# The effects of exercise, oxidants and antioxidants on neurotrophins and oxidative damage on spinal cord of rats

Thesis

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# List of Abbreviations

BDNF: brain derived neurotrophic factor

CNS: Central nervous system

CREB: Cyclic AMP response element-binding protein

EPR: Electron paramagnetic resonance

ERK1/1: extracellular signal kinase

GDNF: glial cell line derived neurotrophic factor

MAPK: mitogen activated protein kinase

mRNA: messenger ribonucleic acid

PBN: N-tert-butyl-a-phenyl nitrone

RCD: Reactive carbonyl derivatives

ROS: reactive oxygen species

RT-PCR: Real time polymerase chain reaction

TBARS: thiobarbituric reactive substances

TrkB: Tyrosine kinase receptor B

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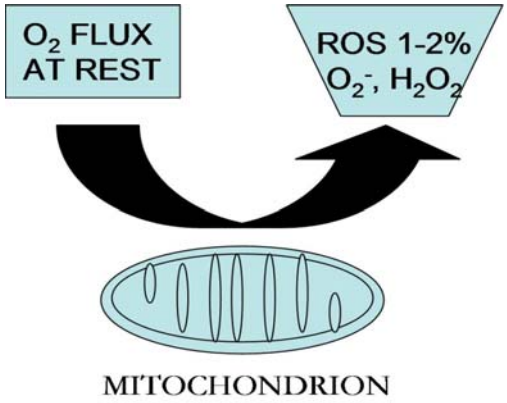
# 1 THE PROBLEM

## *1.1 Introduction*

Oxygen free radicals are products of normal cellular metabolism. Mitochondrion, the organelle of the cell responsible for cellular respiration and energy production is the major site of their production (figure 1.1.1 and figure 1.1.2). A free radical is a molecule with one or more unpaired electrons in atomic or molecular orbitals. The reactivity of free radicals results from their desire to attain an electron of opposing spin direction. As a radical reacts with another molecule in order to pair with another electron to be stabilized, a radical chain reaction begins. The radical chain reaction continues until a termination reaction takes place. The termination reaction occurs when radicals react with each other or with an antioxidant to produce non-radical species (Radak, 2000, chap.1).

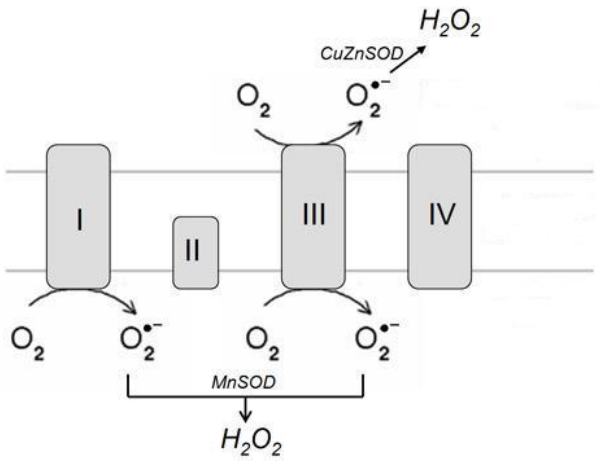
Radicals derived from oxygen represent the most important class of radical species generated in living systems. Among free radicals, the typical ones are: super oxide radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), nitric oxide radical ($NO^{\cdot}$) etc, (see table 1.1.3). Non radical species include among others: singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), ozone ($O_3$) etc, (see table 1.1.3). Free radicals and non radical derivatives of oxygen are collectively termed reactive oxygen species (ROS).

Fig. 1.1.1 Oxygen flux through mitochondria at rest and ROS production




1.1.1 Under resting conditions oxygen flux through mitochondria produces approximately 1-2% ROS.

Fig. 1.1.2 Mitochondrial complexes and superoxide radical production




1.1.2 The figure shows the major production side of superoxide radical ($O_2^{\cdot-}$) in mitochondrial complexes 1 and 3. Immediately after production superoxide radical is buffered by the antioxidant enzyme superoxide dismutase (SOD) and produces the less reactive hydrogen peroxide ($H_2O_2$).

Table 1.1.3 Reactive Oxygen Species Relevant to Biological Systems

| **Reactive Oxygen Species** | **Molecular Formula** |
|---|---|
| **Free radical species** | |
| Superoxide radical | $O_2^-$ |
| Hydroperoxyl radical | HOO· |
| Hydroxyl radical | HO· |
| Nitrogen monoxide | NO |
| Alkoxyl radical | RO· |
| Peroxyl radical | ROO· |
| **Nonradical species** | |
| Singlet oxygen | $^1\Delta_g O_2(^1O_2)$ |
| Hydrogen peroxide | $H_2O_2$ |
| Peroxynitrite | $ONOO^-$ |
| Hydroperoxide | ROOH |
| Ozone | $O_3$ |
| Hypochlorous acid | HC1O |
| Excited carbonyl | RR'CO* |

1.1.3 The table shows the free radical and non-radical species which are collectively called reactive oxygen species (ROS). The table was adopted by (Radak, 2000, chap 1, p. 6).

ROS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or advantageous to living systems (Valko et al. 2006; Radak et al. 2004). The delicate balance between beneficial and harmful effects of ROS is very important aspect of living organisms and is achieved by mechanisms called ‘’redox regulation’’. The redox regulation machinery incorporates enzymatic and non-enzymatic antioxidants as well as repair enzymes (table 1.1.4).

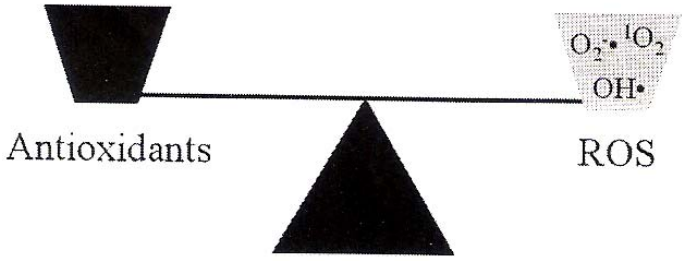
Table 1.1.4 Biological Defense Against Oxidative Stress

| **Antioxidant Defence** | **Occurrence** |
|---|---|
| **Preventive antioxidant enzymes** | |
| Superoxide dismutase | Cu,Zn-SOD Cytosol |
| | Ec-SOD Extracellular fluid |
| | Mn-SOD Mitochondria |
| | Fe-SOD Cytosol, chloroplsts |
| Catalase | Peroxisomes |
| Glutathione peroxidase | Cytosol, extracellular fluid, bio membranes |
| Glutathione S - transferase | Cytosol |
| **Antioxidants** | |
| α – Tocopherol (vitamin E) | Biomembranes, lipoprotein |
| Ascorbic acid (vitamin C) | Cytosol, extracellular fluid |
| Ubiquinol | Biomembranes, lipoprotein |
| Carotenoids | Biomembranes, lipoprotein |
| Reduced glutathione (GSH) | Cytosol, extracellular fluid |
| Uric acid | Cytosol, extracellular fluid |
| **Oxidation damage repair** | **Occurrence** |
| **Repair enzymes** | |
| Nuclease | Nuclei, cytosol |
| Glycosylase | Nuclei, cytosol |
| DNA polymerase | Cytosol |
| Protease | Cytosol |
| Phospholipase | Biomembranes, lipoprotein |

1.1.4 The table shows enzymatic and non-enzymatic antioxidants as well as oxidation damage repair enzymes along with their place of occurrence. Adopted by (Radak, 2000, chap 1, p. 23).

Under normal conditions at rest and in healthy individuals the production of ROS is compensated by the enzymatic and non enzymatic antioxidants (figure 1.1.5).

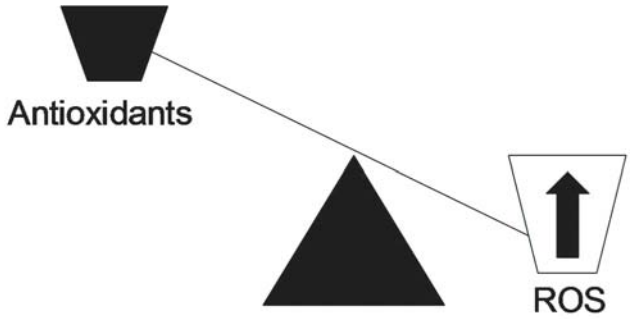
Fig. 1.1.5 Balance between production and removal of ROS




1.1.5 Under normal conditions at rest and in healthy individuals there is still background production of ROS, but these are adequately ‘’buffered’’ by the enzymatic and non-enzymatic antioxidant systems so that there is a balance between production and removal and no net increase in ROS. Adopted by (Deaton and Marlin, 2003).

The harmful effect of ROS causing potential biological damage is termed oxidative stress. Oxidative stress may be developed, despite an adequate antioxidant capacity (enzymatic and non-enzymatic), when a marked increase in ROS production overwhelm the antioxidant system (figure 1.1.6).

Fig. 1.1.6 Marked increase in ROS production consumes endogenous antioxidants

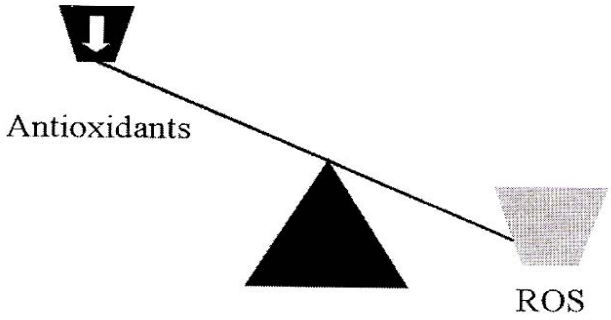



1.1.6 Despite an adequate antioxidant capacity, a marked increase in ROS production may overwhelm the antioxidant system and oxidative stress may be developed. Adopted by (Deaton and Marley, 2003)

Oxidative stress may also occur without any increase in normal background production of ROS due to selective or general reduction in components of the enzymatic or non-enzymatic antioxidant defenses (figure 1.1.7).

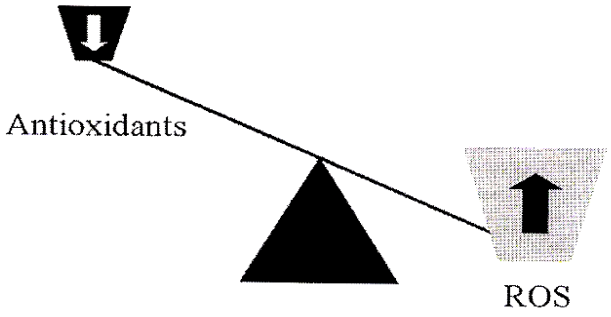
The worst case scenario for oxidative stress to occur is both a reduction in antioxidant capacity combined with increase in ROS production. This may happen in certain disease conditions (figure 1.1.8) (Deaton and Marlin, 2003).

Fig. 1.1.7 Oxidative stress occurrence without increase in normal production of ROS




1.1.7 Oxidative stress may occur without any increase in normal background production of ROS due to selective or general reduction in components of the enzymatic or non-enzymatic antioxidant defenses. Adopted by (Deaton and Marlin, 2003)

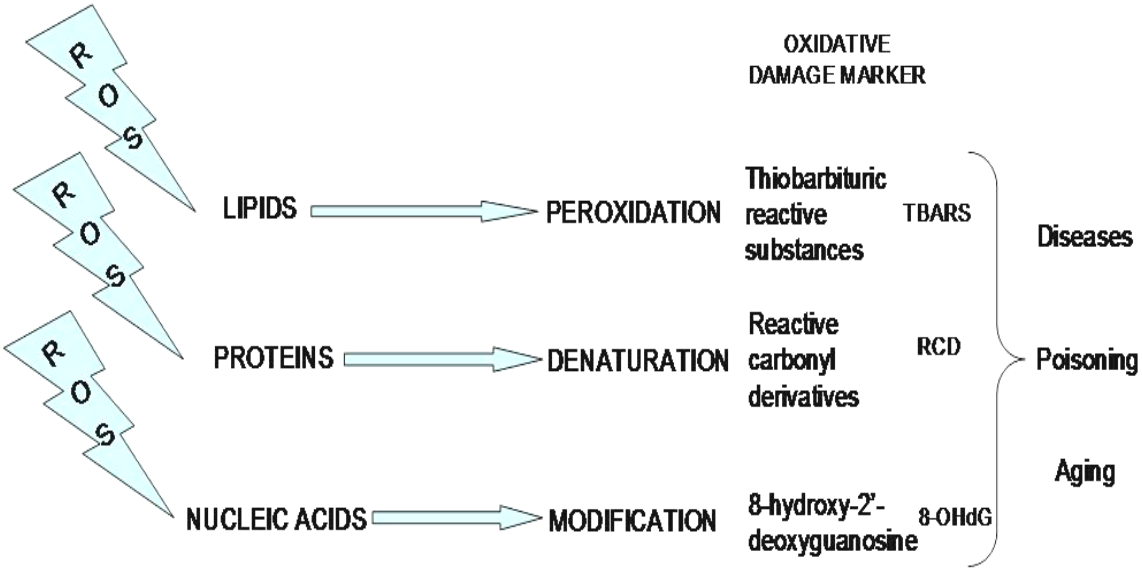
Fig. 1.1.8 Worst case scenario for oxidative stress occurrence




1.1.8 The worst case scenario for oxidative stress occurrence is both a reduction in antioxidant capacity combined with increases in ROS production. This may happen in certain disease conditions. Adopted by (Deaton and Marlin, 2003).

