Chapter 14

Animal Models for Chronic Stress-Induced Oxidative Stress in the Spleen: The Role of Exercise and Catecholaminergic System

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Abstract

We examined the effects of daily exercise on the gene expression of catecholamine biosynthetic enzymes (tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), and phenyl ethanolamine N-methyltransferase (PNMT)), vesicular monoamine transporter 2 (VMAT 2), antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)), concentrations of catecholamines (noradrenaline (NA) and adrenaline (A)) and malondialdehyde (MDA), activities of monoamine oxidase (MAO), and antioxidant enzymes in the spleen of chronically psychosocially stressed rats. Exposure of chronically stressed rats to exercise increased the levels of PNMT protein by 19%, VMAT 2 mRNA by 100%, NA by 160%, and A by 140%; decreased/unchanged MAO enzyme activity; returned concentrations of MDA to control level; and increased CAT and GPx mRNA levels (50% and 150%, respectively). Exercise induced the accumulation of the catecholamines and a decrease of stress-induced oxidative stress in the spleen, which may significantly affect the immune-neuroendocrine interactions in stress conditions. Also, exercise induced the catecholaminergic system and antioxidant defense to become more ready to a novel stressor, which indicates that exercise may induce potentially positive physiological adaptations. Our combined model of chronic social isolation and long-term daily treadmill running in rats may be a good animal model in the research of therapeutic role of exercise in human disease caused by chronic stress.

Keywords: treadmill running, chronic social isolation, catecholamine, antioxidant enzymes, spleen, rats

1. Introduction

Many studies have shown that stress disturbs homeostasis, which induces various disorders. A number of diseases and pathological conditions are related to the long-term adaptive response to stress, particularly under conditions of chronic stress when allostasis can shift from a healthy toward a pathological state [1]. Chronic stress induces behavioral, endocrine, and immune changes in animals [2, 3]. It is known that stress affects a rapid rise of plasma and tissue catecholamines, including the spleen [4]. Data from literature indicate that the sympathetic nervous system (SNS) is one of the major pathways involved in immune-neuroendocrine interactions. The regulation of immunity by sympathetic noradrenergic nerve fibers in the lymphoid organs has been demonstrated by the distribution of tyrosine hydroxylase (TH) nerve fibers, by the presence of adrenoreceptors on the immune system cells, and by immunomodulatory role of noradrenaline (NA) [5]. For example, adrenaline (A) and NA produced by sympathetic nerves may modulate cellular function by acting on β-2 adrenergic receptors of B and Th1 cells [6]. In addition, catecholamine biosynthetic enzymes are expressed in the lymphoid organs [7], as well as in neutrophils and macrophages [8]. It is known that normal catecholaminergic turnover results from balance among synthesis, release, and reuptake of catecholamines. Because of the significant role of catecholamines in neuroendocrine-immune network in stress response, detection of regulatory mechanism for catecholamine synthesis, degradation, release, and reuptake in the spleen in conditions provoked by chronic stress is exceptionally relevant in stress biology, due to its potentially negative impact on immune functions and health. Effective management of stress depends on the ability to identify and quantify the effects of various stressors and determine if individual or combined stressors have distinct biological effects [9]. Animal models have contributed considerably to the current understanding of mechanisms underlying the role of stress in health and disease [10]. It is known that animal model of chronic stress isolation (CSI) produces increased concentrations of catecholamines in the plasma and decreased gene expression of catecholamine biosynthetic enzymes in the spleen, which can modulate the immune function [11]. However, very little is known about the impact of long-term exercise on the catecholaminergic turnover and the antioxidant defense system in the spleen of chronically psychosocially stressed rats. Because of the potential therapeutic role of physical exercise, we investigated whether a combined animal model of chronic isolation and treadmill running in rats (CSITR) may be a good animal model for chronic stress research as well as the benefits of exercise on neuroendocrine and immune functions in stress conditions. Our CSITR animal model was achieved by exposing the individually housed rats to the daily treadmill running for a 12-week period. We opted for long-term daily treadmill running because the short intensive physical activity may induce oxidative stress, while it is not the case with sport-specific activity of longer duration [12]. In addition, we exposed the experimental animals to additional acute immobilization stress, because we wanted to examine whether daily treadmill running induced potentially positive adaptations of the splenic catecholaminergic turnover and antioxidant protection in stress conditions.

We investigated how long-term daily 20 min treadmill running affected the gene expression of key enzymes involved in catecholamine biosynthesis (TH, dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT)), storage (vesicular monoamine

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transporter (VMAT) 2) and degradation (monoamine oxidase (MAO)), as well as the concentrations of catecholamines (NA and A) in the spleen of chronically psychosocially stressed adult rats. Transcription factor cAMP response element-binding protein (CREB) plays a major role in regulation of TH and DBH gene expression during exercise [13]. This chapter discusses the effect of physical exercise on the level of CREB mRNA in the spleen of chronically stressed rats. As the rise in catecholamine catabolism results in increased reactive oxygen species (ROS) production, we measured the concentration of malondialdehyde (MDA), as well as gene expression and activity of the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)). Also, we examined how the additional acute immobilization stress changed the mentioned parameters. In the study, we presumed that physical exercise in chronically stressed rats may induce the potentially positive physiological adaptations of the splenic catecholaminergic turnover, as well as antioxidant protection and oxidative damage repair.

Detecting regulatory physiological mechanism by which physical activity changes catecholaminergic turnover and antioxidant defense system in the spleen in conditions provoked by chronic stress is important in the prevention of immune diseases caused by chronic stress. Also, these results may confirm whether CSITR could be a good animal model in the search for beneficial impact of exercise on neuroendocrine and immune functions in stress conditions.

2. Animal model of chronic social isolation in rats

Many authors have confirmed that animal models are essential to biological research. Chronic individual housing of rats, frequently termed "isolation stress," represents a very strong psychosocial stress [3, 14], which can induce neuroendocrine changes [15] and increased activity of the antioxidant defense system [16, 17] in animals. Also, isolation stress affects different behavioral processes in animals. For example, social isolation led to a reduced duration of grooming and a prolonged latency period to the onset of grooming [18]. In addition, social interactions are an important source of human stress. Social isolation has deleterious effects on health and therefore is regarded as one of the most relevant causes of diseases in mammalian species [14]. For example, it is a risk factor for human depression [19].

Animal models of chronic social isolation (CSI) consisted of 11-week-old Wistar male rats that were subjected to social isolation, with a single animal per cage for 12 or 3 weeks [11, 15]. The visual and olfactory communication among the isolated rats was reduced to the minimal level.

It is known that exposure of an organism to a social isolation leads to the engagement of several hormonal and neurotransmitter systems in the stress response. Chronic social isolation of adult rat males produces a depletion of brain catecholamine stores but no changes in heart auricles and adrenal glands [15]. In addition, CSI of adult rat males decreases the gene expression of catecholamine biosynthetic enzymes in the adrenal medulla [20] and increases concentrations of catecholamines in the plasma [11]. Also, CSI induces an increase in the gene expression of noradrenaline biosynthetic enzymes in stellate ganglia, which may be connected to the increased

risk of cardiovascular diseases [21, 22]. In addition, the gene expression of splenic catecholamine biosynthetic enzymes is decreased after CSI. This might reduce catecholamine synthesis in the spleen and deplete the immunocompetent tissues of catecholamines which cause an impairment of immune response [11]. Key question in adaptive response to stress is how the addition stressor can elicit a variant or altered response depending on prior experience with the current or different stressor. A potentiation of the sympathoadrenal system activity in socially isolated rats upon exposure to novel acute stressors has been reported [23]. The additional acute immobilization does not affect the gene expression of catecholamine biosynthetic enzymes in both auricles of long-term socially isolated rats. This suggests that the response to stress depends on prior experience with stressors [24]. Data from literature indicate a possible adaptation of catecholamine-synthesizing system at the level of gene expression in the heart auricles of chronically socially isolated rats exposed to acute immobilization stress [24]. However, protein levels of catecholamine biosynthetic enzymes in both ventricles of socially isolated rats increased after additional acute stress [25]. With regard to the role of cardiac catecholamines in physiological and pathophysiological processes, it could be hypothesized that increased catecholamine synthesis in the ventricles after acute stress indicates sensitivity of the heart to subsequent stress [25].

It could be concluded that animal model of chronic social isolation (CSI) in rats is a good animal model in the research of neuroendocrine and immune functions in stress conditions. Also, the described results indicate the potential application of CSI animal models in understanding of stress in humans.

3. Animal model of long-term treadmill running in rats

Physical exercise produces modulation of neuroendocrine and immune functions [26] and increases the activity of the antioxidant defense system [27]. Long-term treadmill running in rats is forced exercise which has the propensity to induce both psychological and physical stress [28].

Long-term treadmill running animal model (TR) consists of 11-week-old Wistar male rats that are exposed to long-term treadmill running. Long-term treadmill running is achieved by the rats' daily running on the treadmill for a period of 12 weeks. The treadmill running intensity is gradually increased from week to week and from the initial 10 min—10m/min up to 20 min—20m/min at 0^0 incline [22, 29, 30]. Animals are being exposed to treadmill training 5 days a week for 12 weeks [22].

