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EFFECTS OF SODIUM NITROPRUSSIDE ON SALT STRESS TOLERANCE OF TOCOPHEROL-DEFICIENT *ARABIDOPSIS THALIANA* PLANTS

NADIIA MOSIICHUK

Abstract. In the present study, effects of exogenous sodium nitroprusside (SNP), a nitric oxide ($^{\bullet}$NO) donor, on lipid peroxidation and antioxidant enzyme activities in wild type and tocopherol-deficient lines *vte1* and *vte4* of *Arabidopsis thaliana* subjected to 200 mM NaCl were studied. In wild type plants, pretreatment with SNP did not change level of thiobarbituric acid reactive substances (TBARS), but decreased the activities of dehydroascorbate reductase and guaiacol peroxidase under salt stress. In mutant line *vte1*, which lacks all forms of tocopherols, pretreatment with SNP reduced TBARS level and increases the activities of glutathione reductase and guaiacol peroxidase under salt stress. Ascorbate peroxidase activity decreased under salt stress conditions in both mutant lines, pretreated with SNP. It can be concluded, that pretreatment with SNP could attenuate salt-induced injuries in *A. thaliana* plants via up-regulation of activity of antioxidant enzymes and attenuate lipid peroxidation.

Keywords: antioxidant enzymes, lipid peroxidation, nitric oxide, oxidative stress, tocopherols.

Abbreviations: APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GuPx, guaiacol peroxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; TBARS, thiobarbituric acid reactive substances.

1. INTRODUCTION

Salt stress is one of the most significant abiotic stresses and affects many aspects of plant physiology and homeostasis [2, 23]. The effects of high salinity on plants can be mainly classified from two different points: osmotic stress induced by high salt concentration in the environment and the toxic effect of sodium accumulated in the cell [23]. Along with these primary effects, secondary stress, such as oxidative one, occurs because high concentrations of ions disrupt cellular homeostasis and increase generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO$^{\bullet}$) [2]. The enhanced ROS production during stress induced high salinity can enhance oxidative modification of lipids, nucleic acids, and proteins. Plants possess several mechanisms to detoxify ROS which include non-enzymatic antioxidants as well as antioxidant enzymes [12]. Among non-enzymatic antioxidants, tocopherols (α-, β-, γ- and δ) play a key role because they eliminate singlet oxygen and prevent propagation of lipid peroxidation in membranes by scavenging lipid peroxy radicals. Two major tocopherols possessed by plants are α-tocopherol in green tissues and γ-tocopherol in seeds [21].

In view of a number of studies, salt tolerance often correlates with a more efficient antioxidant system [23, 30]. Therefore, enhancing antioxidant potential in plants may improve plant tolerance to salt stress. Numerous studies reported important role of nitric oxide (•NO) in stress response of plants. Nitric oxide (•NO) is highly reactive free radical with diverse biological functions in plants – either cytotoxic or cytoprotective [18]. The cytoprotection is partly based on its ability to regulate ROS level and toxicity. However, various derivatives of •NO, collectively referred as reactive nitrogen species (RNS), can be toxic [18, 42]. In plants •NO also acts as an important inter- and intracellular signaling molecule involved in many physiological processes, as well as responses to biotic and abiotic stresses [18]. Many reports have shown that exogenous •NO exhibited an antioxidant role during pathogen infection [25], osmotic stress and salinity [39, 44, 47], heavy metal and herbicide toxicity [10, 28, 37, 41, 43, 45]. The possible mechanisms of such protective action include up-regulation of the activity of antioxidant enzymes, including superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR) that reduces oxidative damage and provides protection against ROS-promoted injury. An important role for •NO in regulating salt stress response in plant has already been suggested by several researches [3, 19, 44]. Uchida et al. [39] reported that exogenous •NO enhances antioxidant enzyme activities in rice under salt stress. Same investigators proved that preatreatment with NO-donors elevated seeds germination, root growth and dry weigh accumulation [17, 46]. Based on the above observations, this work aimed to investigate whether exogenous sodium nitroprusside (SNP), •NO donor, may alleviate oxidative damage induced by salt stress in wild type and tocopherol deficient lines *vte1* and *vte4* of *Arabidopsis thaliana* and to elucidate possible involvement of exogenous •NO in improvement tolerance of wild and tocopherol-deficient plants to salt stress. The *vte1* mutant lacks all four tocopherols [27], whereas *vte4* mutant lacks α-tocopherol, but instead posseses γ-tocopherol in leaves [5].

2. MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* wild type (Columbia) and mutant lines *vte4* (SALK_03676) and *vte1* (GABI_11D07), defective in *VTE4* and *VTE1* genes, respectively, were obtained from the Salk Institute [1] and GABI-Kat [29] and selected homozygote plants from the seeds at the Institute of Botany of Kiel University (Germany) were used in the present investigation. The plants were grown in hydroponic system using Rockwool supports as described earlier [38] at 28°C and naturally illuminated environmental conditions. The Gibeau nutrient solution [11] was used and changed every two weeks. Ten-week-old plants were used for experiment. Initially, roots of plants were placed for 24 h in solutions 0.1 mM SNP, $K_4[Fe(CN)]_6$ (additional control to SNP) or distilled water (control). Afterwards, plants from the three groups were exposed to nutrient solution containing 0 or 200 mM NaCl for 24 h. Therefore, the plants of each line were submitted to five treatments: control (pre-treated with H_2O and not NaCl stressed), NaCl (pre-treated with H_2O and NaCl stressed), SNP (pre-treated with SNP and not NaCl stressed), SNP-NaCl (pre-treated with SNP and NaCl stressed), and $K_4[Fe(CN)]_6$ -NaCl (pre-treated with $K_4[Fe(CN)]_6$ and NaCl stressed). After 24 h fully expanded leaves of plants were harvested and frozen with liquid nitrogen.

To measure the level of products of lipid peroxidation and activity of antioxidant enzymes the frozen leaves were powdered in liquid nitrogen with morta and pestle and mixed (1:5, w:v) with 50 mM potassium-phosphate buffer (pH 7.0) that contained 1 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM phenylmethylsulfonylfluoride (PMSF). Ascorbic acid (1 mM) was added to potassium-phosphate buffer in the case of ascorbate peroxidase (APX) assay. The homogenates were centrifuged at 13,000 *g* for 20 min at 4°C in Eppendorf 5415R (USA) centrifuge. The supernatants obtained from each sample was collected and used for further assay.

Supernatants were mixed with an equal aliquot of 40% (w/v) trichloroacetic acid (TCA) and then centrifuged for 10 min at 5000 *g*. The supernatants were used for determination of level of thiobarbituric acid reactive substances (TBARS) as described by Heath and Packer [14].

The activity of ascorbate peroxidase (APX) was measured spectrophotometrically following the decrease of absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) due the oxidation of ascorbic acid to dehydroascorbate [7]. Guaiacol peroxidase (GuPx) activity was assayed spectrophotometrically following the increase in absorbance at 470 nm due to guaiacol oxidation (ε = 26.6 $mM^{-1}cm^{-1}$) [31]. Dehydroascorbate reductase (DHAR) activity was determined by measuring increase in absorbance at 265 nm due the formation of ascorbic acid (ε = 14 mM^{-1} cm^{-1}) [35]. Glutathione reductase (GR) activity was determined as the decrease in absorbance at 340 nm (ε =6.22 mM^{-1} cm^{-1}) due to the oxidation of reduced NADPH [20].

One milliunit of APX, GuPx, DHAR and GR activities is defined as the amount of the enzyme consuming 1 nmol of substrate or generating 1 nmol of product per minute; activities were expressed as international milliunits per milligram of protein.

Protein concentration was determined with Coomassie brilliant blue G-250 according to Bradford's method [6] with bovine serum albumin as a standard.

All values were expressed as means ± S.E.M. of three independent experiments. For statistical analysis, the Student's *t*-test was used to compare values at stress conditions with their corresponding controls values, and to compare *vte4* and *vte1* mutant lines with the wild type.

