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TOXIC EFFECT OF ANTIDEPRESSANTS ON MALE REPRODUCTIVE SYSTEM CELLS: EVALUATION OF POSSIBLE FERTILITY REDUCTION MECHANISM

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Depression is acknowledged as a major public health problem. Pharmacological treatment may cause adverse drug reactions and sexual side effects. At the same time, the knowledge of the molecular mechanisms associated with antidepressant-mediated toxicity to reproductive cells is fragmentary. The aim of this study was the multilevel evaluation of the potential toxicity of several antidepressants or antipsychotic drugs (amitriptyline, 10 μM; escitalopram, 30 μM; fluoxetine, 5 μM; imipramine, 20 μM; mirtazapine, 150 μM; olanzapine, 40 μM; reboxetine, 30 μM; venlafaxine, 250 μM) on the cells of the spermatogenesis pathway. Effects of various drugs were monitored by several methods including mitochondrial activity MTT test, fluorescent staining, real-time PCR, morphology analysis, immunofluorescence, and Western blots. Obtained results suggest the concentration- and the time-dependent cytotoxic effect. The molecular mechanism of cytotoxic effect is mediated by disturbances in the redox balance (increased production of reactive oxygen species and reactive nitrogen species), failure of enzymatic and non-enzymatic cell protection mechanisms (glutathione system, nuclear factor-κB and fibroblast growth factor 2-mediated pathways), and impairment of mitochondrial functions. In addition, we provide for the first time, to our knowledge, evidence that antidepressant treatment may contribute to spindle apparatus assembly defects and organelle distribution during cell division *in vitro* (alterations in the levels of small C terminal domain phosphatase-1 and -3, NuMa, and calnexin protein levels). This study sheds new light on the pathomechanisms of antidepressants action and their associated toxicity towards the reproductive system, emerging issues linked with animal or human reproductive health, and treatment of mood disorders.

Key words: *cell division, drugs, male reproductive biology, spermatogenic cells, toxicity*

INTRODUCTION

According to the National Institute of Mental Health, mood disorders, including major depressive disorders, are a significant global health problem affecting approximately 20% of women and 12% of men at any given point. Nowadays, their treatment is mainly based on the use of individually selected antidepressant medications, as well as in many cases on psychological interventions (1, 2). The mechanism of action of most antidepressants is poorly understood. In general, the efficiency of both generations of antidepressants is at a comparable level with a higher number of adverse drug reactions after treatment with first-generation drugs (3). Common adverse drug reactions associated with treatment with various classes of antidepressants are fluid and electrolyte disturbances, cardiovascular disfunction, extrapyramidal symptoms, falls, bleeding risk, hypersensitive crisis, serotonin syndrome, and anticholinergic burden (4). At the molecular level, antidepressant medications have been shown to affect redox balance, metabolic activity, and cell cycle progression, induce double-strand DNA breaks, telomere damage, micronuclei formation and finally lead to inhibition of proliferation and initiation of apoptotic cell death (5-8). Continuing, recent scientific data points on the possibility of potential negative effects of antidepressant treatments in terms of reproductive biology and general sexual activity. Sexual dysfunctions are reported for all groups of prescribed antidepressants and in general, the clinicians mention the following symptoms: loss of libido, reduced sexual excitability, problems with maintaining an erection, and delayed ejaculation (9). However, the clinical data on specific doses of antidepressants causing first symptoms of toxicity to the reproductive tract is limited and mostly focuses on the general observations related to semen parameters and other markers of male fertility (10). At the same point, the data on the impact of antidepressant drugs on single-cell cellular physiology is fragmentary. Limited but more detailed *in vitro* studies revealed that antidepressants may directly impact the integrity of cell membranes (by binding to sulfhydryl groups of sperm membrane) and/or trigger DNA damage and thus lead to reduced sperm cell count, affected motility, and altered morphological appearance (11, 12). Thus, given the limited data on antidepressant medication use and their widespread and often long-term application, there is a clear need for further data regarding their impact on germ cell quality and male fertility.