Treadmill running may induce physiological adaptations, which can be reflected in increased plasma catecholamine concentration, as well as in the change of the synthesis of catecholamine biosynthetic enzymes in rats [31]. It is a very strong stressor, which activates the sympathoadrenomedullary system and increases the synthesis of splenic PNMT protein catalyzing the conversion of NA to A, which both can modulate the immune functions [31]. It is known that cardiovascular diseases, such as hypertension and heart failure, are often associated with sympathetic nervous system overreactivity [32, 33]. The increase of the noradrenaline biosynthetic enzyme expression in stellate ganglia, which causes the increase of plasma NA levels, due to chronic forced running, may play a role in the growing risk for cardiovascular diseases [22, 34].

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It could be concluded that TR shows adaptations that are indicative of chronic stress and that this animal model in rats is good for the study of neuroendocrine and immune functions in stress conditions.

4. Combined animal model of chronic social isolation and long-term daily treadmill running in rats and "cross stressor adaptation hypothesis"

Data from literature confirm that exercise has been widely used in the last years with therapeutic and preventive purposes in a series of pathophysiological conditions. Exercise training reduces the risk of developing diseases related to chronic stress. For example, a physically active lifestyle is associated with decreased risks of coronary heart disease and high blood pressure [35]. In addition, in humans, regular exercise has a beneficial impact on depression [36]. It is known that the theory of "cross stressor adaptation hypothesis" suggests that exercise training, as a stressor on the body, may alter responsiveness to other types of stressors [37]. Mueller [38] suggests that exercise training appears to reduce sympathoexcitation to a variety of centrally mediated sympathoexcitatory stimuli. Reduction in sympathoexcitation may contribute, in part, to the reduced incidence of cardiovascular disease in physically active individuals [38]. In addition, physical activity prevents splenic NA depletion, or spillover, typically observed in sedentary rats following periods of intense sympathetic drive [39]. Also, physical activity may prevent stress-induced suppression of splenic immunity by reducing sympathetic drive to the spleen during stress [40, 41].

Treatment of chronic social isolation and long-term daily treadmill running (CSITR) consists of exposing the individually housed Wistar male rats to the daily treadmill running during 12 weeks [42].

Understanding the mechanisms by which CSITR training alters control of the SNS in health and disease could be important for developing new strategies in the prevention and treatment of cardiovascular diseases. Treadmill exercise leads to a decreased gene transcription of catecholamine biosynthetic enzymes in stellate ganglia in stressful conditions. This may suggest the beneficial effects of treadmill exercise on cardiovascular system in stressed animals [22].

5. Animal model of acute immobilization stress in rats

Immobilization is a standardized procedure frequently used as an additional acute stressor and is considered as one of the most intensive stressors that significantly changes gene expression [43]. It is known that immobilization results in well-characterized catecholamine responses [44]. In this model, animals were restrained in a prone position on a board for periods of 120 min [45]. The head was restricted from movement by a metal loop over the nose, and the feet were taped to raise supports with bandage tape [45].

It is known that the acute immobilization stressor (IMM) triggers an exaggerated elevation of the plasma catecholamines [42]. One of the key questions in adaptive response to stress is how the additional acute stressor can provoke a variant or altered response depending on prior experience with the current or a different stressor [43]. Additional acute immobilization increases plasma catecholamines in animals previously exposed to chronic social isolation (CSI+IMM) [46] and in animals previously exposed to long-term treadmill running (TR+IMM) [34]. This could mean that prior experience may condition physiological systems to "expect" a problem and, therefore, be more ready to respond to a novel additional acute stressor [43]. In addition, immobilization stress more significantly elevates TH and DBH protein levels in the stellate ganglia in rats previously exposed to long-term treadmill running (TR+IMM). Continuous accumulation of their proteins is an adaptation on applied stress regime [34]. Also, chronically stressed rats exposed to novel stressors exhibit exaggerated responses in gene expression of PNMT enzyme catalyzing conversion of NA to A [34]. The increased release of A from stellate ganglia during the additional acute immobilization in rats previously exposed to long-term treadmill running may be caused by the increased synthesis of PNMT. Increased levels of PNMT enzyme in stellate ganglia may have pathophysiological impact, especially on the cardiovascular system, since A is a powerful β2 adrenergic receptor agonist [34]. Also, the heterotypic novel additional acute immobilization stressor elevates the plasma catecholamines but not excessively in the animals previously exposed to CSITR [42]. This finding might be explained by the quality and especially by the intensity of the stressor used. The novel stressors elicit exaggerated responses in prestressed animals, when the novel stressor is of equal or greater intensity or duration and/or it is repeated [43]. Animals exposed to CSITR are already prepared to manage the new situation evoked by a novel stressor, and the exaggerated response is not necessary [42]. Animals exposed to CSITR treatment have statistically more significant expression of TH, DBH, and PNMT genes in the adrenal medulla after additional acute immobilization stress compared with the animals exposed to acute immobilization stress [42]. The increased catecholamine synthesis in the adrenal medulla of chronically stressed animals after additional acute stress is an important adaptive phenomenon of the sympathoadrenomedullary system in rats [43].

6. Other animal models

6.1. Chemical manipulation

Data from literature indicate that chronic stress produces the activation of SNS and hypothalamus-pituitary-adrenal (HPA) axis. In our previous study, we found that exposure of rats to daily treadmill running increased plasma concentrations of NA, A, and ACTH and decreased CORT concentration [34]. It is known that stress hormones via adrenergic and glucocorticoid receptors of immune cells inhibit secretion of the proinflammatory cytokines, such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and interferon-y (INF-γ), while promoting the secretion of the anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13) [47]. Lasting stress exposure induces HPA "fatigue," glucocorticoid resistance, nuclear factor kappa B (NF-Kb)

activation, and negative feedback, which in turn promote the proinflammatory cytokines [47]. The increased proinflammatory cytokines ultimately cause inflammation, which may induce various diseases [47]. For example, the proinflammatory cytokines alter the metabolic processes of neurotransmitters [48], whose secretion suppression and the reuptake block activity play a role in the pathogenesis of depression [47].

6.2. Genetically modified models

During oxidative stress high concentrations of ROS modify nucleoside triphosphates which are incorporated into the DNA during DNA synthesis and may give rise to mutations. Mutations in the genes of regulatory enzymes, transporters, and receptors of the neurotransmitters in the central nervous system (CNS) have been associated with aggression [49].

6.2.1. Target genes

Mutation in human MAO A gene is associated with impulsive aggression in male humans [50], juvenile delinquency [51], impulsivity [52], and female panic and depressive disorders [53, 54]. In addition, mutations in the TH and DA receptor 4 genes influence impulsivity [55–57], and polymorphism in the glutamate transporter (VGLUT) gene is significantly associated with increased "aggression to strangers" [58]. Polymorphism in the tryptophan hydroxylase (Tph2) gene as a causal factor in 5-hydroxytryptamine (5-HT) deficiency is associated with depression [59].

6.2.2. Transgenic mouse/rat models

Genetically modified mouse and rat models are used in the research of human diseases. It is known that the low activity of MAO A enzyme consequently increases catecholamine levels [49]. Reduced levels of the MAO A enzyme, as well as increased NA levels, were observed in aggressive men [49]. MAO A knockout mice showed increased aggression in adulthood [60] and for this reason were used in the research of behavioral disorders.

7. Materials and methods for studying the protective role of exercise against deleterious effects of oxidative stress

7.1. Animal models

Eleven-week-old Wistar male rats were maintained under standard laboratory conditions with water and food ad libitum and kept three to four per cage [25]. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Vinča Institute of Nuclear Sciences [25], Belgrade, Serbia, which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education." Animals were divided into four groups in accordance with our previous protocol [42]. The **control group** (n = 10) was not exposed to stress. The animals in **CSITR group** (n = 10)

were exposed to chronic combined social isolation and treadmill running. CSITR was achieved by exposing the individually housed rats to the daily treadmill running during 12 weeks [42]. Chronically stressed animals were exposed to treadmill training ran on the treadmill 5 days a week for 12 weeks [22]. During that period, the exercise time was increased from 10 min to 20 min/day and treadmill speed gradually increased from 10 to 20 m/min by the end of the second week, without incline [22]. The animals ran according to the protocol for 10 additional weeks (20 min/day at a speed of 20 m/min, 5 days a week) [22]. The treadmill training protocol used in our studies involved a gradual increase in running intensity and is commonly used in the similar studies [29, 30]. Animals were exposed to a low-intensity treadmill training [31], which is in accordance with the protocol of Erdem et al. [30] who suggested that low exercise intensity is a key factor in the effect of submaximal endurance training on adrenomedullary catecholamine biosynthesis. During the exercise, we monitored the animals continuously. In **IMM group** (n = 10), the animals were exposed to acute stress immobilization, for a period of 2 hours [42]. Immobilization stress was elicited as described by Kvetnansky and Mikulaj [44]. In **CSITR+IMM group** (n = 10), the animals were exposed to CSITR during 12 weeks, and after CSITR, these animals were exposed to additional acute IMM stress for 2 hours [42]. The animals were sacrificed 3 hours after the acute immobilization. Data from literature show that 3 hours after the acute immobilization, changes in gene expression of catecholamine biosynthetic enzymes in the peripheral tissues are expected [43, 61]. To confirm the presence of oxidative stress in chronically stressed animals, we have introduced a **CSI** group. **CSI group** (n = 10) consisted of animals exposed to treatment of chronic social isolation for a period of 12 weeks. The rats were individually housed. The visual and olfactory communication among the isolated rats was reduced to the minimal level. In this group we measured the concentration malondialdehyde (MDA) in the spleen. Measurement of MDA is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases. The spleens were rapidly dissected and frozen. To avoid potentially confounding acute effects of exercise, animals were sacrificed 48 hours after the last training session, which is in accordance with protocol of Gavrilović et al. [31].