3. RESULTS AND DISCUSSION

The involvement of $^{\bullet}NO$ in salinity tolerance has been studied intensively in the past few years. For instance, under salt stress conditions, the exogenous $^{\bullet}NO$ can enhance salt tolerance by alleviating oxidative damage, enhancing activities of proton-pump and Na^+/H^+ antiport in the tonoplast, and K^+/Na^+ ratio (reviewed in [22]). In many cases, the protective role of $^{\bullet}NO$ under salt stress conditions was related with its effects on the ROS elimination. It has been shown that ROS production, particularly $O_2^{\bullet -}$ and H_2O_2, is stimulated under salt stress conditions [15]. Free radical-induced peroxidation of lipids is one of commonly used markers of stress-induced damage [33]. A protective role of $^{\bullet}NO$ against lipid peroxidation was previously reported by many researchers [32, 34, 39, 40]. Nitric oxide can affect lipid peroxidation due to interaction with lipid alcoxyl (LO•) and peroxyl (LOO•) radicals [18]. Decomposition of lipid hydroperoxides results in formation of diverse products including malondialdehyde (MDA). In this work the product of MDA condensation with thiobarbituric acid (TBA) was measured as thiobarbituric acid reactive substances (TBARS). The level of TBARS in leaves of wild type Arabidopsis plants was significantly higher up to 1.5 fold after treatment by salt stress (Fig. 1).

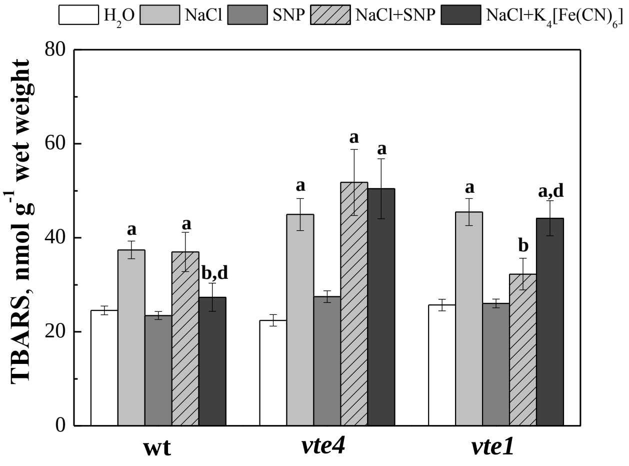

Fig. 1. Effect of SNP pretreatment on TBARS content in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M (n = 3). ^{a}Significantly different from respective control group (H_2O), ^{b}NaCl and ^{d}NaCl+SNP groups ($P < 0.05$).

Exogenous SNP did not changed TBARS content under both normal and salt stress conditions. Salt treatment with addition of potassium ferrocyanide resulted in 1.4-fold decrease of TBARS level in wild type plants. In leaves of mutant line *vte4*, salt stress increased by 2-fold TBARS level (Fig. 1). Under salt stress, the treatment with both potassium ferrocyanide and SNP did not change TBARS level in *vte4* plants. Similarly, in mutant line *vte1* the level of TBARS increased by 1.7-fold under salt stress conditions (Fig. 1). Application of SNP did not change this parameter under normal conditions, but significantly decreased it by 29% under the stress. The treatment with potassium ferrocyanide had no effect on TBARS level under salt stress as compared to plants exposed to 200 mM NaCl only. The *vte1* mutant plants do not synthesize tocopherols, therefore $^{\bullet}$NO can play the key role in a cell as a limiting factor of the chain reaction of lipid peroxidation and thus limit oxidative damage. Two mechanisms which may explain protective $^{\bullet}$NO action against oxidative damage have been widely reported. Firstly, $^{\bullet}$NO may detoxify ROS directly, such as superoxide radicals, to form peroxyntrite, which is less toxic and thus decrease cellular damage [42]. Secondly, $^{\bullet}$NO could function as a signaling molecule, which upregulating cellular antioxidant system [18, 22].

