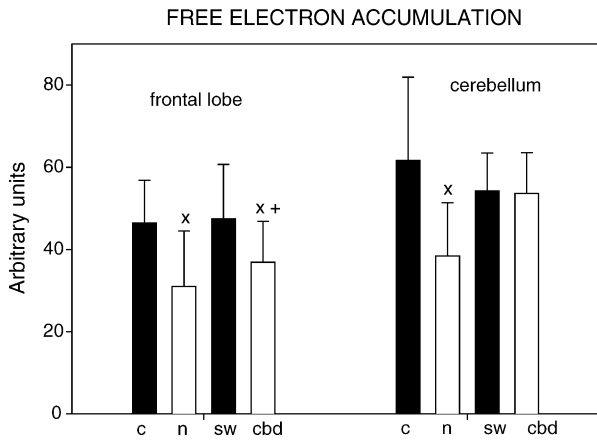


Fig. 1. Bars show the free electron accumulation in frontal lobe and in cerebellum, obtained by EPR measurements. Results are means \pm S.D. for seven animals. Nettle reduced the oxidative stress in both brain regions ($x F < 1$; $p < 0.05$ vs. c), while in the frontal lobe, nettle in combination with exercise could also significantly reduce the free electron accumulation ($+ F < 1$; $p < 0.05$ vs. sw). Group identification: c, control; n, nettle fed; sw, swimmer and cbd, combined (swimmer and nettle fed).

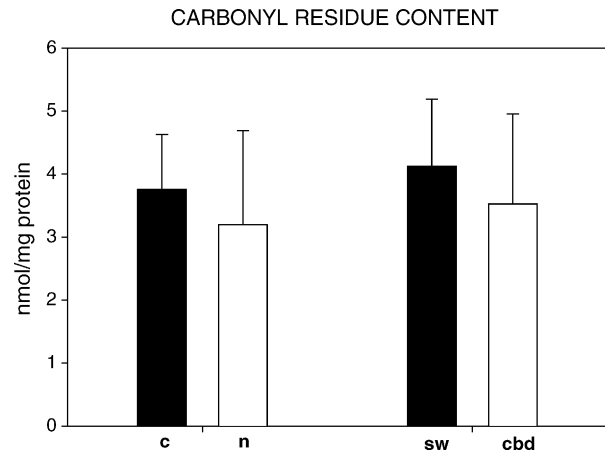


Fig. 2. The graph shows the quantitative measurement, by photometry, for carbonyl residue content in brain. No significant differences were shown among groups. Results are means \pm S.D. for seven animals.

The EPR measurements were carried out as described by Stadler et al. [40]. In brief, the measurements with an X-Band computer-controlled spectrometer, constructed by Magnetech GmbH (Berlin, Germany), were carried out. Approximately 100 mg of tissue samples from forebrain and cerebellum were 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, or an “EPR” simulation program developed by Rockenbauer [37] were used. Sudan Red Oil Red O staining, and neurofibrille-impregnation were also utilized.

Samples from right brain hemispheres, in 1:4 dilution, were suspended in lysis buffer. The carbonyl measurements were done according to the description of Radak et al. [32]. In brief, samples were incubated for 1 h in 500 μ L 10 mN DNPH (dinitro-phenyl hydrazine) with 500 μ L 2N HCl as blanks. Later, 500 μ L of 20% (w/w) trichloro acetic acid (TCA) were added to samples. After centrifuging for 10 min at 20,000 \times g,