Therefore, the aim of this study was the multilevel evaluation of the potential toxicity of several antidepressants or antipsychotic drugs on the cells of the spermatogenesis pathway i.e. type B spermatogonia and spermatocytes. This study will bring new important insight into mechanisms of antidepressant reprotoxicity, emerging issues linked with reproductive health, and treatment of mood disorders.

MATERIALS AND METHODS

Chemicals

The chemicals were of analytical or biotechnology grade and were used without any further modifications. Unless stated otherwise, reagents and drugs were obtained commercially from Thermo Fisher Scientific (Waltham, MA, USA) or Cayman Chemicals (Ann Arbor, MI, USA).

Cell lines

The mouse type B spermatogonia (GC-1 spg) and mouse spermatocytes (GC-2 spd) were obtained from ATCC (Manassas, VA, USA). Routinely, cells were maintained in a CO_2 incubator at 37ºC in a humidified atmosphere. Both cell lines were cultured in basal medium Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose, 1 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 29.2 mg/ml L-glutamine). At 80 to 90% confluency, cells were passaged by trypsinization. For all experiments, cells were seeded at an optimal density of 3.0×10^3 cells/cm^2. The research was conducted on cell lines, therefore did not need any ethical permissions.

Drugs preparation

Antidepressant or antipsychotic drugs: 1) amitriptyline (hydrochloride) (AMI), 2) escitalopram (ESC), 3) fluoxetine (hydrochloride) (FLU), 4) imipramine (hydrochloride) (IMI), 5) mirtazapine (MIR), 6) olanzapine (OLZ), 7) reboxetine (mesylate) (REB), and 8) venlafaxine (hydrochloride) (VEN) were dissolved in high purity dimethyl sulfoxide (DMSO) to obtain 100 mM stock solutions according to characteristic solubility provided by the manufacturer and stored at –20ºC. Further dilutions were prepared in a complete culture medium just before use and added immediately to the cell cultures.

MTT assay

The cytotoxicity MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as described previously (13, 14). Briefly, the cells were seeded at constant density and after 24 h, treated with wide range of antidepressants (depending on drug tested: 1 – 600 µM). After 48 h, the cell culture medium was exchanged with fresh one also supplemented with drugs. The MTT test was performed after 48 h and results are expressed as cell metabolic activity (%), while non-treated control cells are considered as 100%. According to MTT results following concentrations of drugs were chosen for further studies: AMI 10 µM, ESC 30 µM, FLU 5 µM, IMI 20 µM, MIR 150 µM, OLZ 40 µM, REB 30 µM, VEN 250 µM (the concentrations leading to approximately 50% reduction of MTT activity).

Intracellular redox state

Cells were seeded into 96-well plates at a standard density and after 24 h antidepressants at the selected concentrations were added (13, 15). Plates were incubated for 48 and 96 h under standard conditions. Then, a mixture of fluorogenic probes at a final concentration of 5 µM of each prepared in sterile PBS was applied (dihydroethidium DHE, Thiol Tracker Violet - Thermo Fisher Scientific, Waltham, MA, USA, and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate DAF-FM - Cayman Chemicals, Ann Arbor, MI, USA). After 30 minutes of incubation, digital images were captured by InCell Analyzer 2000 (GE Healthcare, Chicago, IL, USA). The signal measurement was made from a single well (in several replications with at least 1000 cells per replication) and results are presented as mean values of relative fluorescence units. The values of the fluorescence signal are not related to the single cell.

Cell morphology analysis

Spermatogenic cells were seeded into flat bottom 6-well plates at a standard density and treated with selected concentrations of antidepressants. Cells were incubated for 96 h, while photos were captured every 48 and 96 h using an inverted microscope equipped with a CCD monochrome camera (Zeiss Axiovert 40 CFL, AxioVs40 software, Oberkochen, Germany).