Wu and colleagues [40] showed that application of SNP slowed down the increase in MDA production in tomato leaves under NaCl treatment. Application of exogenous $^{\bullet}$NO dramatically decreased TBARS level in cucumber root mitochondria under salt stress, whereas sodium ferrocyanide did not affect TBARS level in salt-treated plants [34]. SNP treatment slightly reduced the increase in MDA contents in shoots of *Kosteletzkya virginica* seedlings exposed to 200 mM NaCl [13]. Pretreatment with SNP also decreased levels of lipid peroxidation products in tomato seedlings under osmotic stress induced by drought [24].

Induction of the antioxidant defense system is one of the mechanisms actively employed by plants to survive at high salinity [4, 16]. It was found that $^{\bullet}$NO induced activity of various ROS-scavenging enzymes [17].

An ascorbate-glutathione (AsA-GSH) cycle is the most important H_2O_2-detoxifying system in plant chloroplasts, which operates also in cytosol, peroxisomes, and mitochondria [26]. The enzymes of the ascorbate-glutathione cycle APX, DHAR and GR play an essential role in plant tolerance to the action of various biotic and abiotic stresses by eliminating of H_2O_2, as well as sustaining of reduced status of ascorbate and glutathione [12].

APX which uses ascorbate as a reductant in the first step of the AsA-GSH cycle is the most important plant peroxidase in H_2O_2 detoxification [12, 26]. Salt stress did not affect APX activity in wild type and both mutant lines, *vte4* and *vte1* (Fig. 2).

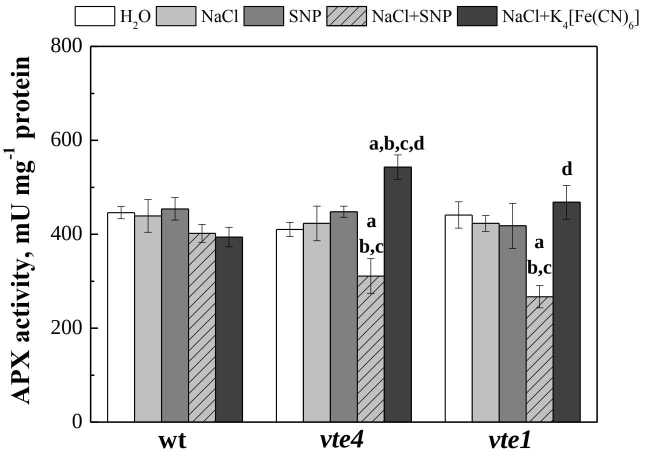

Fig. 2. Effect of SNP pretreatment on the activity of ascorbate peroxidase (APX) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M.(n = 3). ^{a}Significantly different from respective control group (H_2O), ^{b}NaCl and ^{c}SNP groups ($P < 0.05$).

Pretreatment with SNP resulted in a remarkable decrease by 26-36 % in the activity of APX in both mutant lines under salt stress. Pretreatment with potassium ferrocyanide did not significantly influence APX activity in *vte1* mutant plants, and slightly increased by 32 % APX activity in mutant plants *vte4* under salt stress conditions (Fig. 2).

Regeneration of ascorbate via AsA-GSH cycle requires the activity of DHAR and GR [12, 26]. In our study, DHAR activity significantly increased by 47 % in wild type plant exposed to 200 mM NaCl (Fig. 3).

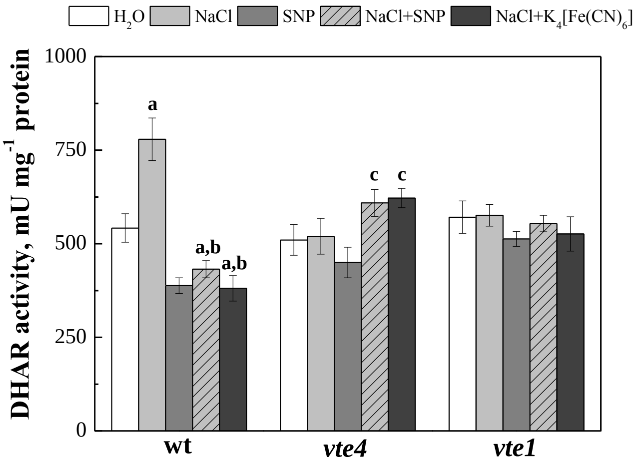

Fig. 3. Effect of SNP pretreatment on the activity of dehydroascorbate reductase (DHAR) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). ^{a}Significantly different from respective control group (H_2O) and ^{b}NaCl groups ($P < 0.05$).

