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EXPERIMENTAL ARTICLES

The Neuroprotective Activity of Tamoxifen and Tibolone during Glutathione Depletion in vitro[1]

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Abstract—Modulators of estrogen receptors play a significant role in cerebral activity and in the regulation of vital functions of the whole organisms. This work objective is to enlarge knowledge of Tamoxifen neuroprotective action and to determine neuroprotective action of Tibolone (both are modulators of estrogen receptors) studying their influence on antioxidant system indices, oxidative stress and mitochondrial dysfunction caused by glutathione depletion of neurons in vitro. Depletion of glutathione by buthionine sulfoximine (BSO) in neurons leads to displacement of the thiol-disulfide equilibrium in vitro. Introduction of BSO into neuron suspension results in a decrease of reduced glutathione (GSH) level and in an increase of oxidized glutathione (GSSG) level. It was also shown an activity suppression of antioxidant enzymes. That results in the onset and development of oxidative (increase in oxidative protein destruction markers level) and nitrosative (increase in 3-nitrotyrosine level) stresses. These disturbances lead to mitochondria dysfunction resulting as a rule in apoptotic neuron death. Tamoxifen and Tibolone (0.1 μM) normalize thiol-disulfide balance and significantly reduce the risk of oxidative and nitrosative stresses. That way, they prevent mitochondria dysfunction and cell death.

Keywords: *glutathione, mitochondrial dysfunction, tamoxifen, thiol-disulfide system, tibolone, Hsp70, protein carbonyls*

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INTRODUCTION

Over the past decade it has been proved that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a significant role in the process of apoptosis/necrosis for various neurodegenerative disorders. Oxidative stress-mediated neuronal loss may be caused by a decline in the antioxidant molecule glutathione (GSH). Glutathione has many physiological functions, such as its participation in the defense against reactive oxygen species [1–7]. Evidence for a role of oxidative stress and diminished GSH status is presented for Lou Gehrig's disease (ALS), Parkinson's disease, and Alzheimer's disease [6]. Significant depletion of the thiol antioxidant glutathione (GSH) may lead to oxidative stress, mitochondrial dysfunction, and ultimately neuronal cell death [8–11]. Glutathione-associated metabolism is a major mechanism for cellular protection against agents which generate oxidative stress [12]. The role of glutathione in regulation of various protective mechanisms in neuron in unfavorable conditions is determined by glutathione as a part of thiol-disulfide system (TDS), which is a prospective target for pharmacological correction at central nervous system (CNS) pathology. Certain results were obtained after using the thiol scavengers of ROS and RNS, precursors of glutathione synthesis, selenium derivatives [13].

Selective estrogen receptor modulators (SERMs) are compounds that bind to estrogen receptors (ER) and produce estrogen-like (agonist) effects in some tissues and estrogen-blocking (antagonist) effects in other tissues [14–15]. SERMs act as ER agonists in the brain. Experimental and clinical data suggest a neuroprotective role for SERMs in normal and injured brain [16–23].

Most of the studies showing neuroprotective action of SERMs have focused on Tamoxifen [24–25]. The neuroprotective action of Tamoxifen indicates that this SERM decreases neuronal damage caused by different forms of neuronal injury in animal models of neurodegenerative diseases. These include animal models of traumatic injury of the central nervous system and peripheral nerves, stroke, multiple sclerosis, Parkinson's disease, Alzheimer's disease, cognitive decline, and mood disorders. A well-documented protective effect of Tamoxifen on the nervous tissue has been realized by means of the following mechanisms: 1—inhibition of excitatory amino acids [26]; 2—inhibition of neuronal nitric oxide synthase (NOS) both in vitro and in vivo [27–28]; 3—scavenging of

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ROS and RNS in vivo [29]; 4—action at estrogen receptors [30].

Tibolone (Livial®) has a mild androgenic and marked estrogenic effect. Being tissue-specific, it has a similar to estrogen effect on central nervous system [31]. Tibolone is a class of ER modulators which acts as a prodrug. Only in the last few years has the number of investigations of the effects of tibolone on brain biochemistry increased [32–34].

This work objective is to enrich knowledge of Tamoxifen neuroprotective action (EBEWE, Austria) and to determine neuroprotective action of Tibolone (Livial, NV Organon, Netherlands) studying their influence on indices of antioxidant system, oxidative stress and mitochondrial dysfunction at glutathione depletion of neurons in vitro.

MATERIALS AND METHODS

Animals

All experimental procedures were carried out on neonatal rats (10–day old) for isolation of neurons and on male rats of Wister line (100–120 g) from the vivarium of the Institute of Pharmacology and Toxicology of National Academy of Medical Science of Ukraine for isolation of mitochondria. The adaptation period, or quarantine period, of animals was 14 days. Animals were housed in cages (size 400 × 320 × 160 mm; five rats per cage) and had free access to water and food and were kept under standard laboratory conditions (room temperature 19–25°C, 50–70% humidity, 12/12 h light/dark cycle). All experiments on animals were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals in Biomedical Research. Rats were sacrificed by decapitation under anesthesia with thiopental (40 mg/kg) and their brains were removed.

Isolation of Neuronal Cell Bodies

Neurons from cerebral cortices were prepared according to procedure described by Sellinger [35]. The method is based on disintegration of cerebral tissue by consecutive filtration through nylon sieves of different pore size. Brains of 10 neonatal rats were put on preliminary cooled glass plate where cerebral cortexes were separated from white matter. Isolated cerebral cortexes were crumbled up by scalpel and transferred to medium containing 7.5% polyvinylpyrrolidone, 1% bovine serum albumin and 10 mM calcium chloride. Then this material was filtered through three nylon sieves of different pore size (286 μm, 82 μm and 58 μm). Filtrates were centrifiiged in the sucrose and ficoll density gradients according the following scheme (Fig. 1).