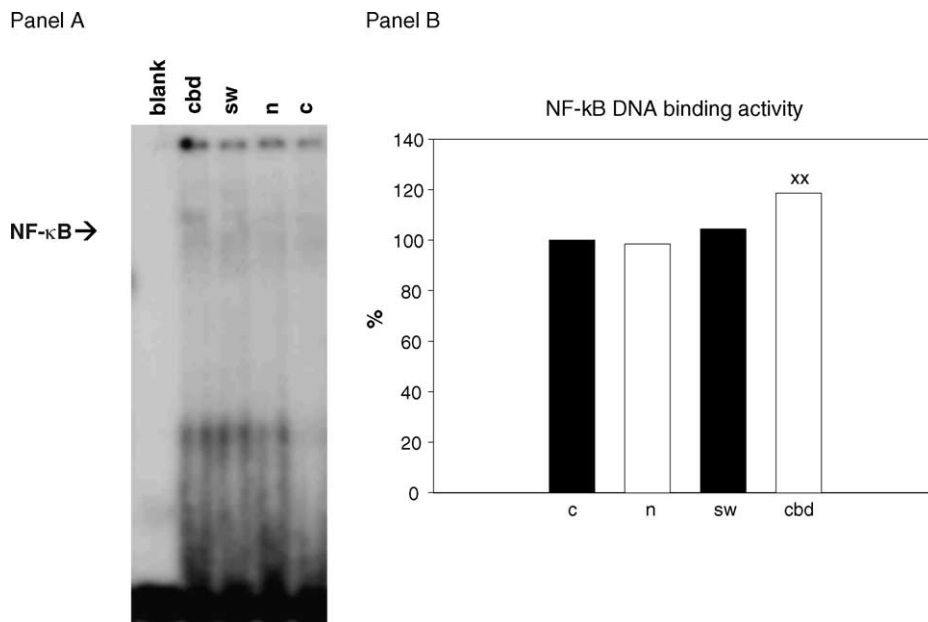


Fig. 3. The NF- κ B binding activity to DNA was measured by EMSA from pooled brain (cerebellum) samples (panel A). Each band demonstrates the pooled sample for seven animals for the cerebellum. Panel B shows the densitometric results of EMSA assay. It appears that only the nettle fed group showed the most increase in NF- κ B binding activity. The difference exceeded $xx \leq 20\%$.

supernatants were removed. Samples were washed in ethanol two times and in acetone once. The remaining pellets were dissolved in 8 M urea. The pellet-urea solution was incubated for half an hour at 37 °C and the absorbance of the samples was detected by electrophotometry at 360 nm.

2.3. Statistical analysis

The statistical significance was assessed by ANOVA, followed by Tukey's post hoc test and Pearson's correlation. The significance level was set at $p < 0.05$.

3. Results

The data obtained by EPR measurements revealed that nettle supplementation significantly reduced the free electron

accumulation, either in the frontal lobe or in the cerebellum compared to control and exercise groups. Regular exercise, on the other hand, did not result in significant alteration in EPR signals (Fig. 1). The levels of carbonyl groups were similar in all groups and the difference was not statistically significant (Fig. 2).

Only the combination of exercise with nettle administration increased the NF- κ B activity. NF- κ B activation (Fig. 3) was higher in both swimming groups when compared to their controls, whereas AP-1 was found to react differently as nettle feeding alone caused immense elevation in AP-1 activity. Six weeks of swimming training had a reduced effect on phosphorylated c-Jun activity while nettle had no influence on c-Jun content (Fig. 4). The transcriptional levels of BDNF and NGF were not affected by regular exercise, nettle supplementation or by the combined effects of exercise and nettle diet (Fig. 5).