7.2. Spleen tissue homogenization, RNA isolation, and cDNA synthesis

Total RNAs were isolated from 0.08 g spleen tissues by using TRIZOL reagent (Invitrogen, USA) as described previously by Gavrilović et al. [31]. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd $(N)_6$ Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol, which is in accordance with protocol of Gavrilović et al. [31].

7.3. Quantitative real-time PCR

TH, DBH, PNMT, CREB, VMAT2, CuZn SOD (SOD1), Mn SOD (SOD2), CAT, and GPx mRNA levels were quantified by quantitative real-time RT-PCR as described previously by Gavrilović et al. [42]. TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1), DBH (Rn00565819_m1), PNMT (Rn01495589_g1), CREB (Rn01441386_g1), VMAT2 (Rn00564688_m1), SOD1 (Rn00566938_m1),

SOD2 (Rn00690587_g1), CAT (Rn00560930_m1), and GPx (Rn00577994_g1). The reference gene (endogenous control) was included in each analysis to correct the differences in the inter-assay amplification efficiency, and all transcripts were normalized to cyclophilin A (Rn00690933_m1) expression [31]. The results are reported as a fold change relative to the calibrator and normalized to cyclophilin A as previously described [31].

7.4. Spleen tissue homogenization and measurement of protein concentration

The spleens were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using bicinchoninic acid (BCA) method (Thermo Scientific Pierce, USA), described by Stich [62].

7.5. Western blot analysis

The TH, DBH, and PNMT proteins were assayed by Western blot analysis as described previously by Gavrilović et al. [42]. Antibodies used for quantification of proteins were for TH the monoclonal primary antibody against mouse TH (monoclonal antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000, Chemicon International, USA); for DBH the anti-dopamine-ß hydroxylase (N-terminal) antibody, sheep (dilution 1:5000, Sigma, USA); for PNMT the polyclonal ant-PNMT primary antibody, rabbit (dilutation 1:1000, Protos Biotech Corporation, USA); and for β-actin the rabbit polyclonal anti-β-actin (ab8227, dilutation 1:5000, Abcam, USA) [31]. After that, the membranes were incubated in the secondary antimouse, anti-rabbit (dilution 1:5000, Amersham ECL™ Western Blotting Analysis System, UK) and anti-sheep (dilution 1:5000, Calbiochem, Germany) antibodies conjugated to horseradish peroxidase [31]. A secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences, UK) [31]. The result was expressed in arbitrary units normalized in relation to β actin, which is in accordance with protocol of Gavrilović et al. [31].

7.6. Concentrations of catecholamines

Spleen tissues were homogenized in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. Catecholamine concentration in spleen fractions was determined using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany) according to the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader (Stat Fax 2100). Concentrations were normalized to 1 g of tissues in homogenate. Values were expressed as ng of catecholamine per g of tissues.

7.7. Monoamine oxidase enzyme activities

Determination of MAO A and MAO B activity was performed using the Amplex Red Monoamine Oxidase Assay (A12214, Molecular Probes, USA), described by Zhou and Panchuk-Voloshina [63]. This assay is based on the detection of H_2O_2 in a horseradish peroxidase-coupled reaction using N-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and stable probe for H_2O_2. Fluorescence was measured with a fluorometer using

excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. Monoamine oxidase activity was expressed as U/mg of protein.

7.8. Malondialdehyde measurement

Malondialdehyde concentration in the spleen fractions was determined using Spectrophotometric Assay for Malondialdehyde BIOXYTECH® MDA-586 (OXIS Health Products, Inc., USA) according to the manufacturer's protocol. The MDA-586 method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. Malondialdehyde concentration was expressed as μM/mg of protein.

7.9. Antioxidant enzyme activities

SOD, CAT, GPx, and GR activities were determined using methods previously described by Stojiljković et al. [64]. Determination of total SOD activity was performed using Oxis Bioxytech SOD-525 Assay (Oxis International, Inc., Portland, OR, USA). CAT activity was determined by the method of Beutler [59], and GPx activity was assessed using the Oxis Bioxytech GPx-340 Assay (Oxis International, Inc., Portland, OR, USA). The final result for enzyme activity was expressed as units per milligram of protein (U/mg).

7.10. Data analysis

The data are presented as means ± S.E.M. Differences of gene expression (mRNA and protein levels) of catecholamine biosynthetic enzymes (TH, DBH, and PNMT); levels of CREB, VMAT 2, SOD 1, SOD 2, CAT, and GPx mRNA; concentration of NA, A, and MDA; as well as enzyme activities (MAO A, MAO B, total SOD, CAT, and GPx) in the spleen were analyzed by one-way ANOVA. The effects of CSITR and IMM compared to control animals, as well as the effects of CSITR+IMM compared to CSITR, were tested by Tukey post-hoc test. Statistical significance was accepted at $p < 0.05$.

Correlations of mRNA levels, protein levels, hormone levels, and enzyme activity were analyzed by the Pearson test, using the Sigma Plot v10.0 (with SigmaStat integration).

8. Results

8.1. Changes of the TH, DBH, PNMT, CREB, and VMAT 2 mRNA levels and TH, DBH, and PNMT protein levels in the spleen

The animals exposed to CSITR showed a decreased level of TH mRNA by 22% ($p < 0.05$, Tukey test, **Figure 1a**), DBH mRNA by 11% ($p < 0.05$, Tukey test, **Figure 1b**), PNMT mRNA by 29% ($p < 0.05$, Tukey test, **Figure 1c**), CREB mRNA by 69% ($p < 0.01$, Tukey test, **Figure 1d**), and increased levels of VMAT 2 mRNA by 100% ($p < 0.01$, Tukey test, **Figure 1e**) and PNMT protein by 19% ($p < 0.05$, Tukey test, **Figure 2c**), whereas levels of TH and DBH protein (**Figure 2a** and **b**) were unchanged compared with the controls.

IMM stress does not change significantly gene expression of catecholamine biosynthetic enzymes (**Figures 1a–c** and **2a–c**) and levels of VMAT 2 mRNA (**Figure 1e**) 3 hours after immobilization. However, the additional exposure of CSITR animals to acute immobilization stress led to increased levels of PNMT protein by 33% ($p < 0.05$, Tukey test **Figure 2c**) and VMAT 2 mRNA by 100% ($p < 0.01$, Tukey test, **Figure 1e**) 3 hours after immobilization.

8.2. Changes of the NA and A concentrations in the spleen

CSITR significantly increased the spleen concentrations of NA by 160% ($p < 0.01$, Tukey test, **Figure 3a**) and A by 140% ($p < 0.01$, Tukey test, **Figure 3b**), compared with control animals. The significant positive correlation was found between the levels of PNMT protein and A concentration in the spleen of animals exposed to CSITR (Pearson $R = 0.631$, $p < 0.05$, **Figure 4a**).

The exposure of the control animals to acute immobilization stress significantly increased NA concentration by 250% ($p < 0.01$, Tukey test, **Figure 3a**) and A concentration by 240%