Sucrose and albumin were washed off the isolated neurons with the help of cooled physiological solution to proceed to the further analysis. For purity control of neurons isolated with this method, neuron suspension was fixed with a mixture of ethanol, formalin and acetic acid (7 : 2 : 1) and was stained with thionine and cresyl violet by the Nissl method and hallocyanine and chromic alum by the Einarson method [36]. The staining showed that neuron fraction contained cell bodies with partially preserved nerve-cell processes. The microscope analysis (Axioskop, Zeiss Germany) determined that neuron enrichment was not less than 90%.

Glutathione Depletion

Depletion of glutathione was carried out by introduction of Buthionine sulfoximine (BSO, Sigma B2640, 500 μM) into neuron suspension with following 24 hours incubation at $t = 37$°C. BSO depletes cellular level of GSH by inhibiting γ-glutamyl-cysteine synthetase, key enzyme in glutathione biosynthesis, and downregulates glutathione-S-transferase level [37, 38].

Depletion of glutathione in mitochondrial fraction was carried out by introduction of chloro-2,4-dinitrobenzene (CDNB, 1 mM) to the incubation medium with following 60 minutes incubation at $t =$ 37°C. It forms conjugates with glutathione by glutathione-S-transferase in cytosolic and mitochondrial fractions [39].

Apoptosis Detection

For apoptosis identification the neurons were stained with fluorescent dye Hoechst 33342 (Sigma, B2261). A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy (Zeiss Axioscope, Germany). The cells exhibiting a high blue fluorescent were interpreted as apoptotic. At least 1000 cells in randomly selected microscopic fields were counted under microscope. Each experiment was performed in triplicate. The obtained results were analyzed for significance by one-way ANOVA test and were presented as mean ±SD.

Determination of Oxidative Stress Markers and Antioxidant System State

Preparation of the cellular extract. Neuron suspension was lysed by two freeze-thaw cycles. After centrifugation of the lysates at 10000 *g* for 10 minutes at 4°C they were stored at −70°C, until analysis. The total protein amount of cell lysates was determined according to Lowry [40].

Determination of protein oxidation. Protein carbonyls can be used as an indicator of protein oxidation, they are assayed by formation of the 2,4-dinitrophenylhydrazone (DNPH) covalent adduct: aldehyde phenylhydrazones (APH) and ketone phenylhydrazones (KPH). APH and KPH were determined spec-

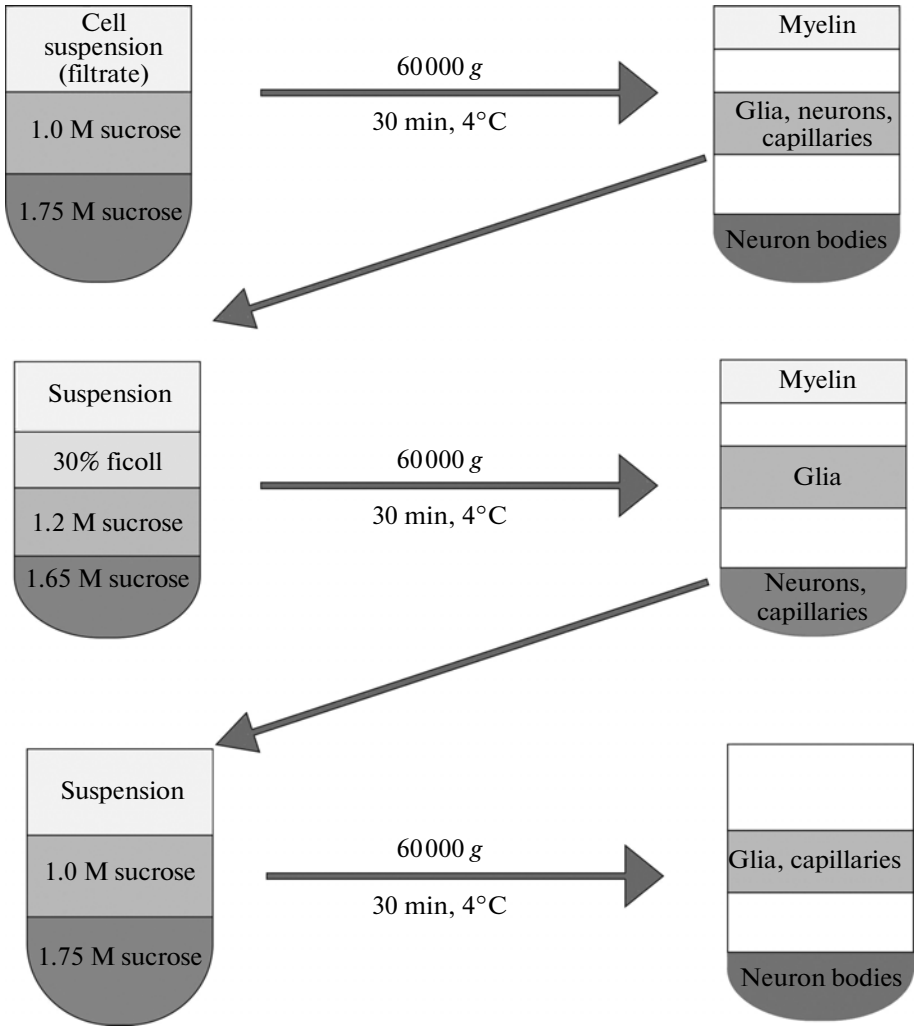

Fig. 1. Scheme of neuron isolation. All steps of neuron isolation were done at 0–4°C. The precipitates after the 1st and the 3d centrifugation include neuron bodies with enrichment not less than 90%.

trophotometrically from the difference in absorbance at 270 nm (for APH) and 363 nm (for KPH). Results were expressed as nmol/mg protein [41].