Under normal conditions, application of NO-donor had no significant effects on DHAR activity. Under salt stress conditions, the treatment with both potassium ferrocyanide and SNP decreased DHAR activity by 44% as compared with NaCl-treated plants. In leaves of *vte4* mutant line, neither NaCl nor SNP changed DHAR after treatment. However, pretreatment with SNP or potassium ferrocyanide increased DHAR activity by 19% under NaCl stress as compare with corresponding controls. None of the treatments had effects on DHAR activity in *vte1* mutant line (Fig. 3).

The activity of GR did not change in wild type plants after all treatments (Fig. 4). Exposure to 200 mM NaCl enhanced GR activity by 27-39 % in leaves of both mutant lines. In mutant line *vte4*, pretreatment with SNP or potassium ferrocyanide slightly attenuated increase in GR activity under salt stress condition. At the same time, pretreatment with both potassium ferrocyanide and SNP did not change GR activity under salt stress conditions in *vte1* mutant line (Fig. 4).

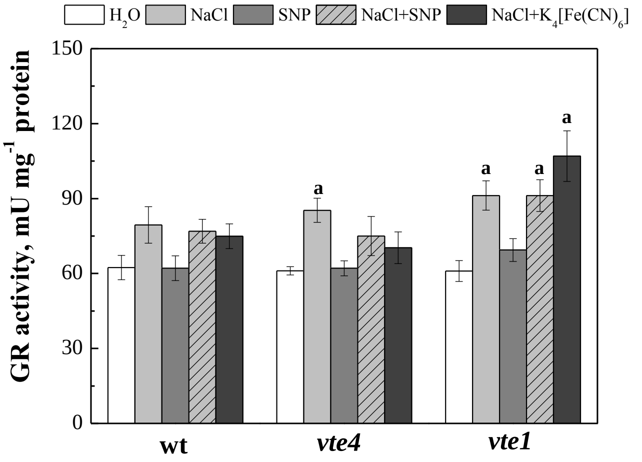

Fig. 4. Effect of SNP pretreatment on the activity of glutathione reductase (GR) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). [a]Significantly different from respective control group (H_2O) ($P < 0.05$).

It is known that in plants guaiacol peroxidases (GuPx) also may participate in H_2O_2 detoxification. Similarly to APx, GuPx scavenge H_2O_2 using plant phenolic compounds, in particular guaiacol (*o*-methoxyphenol) as an electron donor [12]. In our experiments, salt stress strongly increased GuPx activity in all three plant lines (Fig. 5).

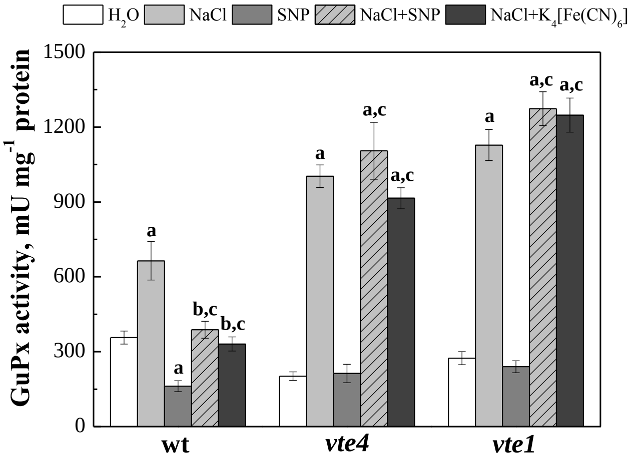

Fig. 5. Effect of SNP pretreatment on the activity of guaiacol peroxidase (GuPx) in leaves of wild type (wt), vte4 and vte1 plants of A. thaliana under salt stress, induced by 200 mM NaCl. Data are means ± S.E.M. (n = 3). [a]Significantly different from respective control group (H_2O), [b]NaCl and [c]SNP groups ($P < 0.05$).