Disturbance in the equilibrium status of pro-oxidant/anti-oxidant reactions in living organisms leads to oxidative damage. It is well established that the uncontrolled generation of ROS in vivo oxidizes bio-molecules, such as nucleic acids, proteins, and lipids, which alters genetic information, denaturates proteins, inactivates enzymes, and disorders bio-membranes (figure 1.1.8) (Radak, 2000, chap.1). Oxidative damage can only be verified by the direct measurement of markers of oxidative damage (figure 1.1.9) (Deaton and Marlin 2003).

Fig. 1.1.9 Uncontrolled generation of ROS and cell's macromolecules




1.1.9 The figure shows the effect of excess ROS production on cell's lipids, proteins and nucleic acids and the general effect on the organism. In addition, it is shown which damage marker is investigated to determine oxidative damage for each macromolecule.

Excess ROS in one hand, as has been mentioned, can damage cell structures and macromolecules and may cause cell death (Deaton and Marlin 2003; Valko et al. 2006).

On the other hand, beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in response to defense against infectious agents and in the function of a number of signaling systems (Valko et al. 2006). It is known, that ROS, but especially $H_2O_2$ have a concentration dependent diverse effect on spinal cord, at high concentration they can induce apoptosis and serious cell damage (Liu et al. 1999), although recent findings suggest that low levels of endogenous $H_2O_2$ is required for wound healing in different tissues (Sen and Roy 2008; Roy et al. 2008). In addition, oxidative challenge, such as ischemic preconditioning has been shown to attenuate oxidative stress at spinal cord (Lee et al. 2008).

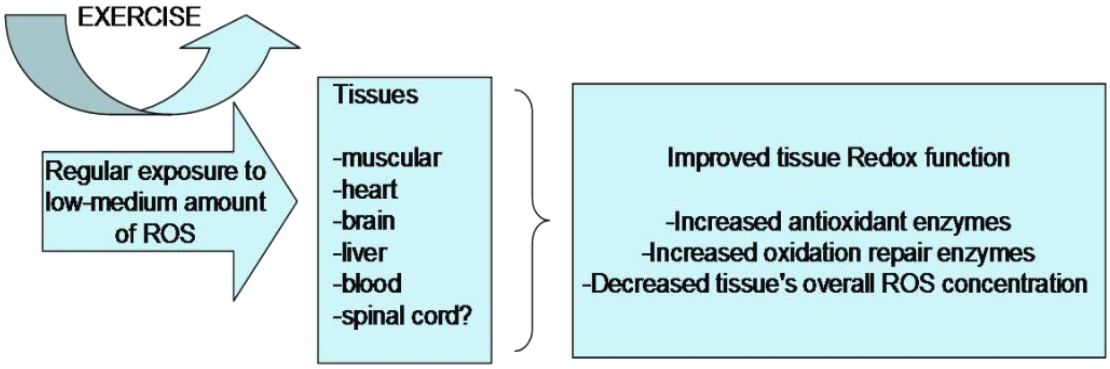
It is well known that oxygen consumption can increase several fold with exercise and that free radical production measured by electron spin resonance spectroscopy correlates strongly with maximal oxygen consumption (Deaton and Marlin 2003).

Regular exercise seems to posses the capability of generating such an amount of reactive oxygen species (ROS) that induces those signaling pathways that drive the biological systems to up-regulated processes and such adaptive responses necessary for survival and better function (figure 1.1.10) (Radak et al. 2005; Arumugam et al. 2006; Radak et al. 2008). Evidence suggest that chronic exposure to reactive oxygen species (ROS) such as during exercise decreases ROS concentration in cerebellum (Radak et al. 2006), reactive carbonyl derivatives in rat's brain (Radak et al. 2000b; Ogonovszky et al. 2005) and rat's myocardium (Radak et al. 2000a). In addition, it induces up-regulation of anti-oxidant enzymes (Somani et al. 1994; Venditi and Meo 1996; Nakatami et al. 2005) increases DNA repair (Radak et al. 2002), proteosome activity (Radak et al. 2000) and neurotrophins release (Oliff et al. 1998; Adlard et al. 2004; Cotman and Brechtold 2002; Berchtold et al. 2005; Neeper et al. 1996). In addition, regular exercise has been shown to up-regulate overall brain function and improve sensory recovery after spinal cord contusion (Hutchinson et al. 2004) and this has been associated to neurotrophins release.

This phenomenon coincides with the hormesis theory, (dose response phenomenon characterized by a low dose of stimulation, high dose of inhibition, resulting in either a J-shaped or an inverted U-shaped dose response, which is a non-monotonic response), which was developed after observing that low doses of toxins

and/or radiation can exert beneficial effects on lower organisms (Radak et al. 2005). Increasing evidence suggests that hormesis may operate in higher animals, as well (Radak et al. 2005).

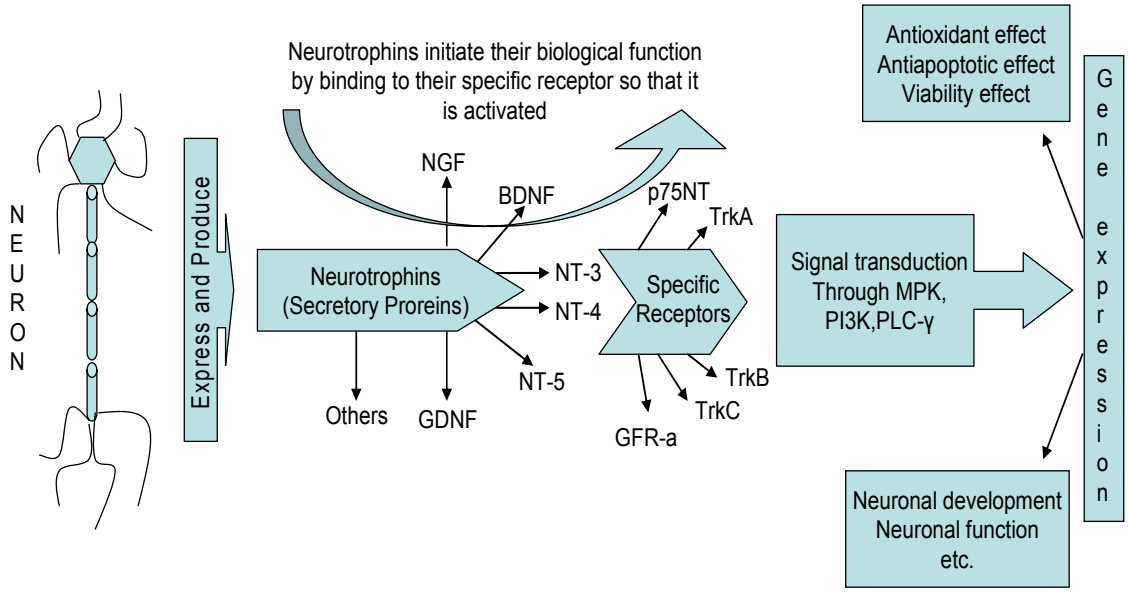
Fig. 1.1.10 Regular exercise drives biological systems to adaptive responses




1.1.10 The figure shows the positive effect of regular exercise which is translated as a regular exposure to low-medium amount of ROS. As can be seen from the figure, exercise influences positively several tissues and results in up-regulated redox machinery function. Spinal cord is marked with a question mark since before this study some points referring to this tissue redox machinery were still vague.

Neurotrophins (figure 1.1.11) especially, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), are important functional regulators of neurons (Bramham and Messaoudi 2005 ) and cell survival and it has been shown to rescue CNS cells from damage and cell death when exposed to ROS (Gabaizadeh et al. 1997; Giehl et al. 1998). This phenomenon supports the capacity of these neurotrophins in exerting an anti-oxidant and anti-apoptotic effect (Akaike and Kume 1998; Chao and Lee 1999; Joosten et al. 2004; Petersen et al. 2001).

Fig. 1.1.11 Neurotrophins, their specific receptors, signal transduction and neuronal effects




1.1.11 The figure shows the expression and production site of neurotrophins which takes place in neuron. Neurotrophins belong to a family of secretory proteins that include: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and glial cell line neurotrophic factor (GDNF). All neurotrophins bind to the p75 neurotrophin receptor (p75NR), and each neurotrophin also binds to a specific Trk receptor tyrosine kinase: NGF binds to TrkA; BDNF and NT-4/5 binds to TrkB; NT-3 to TrkC; and GDNF to GDNF receptor alpha (GFR-a). Ligand binding results in dimerization and autophosphorelation of the Trk receptors, leading to activation of the tyrosine kinases. Activated receptors then are capable of triggering a number of signal transduction cascades including the mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the phosholipase C-γ (PLC- γ ) pathway. These signals then pass on to the nucleus to activate transcription factors that alter gene expression and leads to up-regulated function.

Evidence suggests that, in general, concentration of BDNF and GDNF can be modified by exercise and an altered redox homeostasis (figure 1.1.12) (Chao and Lee 1999; Cotman and Berchtold 2002; Bramham and Messaoui 2005; Wang et al. 2006) and can play a role in tissue preconditioning against oxidative stress (Lee et al. 2008).

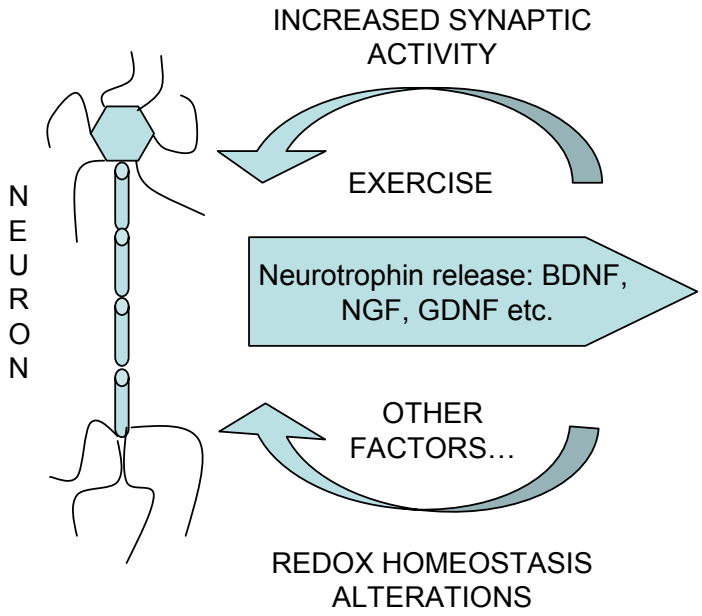
Furthermore, neurotrophins exert complex modulation of dendritic and axonal growth in the brain and spinal cord (Lu et al. 2003; Mills et al. 2007) and participate in long-term-potentiation (LTP) which is associated to memory and learning (Lu 2003; Cotman and Berchtold 2002; Bramham and Messaoui 2005).

BDNF and its tyrosine kinase receptor TrkB have been extensively studied in different brain regions in response to exercise. Results of such studies demonstrate an increase in TrkB and its ligand with exercise in both transcriptional and translational level (Klintsova et al. 2004; Kim et al. 2005; Neeper et al. 1996; Cotman and Berchtold 2002; Johnson and Mitchell 2003; Johnson et al. 2003). On the other hand, a down-regulation of these factors has been observed when animals are deprived of regular physical activity (Widenfalk et al. 1999).

Neural activity induced by exercise has been also studied in spinal cord tissue in response to neurotrophin release. Although spinal cord is generally considered to be less prone to up-regulation of trophic factors than the brain following injury or other experimental interventions (Widenfalk et al. 1999) recent data reports that voluntary exercise has the capability of inducing/restoring levels of neurotrophins both in intact or injured spinal cord and promote neuroplasticity (Ying et al. 2005; Gomez-Pinilla et al. 2002; Molteni et al. 2004; Skup et al. 2002).

However, most of the exercise-studies which examine neurotrophin response in spinal cord are restricted to the lumbar region since it is believed that this is the part where the most neural activity takes place during exercise (Gomez-Pinilla et al. 2000). It has been demonstrated that neural activity plays a vital role in BDNF production and release from neurocells (figure 1.1.12).

Fig. 1.1.12 Factors influencing Neurotrophin release from neurocells




1.1.12 The figure indicates several factors that have been shown to influence neurotrophin release from neurocells in different brain regions and spinal cord.

Consequently, in the present exercise-study, focus will be centered in the neurotrophin response of cervical spinal cord and how this response is related to ROS since this is one of the most often injured parts of spinal cord, and injury is known to be associated with ROS production (Kass et al. 2008).