3-Nitrotyrosine (3-NT) is a product of irreversible nitration of tyrosine residues by peroxynitrite ($ONOO^-$). Protein oxidation involving RNS was measured by determination of 3-NT level (Nitrotyrosine ELISA kit, HK501). Results were expressed as nmole/mg protein.

Reduced/oxidized glutathione assay. Reduced and oxidized glutathione (GSH and GSSG) concentration was determined by a fluorometric method based on the reaction of reduced glutathione (GSH) with o-phthalic anhydride. Firstly, GSH was measured by its fluorescent complex with o-phthalic anhydride. Then GSH was masked by l-methyl-2-vinylpyridinium. For measuring of GSSG it was previously reduced by reaction with NADPH by glutathione reductase. Results were expressed as μmole/g tissue [42].

Assay of Mn-SOD. Superoxide dismutase (Mn-SOD) activity determination (principle of the method): the method is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon measured at 560 nm. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg protein [43].

Mitochondria isolation. Isolated cerebral cortexes of male rats of Wistar line (100–120 g) were crumbled up by scalpel and homogenized with medium containing 250 mM sucrose, 5 mM HEPES, 500 μM EDTA (pH 7.4).

For mitochondria isolation, this suspension was centrifuged for 7 minutes at 700 *g* (4°C). Then supernatant was centrifuged for 15 minutes at 11000 *g* (4°C). The mitochondria fraction was washed and resuspended in a medium, which does not containing EDTA, and kept on ice for further research [44].

Mitochondrial dysfunction. The mitochondria dysfunction level was estimated on mitochondrial permeability transition pore (mPTP) opening and on mitochondrial membrane potential ($\Delta\psi$). Preliminary mitochondrial suspension was incubated with 1 mM CDNB for 60 minutes at $t = 37°C$.

For measurement of mPTP opening, mitochondria were suspended in freshly prepared swelling buffer (120 mM KC1, 20 mM Tris-HCl, 1 mM KH_2PO_4, 2 mM glutamate, 1 mM malate, pH 7.4) at 1 mg/mL, and swelling of mitochondria was monitored by decrease in absorbance at 540 nm in the presence of $CaCl_2$ (5–200 μM). Cyclosporin A (CsA, 0.2 μM) was added 2–3 min before $CaCl_2$ to prevent the swelling of mitochondria. Extent of pore opening was expressed in terms of changes in absorbance at 540 nm per minute in the presence and absence of Ca^{2+} and CsA (Fluka, Sweden).

The electrical mitochondrial membrane potential difference ($\Delta\Psi$) was monitored spectrofluorimetrically using 5 μM safranine O (Aldrich, USA) as an indicator. $\Delta\Psi$ was determined as difference in absorption of the sample at 515 nm and 525 nm [45].

Western blot. After determination of total protein amounts of cell lysates, 25 μg total protein were loaded and separated in 10% polyacrylamide gel under denaturating conditions [46] at constant 200 V. Proteins were transfered from SDS-polyacrylamide gel to nitrocellulose membranes by using method of [47]. Membranes were washed four times for 5 min with 1 × TBST buffer (10 mM Tris, pH: 8.0; 150 mM NaCl; 0.5% Tween-20) after transfer step. Non specific sites were blocked using western blocker solution (Sigma, W0138). The membranes were incubated overnight with a monoclonal anti-heat shock protein 70 (Hsp70, Sigma, H5147; dilution 1 : 5000). Then, membranes were washed with cold 1 × TBST (5 min, 4 times). After rinsing of membranes, the membranes were incubated with secondary biotinylated anti-mouse IgG (Sigma, Mouse ExtrAvidin Peroxidase Staining Kit, EXTRA2; dilution 1 : 1000) for 1 hour. After the secondary antibody incubation, the membranes were treated with ExtrAvidin peroxidase (Sigma, Mouse ExtrAvidin Peroxidase Staining Kit, EXTRA2). For detection, blots were processed with 3-amino-9-ethylcarbazole (Sigma, AEC, A6926).

Study of Neuroprotective Activity of Tamoxifen and Tibolone in vitro

Tamoxifen and Tibolone in 0.1 μM; 10 μM; 100 μM concentrations were introduced into neuron suspension and mitochondrial suspension 30 minutes before CDNB/BSO for the study of neuroprotective activity and also effective doses of these agents. So we divided neuron suspension into 6 groups. The first group was incubated for 30 min with 0.1 μM Tamoxifen, than for 1 hour with 500 μM BSO. The second group was incubated for 30 min with 10 μM Tamoxifen, than for 1 hour with 500 μM BSO. The third group was incubated for 30 min with 100 μM Tamoxifen, than for 1 hour with 500 μM BSO. The fourth group was incubated for 30 min with 0.1 μM Tibolone, than for 1 hour with 500 μM BSO. The fifth group was incubated for 30 min with 10 μM Tibolone, than for 1 hour with 500 μM BSO. The sixth group was incubated for 30 min with 100 μM Tibolone, than for 1 hour with 500 μM BSO.

The mitochondrial suspension were also divided into 6 groups. The first group was incubated for 30 min with 0.1 μM Tamoxifen, than for 30 min with 1 mM CDNB. The second group was incubated for 30 min with 10 μM Tamoxifen, than for 30 min with 1 mM CDNB. The third group was incubated for 30 min with 100 μM Tamoxifen, than for 30 min with 1 mM CDNB. The fourth group was incubated for 30 min with 0.1 μM Tibolone, than for 30 min with 1 mM CDNB. The fifth group was incubated for 30 min with 10 μM Tibolone, than for 30 min with 1 mM CDNB. The sixth group was incubated for 30 min with 100 μM Tibolone, than for 30 min with 1 mM CDNB.