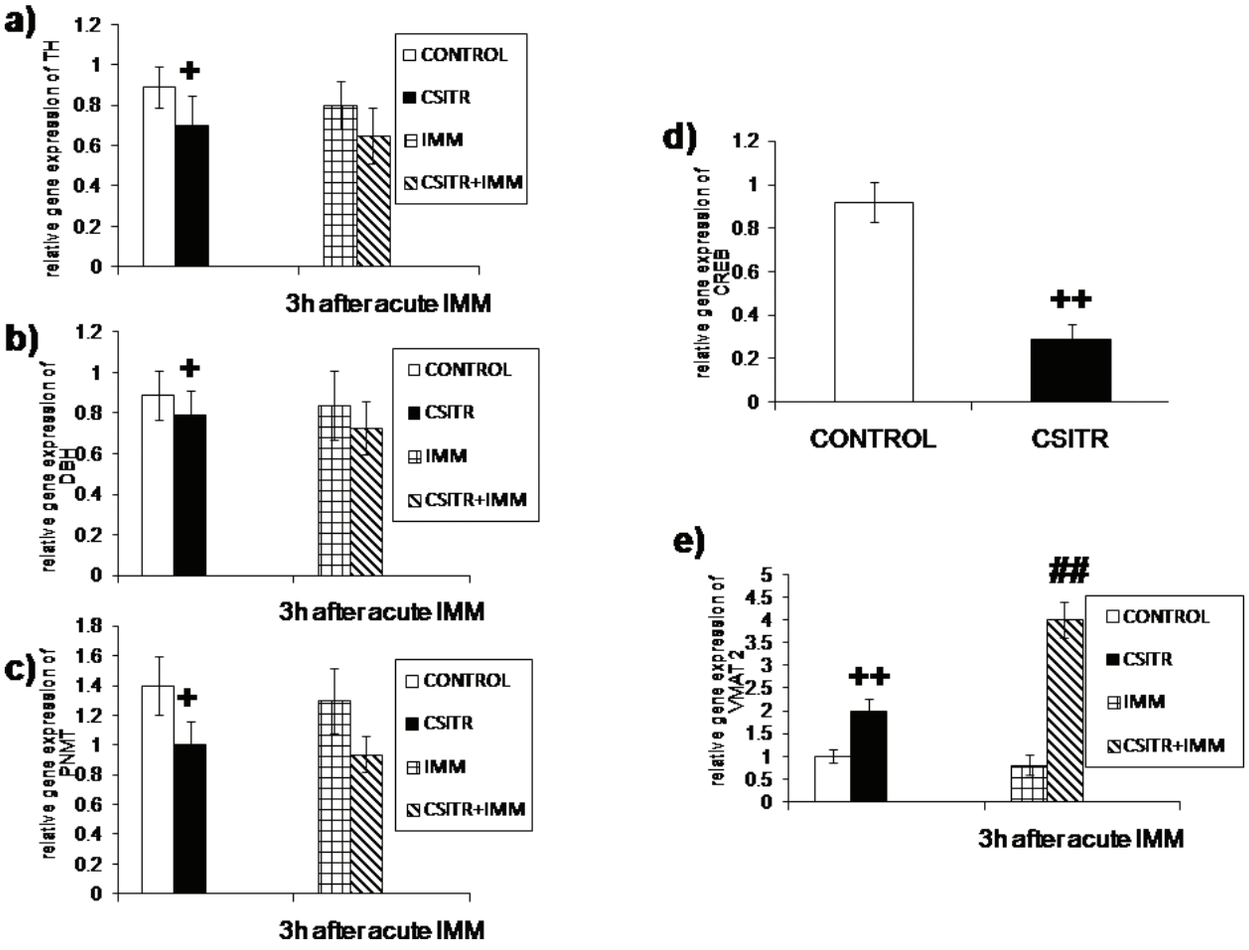

Figure 1. Effects of CSITR and CSITR+IMM models on tyrosine hydroxylase (TH) [a], dopamine-ß-hydroxylase (DBH) [b], phenylethanolamine N-methyltransferase (PNMT) [c], cAMP response element binding (CREB) [d], and vesicular monoamine transporter 2 (VMAT2) [e] mRNA levels in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$, ++$p < 0.01$ CSITR animals compared to control animals (Tukey test) and ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

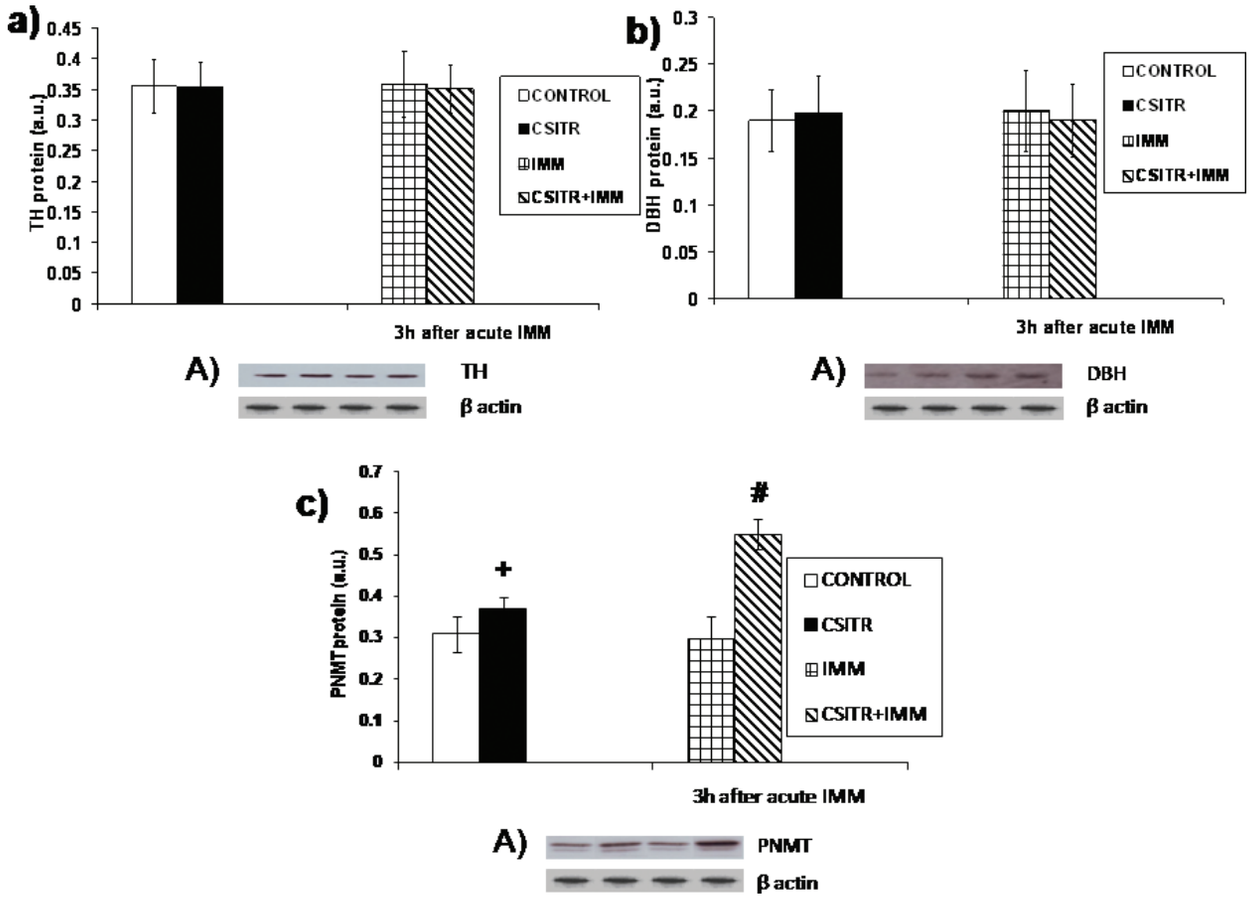

Figure 2. Effects of CSITR and CSITR+IMM models on tyrosine hydroxylase (TH) [a], dopamine-ß-hydroxylase (DBH) [b], and phenylethanolamine N-methyltransferase (PNMT) [c] protein levels in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +p < 0.05 CSITR animals compared to control animals (Tukey test) and #p < 0.05 CSITR+IMM animals compared to CSITR animals (Tukey test).

($p < 0.01$, Tukey test, **Figure 3b**), whereas the additional acute immobilization of CSITR animals decreased NA concentration by 17% ($p < 0.05$, Tukey test, **Figure 3a**) and increased A concentration by 15% ($p < 0.05$, Tukey test, **Figure 3b**) 3 hours after immobilization. The significant positive correlation was found between the levels of PNMT protein and A concentration in the spleen of animals exposed to CSITR+IMM (Pearson $R = 0.721$, $p < 0.05$, **Figure 4b**). However, the significant negative correlation was found between the levels of NA concentration and A concentration in the spleen of animals exposed to CSITR+IMM (Pearson $R = -0.661$, $p < 0.05$, **Figure 4c**).

8.3. Changes of the MAO A and MAO B activity in the spleen

The animals exposed to CSITR showed a decreased enzyme activity of MAO B by 34% ($p < 0.05$, Tukey test, **Figure 5b**), whereas enzyme activity of MAO A (**Figure 5a**) was unchanged, compared with control animals.

IMM stress significantly increased the enzyme activities of MAO A by 1000% ($p < 0.001$, Tukey test, **Figure 5a**) and MAO B by 376% ($p < 0.001$, Tukey test, **Figure 5b**) 3 hours after the cessation