The exercise-induced adaptive responses described above (neurotrophin response to exercise and up-regulation of redox machinery), consequently, result in tissue's resistance to oxidative stress (Nakatani et al. 2005; Radak et al. 2000; Cotman and Berchtold 2002; Radak et al. 2008) and enhanced physiological and functional capacity (Radak et al. 2000; Cotman and Berchtold 2002; Radak et al. 2008).

On the other hand, overproduction of ROS such as during pathological conditions or even generation of ROS in response to acute or single bout of exercise, our body can not adapt to the oxidative challenge because of the shortness of exercise

duration and the physiological demands of intensity. Under these conditions ROS can cause potential biological damage to cell structures and macromolecules including lipids and membranes, proteins and nucleic acids (figure 1.1.9) (Deaton and Marlin 2004; Valko et al. 2006).

To challenge this overproduction of ROS and to diminish disturbance in the equilibrium status of pro-oxidant/anti-oxidant reactions in living organisms which causes oxidative damage many investigations have focused their interest on antioxidant intervention studies. These studies provide anti-oxidant administration in several conditions associated with increased production of ROS including exercise, sedentary life style conditions (obesity, over-eating etc); smoking and pathological neurodegenerative states all showing confounding results in the effectiveness of anti-oxidants (Gilgun-Sherki et al. 2002). It has been also postulated that an antioxidant administration during exercise may attenuate the exercise-induced adaptation to oxidants in skeletal muscle (Gomez-Cabrera et al. 2006).

One synthetic anti-oxidant capable of scavenging many types of free radicals, including oxygen and carbon based radicals, is the spin trapping compound N-tert-butyl-α-phenylnitrone (PBN). Specifically, several studies report decrease in brain protein oxidative damage (Carney et al. 1991; Dubey et al. 1995; Ernst et al. 2003) and an improved cognitive performance and survival especially in aged rats after chronic administration of PBN (Socci et al. 1995; Sack et al. 2003) as well as suppressed ROS production by mitochondria (Floyd 1999). Up to date, no investigations have been reported for the effect of PBN in spinal cord or in an exercise study in general.

Since, most of these observations described briefly above are tissue dependent, and according to our knowledge, the response of spinal cord to regular exercise, oxidative challenge is poorly understood, we aimed to study the response of neurotrophins and free radical concentration at spinal cord to asses the response of these important components.

Based on the review of related literature we decided to test the following hypotheses in order to fill out the vague points of the existing data on the subject.

## 1.2 Hypotheses

Within the limitations of this study, the following null hypotheses will be tested at .05 level of significance ($p < 0.05$):

1. $H_0$: There will be no difference in free radical concentration among groups.

We chose to investigate the effect of exercise and oxidant, anti-oxidant administration in spinal cord since there is no literature associated to the effect of the above mentioned treatments in this tissue. Although free radical concentration has been previously tested in myocardium and brain this has never been tested in spinal cord. It is then of primary interest to know how redox alterations induced either by exercise or oxidant antioxidant treatments modulate free radical level in spinal cord since the resistance against oxidative stress, could be vitally important at the prevention of oxidative stress-associated spinal cord alterations. We hypothesized that regular exercise and PBN may decrease free radical level in spinal cord since other studies in myocardium and brain support this statement. In addition we hypothesized that an additional stress to exercise such as oxidant intervention may lower free radical concentration even more. We further hypothesized that PBN intervention in exercised animals may not have the same effect as exercise alone but rather may have negative effect in terms of decreasing free radical concentration since may interfere with exercise-induced adaptation to oxidants.

2. $H_0$: There will be no difference in BDNF concentration among groups.

We hypothesized that exercise and redox alterations induced by oxidant-antioxidant administration may induce alterations in BDNF's mRNA and protein concentration since evidence suggests that the concentration of this trophic factor is reported to be modulated by physical activity and redox alterations.

3. $H_0$: There will be no difference in GDNF concentration among groups.

Since GDNF is reported to have antioxidant properties and its concentration may be modulated by redox alterations we hypothesized that the present treatments may alter GDNF level in spinal cord.

4. $H_0$: There will be no difference in TrkB mRNA expression among groups.

It has been reported that BDNF's receptor TrkB mRNA is increased by exercise. On the other hand, it is well established that exercise induces alterations in redox homeostasis. Thus, we hypothesized that exercise and exogenous administration of redox balance alteration inducers may modify TrkB's mRNA level of expression in spinal cord.

5. $H_0$: There will be no difference in BDNF mRNA expression among groups.

It has been shown that exercise increases the expression of BDNF mRNA in lumbar region of spinal cord. In addition, evidence suggests BDNF's mRNA can be modified by an altered redox balance in neouro-cells environment. Therefore, we hypothesized that exercise and oxidant antioxidant administration may induce alterations on BDNF's mRNA level.

6. $H_0$: There will be no difference in oxidative protein damage among groups.

It has been shown that regular exercise decreases the oxidative protein damage in several tissues. In addition, it is also know that by manipulating redox alterations through exogenous oxidant antioxidant administration may also induce changes on tissue's oxidative protein damage. However, this has never been tested in spinal cord. Thus, we hypothesized that exercise and oxidant antioxidant interventions may induce changes on oxidative protein damage in spinal cord.

7. $H_0$: There will be no relationship between free radical concentration and BDNF concentration among groups.

It has been shown that oxidative challenge on a specific type of neuro-cells could increase BDNF concentration while on the other hand, treatment of those cells with an antioxidant resulted in inhibition of BDNF concentration. Therefore, we hypothesized that there will be a possibility of a positive relationship between free radical and BDNF concentration in spinal cord.

8. $H_0$: There will be no relationship between free radical concentration and oxidative protein damage among groups.

Since free radicals concentration plays crucial role in inducing oxidative damage, we hypothesized that there could be a positive correlation between free radical concentration and tissue's protein oxidative damage.

9. $H_0$: There will be no difference in ERK's activation among groups.

Studies report that exercise and redox alterations increase ERK's activation signal and the increase is associated to BDNF induction. Therefore, we hypothesized that both exercise and oxidant antioxidant treatments may increase ERK's activation signal in spinal cord.

10. $H_0$: There will be no difference in BDNF mRNA expression level among groups.

Previous studies report that exercise increases the BDNF's mRNA expression level both in brain and spinal cord. In addition, since redox alterations may modify BDNF's mRNA level we hypothesized that exercise and oxidant antioxidant administration may modify BDNF's mRNA level in spinal cord.

## *1.3 Objectives*

Since, most of these observations described briefly above are tissue dependent, and according to our knowledge, the response of spinal cord to regular exercise, oxidative challenge is poorly understood, we aimed to study the response of neurotrophins and free radical concentration at spinal cord to asses the response of these important components. Since the resistance against oxidative stress, could be vitally important at the prevention of oxidative stress-associated spinal cord alterations. We suggested that low level of oxidant, like exercise has a beneficial, while high level of oxidant and/or antioxidant treatment would have unfavorable effect on BDNF and GDNF according to the hormesis theory. Moreover, adaptation to moderate level of ROS treatments, which involves regular exercise, would stimulate neurotrophins, which could be an important mean to cope with oxidative challenge. Therefore, it cannot be excluded that treatment with low level of H2O2 or physical exercise could have beneficial effects on preconditioning of the spinal cord against oxidative challenge.

## 1.4 Delimitations of the study

It should be noted that the results of the present investigation are confined within certain conditions, which include:

1. Age and gender of animals (young, healthy, male Wistar rats)
2. Cervical region of spinal cord
3. Chronic, moderate intensity of enforced running (10 weeks, 27m/min, 5 days/ week, 1hour/day).

## 1.5 Limitations of the study

The present investigation may be limited by the following factors:

1. Relatively small sample size.

## 1.6 Definition of terms

Antioxidant: antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including removal of $O_2$, scavenging reactive oxygen species or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation. The natural antioxidant system can be classified into two major groups: enzymes and low molecular weight antioxidants (LMWA). The enzymes include SOD, catalase, peroxidase, and some supporting enzymes. The LMWA group of molecules can be further classified into directly acting antioxidants (e.g., scavengers and chain breaking antioxidants) and indirectly acting antioxidants (e.g., chelating agents) (Gilgun-Sherki et al., 2002).

Electron Paramagnetic Resonance (EPR): EPR is a spectroscopic technique that detects chemical species that have unpaired electrons (http://www.chem.queensu.ca/eprnmr/EPR_summary.htm). Electrons, and other particles, have an intrinsic angular momentum, known as spin. This creates a magnetic dipole moment. When the electron is placed in a magnetic field, the intrinsic magentic dipole can align in one of two ways, parallel or anti-parallel to the field. The anti-

parallel state is of lower energy. However, applying radiation of a certain frequency to the electron can raise it to the higher energy state, in which its magnetic dipole is parallel to the applied magnetic field. It will then fall back to the lower energy state, emitting a photon. If radiation continues to be applied, then the electron will "resonate" between the two energy states. This is known as electron spin resonance, and is used to identify compounds, which each have a unique spectrum of radiation absorption (http://en.wikipedia.org/wiki/Electron-spin_resonance).

Free radicals: free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Valko et al., 2006).

Neurotrophic factor/Neurotrophin: any of a group of neuropeptides (as nerve growth factor) that regulate the growth, differentiation, and survival of certain neurons in the peripheral and central nervous systems (http://www2.merriam-webster.com/cgi-bin/mwmednlm?book=Medical&va=neurotrophic+factor).

Oxidant/oxidizing agent: a substance that oxidizes something especially chemically (as by accepting electrons) (http://www2.merriam-webster.com/cgi-bin/mwmednlm?book=Medical&va=oxidizing+agent).

Oxidative damage marker: a marker that verifies that oxidative damage has occurred (e.g. reactive carbonyl derivatives, malondialdehyde, 8-hydroxydeoxyguanosine) (Deaton and Marlin, 2003).

Oxidative stress: physiological stress on the body that is caused by the cumulative damage done by free radicals inadequately neutralized by antioxidants and that is held to be associated with aging (http://www2.merriam-webster.com/cgi-bin/mwmednlm).

Redox homeostasis: is the delicate balance between beneficial and harmful effects of free radicals (Valko et al., 2006). In such a state, ROS are adequately buffered by the nonenzymatic and enzymatic antioxidant systems so that there is a balance between production and removal and no net increase in ROS. This also implies that oxidized antioxidants are regenerated (reduced) to keep pace with ROS production (Deaton and Marlin, 2003).

Reactive oxygen species: Reactive intermediate oxygen species including both radicals and non-radicals. These substances are constantly formed in the human body and have been shown to kill bacteria and inactivate proteins, and have been implicated in a number of diseases. Scientific data exist that link the reactive oxygen species produced by inflammatory phagocytes to cancer development (http://cancerweb.ncl.ac.uk/cgi-bin/omd?reactive+oxygen+species).

# 2 METHODS

## *2.1 Animals*

Thirty-six, five month old, healthy, male Wistar rats, with mean weight 258.53 grams, 30.66 ± SD at the start of the experiment, were used in the study and were cared for according to the guiding principles for the Care and Use of Animals based upon the Helsinki Declaration, 1964 (local license number: 28/1999; 1794/003/2004). The study was approved by the local Animal Welfare Committee. Animals were housed in standard polyethylene cages with food and water ad libitum. Animal's weight was registered at the beginning of each experimental week (table 2.1.1 and table 2.1.2).

Table 2.1.1 Mean pre and post-treatment body-weight and standard deviation in grams

| Experimental Groups | Mean pre-treatment body weight and standard deviation in grams | Mean post-treatment body weight and standard deviation in grams |
|---|---|---|
| Non-exercised control injected with saline (NEC) | 245,83 ±17,15 | 409,16 ±45,54 |
| Exercised control injected with saline (EC) | 254,16 ±29,39 | 353,33 ±35,02 |
| Non-exercised injected with hydrogen peroxide (NEP) | 255,83 ±19,6 | 375,83 ±21,07 |
| Exercised injected with hydrogen peroxide (EH) | 262,5 ±26,78 | 353,33 ±29,43 |
| Non-exercised injected with PBN (NEP) | 234,16 ±9,7 | 372,5 ±27,34 |
| Exercised injected with PBN (EP) | 307,5 ±18,37 | 388,33 ±28,4 |

2.1.1 The table shows mean body-weight and standard deviation of the groups at beginning of the experimental period during the first week and the respective body-weight during the last week of the experimental period. The weight measures are indicated in grams.

Table 2.1.2 Difference in grams and in percentage of body-weight increment between pre and post-treatment periods among non-exercised and exercised groups.

| Experimental Groups | Mean body-weight in grams that has been added between pre and post-treatment period | Percentage of increment in each group |
|---|---|---|
| Non-exercised control injected with saline (NEC) | 163,33 | 68% |
| Exercised control injected with saline (EC) | 99,17 | 39% |
| Non-exercised injected with hydrogen peroxide (NEP) | 120 | 47% |
| Exercised injected with hydrogen peroxide (EH) | 90,83 | 35% |
| Non-exercised injected with PBN (NEP) | 138,34 | 59% |
| Exercised injected with PBN (EP) | 80,83 | 26% |

2.1.2 The table shows the increase in mean body-weight in grams and in percentage between the pre and post-treatment period in each experimental group. The measurements suggest that non-exercised animals have increased their body-weight in higher percentage than their respective exercised groups.