Statistical Analysis

Results were expressed as mean ± standard deviation (mean ±SD). The Kolmogorov-Smirnov test was chosen to analyze data distribution. According to these results, appropriate parametric or non-parametric tests were used in further analyses. Unpaired Student's *t*-test or Mann–Whitney *U* test was performed to assess differences among quantitative variables between groups. Chi square test was applied to analyze differences in qualitative and categorical variables. Data was analyzed using application software "Biostatistics for Windows, version 4.03" and "Microsoft Excel 2007". All p values below 0.05 ($p \le 0.05$) were considered statistically significant.

RESULTS

The effect of Tamoxifen and Tibolone on Apoptosis in Neurons Preincubated with 500 μM BSO

A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy. Treatment of neurons with 500 μM BSO resulted in significant increase of apoptotic neurons, stained with Hoechst 33342.

Tamoxifen and Tibolone in the concentrations of 0.1 μM; 10 μM; 100 μM prevent cell death in the neuron suspension preincubated with 500 μM BSO. The most effective concentration for both agents is 0.1 μM (Fig. 2). At this concentration, Tamoxifen reduced apoptotic neurons by 50%, and Tibolone—more than 77% in comparison to control (Fig. 2). It should be noted that Tibolone reduced the apoptotic index more than Tamoxifen ($p \le 0.05$).

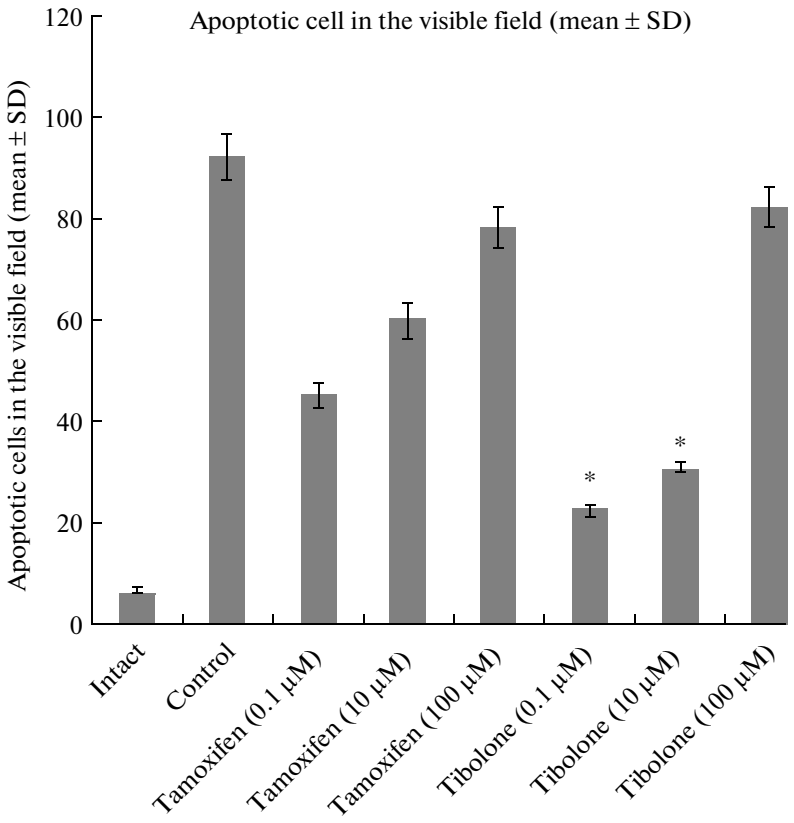

Fig. 2. Influence of Tamoxifen and Tibolone on apoptotic cells in the neuron suspension preincubated with BSO (500 μM). *—$p \leq 0.05$ in comparison to Tamoxifen.

Influence of Tamoxifen and Tibolone on Glutathione System in Neuron Suspension Preincubated with BSO (500 μM)

Neuron incubation with 500 μM BSO during 24 hours resulted in the displacement of thiol-disulfide equilibrium in the oxidized thiol direction. It was evidenced from a critical decrease of GSH on 85%) and an increase of GSSG on 72% in comparison to intact samples. We found that treatment of neurons with 0.1 μM (effective dose) Tamoxifen and Tibolone resulted in glutathione system rebalancing (an increase of GSH and a decrease of GSSG). It is important to mark that Tibolone statistically reliably ($p \leq 0.05$) exceeded Tamoxifen on the majority of studied parameters (Fig. 3).

Influence of Tamoxifen and Tibolone on Oxidative Stress in Neuron Suspension Preincubated with BSO (500 μM)

Treatment of neurons with 500 μM BSO leads to the increase of markers of protein oxidative damage: APH, KPH and also 3-NT were increased. We found that Tamoxifen and Tibolone at 0.1 μM treatment of neurons preincubated with BSO led to a decrease of oxidative stress intensity (a decrease of APH, KPH, 3-NT) (Fig. 4).

Influence of Tamoxifen and Tibolone on Hsp70 Content in Neuron Suspension Preincubated with BSO (500 μM)

Late data showed the protective role of HSP at a cerebral ischemia, which is accompanied by intensification of free-radical oxidation processes, displacement of thiol-disulfide balance, development of nitrosative stress and glutamate excitotoxicity [48, 49]. HSP are induced in cells of all living organisms in response to the action of various stress factors, such as thermal shock, hypoxia, ischemia, metabolic disturbance, viral infection and influences of pharmacological agents. It is known that HSP take part in vital functions of cell. Most protective functions of HSP are related to chaperone activity that is their ability to find out damaged or resynthesized polypeptides and correct their structure by ATP-dependent mechanism or delete uncorrectable proteins through a proteosomic apparatus. Due to stabilization of the oxidation damaged macromolecules HSP are able to prevent mPTP opening that blocks release of cytochrome c from mitochondria. It shows its antiapoptotic action.