Under normal conditions, application of SNP significantly decreased GuPx activity in wild type plants. Pretreatment with SNP and potassium ferrocyanide significantly reduced the increase in GuPx activity in wild type plants under salt stress conditions. In mutant lines *vte4* and *vte1*, pretreatment with both SNP and sodium ferrocyanide did not change GuPx activity under NaCl stress.

Some authors supposed that $^\bullet$NO could increase the activity of antioxidant enzymes by stimulation of H_2O_2 producing system(s) [8]. Guo and colleagues [13] proposed that exogenously applied SNP indirectly enhance activities of antioxidant enzymes under salt stress by the increasing proline content.

Previously Shi et al. [34] reported that application of SNP greatly induced the H_2O_2-scavenging enzymes CAT, APX and GuPx under salt stress. In the experiment, application of SNP also promoted DHAR and GR activities under salt stress, and such promotion was important for the efficient H_2O_2-scavenging by APX in cucumber mitochondria [34]. Tanou et al. [36] showed that exogenously introduced $^{\bullet}NO$ (as SNP) effectively induced antioxidant enzyme activities, particularly ones of APX and GR, promoted the maintenance of the cellular redox homeostasis and mitigated the oxidative damage caused by $HO^{\bullet}$ under high salinity. The study of Uchida et al. [39] also revealed that pretreatment with SNP enhanced not only ROS scavenging enzymes activities, but also expression of transcripts for stress-related genes under salt stress conditions. SNP improved the activity of antioxidant enzymes of cucumber seedling leaves under NaCl-induced stress to different extent, and reduced the rate of $O_2^{\bullet -}$ production, membrane permeability, H_2O_2 and MDA contents simultaneously [9].

4. CONCLUSION

It can be concluded that pretreatment with SNP attenuated salt stress induced injuries in *Arabidopsis thaliana* plants via up-regulation of the activities of antioxidant enzymes and prevention of lipid peroxidation. The most pronounced SNP effects were observed in tocopherol-deficient *vte1* mutant plants, in which pretreatment with SNP reduced TBARS level and increased activities of GR and GuPx.

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Address: Nadiia Mosiichuk, Vasyl Stefanyk Precarpathian National University, 57, Shevchenko Str., Ivano-Frankivsk, 76018, Ukraine.
E-mail: sem_nad@ukr.net.

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Мосійчук Н. М. Вплив нітропрусиду натрію на стійкість до сольового стресу дефектних за токоферолом рослин *Arabidopsis thaliana*. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 123–132.

У даній роботі досліджено влив екзогенного донора оксиду азоту (•NO) нітропрусиду натрію (НПН) на пероксидне окислення ліпідів та активність антиоксидантних ферментів у дикого типу і дефектних за біосинтезом токоферолу ліній *vte1* та *vte4* *Arabidopsis thaliana* за дії 200 мМ NaCl. У рослин дикого типу, попередня обробка НПН не впливала на рівень ТБК-активних продуктів, але знижувала активність дегідроаскорбатредуктази та гваяколпероксидази в умовах сольового стресу. У безтокоферольної лінії *vte1* попередня обробка НПН знижувала вміст ТБК-активних продуктів та підвищувала активність глютатіонредуктази та гваяколпероксидази за дії сольового стресу. Активність аскорбатпероксидази знижувалася за дії сольового стресу у рослин двох мутантних ліній, попередньо експонованих з НПН. Можна дійти висновку, що попередня обробка НПН може послаблювати дію сольового стресу у рослин *Arabidopsis thaliana* шляхом збільшення активності антиоксидантних ферментів та ослаблення пероксидного окислення ліпідів.

Ключові слова: антиоксидантні ферменти, пероксидне окислення ліпідів, оксид азоту, оксидативний стрес, токофероли.