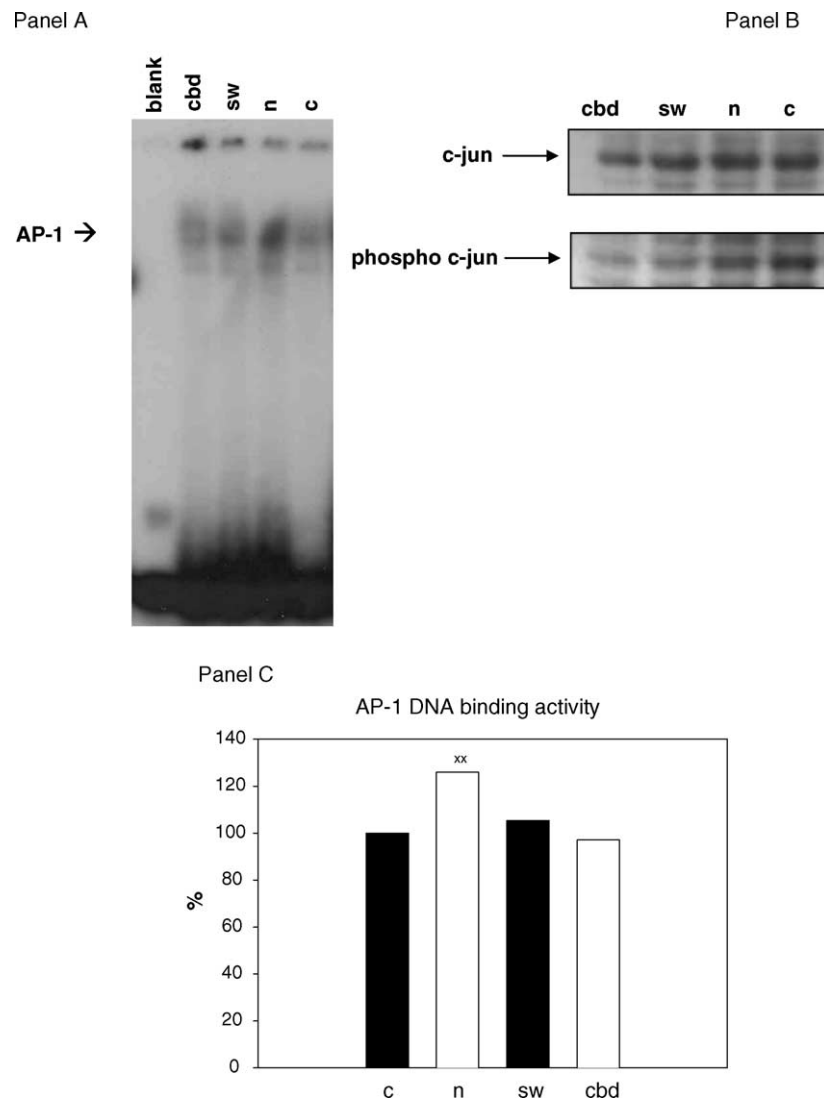


Fig. 4. Panel A shows the AP-1 DNA-binding activity, and panel B shows the c-Jun and phosphorylated c-Jun protein content. Each band demonstrates the pooled sample of seven animals for the cerebellum. The panel C shows the densitometric result of EMSA assay. The greatest increase in AP-1 binding activity happened in the combined group. The difference exceeded $^{xx} \leq 20\%$.

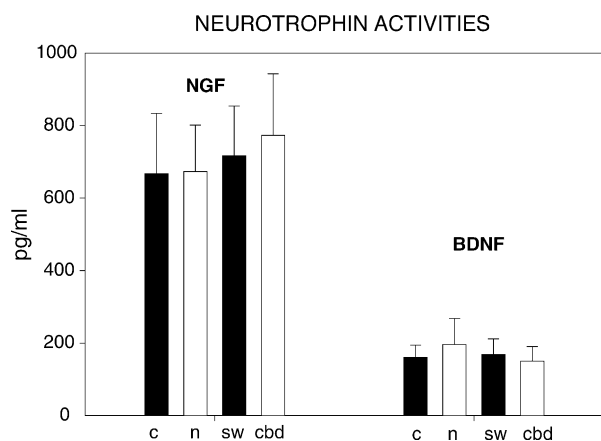


Fig. 5. BDNF and NGF were determined using the E-Max ImmunoAssay System. Samples were obtained from brain homogenates. No significant changes were found among groups. Results are means \pm S.D. for seven animals.

When the correlation of groups, among factors, was examined, a positive correlation was demonstrated between carbonyl and BDNF content ($r = 0.87$; $p < 0.01$).

4. Discussion

In this study, the effect of chronic exercise and nettle supplementation were investigated, on the oxidative stress markers in several parts of the brain. According to the results, it can be concluded that swimming training has no significant effect on the level of reactive oxygen species, assessed by EPR. To our knowledge, this is the first study, which measured the ROS content in the brain after chronic exercise training. Further signals — as reported in the literature — such as altered activity of antioxidant enzymes [39] or oxidative damage of lipids and proteins [34,41], suggest that exercise has a capability to significantly alter the generation of ROS. However, it has to be mentioned that the effect of swimming and running can be quite different in the brain.