Treatment of neurons with 500 μM BSO leads to the decrease of Hsp70 content. We found that Tamoxifen and Tibolone at 0.1 μM treatment of neurons preincubated with BSO led to an increase of Hsp70 content (Fig. 5).

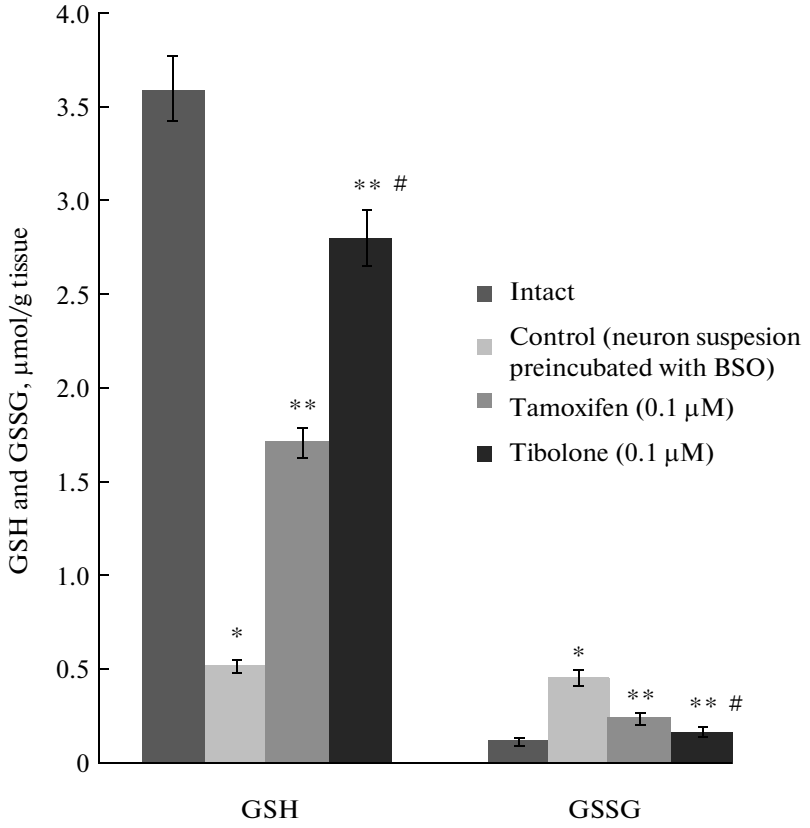

Fig. 3. Influence of Tamoxifen and Tibolone on glutathione system in neuron suspension preincubated with BSO (500 μM). Results represent mean ±SD ($n = 6$). *—$p \le 0.05$ in comparison to intact samples; **—$p \le 0.05$ in comparison to control; #—$p \le 0.05$ in comparison to Tamoxifen.

Influence of Tamoxifen and Tibolone on the Indices of Antioxidant System in Mitochondria Preincubated with CDNB (1 mM)

GSH forms a conjugate with CDNB in the mitochondrial matrix, a process believed to be catalyzed by GST [39]. The addition of 1 mM CDNB for 60 minutes to isolated neuron mitochondria caused a loss of GSH on 62%. GSH depletion caused a change in activity of Mn-SOD activity: Mn-SOD activity was significantly decreased after CDNB treatment. Tamoxifen and Tibolone at 0.1 μM show a positive effect on the antioxidant system of mitochondria by increasing of the amount of mitochondrial glutathione and also promoting Mn-SOD activity (Fig. 6).

Influence of Tamoxifen and Tibolone on the Functional State in Mitochondria Preincubated with CDNB (1 mM)

Mitochondria play critical roles in both the life and death of cells. In healthy neurons, their primary function is the provision of ATP through oxidative phosphorylation. However, latent within mitochondria, there exist mechanisms that, once activated, convert the mitochondria from organelles that support the life of the cell to those the actively induce both apoptotic and necrotic cell death. The switch in roles is mediated by the opening of a nonspecific pore in the mitochondrial inner membrane, known as the mitochondrial permeability transition pore (mPTP). This normally remains closed, but can open under conditions of cellular stress with dire consequences.

It was determined that mitochondrial membrane potential ($\Delta\psi$) was decreased and difference between light absorption at 540 nm was increased. Therefore, we can assume mPTP formation (Fig. 7). Tamoxifen and Tibolone limited development of mitochondrial dysfunction by suppression of mPTP formation and saving of mitochondrial membrane potential ($\Delta\psi$).

DISCUSSIONS

GSH is the major nonprotein thiol in cells and plays a key role in detoxification of reactive oxygen metabolites (peroxides) and reactive electrophilic compounds [50]. There exist two major GSH pools in cells, a cytoplasmic and mitochondrial pool.