Six rats were randomly assigned to each of six groups: non-exercised control injected with saline (NEC), non-exercised injected with $H_2O_2$ (NEH), non-exercised injected with N-tert-butyl-α-phenylnitrone PBN (Sigma) (NEP), exercised control injected with saline (EC), exercised injected with $H_2O_2$ (EH) and exercised injected with PBN (EP). Non-exercised groups remained sedentary for the whole 10 weeks of the study.

After one week adaptation period consisted of 1hour running/day with the intensity starting from 17m/min and reaching 27m/min the last day of the adaptation period, exercised groups were subjected to forced treadmill running for 9 more weeks, five days/week, 1hour/day at 27 m/min intensity on a treadmill apparatus consisted of six lanes separated by glass. At the rear, each lane was equipped with metal bars providing motivation to the animals by 1-3V electrical current.

During weeks 9th and 10th all animals were injected subcutaneously day after day, one hour prior to exercise with saline (NEC & EC), with 0.5 Mm $H_2O_2$ (NEH & EH). The remaining groups (NEP & EP) were injected in similar manner during the 9th and 10th week, every other day and one hour prior to exercise, with 13mg/100gr of body weight of PBN diluted in saline (Janzen et al. 1995). One day after the last training animals were sacrificed by decapitation and spinal cord was extracted and the cervical region was separated (The entire posterior part of spinal column was carefully chopped apart until spinal cord was clearly visible and the cervical region was identified and separated by thoracic by counting the anterior inner articular surfaces of the vertebrae) and frozen in liquid nitrogen and stored at -80 $^{o}$ C until analyses.

## *2.2 Pilot study*

To establish the $H_2O_2$ dose a pilot study was designed (data not shown) in which rats were injected with 1 mM or 0.5 mM of $H_2O_2$. Most animals injected with 1 mM of $H_2O_2$ died within a few seconds and survivors were not able to exercise on the treadmill within an hour. Those animals that received a dose of 0.5 mM of $H_2O_2$ were able to exercise within an hour after the injection and the dose was sufficient in inducing lipid peroxidation in spinal cord homogenates. For the estimation of lipid peroxidation marker malondialdehyde (MDA) the thiobarbituric acid-reactive substances (TBARS) were utilized as described earlier by (Ohkawa et al. 1979).

## 2.3 Instrumentation and collection of data (Biochemical assays and equipments)

*2.3.1 Treadmill apparatus:*

consisted of six lanes separated by glass. At the rear, each lane was equipped with metal bars providing motivation to the animals by 1-3V electrical current.

*2.3.2 Treadmill apparatus*

*BDNF and GDNF determination*: spinal cord samples were homogenized in 8 volumes of lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl (Ph 8.0), 1% NP40, 10% glycerol, 1mM PMSF, aprotinin (10 mg/ml), leupeptin (1μg/ml), and 0.5 mM sodium vanadate. The protein determination was performed in triplicates as described earlier by Lowry et al. (1951).

The concentrations of BDNF and GDNF were determined, from the spinal cord, 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 monoclonal anti-BDNF or monoclonal anti-GDNF over-night at 4 $^{o}$ C. The next day, the plates were blocked with 1 x B&S buffer for 1 h at room temperature. Serial dilutions of known BDNF ranging from 500 to 0 pg and GDNF ranging from 1000 to 0 pg, were performed in duplicate for the standard curve for each set of tissue. For both the standards and the samples, 100 μl was added in each well in duplicate, and incubated for 2 h (BDNF) or 6 h (GDNF) at room temperature. The wells were then incubated with a secondary antihuman BDNF polyclonal antibody (1 h at room temperature) or antihuman GDNF polyclonal antibody (overnight at 4 $^{o}$ C). Then, the wells were incubated with anti-IgY (BDNF) or anti-chicken IgY (GDNF) conjugated to HRP for 1 h (BDNF) or 2 h (GDNF) at room temperature. A TMB was used to develop color in the wells for ten minutes at room temperature. The reaction was stopped with the addition of 1N HCl to the wells. The absorbance was read at A450 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1 software, Sunnyvale, CA).

*Determination of reactive carbonyl derivatives (RCD)*: was performed by Western blot. In brief, homogenates of pooled spinal cord samples were incubated for 1 h in 400 μl of 10 mM DNPH (dinitro-phenyl hydrazone) with 400 μl of 2N HCl as blanks. Later, 500 μl of 20% trichloro acetic acid (TCA) were added to samples. Samples were then centrifuged for 10 min, on 4 $^{o}$ C, at 20,000 x g, and supernatants were removed. Followed, samples were washed twice in ethanol and once in acetone. The remaining pellets were dissolved in 8 M urea and the pellet-urea solution was then incubated at 37 $^{o}$ C for half an hour. Protein concentration was then set equal among the samples followed by polling the samples of each group. Protein concentration was once

again set until we achieve equal protein concentration among the pooled samples of the groups. Equal amount of protein from each group was then loaded on a 12% polyacrylamide gel and electrophoresis was performed. After electrophoresis the proteins were transferred to PVDF membrane. Then the membrane was incubated for 1 h in TBST [20 mM Tris-HCl (pH 7,4), 1,5 mM NaCl and 0.01% Tween 20) blocking buffer containing 5% skimmed milk. Membrane was then treated with anti-DNPH antibody (1:5000) in blocking solution for 1 h and then washed 3 x 10 min in TBST. Incubation of the membrane with the secondary antibody was followed (anti-rabbit HRP, 1:5000) in blocking solution for 1 h and then washing again 3 x 10 min. Exposure to hyper-film (CL-XPosure$^{TM}$ #34089) was carried out using enhanced chemiluminescence (PIERCE #34080). Exposure time was set to 1 min.

*Determination of extracellular signal kinase (ERK1/2)*: homogenates of spinal cord samples were set equal among groups utilizing the Bio-Rad protein assay for cytocolic proteins according to the manufacturer's protocol. Groups were then pooled and subsequent protein determinations utilizing Bio-Rad protein assay (3 protein assays were performed and the mean value was calculated) were once more performed in order to set equal protein concentration among the pooled groups. Once protein content set equal an end volume of 20ul was removed from each pooled sample and 2ul of sample buffer containing 1% bromphenol blue was added. The samples were then vortex and incubated at 95$^{o}$C for 5 min. Followed, samples were centrifuged for 5 min on 16 000 g at 22$^{o}$C and loaded to an 8% gel along with 20ul of molecular weight marker indicator (BenchMark$^{TM}$, Pre-Stained Protein Ladder). Electrophoresis was then carried out and subsequent separation of proteins was performed. Once electrophoresis was over the protein transferred to a PVDF membrane. Then the membrane was incubated for 1 h in blocking buffer containing TBST + 5% skimmed milk and then washed 3 times by TBST. Membrane was then treated with primary phosphor-ERK1/2 antibody, mouse monoclonal (Cell Signaling, #9106S) and incubated overnight at 4oC. Primary antibody concentration was 1:500. The next day membrane was washed 3 times for 10 min in TBST and incubated for 1 h in mouse secondary-HRP antibody (1:5000). The membrane was once again washed 3 times for 10 min in TBST. Exposure to hyper-film (CL-XPosure$^{TM}$ #34089) was carried out using enhanced chemiluminescence (PIERCE

#34080). Exposure time was set to 20 min. For the detection of unphosphorelated-ERK1/2 the same membrane was used. First the membrane was treated with 50 ml stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM b-mercapto ethanol added only before use) at 40Oc shacking for 30 min. Then the membrane was washed 3 times with TBST for 10 min or until the smell of b-mercapto ethanol wash away. After this step the immunostaining procedure was as described for the detection of phosphor-ERK. The primary antibody used was a rabbit polyclonal (Cell Signaling, #9102) with concentration 1:500. The secondary antibody was an anti-rabbit-HRP (1:5000). Exposure to hyper-film (CL-XPosure$^{TM}$ #34089) was carried out using enhanced chemiluminescence (PIERCE #34080). Exposure time was set to 30 seconds.

*RNA isolation*: was carried out by the FastRNA Pro Green Kit (6045-050 QBioGene) using the FastPrep Instrument (6001-120 QBioGene) according to the manufacturer's protocol. In brief, approximately 100 mg of spinal cord tissue was placed to a green-cap tube containing Lysing Matrix D provided by the kit and 1 ml of RNApro solution was added to the sample. Matrix tube was then processed in the FastPrep Instrument for 40 seconds at a setting of 6.0. Matrix tube was then centrifuged at maximum speed ( > 12,000 x g) for 5 min at 4 $^{o}$ C. Supernatants were then transferred to a new microcentrifuge tube and incubated at room temperature for 5 min. Then 300 μl of chloroform (NO isoamyl alcohol) was added, vortexed for 10 seconds and incubated at room temperature for 5 min. Centrifugation again at maximum speed for 5 min at 4 $^{o}$ C and transfer of supernatants to a new microcentrifuge tubes. Cold absolute ethanol 500 μl was added to the samples and stored in -20 $^{o}$ C for at least 30 min. Sample were then centrifuged for 10 min at maximum speed at 4 o C and supernatants were removed. Pellets were then washed with 500 μl of cold 75% ethanol (made with DEPC-$H_2O$). Ethanol was then removed and pellets were air dried for 5 min at room temperature and RNA resuspended in 50 μl of DEPC-$H_2O$ followed by a 5 min incubation period at room temperature. Finally, 2 μl of RNA was measured form each sample at 260/280 absorbance ratio (NanoDrop® ND-1000 Spectrophotometer) to determine RNA concentration and purity. RNA was stored at -80 C° for few days until cDNA synthesis.

*Reverse transcription reaction-cDNA synthesis*: Two micrograms of RNA from each sample were reverse transcribed in a total volume of 40 µl. Reverse transcription reaction mixture [8 µl of 5X reverse transcription buffer, 8µl of $MgCL_2$, 4µl of dNTP, 1µl of Random Primers, 1µl of RNase-inhibitors, 1µl of Reverse Transcriptase] was incubated at 45C˚ for 50 minutes, heated at 90C˚ for 10 minutes in order to discontinue the reaction and then quick-chilled at -80C˚ for 10-20 minutes. cDNA samples were stored at -20C˚ for subsequent RT-PCR analysis.

*Real time quantitative RT-PCR*: The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify mRNA transcription for each gene. The real time RT-PCR reaction mixture consisted of [2µl of cDNA, 1.3µl primer, 12.5 µl of TaqMan universal PCR master mix, 9.2µl RNase-free water] for a total volume of 25µl. The amplification profile involved an initial step at 50 ˚C for two minutes, a second step at 95 ˚C for ten minutes and followed by 40 cycles at 95 ˚C for 15 seconds and at 60 ˚C for one minute. All reactions were set up in duplicates and repeated once.

Analyses of the real-time quantitative PCR data were performed using the comparative threshold cycle [Ct] method as suggested by Applied Biosystems (User Bulletin #2). Ct values are defined as the PCR amplification cycle in which the reporter signal is greater than the minimal detection level, and Ct is inversely related to the relative abundance of a particular transcript. During PCR, the template DNA quantity doubles at each cycle. Therefore, the target quantity $X_{target}$ at threshold cycle $C$t is given by: $X_{target} = 2^{-\Delta CT}$.

The relative expression of different mRNAs was determined by relative quantification $\Delta CT = C_T$target $- C_T$β-actin, where β-actin represents the reference housekeeping gene.

Pairs of primers and TaqMan probes were designed by Applied Biosystems (inventoried TaqMan® gene expression assays). All assays were purchased from Applied Biosystems.

The sequences of probe, forward and reverse primer for TrkB was designed by Applied Biosystems: TrkB: (5'- CCAGGGCAGAGTCCTTCAG-3'); forward: (5'- TTCCCGCTGCCAGCAT-3'); reverse: (5'- CATCAGCTCGTACACCTC-3'). The assay ID of BDNF is the following (AppliedBiosystems, Rn00560868_m1). As an

internal standard housekeeping gene we utilized the expression of b-actin (AppliedBiosystems, Rn00560868_m1).

*The EPR measurements:* were carried out as described by Stadler et al. (2003). In brief, the measurements were carried out with an X-Band computer-controlled spectrometer, constructed by Magnetech GmbH (Berlin, Germany). Approximately 100 mg of tissue sample from the cervical region of spinal cord was frozen into a rod-shaped form and spectra of the sample was recorded at 77K using a quartz finger Dewar, filled with liquid nitrogen. Instrument settings were: 100 kHz modulation frequency, 0.7050 mT modulation amplitude, 18mW microwave power, 1 min scan time, and 20.63mT field sweep. For evaluation, double integration method of the EPR signals, with Mn/MnO as an internal standard, was used, and the data were expressed as arbitrary units.