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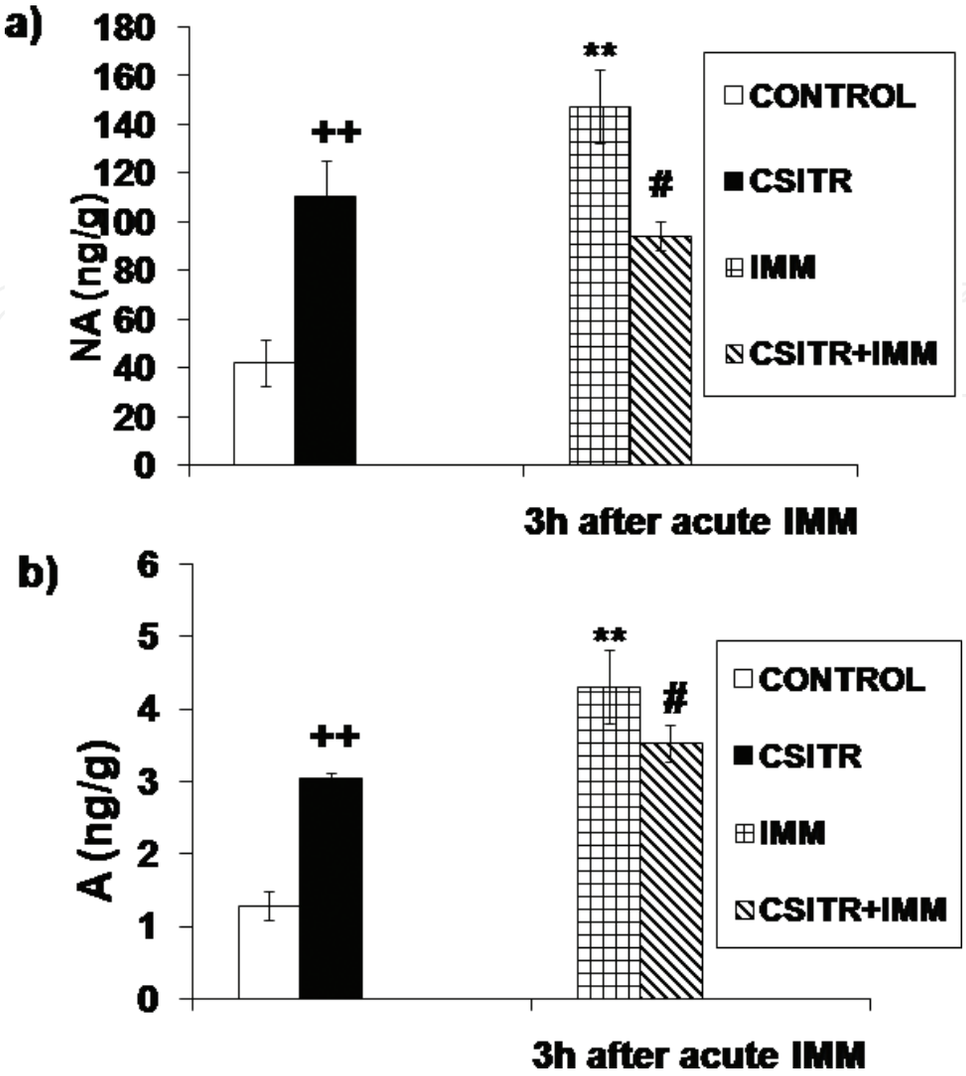

Figure 3. Effects of CSITR and CSITR+IMM models on the concentration of noradrenaline (NA) [a] and adrenaline (A) [b] in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: ++$p < 0.01$ CSITR animals compared to control animals (Tukey test); **$p < 0.01$ IMM animals compared to control animals (Tukey test); and #$p < 0.05$ CSITR+IMM animals compared to CSITR animals (Tukey test).

of immobilization. The additional acute immobilization of CSITR animals increased enzyme activities of MAO A by 116% ($p < 0.01$, Tukey test, **Figure 5a**) and MAO B by 107% ($p < 0.01$, Tukey test, **Figure 5b**) 3 hours after the cessation of immobilization.

8.4. Changes of the MDA concentrations in the spleen

Chronic social isolation (CSI) significantly increased concentrations of MDA by 21% ($p < 0.05$, Tukey test, **Figure 6**) compared with control animals. The animals exposed to CSITR showed unchanged levels of MDA compared with control animals (**Figure 6**).

The exposure of the control animals to acute immobilization stress significantly increased MDA concentration by 26% ($p < 0.05$, Tukey test, **Figure 6**), whereas the additional acute immobilization of CSI animals increased MDA concentration by 50% ($p < 0.01$, Tukey test,

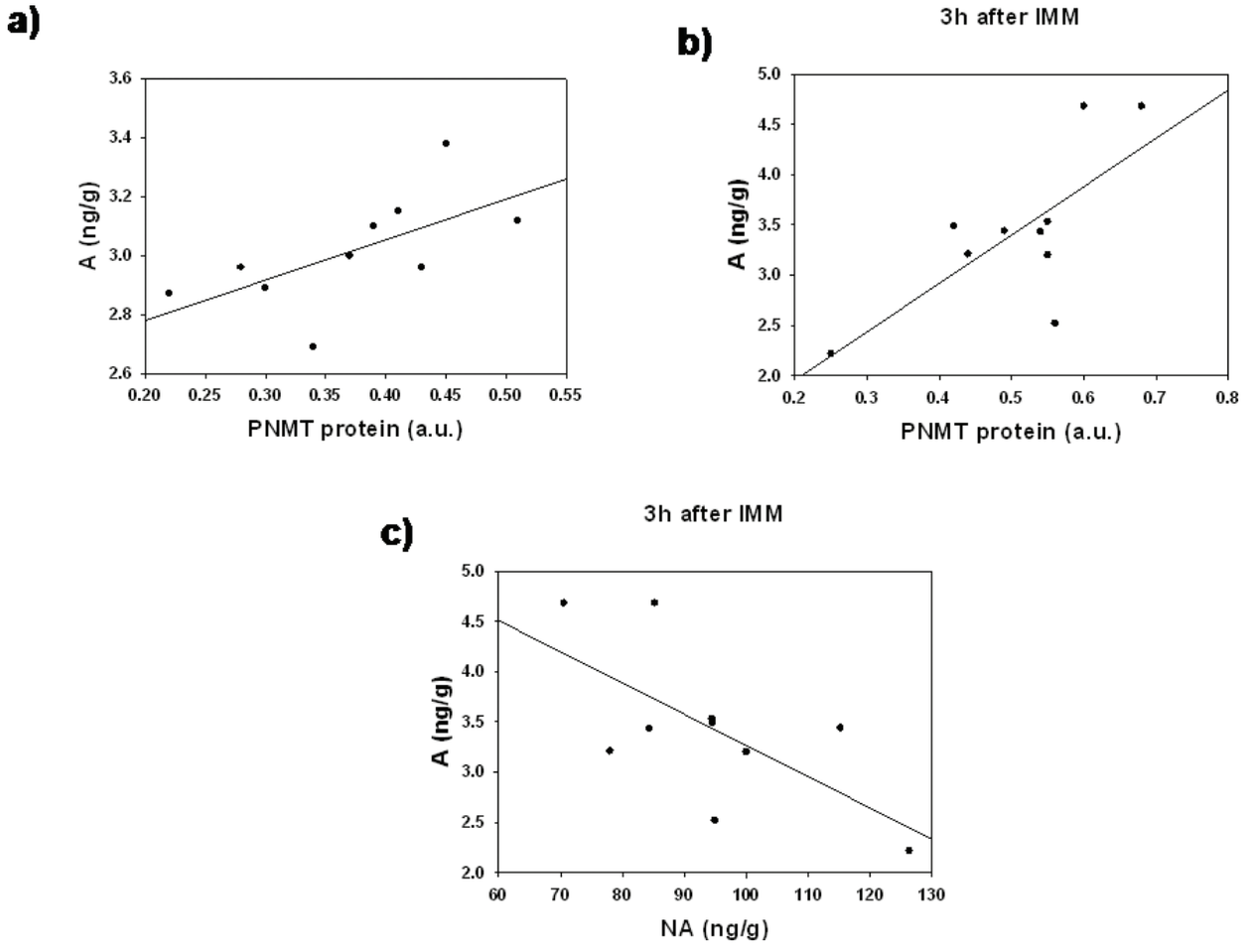

Figure 4. The correlation between PNMT protein level and concentrations of A and NA in the spleen of animals exposed to chronic social isolation and daily treadmill running, as well as of animals exposed to additional acute 2h immobilization stress after chronic social isolation and daily treadmill running (Pearson). **(**a) The correlation in the levels of PNMT protein and A concentrations in the spleen of animals exposed to CSITR (Pearson). (b) The correlation in the levels of PNMT protein and A concentrations in the spleen of animals exposed to CSITR+IMM (Pearson). (c) The correlation between NA and A concentrations in the spleen of animals exposed to CSITR+IMM (Pearson).

Figure 6) 3 hours after the cessation of immobilization. Also, the additional acute immobilization of CSITR animals increased MDA concentration by 16% ($p < 0.05$, Tukey test, **Figure 6**) 3 hours after the cessation of immobilization.

8.5. Changes of the SOD 1, SOD 2, CAT, and GPx mRNA levels as well as total SOD, CAT, and GPx activity in the spleen

The animals exposed to CSITR showed unchanged levels of SOD 1 and SOD 2 mRNA (**Figure 7a** and **b**), as well as significantly increased levels of CAT mRNA by 50% ($p < 0.05$, Tukey test, **Figure 7c**) and GPx mRNA by 150% ($p < 0.01$, Tukey test, **Figure 7d**) compared with control animals. However, CSITR treatment significantly decreased the enzyme activities of total SOD by 36% ($p < 0.05$, Tukey test, **Figure 8a**) and GPx by 30% ($p < 0.05$, Tukey test, **Figure 8c**) compared with control animals, whereas CAT activity remained unchanged (**Figure 8b**).