Neurons treated with BSO at 500 μM for 24 hours resulted in critical depletion of GSH in cytosolic fractions of neuron. That in turn, causes uncontrolled production of ROS and RNS and development of oxidative and nitrosative stresses. It was established, that

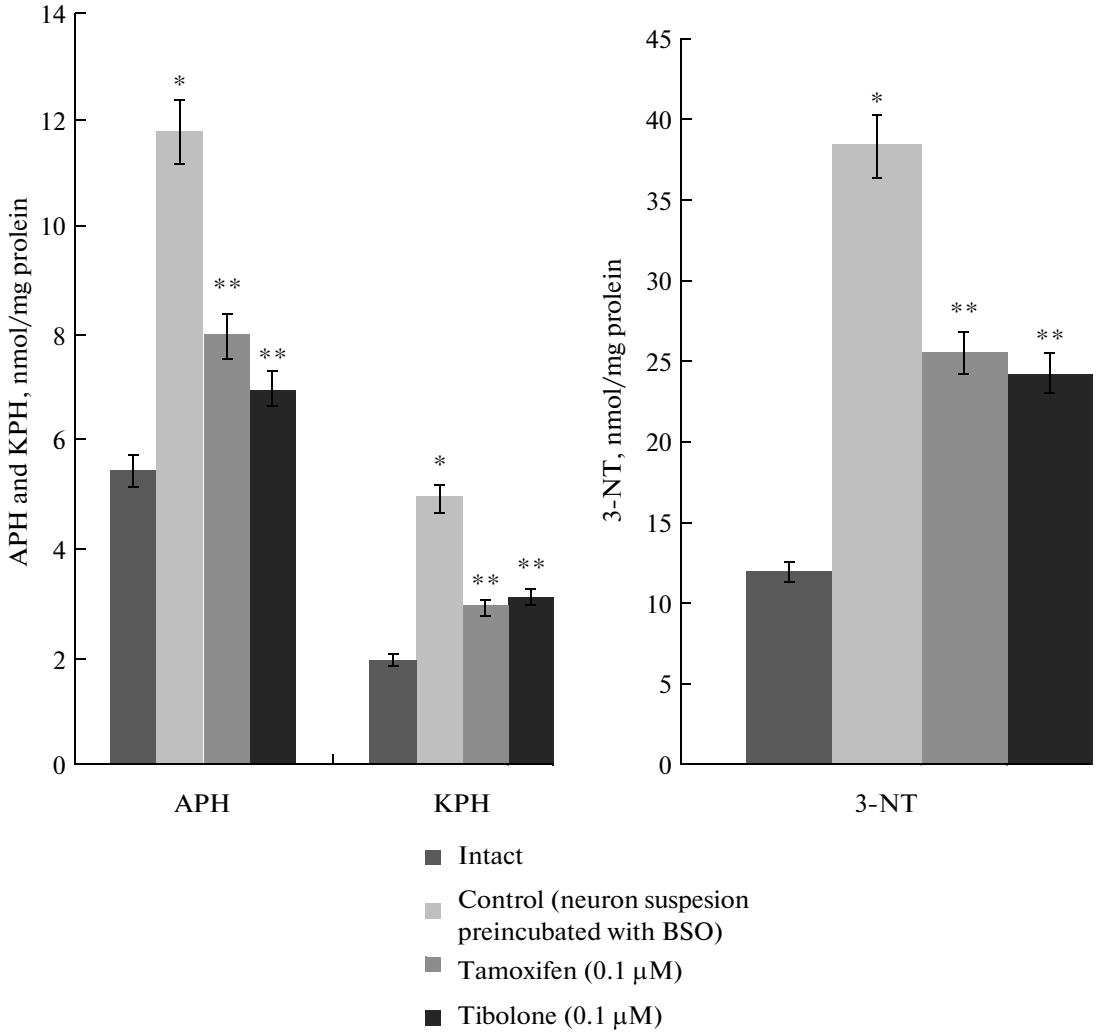

Fig. 4. Influence of Tamoxifen and Tibolone on oxidative stress in neuron suspension preincubated with BSO (500 μM). Results represent mean ±SD ($n = 6$). *—$p \le 0.05$ in comparison to intact samples; **—$p \le 0.05$ in comparison to control.

GSH deficiency leads to an increase of APH and KPH and an increase of 3-NT. The cytotoxic NO derivatives react with aromatic and aliphatic amines and form N-nitroamines. The possible proof of that is an increase of 3-NT. N_2O_3 reacts with a cysteine to form S-nitrocysteine and with GSH to form S-nitroglutathione. S-nitroglutathione is a main transport form for NO. Some research has established that the NO transport occurs with a formation of N_2O_3, which after nitrosilates thiols. Therefore GSH is more depleted that leads to more disruption of the thiol-disulfide equilibrium [51, 52]. The mechanism of an increase of cytosolic GSH by Tamoxifen and Tibolone after GSH depletion using BSO is unclear. Probably it's associated with induction of expression of enzymes participated in GSH synthesis de novo or in reduction of GSSG (glutathione reductase). It could be realized through activation of estrogenic receptors by these SERMs.

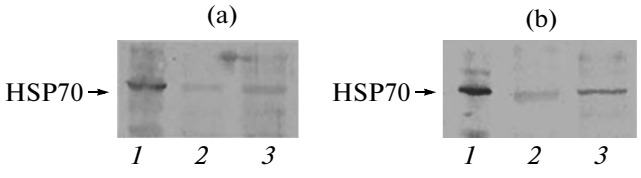

Fig. 5. Influence of Tamoxifen and Tibolone on HSP70 content in neuron suspension preincubated with BSO (500 μM). *1*—intact; *2*—control, neuron suspension preincubated with 500 μM BSO; *3*(a)—0.1 μM Tamoxifen; *3*(b)—0.1 μM Tibolone.