## 2.4 Independent variables

In the present investigation, independent variables include the following:

1. There will be three non-exercised and three exercised groups. Non-exercised animals will be kept in sedentary state within their cages for ten weeks. On the other hand, exercised animals will be assigned to enforced exercise training (treadmill running) for ten weeks. Exercise frequency, intensity and duration will be as followed: during the first week animals will be exposed to treadmill running of low intensity 17m/min, for one hour, 5 days/week as an adaptation period. During the remaining nine weeks the intensity will be increased to 27m/min, for one hour, 5 days/week until the end of the study.
2. Oxidant, antioxidant and placebo administration: during the last two weeks of the study (weeks $9^{th}$ and $10^{th}$) all animals will be injected with either $H_2O_2$ (0.5 mM/kg), PBN (13mg/100gr of body weight) or placebo (saline) according to the experimental group each animal belong, as described earlier. All animals will be injected with the aforementioned substances, every other day for two weeks, one hour prior to exercise.

## 2.5 Dependent variables

In the present investigation the level of change induced by the effect of independent variables is expected in the following factors:

1. Free radical accumulation (EPR)
2. BDNF concentration (ELISA)
3. GDNF concentration (ELISA)
4. BDNF mRNA expression (RT-PCR)
5. TrkB mRNA expression (RT-PCR)
6. ERK1/2, phosphor-ERK1/2 (Western blot)
7. Oxidative protein damage (Western blot).

## 2.6 Treatment of Data

In the present investigation, thirty-six (N=36), healthy, male, Wistar rats were randomly assigned to one of six experimental groups, each group consisted of six animals (n=6): non-exercised control injected with saline (NEC), non-exercised injected with $H_2O_2$ (NEH), non-exercised injected with N-tert-butyl-α-phenylnitrone PBN (NEP), exercised control injected with saline (EC), exercised injected with $H_2O_2$ (EH) and exercised injected with PBN (EP).

## 2.7 Statistical analysis

Statistical tests were performed using STATISTICA 7, software. Initially, all dependent variables underwent a Normality test (Shapiro-Wilk's W test). Non-normally distributed variables include: free radical concentration and TrkB mRNA. To test differences among normally distributed variables we use one way ANOVA followed by Tukey HSD post-hoc test. Non-parametric test Kruskal-Wallis ANOVA by ranks was utilized to test differences among the non-normally distributed followed by Mann-Whitney U test when significant. Non-parametric Kendall Tau correlation test (non-parametric alternative to parametric Pearson's correlation) was utilized to test

relationships among groups since the variables of interest were not normally distributed. The significance level was set at $p<0.05$.

# 3 ANALYSIS OF RESULTS

## *3.1 Free Radical Accumulation (EPR Results):*

Statistical analysis of EPR data was performed by the non-parametric Kruskal-Wallis test ANOVA by ranks since the variable of interest was not normally distributed. Statistical analysis showed that significant differences existed among groups Kruskal-Wallis test ($H (5, N = 32) = 13.98$, $p<.05$), (Fig. 3.1). Statistical analysis was then followed by Mann-Whitney U test. EC group was shown to have significantly lower free radical concentration than the respective sedentary control NEC and NEH group ($p<0.05$). NEP group had significantly lower free radical concentration than both NEC and NEH groups but no significant difference observed between NEP and its respective EP group ($p>0.05$). A strong tendency of exercise decreasing free radical concentration was observed between EH and NEH groups ($p=0.06$) but it was not statistically significant (Fig. 3.2). Results expressed in percentage: The level of free radical concentration obtained from EPR measurements in cervical spinal cord of EC and NEP groups decreased to 73% and 79% ($p<0.05$) of control (NEC) respectively. In addition, NEP group decreased to 74% of NEH group ($p<0.05$). A strong tendency was observed in the cervical spinal cord of EH in which free radicals decreased to 81% of NEH group ($p=0.06$).

## *3.2 BDNF Protein Content:*

Statistical analysis of BDNF protein concentration was carried out by one way ANOVA. Statistical analysis indicated that significant differences existed among groups ($F (5, 30) = 6.49$, $p<0.05$). Tukey HSD post hoc test was then followed revealing the following significant differences among groups: EC group was shown to have significantly lower BDNF concentration than both NEC and NEH groups ($p<0.05$). Similarly, NEP group had significantly lower BDNF concentration than NEC, NEH and EH group respectively ($p<0.05$) (Fig. 3.3). Results expressed in percentage: BDNF

protein level in EC and NEP groups decreased to 49% and 48% ($p<0.05$) of control (NEC).

## 3.3 Free Radical accumulation and BDNF protein concentration:

When free radical concentration and BDNF protein content were plotted together a significant positive correlation was observed. Statistical analysis was carried out by the non parametric equivalent of analyzing relationships among variables, Kendall Tau test (0.50, $p<0.05$), (Fig. 3.4).

## 3.4 Protein oxidative damage (Western blot):

Protein damage in cervical spinal cord proteins was performed by Western blot. No significant differences were observed in the level of protein oxidation among groups (Fig. 3.5).

## 3.5 TrkB mRNA expression (RT-PCR data):

BDNF's receptor TrkB data analyzed by the non parametric test Kruskal Wallis ANOVA by ranks since the variable of interest was not normally distributed. Statistical analysis showed that no significant differences could be observed among groups ($p>0.05$), (Fig. 3.6, Fig. 3.7).

## 3.6 GDNF protein content:

Statistical analysis of GDNF protein concentration was carried out by one way ANOVA. Statistical test indicated significant differences among groups ($F(5, 30) = 3.89$, $p<0.05$). Post hoc test Tukey HDC specified the following significant group differences: NEH group was shown to have significantly higher GDNF protein concentration than the two other sedentary groups NEC and NEP ($p<0.05$) respectively. Results expressed in percentage: The level of GDNF protein in the cervical spinal cord

of the NEH group increased beyond 153% of control (NEC) and 140% of NEP group ($p<0.05$) (Fig. 3.8).

## *3.7 ERK1/2 Activation:*

Immunoblots demonstrated that ERK1 was mainly activated in groups EH and EC suggesting a possible exercise-associated ERK1 phosphorelation. On the other hand, ERK2 remained unaffected by the treatments utilized (Fig. 3.9).

Figure: 3.1 Free radical concentration

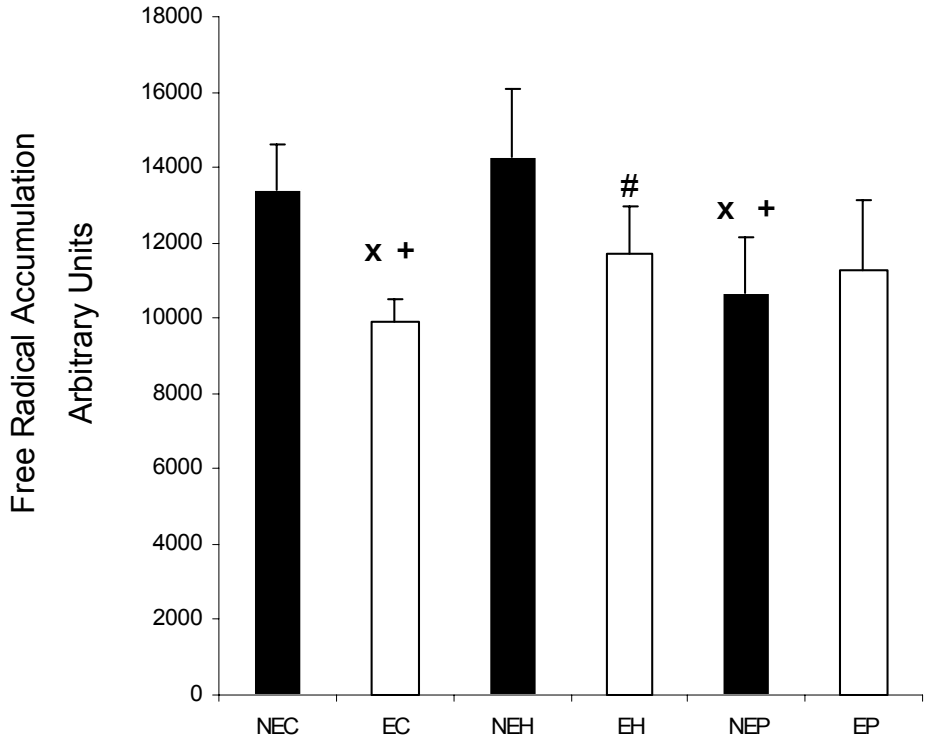



Fig: 3.1 Values are means ± S.D. for six animals. Exercise reduced significantly the ROS concentration in EC group ($^{x}$ $p<0.05$ vs. NEC and $^{+}$ $p<0.05$ vs. NEH) and with a strong tendency of reducing ROS concentration in EH group ($^{\#}$ $p=0.06$ vs. NEH). PBN administration reduced significantly the ROS concentration in sedentary animals ($^{x}$ $p<0.05$ vs. NEC and $^{+}$ $p<0.05$ NEH). Group identification: NEC, non-exercised control;

EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

Figure: 3.2 EPR Spectra

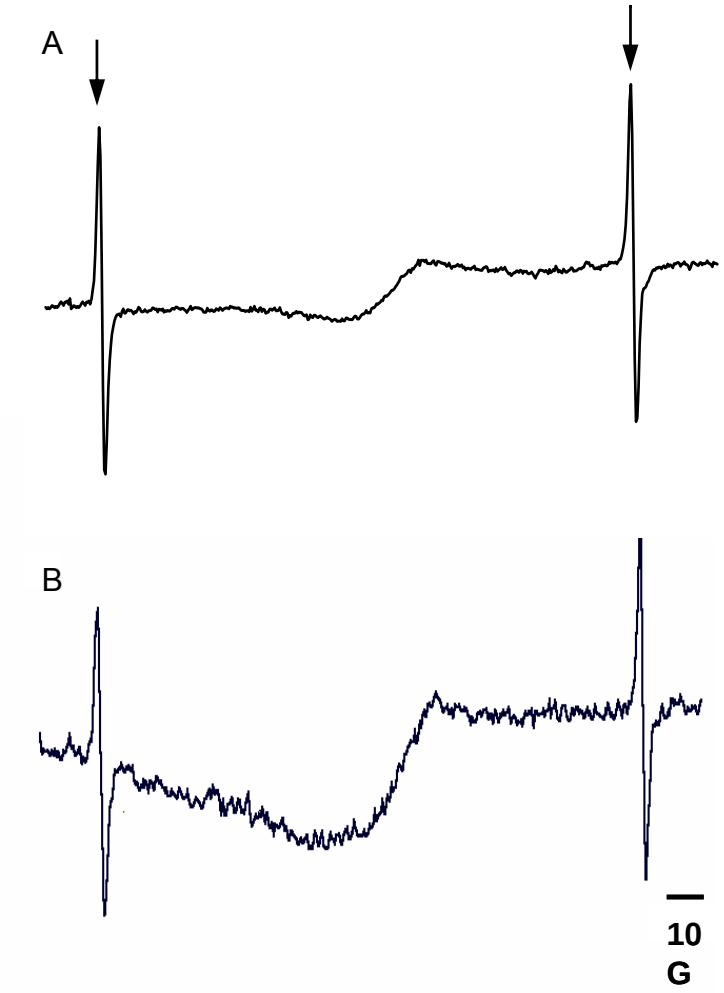



Fig: 3.2 EPR spectra taken *ex-vivo* at 77 K showing steady state native free radical concentrations in the cervical spinal cord of (A) exercised and (B) non exercised rats treated with $H_2O_2$. Spectra contain two Mn/MnO signals as internal standards, indicated by arrows. The area observed between the two signals of standards was double integrated for evaluation.