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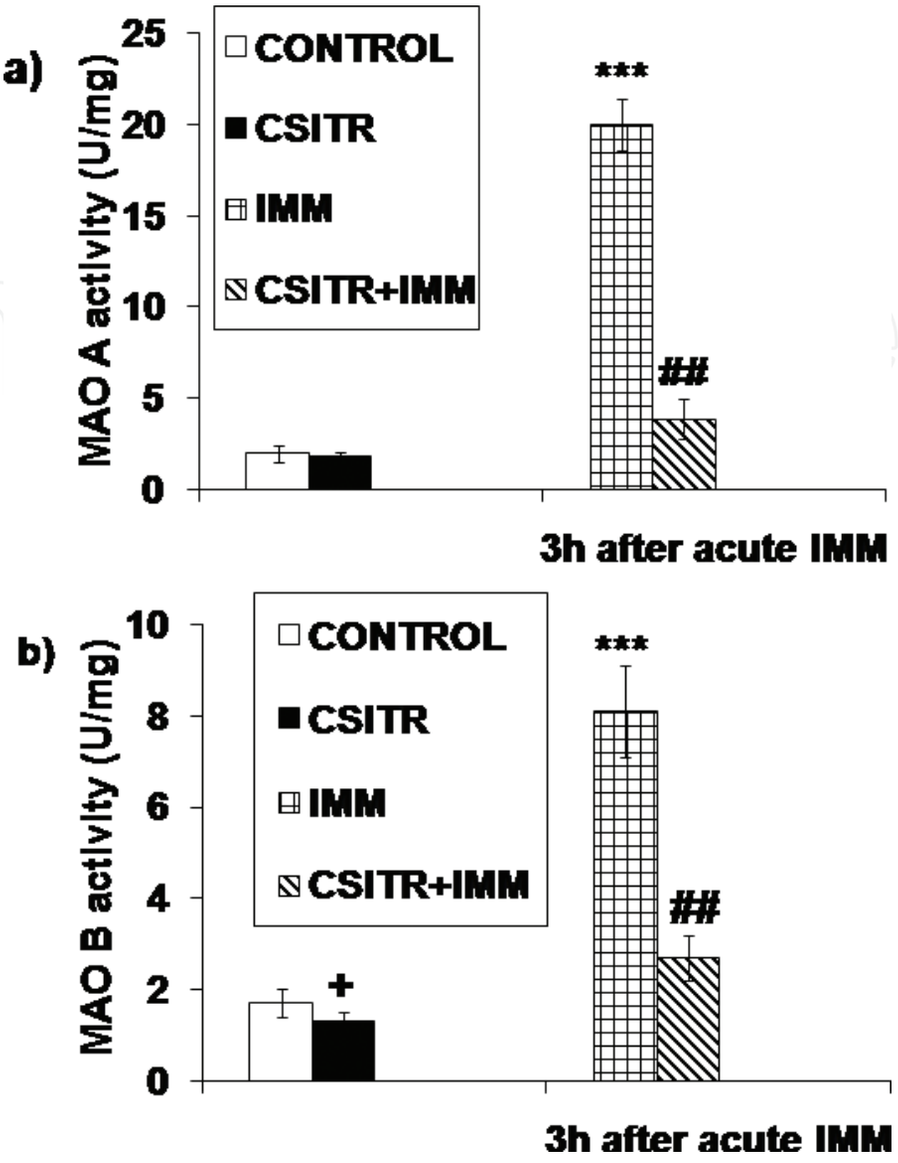

Figure 5. Effects of CSITR and CSITR+IMM models on the enzyme activity of the monoamine oxidase A (MAO A) [a] and monoamine oxidase B (MAO B) [b] in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$ CSITR animals compared to control animals (Tukey test), ***$p < 0.001$ IMM animals compared to control animals (Tukey test), and ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

IMM stress does not change mRNA levels of SOD 1, SOD 2, and CAT (**Figure 7a–c**) as well as enzyme activity of total SOD and CAT (**Figure 8a** and **b**) 3 hours after the cessation of immobilization. However, IMM treatment significantly increased mRNA levels of GPx by 20% ($p < 0.05$, Tukey test, **Figure 7d**) as well as enzyme activity of GPx by 135% ($p < 0.01$, Tukey test, **Figure 8c**) 3 hours after the cessation of immobilization. The additional acute immobilization of CSITR animals increased mRNA levels of SOD 1 by 37% ($p < 0.05$, Tukey test, **Figure 7a**), SOD 2 by 115% ($p < 0.01$, Tukey test, **Figure 7b**), CAT by 57% ($p < 0.05$, Tukey test, **Figure 7c**), and GPx by 18% ($p < 0.05$, Tukey test, **Figure 7d**) as well as enzyme activities of total SOD by 68% ($p < 0.05$, Tukey test, **Figure 8a**), CAT by 13% ($p < 0.05$, Tukey test, **Figure 8b**), and GPx by 576% ($p < 0.01$, Tukey test, **Figure 8c**) 3 hours after the cessation of immobilization.

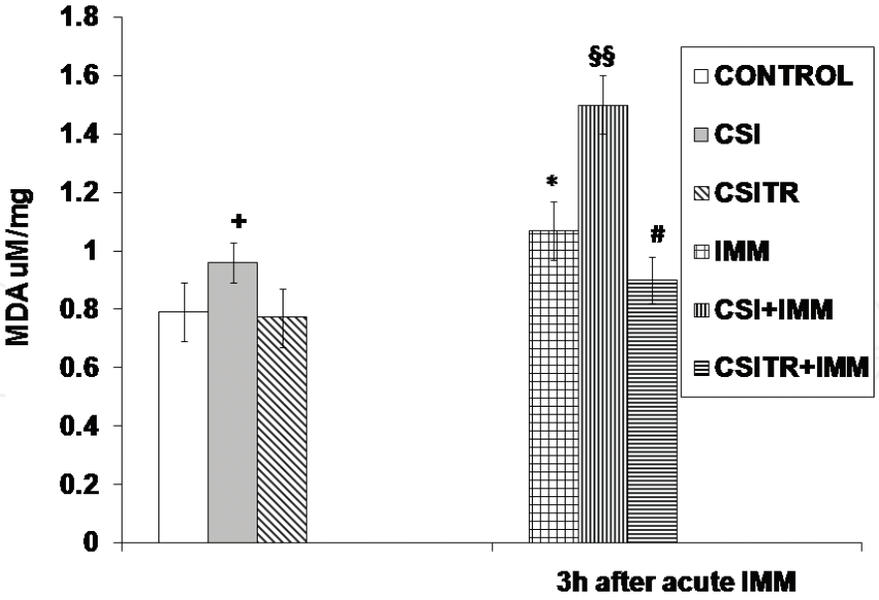

Figure 6. Effects of CSITR and CSITR+IMM models on the concentration of malondialdehyde (MDA) in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +p < 0.05 CSI animals compared to control animals (Tukey test), *p < 0.05 IMM animals compared to control animals (Tukey test), §§p < 0.01 CSI+IMM animals compared to CSI animals, and #p < 0.05 CSITR+IMM animals compared to CSITR animals (Tukey test).