Mitochondrial GSH is important protection against oxidative stress is supported by (a) a direct reaction with reactive oxygen and nitrogen species and (b) as an electron donor for enzyme linked systems, peroxiredoxins and glutathione peroxidases [53]. Mitochondrial GSH depletion occurs in different neurodestructive disorder such as cerebral ischemia

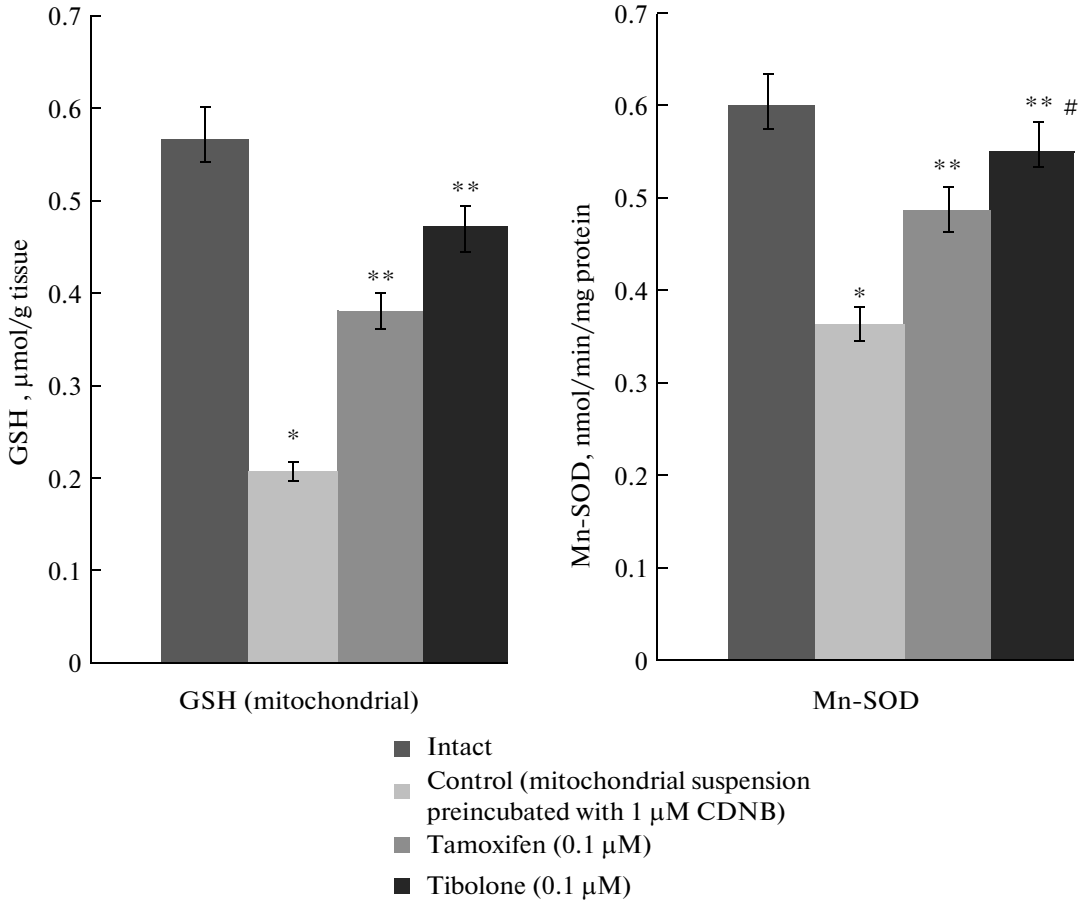

Fig. 6. Influence of Tamoxifen and Tibolone on antioxidant system in mitochondria preincubated with CDNB (1 mM). Results represent mean ±SD ($n = 6$). *—$p \le 0.05$ in comparison to intact samples; **—$p \le 0.05$ in comparison to control; #—$p \le 0.05$ in comparison to Tamoxifen.

[54]. Oxidative injury of neurons is possibly stimulated when mitochondrial GSH is depleted by 1 mM CDNB during 1 hour [55, 56].

Excess RNS (peroxynitrite, nitrosonium ion) produced by GSH deficiency in mitochondria, results in Mn-SOD oxidative modification that decreases its activity. Another possible explanation for the change in Mn-SOD activity might be that with less GSH available to conjugate H_2O_2, the H_2O_2 becomes elevated to the point that it provides negative feedback on Mn-SOD [57]. The decrease of Mn-SOD activity leads to the second "boom" of free-radical reactions and increase oxidative destruction of redox-sensitive sites of mitochondrial membrane and forming of stable mitochondrial dysfunction [58]. Besides, uncontrolled growth of active NO derivatives leads to oxidation of mitochondrial respiratory chain proteins and inactivation of mitochondrial Mn-SOD, that more depletes antioxidant system of neuron. Tamoxifen treatment induced Mn-SOD expression. The ability of tamoxifen to enhance MnSOD is an important effect underlying its antioxidant and neuroprotective actions [59].

Mitochondrial GSH is considered vital for cell survival. Cell necrosis caused by GSH-depleting agents is believed to occur only after mitochondrial GSH depletion, with cytoplasmic GSH depletion being less consequential [55, 60]. Cell necrosis after severe mitochondrial GSH depletion has been ascribed to increase steady-state levels of reactive oxygen species that lead to mitochondria damage and dysfunction [55].

The GSH deficiency in mitochondria leads to an increase of ROS and RNS production and oxidation of cysteine-containing sites of proteins, which form a mitochondrial pore. The rise of NO active forms causes oxidation of SH-groups of ATP/ADP-antiporter which is a protein of inner membrane of mitochondria. That leads to conversion of this carrier of adenine nucleotides to a nonspecific channel which is permeable for any low-molecular compounds. mPTP opening converts mitochondria from "power house" to "firebox" of substrates for oxidation without ATP production. Due to mPTP formation the mitochondria becomes permeable for K^+ and Cl^- and only proteins form its osmotic pressure. There are more proteins in matrix than in intermembrane space and that