Figure: 3.3 BDNF Content

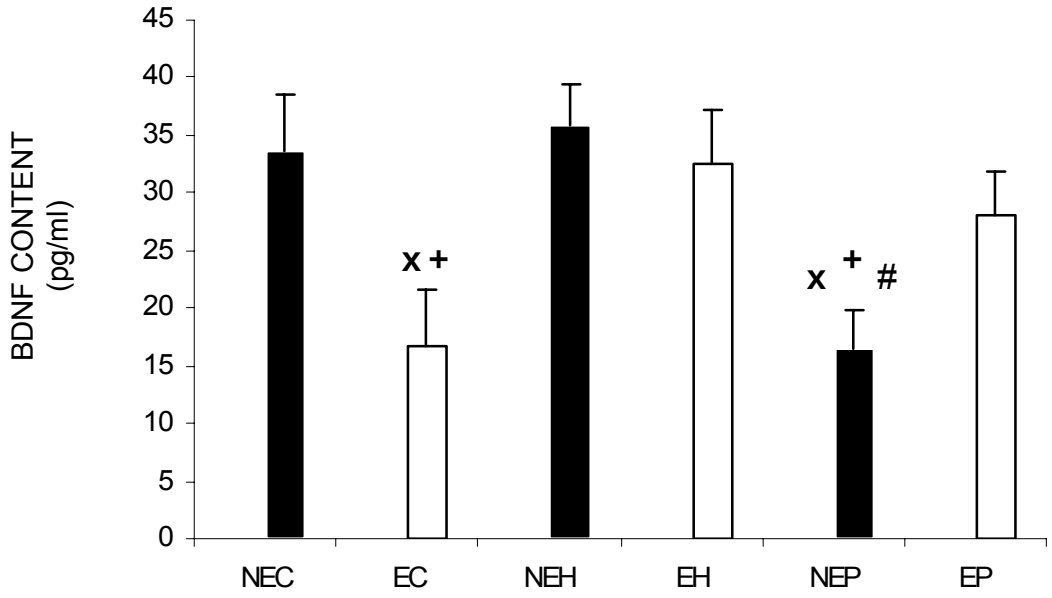



Fig: 3.3 Values are means ± S.D. for six animals. Exercise alone and PBN in sedentary animals significantly reduced BDNF content in cervical spinal cord region ($^{x}$ $p<0.05$ vs. NEC and $^{+}p<0.05$ vs. NEH). BDNF content remained significantly elevated in NEC and $H_2O_2$ treated groups NEH and EH ($^{x}$ $p<0.05$ vs. NEC, $^{+}$ $p<0.05$ vs. NEH, $^{\#}$ $p<0.05$ vs. EH). Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

Figure: 3.4 Free radical concentration/BDNF content

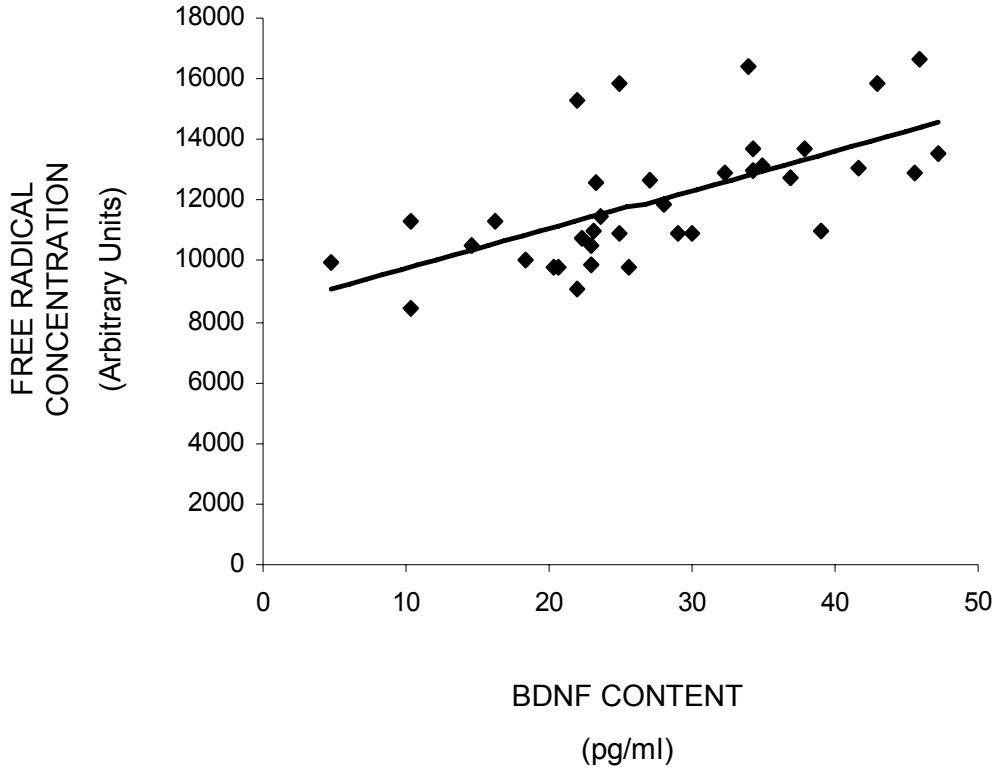



Fig. 3.4 The plot shows the positive correlation between Free Radical concentration and BDNF content in the cervical spinal cord region. Statistical analysis was performed with the non-parametric equivalent of analyzing relationships among variables Kendall Tau (0.50, $p<0.05$). Animals with higher ROS concentration had higher BDNF content and vice versa.

Figure: 3.5 Oxidative protein damage

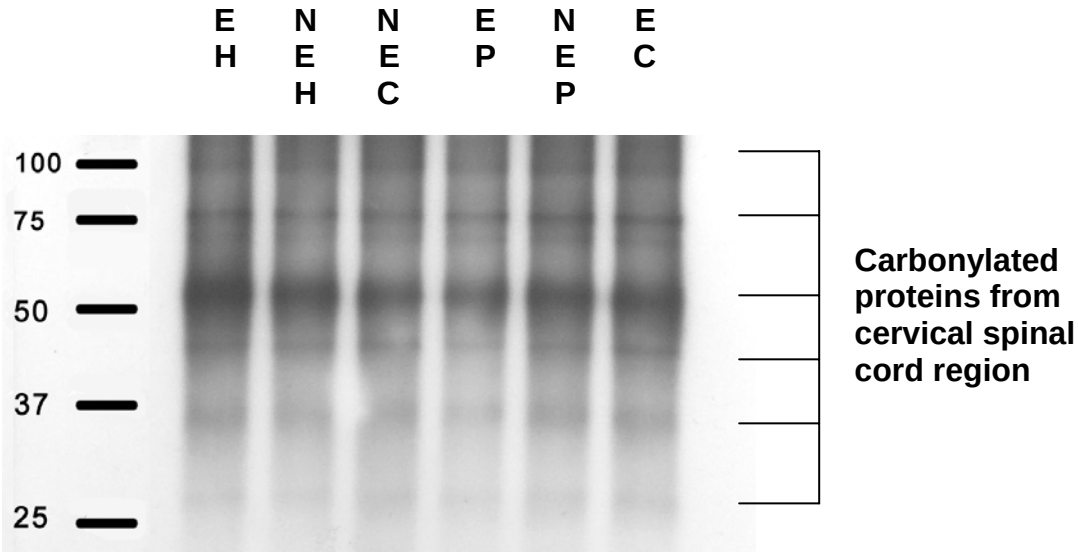



Fig. 3.5 The carbonylated proteins from cervical spinal cord region were determined from pooled tissue samples by Western Blot. The same amount of protein concentration from each group's pooled sample was loaded on a 12% polyacrylamide gel. No significant differences were observed in the level of carbonylated proteins among groups. Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

Figure: 3.6 TrkB mRNA expression

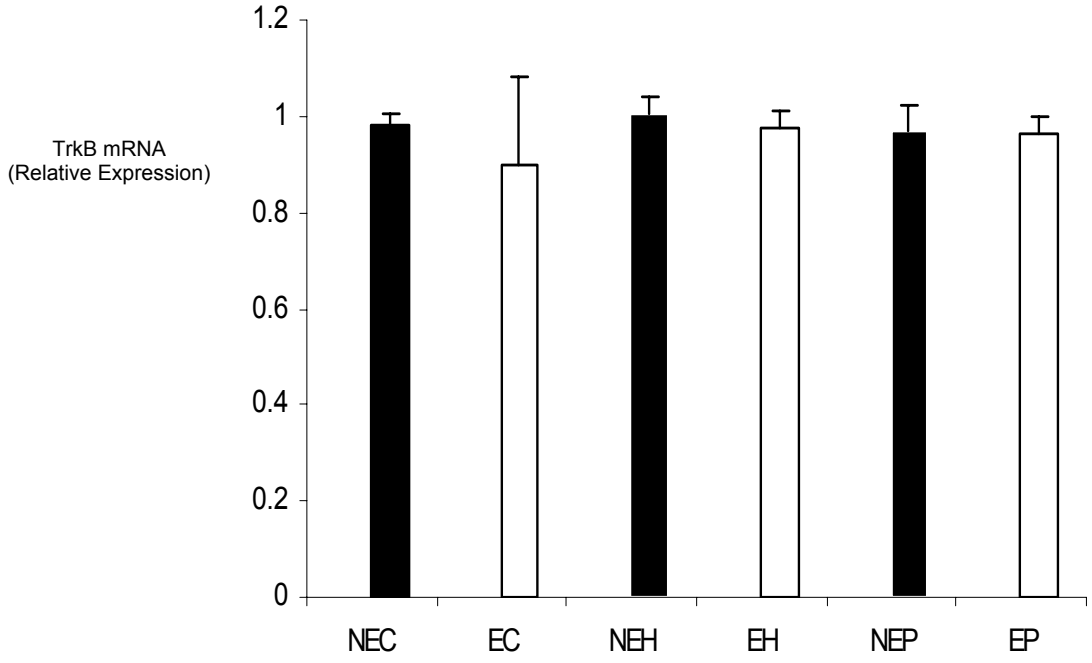



Fig.3.6 The bars show the mean relative expression of TrkB mRNA from cervical spinal cord homogenates. No significant changes were found among groups. Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

Figure: 3.7 TrkB mRNA expression in percentage of control (NEC)

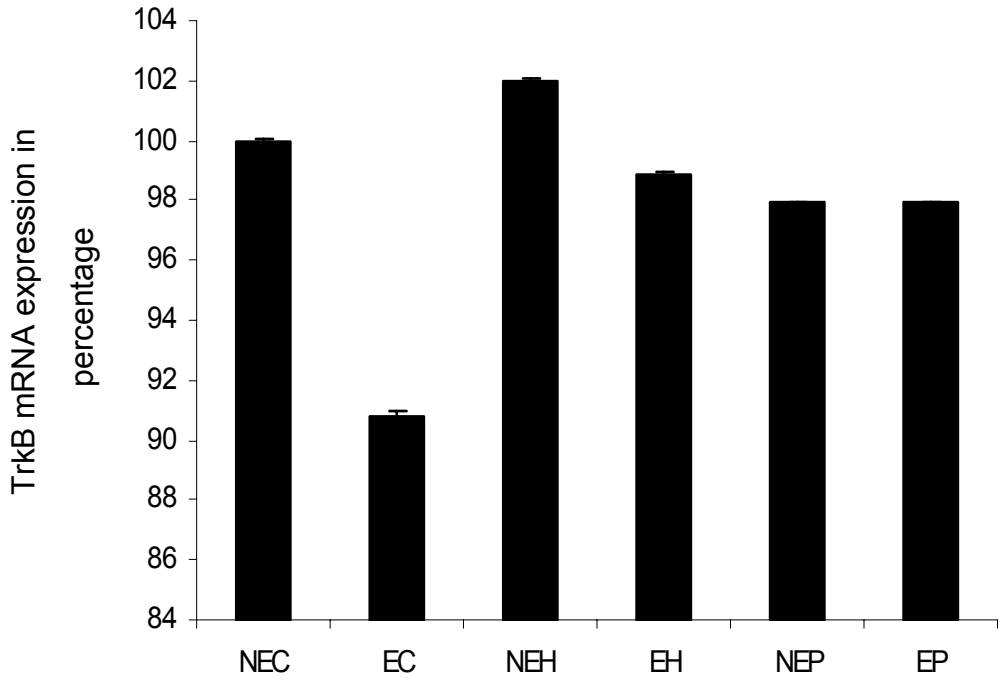



Fig. 3.7 The level of TrkB mRNA showed no significant differences among experimental groups.

Figure: 3.8 GDNF Content

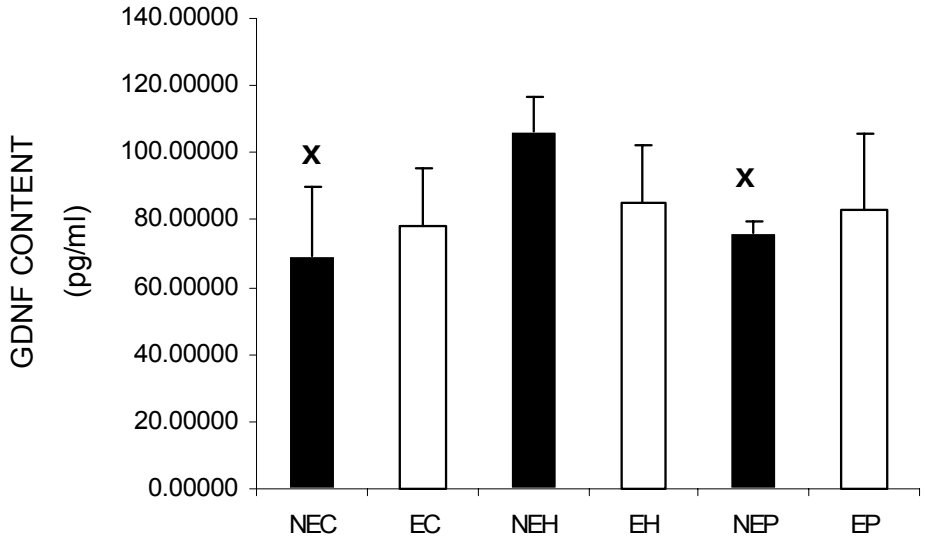



Fig. 3.8 Values are mean ± S.D. for six animals. $H_2O_2$ increased significantly GDNF content in cervical spinal cord region while PBN in sedentary animals kept GDNF production at control levels ($^{x}$ $p<0.05$ vs. NEH). Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