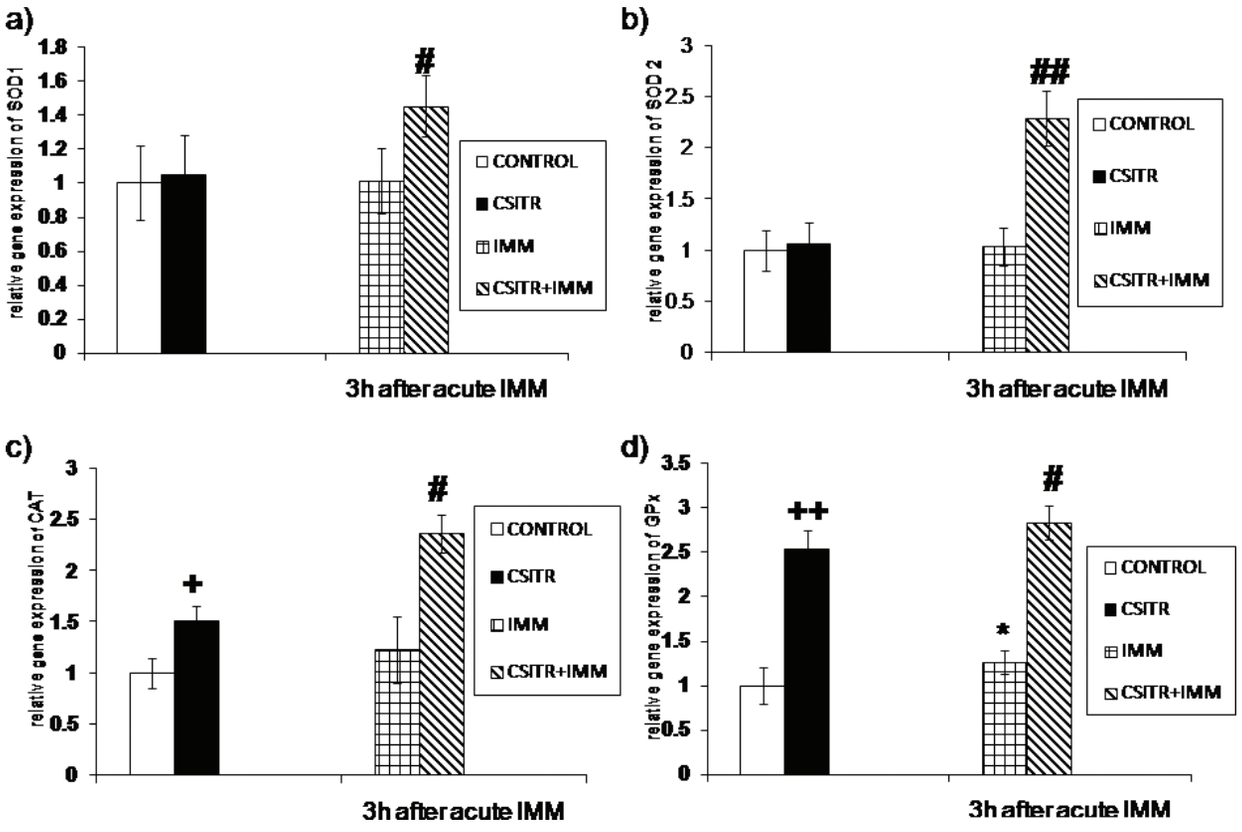

Figure 7. Effects of CSITR and CSITR+IMM models on CuZn superoxide dismutase (SOD1) [a], Mn superoxide dismutase (SOD2) [b], catalase (CAT) [c], and glutathione peroxidase GPx [d] mRNA levels in the spleen. Data are shown as mean ± S.E.M. of 10 rats. Symbols: +p < 0.05, ++p < 0.01 CSITR animals compared to control animals (Tukey test), *p < 0.05 IMM animals compared to control animals (Tukey test), and #p < 0.05, ##p < 0.01 CSITR+IMM animals compared to CSITR animals (Tukey test).

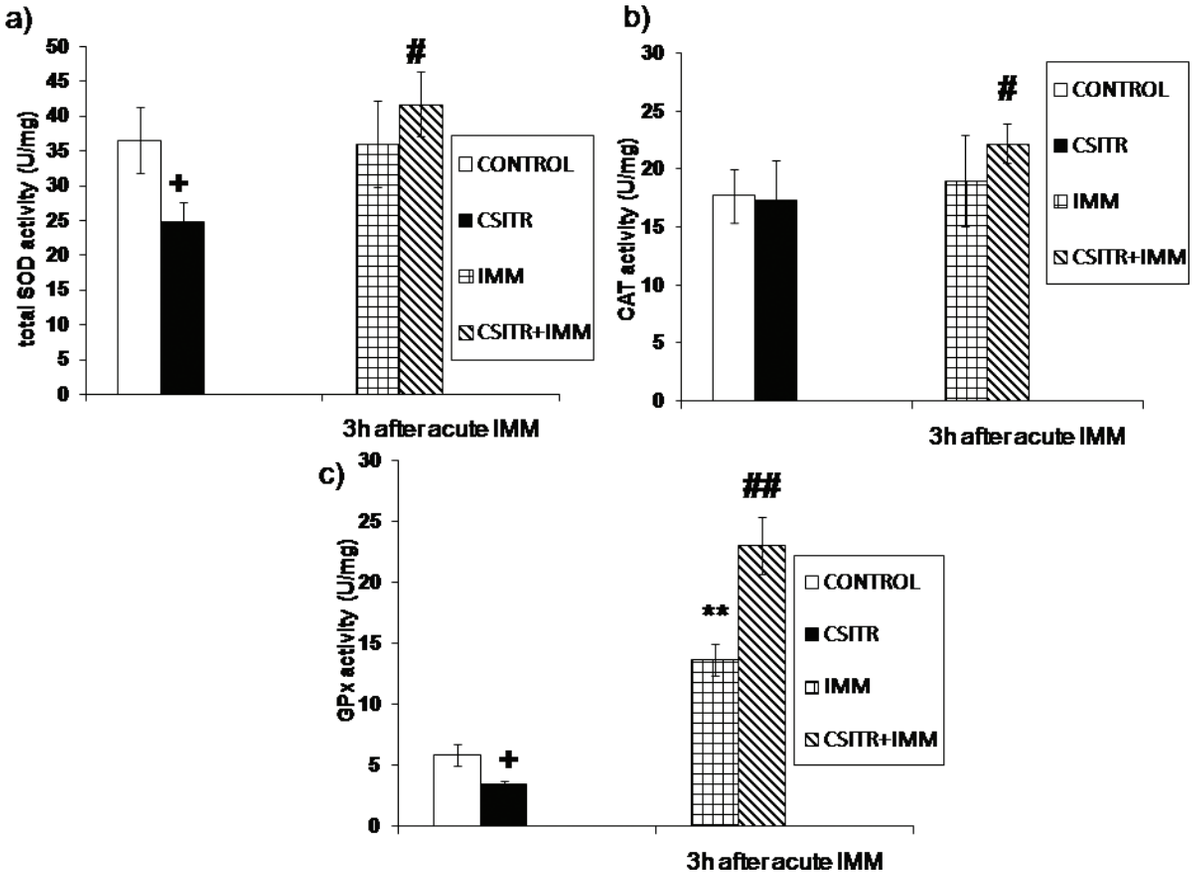

Figure 8. Effects of CSITR and CSITR+IMM models on total superoxide dismutase (SOD) [a], catalase (CAT) [b], and glutathione peroxidase GPx [c] enzyme activity in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$ CSITR animals compared to control animals (Tukey test), **$p < 0.01$ IMM animals compared to control animals (Tukey test), and #$p < 0.05$, ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

9. Discussion

It is known that chronic social isolation induces a reduction of gene expression of noradrenaline biosynthetic enzymes in the spleen [11]. Since the data from literature confirm that the treadmill running stimulates concomitantly peripheral catecholamine secretion and central noradrenergic activity, i.e., NA turnover and release [65], it was tentative to expect that treadmill running would change the splenic catecholamine synthesis of chronically psychosocially stressed rats. However, the results presented in this chapter show that the treadmill running does not lead to further modulation of gene expression of splenic noradrenaline biosynthetic enzymes (TH and DBH) and that reduced level of CREB mRNA coincides with the reduced TH and DBH mRNA levels of chronically psychosocially stressed rats. Also, the treadmill running does not change levels of splenic TH and DBH protein of chronically stressed rats. This finding indicates the decrease of de novo synthesis of NA in the spleen and that the CREB plays a major role in regulating the expression of TH and DBH genes during treadmill running, which is in accordance with the reports of Erdös et al. [13]. Therefore, the treadmill exercise does not affect the synthesis of splenic NA biosynthetic enzymes of chronically stressed rats. Although levels of splenic noradrenaline biosynthetic enzymes are unchanged,

concentration of NA in the spleen of chronically stressed animals exposed to daily exercise is increased. This finding indicates exogenous source of NA in the spleen of chronically stressed rats exposed to daily exercise. These findings strengthen the idea that the sympathetic nervous system (SNS) participates in the NA response to CSITR, which is in accordance with results of Blandino et al. [66], who have confirmed that the noradrenergic system plays an integral role in modulations of splenic IL-1 beta response to stress. In addition, exposure of chronically stressed rats to daily treadmill running reduces PNMT mRNA level. However, CSITR treatment leads to continuous accumulation of PNMT protein catalyzing the conversion of NA to A, suggesting the possibility of the conversion of sympathetic neurotransmitter NA to A in the spleen (**Figure 4a**). This is indicated by significant positive correlation between the levels of PNMT protein and A in the spleen. It is known that catecholamine via adrenergic receptors induces modulation of many immune functions like splenic cytokine production [4]. Moreover, catecholamines might be stored into vesicles by VMAT or degraded by MAO and catechol-O-methyltransferase (COMT) [67]. Expression of VMAT, which plays an important role in the transport of newly synthesized catecholamines into vesicles, positively correlated with norepinephrine levels in both T and B cells which might suggest increased capacity for intracellular catecholamine production [68]. Endogenous catecholamines can modulate function of lymphocytes themselves by a paracrine and autocrine pathway [69]. O'Donnell et al. [70] found that increase of catecholamine levels coincided with reduction of splenic B and NK cells and a concomitant increase in T cells. As reported in this chapter, the treadmill running increases splenic VMAT 2 gene expression, and that increased level of VMAT 2 mRNA coincides with the increased splenic NA and A levels of chronically psychosocially stressed adult rats. A high splenic VMAT 2 transcript level suggests increased capacity of the splenic catecholamines. Therefore, exercise induces accumulation of catecholamines in the spleen of chronically stressed rats, indicating higher readiness of catecholaminergic system to a novel stressor (**Figure 1e**). Brown et al. [71] found that endogenous catecholamines might further initiate intracellular oxidation and apoptosis. However, daily treadmill running does not change enzyme activity of MAO A and decreases enzyme activity of MAO B in the spleen of chronically stressed rats (**Figure 5**). Decreased or unchanged enzyme activities of MAOs indicate that daily treadmill running decreases catecholamine degradation of chronically stressed rats. Therefore, these results indicate that the treadmill running induces accumulation of the splenic catecholamines and that the SNS probably plays a major role in accumulation of the splenic catecholamines in chronically stressed rats.