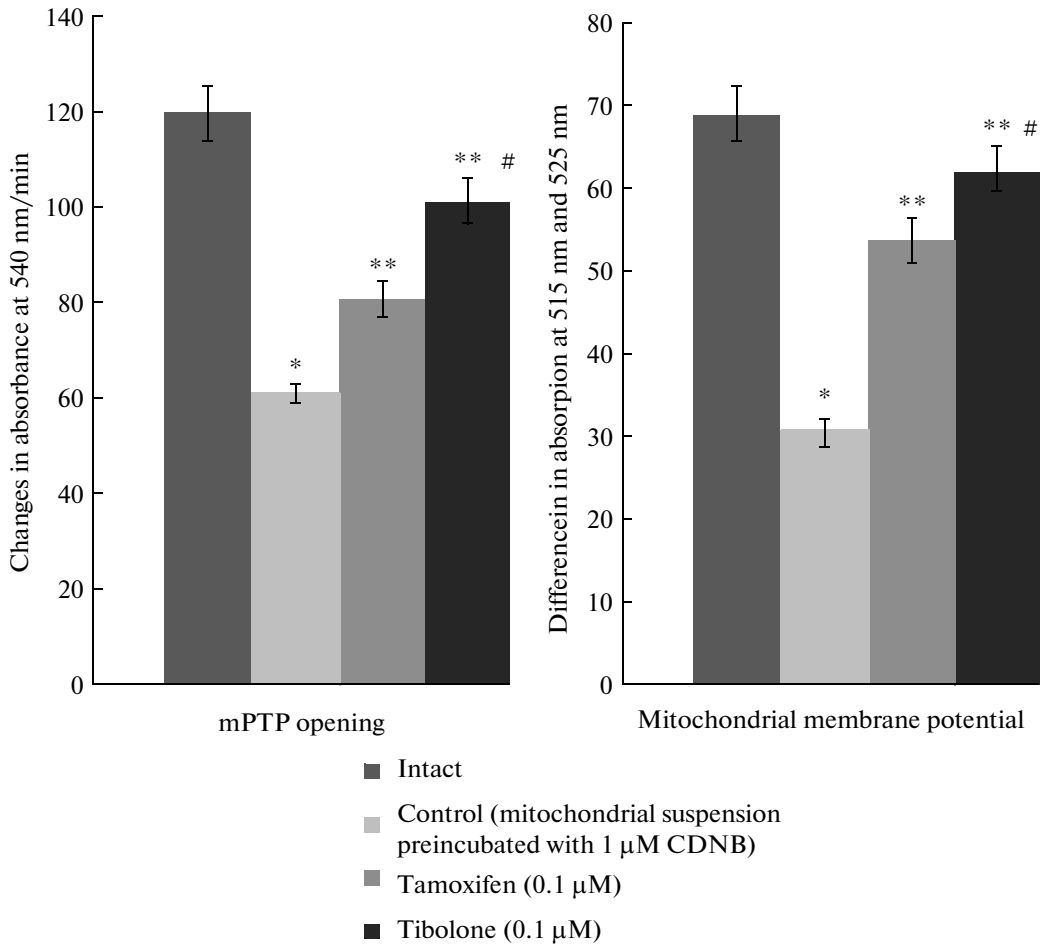

Fig. 7. Influence of Tamoxifen and Tibolone in concentration 0.1 μM on mitochondrial dysfunction in the neuron mitochondria suspension preincubated with CDNB (1 mM). Results represent mean ±SD ($n = 6$). *—$p \le 0.05$ in comparison to intact samples; **—$p \le 0.05$ in comparison to control; #—$p \le 0.05$ in comparison to Tamoxifen.

is why water begins to penetrate into the matrix, in an effort to dilute the protein solution there. This causes mitochondria to swell. Also unfolding of the cristae allows the matrix to expand without rupture of the inner membrane, the outer membrane will break. As a result apoptotic proteins get out to cytosol develop so-called "mitoptosis" initiating apoptotic death of neuronal cell [52]. Also, the inner membrane becomes freely permeable to protons. This uncouples oxidative phosphorylation, causing the proton-translocation ATPase to reverse direction and so actively hydrolyse ATP, rather than synthesis it. Under such conditions, intracellular ATP concentrations rapidly decline, leading to the disruption of ionic and metabolic homeostasis and the activation of degradative enzymes such as phospholipases, nucleases, and proteases. Unless pore closure occurs, these changes will cause irreversible damage to the cell, resulting in cell death.

It was found that mitochondrial $\Delta\psi$ was increased by Tamoxifen and Tibolone. The inhibition of CDNB-induced mitochondrial depolarization by Tamoxifen and Tibolone is consistent with their reported action as an inhibitor of mPTP formation.

In our opinion, similar action of estrogen receptors modulators is explained at first by their direct antioxidant effects [18, 61, 62]. Secondly, it is known that activation of receptors by estrogens includes detaching of heat shock proteins (HSP) from ER that provides entering of HSP into a cell [20]. It was shown in our work that pretreatment of neurons with Tamoxifen and Tibolone increases Hsp70 content in GSH depleted by BSO neurons.

In the third, by researches of Brann and Dubai [30, 63], it was found out that estrogens modulate expression of global transcription factors, in particular AP-1, which is responsible for the synthesis of main enzymes of antioxidant system and TDS. Possibly this explains a significant ability of Tamoxifen and Tibolone to restore the activity of Mn-SOD [30, 63]. Besides, the antiapoptotic effect of estrogens explained by stimulation of expression of Bcl-xL family antiapoptotic proteins was marked [22].

Thus, the results we received expose the role of the glutathione system of neuron as the important target for neuroprotective therapy. Tamoxifen is well-known as neuroprotective agent. We extended knowledge about Tamoxifen neuroprotective action and studied neuroprotective action of Tibolone realized through normalization of the thiol-disulfide system, reducing of oxidative and nitrosative stresses development resulted in prevention of mitochondria dysfunction and cell death.

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