Figure: 3.9 ERK1/2 Activation

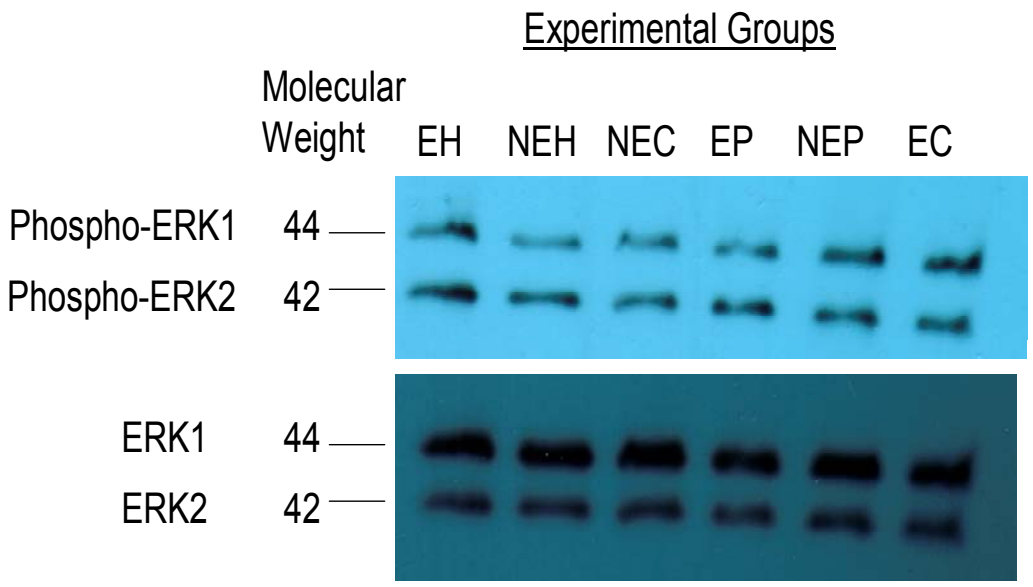



Fig.3.9 Representative immunoblots showing the activation of ERK1/2 in the respective experimental groups. The upper immunoblot indicates the phosphorelated form (activated form) of ERK1/2. The lower immunoblot indicates the un-phosphorelated form of ERK1/2 and serves as the internal standard. The immunoblot showing the activated form of ERK1/2 indicates that ERK1 was mainly activated in groups EH and EC suggesting a possible exercise-associated ERK1 phosphorelation. On the other hand, ERK2 remained unaffected by the treatments utilized. Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised $H_2O_2$ injected; EH, exercised $H_2O_2$ injected; NEP, non-exercised PBN injected and EP, exercised PBN injected.

# 4 DISCUSSION

## *4.1 Interpretation of Results*

The spinal cord is the main pathway for information connecting between brain, peripheral nervous system and musculature, and its ability to cope with oxidative stress related challenges could have vital importance. Regular exercise results in systemic adaptation to the body (Radak et al. 2008) although the oxidative stress associated adaptation of spinal cord is poorly investigated. Therefore, the present investigation was designed to evaluate the effects of regular exercise, oxidant and antioxidant treatment of neutrotrophin level of spinal cord.

The results revealed that chronic exercise training decreases the level of free radicals in the cervical section of the spinal cord of the exercised control (EC) group, as was initially hypothesized, based on our earlier results. A previous investigation from our laboratory showed that chronic swimming training decreased free radical concentrations in the cerebellum (Radak et al. 2006). As well, habitual treadmill running has been demonstrated to decrease hydroxyl radical concentrations in plasma and soleus muscle (Itoh et al. 1998). This decrease in free radical concentration in the spinal cord of the exercised group could be related to an up-regulated antioxidant enzyme capacity as a result of chronic training (Itoh et al. 1998; Somani et al. 1994) or a result of more tightly controlled ROS generating systems.

On the other hand, our hypothesis that a higher level of oxidative challenge through exercise and oxidant administration could further diminish free radical concentration could not be supported. Even though oxidant addition did not result in further lowering of free radical concentration in the exercised groups, a strong tendency of exercise to decrease free radical concentration was observed in the exercised-$H_2O_2$ injected group (EH).

The magnitude of the oxidative stress which can be tolerated by the spinal cord is vital, since spinal cord injuries easily cause massive oxidative stress resulting in apoptosis and necrosis and this can be attenuated by pre-conditioning (Li et al. 2007). Mild oxidants challenged cells are increasing their viability through complex

adaptation, which results in maintained physiological function in a condition that induces adaptation to normal cell population (Contreras et al. 2005).The strong tendency of lowering free radical concentration may suggest a preconditioning effect of exercise against $H_2O_2$ (Radak et al. 2000).

PBN is reported to have multiple pharmacological activities to reduce ROS, including the ability to trap alkoxyl radicals, superoxide radicals, hydroxyl radicals and decrease inducible cytochrome (cyclooxygenase-2) as well as inhibit nuclear factor-κB transduction (Lapchak et al. 2001). As suspected PBN intervention in the non-exercised group (NEP) was shown to be effective in decreasing free radical concentration as compared to the non-exercised control (NEC) and non-exercised $H_2O_2$ groups (NEH). This finding is supported by other studies, which have reported a neuroprotective effect of PBN which was derived by successfully attenuating hydroxyl radical in rat striatum (Lancelot et al. 1997) and by suppressing $H_2O_2$ production in isolated brain mitochondria (Foyd 1999).

On the other hand, the combined effects of exercise and PBN did not result in a decreased free radical level, possibly due to an antioxidant interaction in the exercise-induced adaptation process. Although, data on spinal cord is not known, in skeletal muscle it has also been shown that allopurinol, a potent inhibitor of xanthine oxidase, attenuates the exercise-induced adaptation to ROS (Gomez-Cabrera et al. 2006).

The level of oxidative protein damage, assessed by RCD was not changed as a result of regular exercise, oxidant and antioxidant treatment. Toldy et al. (Toldy et al. 2005) have similarly reported no effect of regular exercise on brain RCD. The significant differences observed in EPR data and the unaltered RCD suggest that the level of free radicals is not the only determinant of oxidative damage occurrence, but rather, the shift of balance between endogenous oxidants and antioxidants is what determines oxidative damage. It should be noted that absence of significant differences does not necessarily imply the lack of a biological effect, since redox homeostasis mechanisms have several levels of control.

Evidence suggests that BDNF and its cognate receptor are up-regulated by physical activity in both brain and spinal cord (Cotman and Berchtold 2002) and linked to better function. Actually, we have also observed improved spatial memory by regular exercise (unpublished data), but it appears that it is not associated with higher

concentration of BDNF protein or mRNA level of TrkB in the spinal cord, emphasizing the importance of local environment.

In the present study, to our surprise, BDNF actually decreased significantly as a result of exercise (EC vs. NEC). Similar findings have been reported recently by Engesser-Cesar et al. (Engesser-Cesar et al. 2007) who reported a decrease in the level of BDNF in the thoracic region of spinal cord of mice, with exercise. One explanation for this chronic exercise-induced BDNF decrease could be associated with improved motor coordination as a result of motor learning. Improved motor coordination would lead to a more economic function resulting, consequently, in a fewer number of motor units being activated and resulting in less neural activity and thus decreased BDNF production.

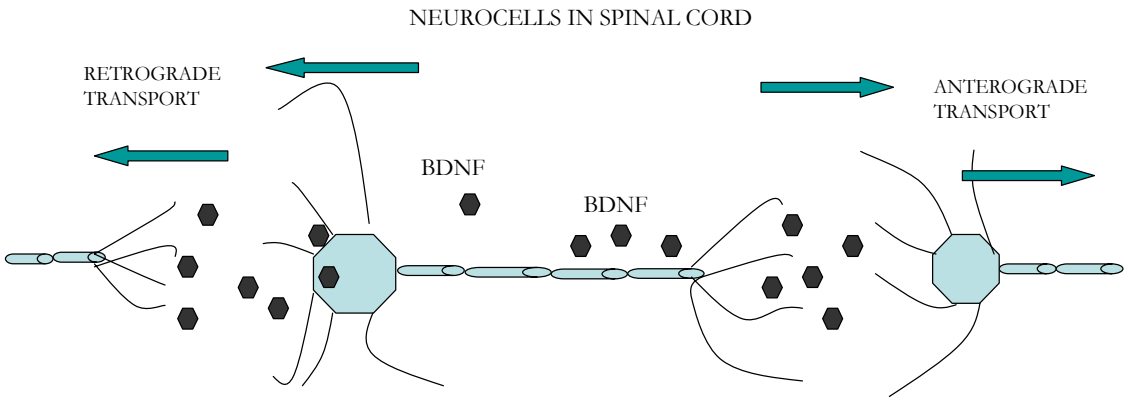
This hypothesis requires a great deal of further investigation especially since BDNF was similarly decreased in the non-exercised group treated with PBN. In addition, it appears that exercise with low intensity increases, but with high intensity decreases the BDNF content in frontal cortex of the brain (Aguire et al. 2007; Aguire et al. 2008) suggesting that exercise with moderate intensity could be more beneficial to brain in terms of BDNF level. It cannot be excluded that similar phenomenon could occur at the spinal cord, which explains why BDNF concentration decreased in our study.

The sensitivity of BDNF on ROS could be due to fact the cAMP-responsive element-binding protein which is one of the transcription factors of BDNF is redox sensitive (Galeotti et al. 2005). Indeed, groups with the lowest BDNF levels have been shown to have the lowest ROS concentrations and positive significant correlation was acquired between BDNF and ROS concentration. This is in accordance with the finding of Wang et al. (Wang et al. 2006a ; Wang et al. 2006b) which reveals, at least in cell culture, BDNF production from PC12 and brain microvascular cells was stimulated by ROS, while antioxidants prevented this increase (Wang et al. 2006b).

Interestingly, in the present study, although BDNF protein was present in the cervical portion of spinal cord BDNF mRNA could not be detected by RT-PCR. Double checking of BDNF expression revealed that indeed the cervical part of spinal cord did not express BDNF in any of the groups. Our results, reporting the presence of BDNF protein in the area but absence of BDNF mRNA suggest that BDNF protein could have

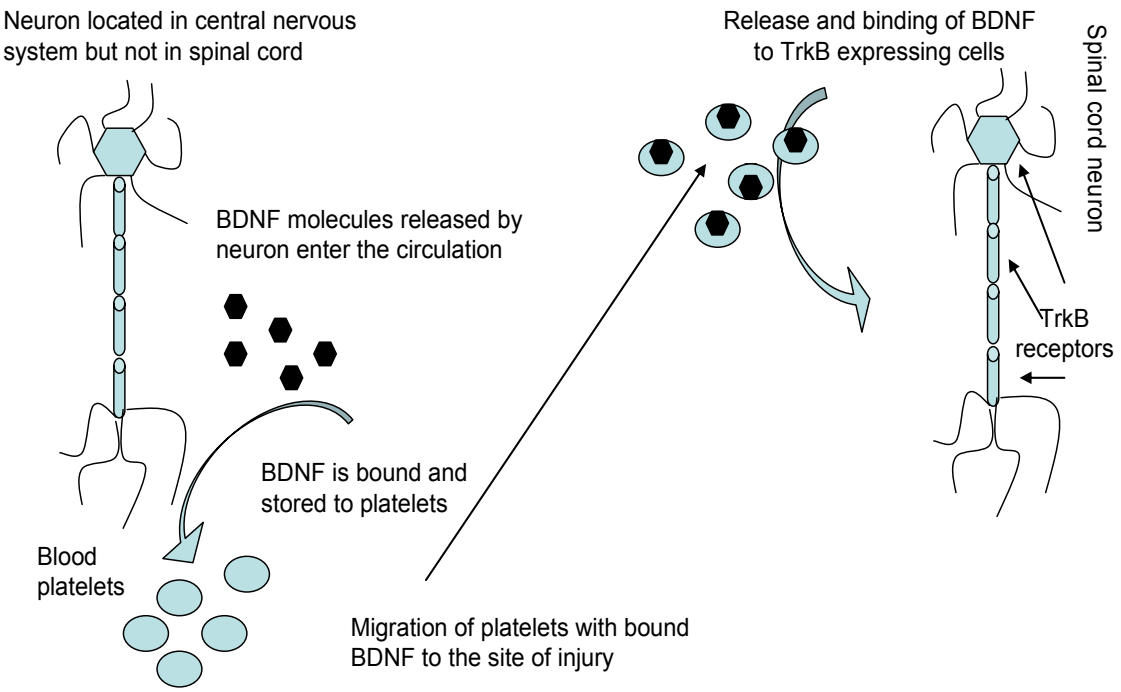
been expressed elsewhere and transported to cervical region possibly by two ways: 1) since BDNF can be both autocrine and paracrine agent (Cotman and Berchtold 2002), it is possible to be expressed in the lumbar region as most of the studies report and transported retrogradely to cervical region (figure 4.1.1), 2) BDNF could have been expressed elsewhere in the CNS and transported to spinal cord through the blood, specifically, bound to platelets and released to cells expressing TrkB (figure 4.1.2) (Radka et. al. 1995; Fujimura et. al. 2002). Platelets appear to bind, store and release BDNF upon activation at the site of traumatic injury to facilitate the repair of peripheral nerves or other tissues that contain TrkB (Fujimura et. al. 2002).