Chronic social isolation significantly increases concentrations of MDA in the spleen (**Figure 6**). The literature data confirm that exercise training has beneficial effects on oxidative stress and antioxidant defense systems in multiple organs [72]. Meguid et al. [73] showed a significant decrease in serum level of malondialdehyde (MDA) in Down syndrome individuals after treadmill exercise for 3 months. Exposure of chronically stressed rats to daily treadmill running induces return of MDA concentration in the spleen to basal level (**Figure 6**). This confirms that the chronic exercise training induces adaptations that decrease stress-induced oxidative stress. It is in line with the reports of Belviranli et al. [74], who observed that chronic exercise has protective role because the decreased oxidative damage is associated with improved aerobic metabolism induced by physical training.

The decreased oxidative stress resulting from chronic training may originate from the elevated antioxidant system [75]. Powers et al. [76] observed that different combinations of intensity (low, moderate, and high) and duration (30, 60, and 90 min/day) produced different effects on the regulation of the antioxidant enzymes SOD, CAT, and GPx in the left ventricle. Exposure of chronically stressed rats to daily treadmill running induces an increase in CAT and GPx mRNA levels, while SOD1 and SOD2 mRNA levels remain unchanged (**Figure 7**). It is known that the adaptive response of the antioxidant system is specific to either the type of tissue or the different antioxidant systems involved [77, 78]. Ordonez et al. [79] found that a 12-week exercise significantly increased erythrocyte glutathione peroxidase activity which resulted in reduced oxidative damage. Sprint training caused an increase in the cardiac activity of glutathione redox cycle-related enzymes (GPx and GR) without inducing any changes in glutathione S-transferases (GST) and SOD activities or glutathione (GSH) levels in the myocardium [80]. It is important to notice that in CSITR the level of CAT activity remains unchanged, whereas total SOD and GPx activities are decreased (**Figure 8**). After 12 weeks of training process, changes in mRNA levels of antioxidant enzymes are not consistent with the changes in enzyme activities in the spleen of chronically stressed rats. Discrepancies between mRNA levels and activities may be related to differences in mRNA stability or translational efficiency [81]. García-López et al. [82] suspect that it is possible that the expressions of antioxidant enzymes mRNA were initially upregulated and then downregulated. In addition, regulation of expression might act on individual mRNAs to block their translation and thereby lead to their degradation [82]. Therefore, message degradation may be the primary target of regulation of expression [82]. Discrepancies between mRNA levels and activities of MnSOD may be in a kinase/phosphatase signal transduction pathway that may exert a fine control over posttranscriptional regulation of MnSOD expression [83]. In addition, CAT may be inactivated by its substrate, hydrogen peroxide, due to formation of complex II or complex III of CAT at high peroxide concentrations [84]. Nilakantan et al. [85] found that NO or NO-derived products inhibit both CAT and GPx enzyme activities. The results presented in this chapter confirm that daily treadmill running induces high splenic antioxidant enzyme transcript levels probably for immediate translation whenever necessary in chronically stressed rats, which is in accordance with the results of García-López et al. [82]. A high splenic CAT and GPx transcript levels suggest that exercise could induce the antioxidant defense system to become more ready to a novel stressor.

To confirm whether exercise is optimal stimulus to regulate expression levels of splenic catecholamines and antioxidant enzymes and whether the exposure of chronically stressed rats to daily treadmill running induces potentially positive adaptations of the splenic catecholamines and antioxidant protection, this chapter discusses the effects of additional acute immobilization stress. Detection of regulatory mechanism for catecholamine metabolism and antioxidant protection in the spleen in conditions provoked by the additional acute immobilization of chronically stressed animals exposed to daily exercise is exceptionally relevant in stress biology, because of the significant role of catecholamines and oxidative stress in modulation of immune function. The acute immobilization (IMM) and additional acute immobilization (CSITR+IMM) do not affect the synthesis of splenic noradrenaline biosynthetic enzymes (TH and DBH) 3 hours after a termination of immobilization stimulus (**Figure 2**). Also, acute

immobilization (IMM) does not change the level of PNMT mRNA and PNMT protein 3 hours after the termination of immobilization stimulus (**Figure 2**). Wong et al. [86] reported that PNMT protein and enzyme activity changes require additional time of approximately 18–20 hours to reach maximum stimulated levels. Three hours after the additional acute immobilization (CSITR+IMM), the increased synthesis of splenic PNMT protein (**Figure 2**) affects the increase of A (**Figure 3**) in the spleen of chronically stressed animals exposed to daily exercise. These data raise the possibility that 3 hours after additional acute stress, the spleen only converts sympathetic neurotransmitter NA to A of chronically stressed animals exposed to daily exercise. This is confirmed by the significant positive correlation between the levels of PNMT protein and A (**Figure 4b**), as well as negative correlation between the levels of NA and A in the spleen (**Figure 4c**). The acute immobilization (IMM) triggers an exaggerated elevation of splenic catecholamines, while the additional acute immobilization (CSITR+IMM) elevates only splenic A in chronically stressed animals exposed to daily exercise. This data confirm that the chronically stressed animals exposed to daily exercise show high readiness to convert sympathetic neurotransmitter NA to A. In addition, significantly elevated levels of VMAT 2 mRNA 3 hours after additional acute immobilization in chronically stressed animals exposed to daily exercise were found (**Figure 1**). Chronically stressed animals exposed to daily exercises have statistically less significant activation of MAO enzymes after additional acute immobilization compared with the animals exposed only to acute immobilization stress (**Figure 5**). These results confirm that the additional acute immobilization (CSITR+IMM) reveals high readiness of chronically stressed animals exposed to daily exercise for the accumulation of splenic A.

Additionally, it was proven that 3 hours after the acute immobilization, concentration of splenic MDA increased, which is in accordance with the reports of Belviranli et al. [74], who showed that the acute stress triggers oxidative stress. The acute immobilization (IMM) does not change either the levels of SOD 1, SOD 2, and CAT mRNA (**Figure 7**) or the total SOD and CAT enzyme activity (**Figure 8**) in the spleen 3 hours after a termination of immobilization stimulus. This finding is in line with the reports of Pajović et al. [16], who confirm that the acute immobilization does not change the levels of SOD enzyme activity. The increased oxidative stress produces inhibitory effects on CAT and SOD activity, which is evident from decreased enzyme activity of CAT and SOD (**Figure 8**) and increased concentration of MDA (**Figure 6**). These results are in accordance with the reports of Haider et al. [87] who showed that increased oxidative stress produced inhibitory effects on CAT activity. However, the acute immobilization (IMM) increases only the levels of mRNA and enzyme activity of GPx (**Figures 7** and **8**), but that increase was not sufficient to reduce oxidative stress. These results, together with the above mentioned data, confirm that acute immobilization induces oxidative stress. In addition, elevated levels of MDA 3 hours after the cessation of immobilization in chronically stressed animals exposed to daily exercise are observed (**Figure 6**). However, additional acute immobilization (CSITR+IMM) induces an increase of SOD 1, SOD 2, CAT, and GPx mRNA (**Figure 7**), as well as total SOD, CAT, and GPx enzyme activity (**Figure 8**) 3 hours after the cessation of immobilization in chronically stressed animals exposed to daily exercise. These data suggest high readiness of splenic antioxidant enzymes to repair or prevent damage by reactive oxygen species in chronically stressed animals exposed to daily exercise after additional acute immobilization stress. This could mean that exercise may condition physiological systems to “expect” a problem and, therefore, be more ready to respond

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to a novel additional acute stressor by increased antioxidant protection. The readiness of the chronically stressed organism exposed to exercise to respond to a heterotypic stressor by an exaggerated expression of splenic antioxidant enzymes is an important adaptive phenomenon of the antioxidant defense system. Therefore, these results confirm that exercise have an important protective role in the splenic antioxidant defense system.

10. Conclusions

The exposure of chronically stressed rats to daily exercise induces the increase in the synthesis of splenic PNMT protein catalyzing the conversion of sympathetic neurotransmitter NA to A. In addition, the increased levels of splenic VMAT 2 mRNA and decreased/unchanged MAO enzyme activity suggest that daily exercise leads to accumulation of splenic catecholamines in chronically stressed rats. The accumulation of the splenic catecholamines provoked by exercises may have an important impact on the immune-neuroendocrine interactions in stress conditions. The return of the splenic MDA concentrations to basal levels confirms that exercise may decrease stress-induced oxidative stress, while the increased splenic antioxidant enzyme (CAT and GPx) transcript levels suggest that exercise could induce the antioxidant defense system to become more ready to a novel stressor, which indicates that exercises may repair oxidative damage in chronically stressed rats. Moreover, it can be concluded that exposure of chronically stressed rats to daily exercise causes high splenic antioxidant enzyme transcript levels and catecholamine levels and that the exercise can be beneficial, inducing an adaptive response to possibly other stressors that may be encountered later.

The remarkable anatomical and physiological similarities between humans and animals, particularly mammals, have prompted researchers to investigate a large range of mechanisms and assess novel therapies in animal models before applying their discoveries to humans [88]. Our combined model of chronic social isolation and long-term daily treadmill running may be a good animal model in the research of the preventive role of exercise on neuroendocrine and immune functions in stress conditions, suggesting the potential application of CSITR animal model in understanding of human stress, as well as the potential therapeutic role of exercise in human diseases.

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Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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