In the present study the level of carbonyl groups was unaltered by swimming exercise and this is in accordance with the EPR data. Nettle leaf supplementation, on the other hand, decreased the level of ROS, suggesting an antioxidant role. This finding is in accordance with one previous study's observation where the nettle leaf extract reduced significantly the free electron accumulation in several brain areas, as an antioxidant agent [29].

NF- κ B and AP-1 are redox-sensitive and oxidant activated transcription factors, which regulate inflammation-related gene expression, viral replication, cell–cell interactions, apoptosis, proliferation, etc. [3]. The activation of NF- κ B can be induced by cytokines that activate kinases specific for I- κ B, an inhibitor protein in the complex with NF- κ B, from which it is dissociated upon phosphorylation [4]. Riehemann et al. [36] demonstrated the anti-inflammatory effect of nettle on NF- κ B activation.

However, we found, on the contrary, that nettle did not change the level of NF- κ B activation. The discrepancy between these two studies could be due to the different experimental conditions related to the amounts and kinds of nettle supplementation. The combined effect of swimming and nettle diet resulted in increased NF- κ B binding to the DNA. This is interesting since neither nettle nor swimming showed significant effects on NF- κ B activation.

AP-1 is composed of homo- or heterodimers of the protein products of individual members within the Jun (c-Jun, c-Jun-B, and c-Jun-D) and Fos (c-Fos, FOS B, Fra-1, and Fra-2) immediate-early response gene families. The outcome of AP-1 activation is dependent on the complex combination of AP-1 dimers. NGF withdrawal leads to increased c-Jun levels and c-Jun phosphorylation in neurons in culture, and microinjection of a dominant-negative c-Jun isoform blocks cell death indicating that AP-1 activity is necessary for neural cell death [15]. Numerous subsequent studies have provided further evidence regarding the essential role of JNK and c-Jun activation in neural cell death induced by diverse stimuli (withdrawal of trophic support, DNA damage, oxidative stress, β -amyloid exposure, and excitotoxic stress) [43].

The fact that AP-1 content is significantly increased by nettle supplementation in this study has a very important physiological meaning. Exercise alone and in combination reduced the amount of phosphorylated c-Jun. As c-Jun plays an important role in apoptosis, it can be suggested that both exercise and nettle reduce the risk of apoptosis. The c-Jun was down-regulated with nettle supplementation, but 6 weeks of swimming training had the strongest reducing effect on c-Jun protein content. Therefore, swimming elevated the NF- κ B activity, which could mean an antiapoptotic effect [1,21], together with the down-regulation of c-Jun. Nettle, as an up-regulator of AP-1 and as a down-regulator of c-Jun may mean antiapoptotic and cell survival supporting effect [19].

The content of BDNF and NGF were measured in chronic swimming training and nettle feeding for 6–8 weeks. BDNF and NGF are two of the most abundant neurotrophins, and are widely distributed in the central nervous system and associated with memory, some learning processes, and brain-insults, including a variety of stresses [22]. Voluntary exercise has been shown to up-regulate the content and mRNA of these trophic factors [7]. However, in the current study, we did not detect any significant alterations at translation level. One of the reasons could be that it appears that running has a more powerful effect on BDNF and NGF content than swimming [11]; and other factors such as age, and strain of the animals could play a role.

The results of our study suggest that both exercise and nettle-feeding influence physiological brain functions. Nettle supplementation decreases the level of ROS and increases the DNA binding of AP-1. Nettle was found to be an effective antioxidant and possibly antiapoptotic supplement promoting cell survival in brain. Since the animals in the present study were healthy it can be suggested that the shift of intracellular molecular machineries towards antiapoptosis and

antioxidant state may be important in the outcome of later sudden brain injury (the balance in the neurons was shifted toward antiapoptotic and antioxidant state, which can be preventive against later injuries.) Exercise, as a downregulator of c-Jun and in combined group as an upregulator of NF- κ B, may play a particular role in antiapoptotic processes, which merits further studies.

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