Fig. 4.1.1 BDNF can function as both autocrine and paracrine agent




4.1.1 BDNF could have been expressed in the lumbar region of spinal cord and transported retrogradely to cervical region. This assumption could explain the presence of BDNF protein in the cervical region while absence of BDNF expression was evidenced as shown by the RT-PCR measurement.

Fig. 4.1.2 BDNF's transportation bound to blood platelets




4.1.2 The figure shows a possible explanation referring to the presence of BDNF protein in cervical spinal cord although no expression of BDNF mRNA was detected. It has been demonstrated that BDNF protein can enter circulation where it is bound and stored to platelets. Further on, blood platelets with the stored BDNF protein molecules migrate to the site of injury where BDNF is released and bound of course only to TrkB expressing cells.

Previous investigations have shown that ERK1/2 phoshorylation is associated to exercise training and is has been connected to cyclic AMP response element binding protein activation whose phosphorylation leads to BDNF gene transcription (Shen et al. 2001; Vaynman et al. 2003). It has been shown that physical activity elicits sustained activation of the cyclic AMP response element-binding protein and mitogen-activated protein kinase in the rat hippocampus (Shen et al. 2001).

However, results of these studies report a confused time-course of induction of this signaling molecule and a variable effect over CREB activation. Specifically it has been shown that MAPK was significantly necessary for exercise-induced CREB phasphorylation but not as significantly important for exercise-induced BDNF induction. In addition, the time-course of MAPK activation was shown to be insensitive as early as 4 h and 3 days after exercise while its activation persisted for 1 month and became insignificant after 2 months of continuous exercise.

In the present investigation, our data shows that after 10 weeks of forced exercised training the level of activated ERK1 was higher in EC and EH groups while ERK2 was not different among groups. This result suggests that the exercised group's BDNF content was based upon ERK1 signaling pathway. On the other hand, the level of ERK1 activation and the overall BDNF content do not match each other possibly suggesting that the BDNF induction was carried out by another pathway of CREB's phosphorylation in non-exercised and EP groups.

GDNF protein has been shown to be produced in significantly greater extent in the non-exercised group injected with $H_2O_2$ (NEH) than in the other non-exercised groups (NEC and NEP). A number of studies associate GDNF elevation with a neuroprotective effect in neurons undergoing oxidative stress. Accordingly, GDNF has been previously shown to rescue neuro cells exposed to $H_2O_2$ (Onyango et al. 2005) and free radical-mediated cell damage (Lingor et al. 1999). The neuro-protective effect of GDNF has been linked to increased antioxidant enzyme activities (Chao and Lee 1999). Exercise, on the other hand, induced no alteration in spinal cord GDNF content. Widenfalk et al. (Widenfalk et al. 1999) similarly reported insensitivity of this neurotrophin to exercise stimulus in hippocampus.

The results of this study suggest that regular exercise and PBN administration in non-exercised animals are effective means of decreasing free radical concentrations in

the cervical region of the spinal cord. This is of primary clinical importance since exercise can provide an effective preconditioning stimulus over oxidative stress-associated diseases or oxidative stress associated with traumatic spinal cord injury. PBN may as well serve as a direct means of attenuating ROS.

Apparently, at least in the present investigation, the levels of ROS seem to play a role in determining BDNF protein content in the cervical spinal cord and GDNF release seems to be $H_2O_2$ sensitive and its production may be associated with survival promoting effects. Absence of BDNF mRNA expression in cervical region of spinal cord suggests that the detected BDNF protein was possibly transported there either retrogradely from lumbar region or expressed elsewhere in CNS and transported there through the blood. ERK activation signaling pathway appears to be not the only pathway of BDNF induction under the present experimental conditions. The data obtained in the present study, do support the hormesis theory.

# 5 CONCLUSIONS

## *5.1 Conclusions*

Following our results and the limitations of our study we conclude that:

1. Chronic exercise decreased the free radical concentration in the cervical spinal cord of exercised control rats. PBN was effective in decreasing free radical concentration only in non-exercised animals while PBN in exercised animals seems to attenuate the ROS-induced adaptive responses to exercise. Thus, chronic exercise, and PBN administration in non-exercised animals are effective means in reducing oxidative stress in cervical spinal cord of young male Wistar rats.
2. In contrast with other studies, the present investigation reported a decrease rather than increase in BDNF concentration with exercise. PBN in non-exercised animals could similarly induce significant reduction in BDNF protein level. In the present study chronic exercise alone and PBN administration in non-exercised animals induced down-regulation in BDNF protein concentration. Interestingly, ROS concentration and BDNF protein concentration are significantly and positively correlated. That is, animals with higher ROS concentration had higher BDNF protein concentration and vice versa. Apparently, at least in the present study the level of ROS concentration in the cervical spinal cord of young male Wistar rats seems to determine the level of BDNF protein concentration.
3. No differences were observed in oxidative protein damage among group's pooled samples indicating that the accumulation of free radicals were tolerated by the spinal cord cells and that the increase was still in the physiological range of the redox homeostasis. This suggests that the level of ROS is not the only determinant for oxidative damage to occur.
4. According to the findings of the present study, activation of ERK is not the only pathway responsible for BDNF protein induction under the present experimental conditions.

5. In the present investigation $H_2O_2$ seems to be the major determinant of GDNF protein concentration in cervical spinal cord. Exercise showed to have no effect in inducing GDNF release.
6. Lack of BDNF mRNA expression in cervical region suggests that the detected BDNF protein in the area was transported there either retrogradely form lumbar region or BDNF was possibly expressed elsewhere in CNS and transported there through the blood.

## *5.2 Recommendations for Further Research*

Based on the findings of this study, the following recommendations are offered for further research.

1. Along with free radical concentration and protein oxidative protein markers assessment, it is recommended that assays of major antioxidant enzymes detection should be utilized in order to examine the contribution of enzymatic antioxidants in the level of free radicals and protein carbonyls level.

# 6 SUMMARY

## *6.1 Summary*

The purpose of this study was to investigate the effect of chronic enforced exercise and administration of hydrogen peroxide ($H_2O_2$), N-tert-Butyl-α-phenyl-nitrone (PBN) on reactive oxygen species concentration (ROS), oxidative damage markers and neurotrophins (NTs) release in cervical spinal cord of rats. Specifically, NTs investigated include the brain-derived neurotrophic factor (BDNF), glial cell lined-derived neurotrophic factor (GDNF) and the mRNA of BDNF and BDNF's tyrosine kinase receptor (TrkB) with the mitogen-activated protein kinase (MAPK) superfamily signaling molecule, extracellular signal kinase (ERK1/2). One of our major goals was to elucidate whether there is any kind of relationship (causative or associative) between ROS concentration and BDNF release and that antioxidant treatment might attenuate exercise-induced adaptive responses. In order to test our hypothesis and examine the physiological response of spinal cord to exercise and oxidant-antioxidant administration, thirty-six, five month old, healthy, male Wistar rats were used in the study. Six rats were randomly assigned to each of six groups: non-exercised control injected with saline (NEC), non-exercised injected with $H_2O_2$ (NEH), non-exercised injected with N-tert-butyl-α-phenylnitrone PBN (NEP), exercised control injected with saline (EC), exercised injected with $H_2O_2$ (EH) and exercised injected with PBN (EP). For the determination of free radical concentration we used an Electron Paramagnetic Resonance (EPR), for the detection of BDNF and GDNF proteins we utilized the enzyme linked immunoassay (ELISA), the estimation of oxidatively modified spinal cord proteins was performed by Western blot and for the detection and determination of the relative mRNA expression of BDNF and TrkB we utilized RT-PCR. Results of our study indicated that the data obtained from EPR measurement revealed that EC group had significantly lower accumulation of free radicals as compared to NEC group. Similarly there was a tendency of significantly lower free radical accumulation in EH group as compared to NEH. NEP had significantly lower concentration of free radical accumulation than the other non-exercised groups NEC and NEH. In contrast to the

other exercised groups EP group had higher free radical accumulation from its respective NEP group but no significant difference was observed. Oxidative damage of proteins as indicated by Western blot showed no differences among the pooled samples of the groups, indicating that the accumulation of free radicals were tolerated by the cells and that the increase was still in the physiological range of the redox homeostasis. BDNF protein level was significantly decreased in EC and NEP groups as compared to NEC and NEH groups respectively and in NEP group as compared to EH group. Interestingly when free radical concentration and BDNF protein concentration are plotted together in a correlation plot a significant positive correlation is acquired, suggesting either an associative or a causative relationship. The mRNA level of BDNF receptor, TrkB, was expressed in all groups but no significant differences were observed. In contrary, BDNF mRNA expression could not be detected with RT-PCR. The pattern of TrkB expression was significantly and positively correlated with the BDNF protein concentration. Exercise-induced ERK activation was observed only in EC and EH group while EP and non-exercised groups BDNF induction seemed to be independent of ERK pathway activation. The level of GDNF protein was significantly increased in NEH group as compared to NEC and NEP groups suggesting that $H_2O_2$ injection altered the content of GDNF.

## 6.2 Összefoglalás

Kutatásunk célja az volt, hogy megállapítsuk, milyen hatást gyakorol a rendszeres fizikai aktivitás, valamint a hidrogén-peroxid és a N-tert-Butyl-α-phenyl-nitrone (PBN) adagolás a szabadgyök (ROS) koncentrációra, illetve az oxidatív károsodást jelölő markerek és neurotrofinok (NTs) felszabadulására, patkányok nyaki gerincvelői szakaszán. A specifikusan vizsgált anyagok közé tartozott az agy eredetű neurotrofikus faktor (BDNF), a glia sejtvonal-eredetű neurotrofikus faktor (GDNF), valamint a BDNF mRNS-e és tyrozin kineáz receptora (TrkB), továbbá a MAP-kinázokhoz tartozó, sejten kívüli jelző kinázok (ERK 1/2). Egyik fő célunk volt annak tisztázása, hogy van-e bármiféle kapcsolat (okozati vagy asszociatív) a szabadgyök koncentráció és a BDNF felszabadulás között, illetve annak a feltételezésnek az alátámasztása, hogy az antioxidáns kezelés csökkentheti az edzéshatásra kialakuló adaptív választ. Hipotézisünk vizsgálatára harminchat, öt hónapos, egészséges, hím Wistar patkányt vontunk be a kísérletbe. Minden csoportba random módon hat állatot választottunk ki: salinnal kezelt, nem edző kontrol állatok (NEC), hidrogén-peroxiddal kezelt, nem edző állatok (NEH), PBN-nel kezelt, nem edző állatok (NEP), salinnal kezelt, edző állatok (EC), hidrogén-peroxiddal kezelt, edző állatok (EH), PBN-nel kezelt edző állatok (EP). A szabadgyök koncentráció meghatározására EPR-t használtunk, a BDNF és GDNF fehérje koncentrációt E-MAX Immuno Assay System segítségével határoztuk meg a gerincvelőből. A reaktív carbonyl gyökök mennyiségi meghatározása Western blot útján történt, míg a BDNF- és a TrkB relatív mRNS mennyiségének detektálására és meghatározására RT-PCR-t használtunk. Vizsgálatunk eredménye azt mutatja, hogy a cervikális szakaszon történő EPR mérésekből megállapított szabadgyök koncentráció értéke az EC csoport estében szignifikánsan alacsonyabb a NEC csoporthoz képest. Hasonlóképp, az EH csoport gerincvelőjében mért szabadgyökök mennyisége szignifikánsan kevesebbnek bizonyult, összehasonlítva a NEH csoporttal. A nem edző csoportok közül a legalacsonyabb ROS koncentrációt a NEP csoport estében lehetett kimutatni. Az oxidált fehérjék mennyisége – melynek megállapítására a Western blot méréssel kimutatott carbonylált proteinek szolgáltak – nem mutatott különbséget a csoportok között, ami feltételezi a sejtek toleranciáját a felgyülemlett szabadgyökökkel szemben, illetve, hogy az emelkedett oxidáns szint a redox homeosztázis fiziológiás

tartományán belül maradt. A BDNF fehérje szint alakulása az EC, valamint a NEP csoportok estében szignifikáns csökkenést mutatott a NEC és NEH csoportokhoz képest, valamint a NEP csoport az EH csoporthoz képest is. Érdekes módon, a szabadgyök koncentráció, valamint a BDNF koncentráció közös grafikonja szignifikáns pozitív korrelációt mutat, mely asszociatív kapcsolatot feltételez. A BDNF receptor mRNS szintjét (TrkB) meghatároztuk az összes csoport estében, de szignifikáns különbséget nem találtunk. Viszont BDNF mRNS-t egyik csoportban sem tudtunk kimutatni a cervikális régióból. Az immunoblot eredmények azt mutatják, hogy az ERK1 aktivitás leginkább az EH és az EC csoportok esetében növekedett, míg az EP és a nem edző csoportok esetén a BDNF indukció független az ERK aktivitástól. A gerincvelői GDNF fehérje a NEH csoport esetében szignifikáns emelkedést mutatott a NEC és NEP csoporthoz képest, mely eredmény alapján megbízhatóan feltételezhetjük a hidrogén-peroxid stimuláló hatását a GDNF szintre.

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