Gene quantification with real-time PCR

Cell samples were homogenized and total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions. RNA concentration and purity were estimated by measuring the absorbance at A260/A280 nm wavelength on Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, 0.8% agarose gel electrophoresis was applied to estimate RNA quality. cDNA synthesis was performed using a commercial High Capacity cDNA Reverse Transcription Kit in a total volume of 20 µl, as described in manufacturer's instructions. Briefly, 2 µg of RNA was diluted to 10 µl with sterile water and mixed with 10 µl of freshly prepared master mix (2 µl of 10×RT buffer, 0.8 µl of 25×dNTP Mix, 2 µl of 10×RT Random Primers, 1 µl of Multiscribe Reverse Transcriptase and 3.2 µl H_2O). The TaqMan probes [*NuMa* (#4331182, ID Mm00659817_m1), *Scp1* (#4351372, ID Mm01298009_m1), *Scp3* (#4331182, ID Mm00488519_m1), *18S ribosomal* RNA (#4331182, ID Mm03928990_g1)] and the TaqMan Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA) were used for the real-time PCR reactions in a final volume of 10 µl (5 µl Master Mix, 0.5 µl probe, 4.5 µl cDNA). The real-time PCR was run for 40 denaturing cycles at 95ºC for 15 s followed by extension at 60ºC for 60 s (StepOnePlus Real-time PCR System). The experiment was normalized to the *18S rRNA* reference gene. The relative mRNA level analysis of each target gene was evaluated with comparative quantification $2^{-\Delta Ct}$ formulae.

Western blot analysis

Specific protein molecules from among a mixture of proteins were identified by Western blotting as described elsewhere (5, 16). Briefly, cell homogenates were obtained with RIPA buffer and total protein concentration was assessed with BCA assay and BSA as standard. Then, 40 µg of proteins were resolved onto 10% SDS-PAGE and then electroblotted into PVDF membranes. Membranes were blocked with 1% BSA and the following antibodies were used: rabbit anti-β-actin (1:10,000; #PA1-16889, RRID: AB_568434), rabbit anti-p-NF-κB (1:1000. #PA5-37658, RRID: AB_2554266), mouse anti-FGF2 (1:1000, #MA1-24682, RRID: AB_780591), mouse anti-calnexin (1:1000, #MA3-027, RRID: AB_2069043), rabbit anti-NuMa (1:1000, #PA1-32451, RRID: AB_2154615), rabbit anti-alpha-2A (1:500, #PA1-048,

RRID: AB_2225243), goat anti-SERT (1:500, #PA5-18374, RRID: AB_10981912), rabbit anti-SCP1 (1:100, #PA1-46254, RRID: AB_2302734), rabbit anti-SCP3 (1:2000, #PA1-16766, RRID: AB_2197343) (Thermo Fisher Scientific, Waltham, MA, USA). After overnight incubation, membranes were washed and incubated with HRP-conjugated secondary antibody for 1 h at RT: anti-mouse (1: 40,000, #A9044, RRID: AB_258431) and anti-rabbit (1:40,000, #A0545, RRID: AB_10689821) (Sigma, Saint Louis, MO, USA). The data represent the relative density normalized to the levels of β-actin, while densitometry measurements were done with the use of GelQuantNET software (http://biochemlabsolutions.com).

Hematoxylin-eosin staining and immunohistochemistry

Routine hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) protocols were performed as described previously (17). Briefly, tissues were fixed in 4% formalin buffer, dehydrated, embedded in Paraplast, cut in 4 µm sections on microtome, placed on slides, dehydrated, stained with hematoxylin and eosin (HE staining) or for IHC boiled to retrieve antigens, blocked with BSA and incubated overnight with primary antibodies: anti-alpha-2A (1:1000, #PA1-048, RRID: AB_2225243), anti-SERT (1:1000, #PA5-18374, RRID: AB_10981912). Next, slides were incubated with goat anti-rabbit/mouse secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h in RT followed by detection with DAKO EnVision-HRP System and mounted with DPX (dibutylphthalate polystyrene xylene). Photographs were taken with the Olympus CX41 microscope equipped with Motic Images Plus 3.0 software.

Immunostaining

Standard immunostaining protocols (18) were applied. Briefly, cells were fixed with 4% formaldehyde, permeabilized with 0.25% Triton-X, blocked with 1% BSA and probed with the primary antibody anti-alpha-2A (1:1000, #PA1-048, RRID: AB_2225243), anti-SERT (1:1000, #PA5-18374, RRID: AB_10981912), and a secondary antibody conjugated to FITC (fluorescein-5-isothiocyanate) or Texas Red (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were counterstained

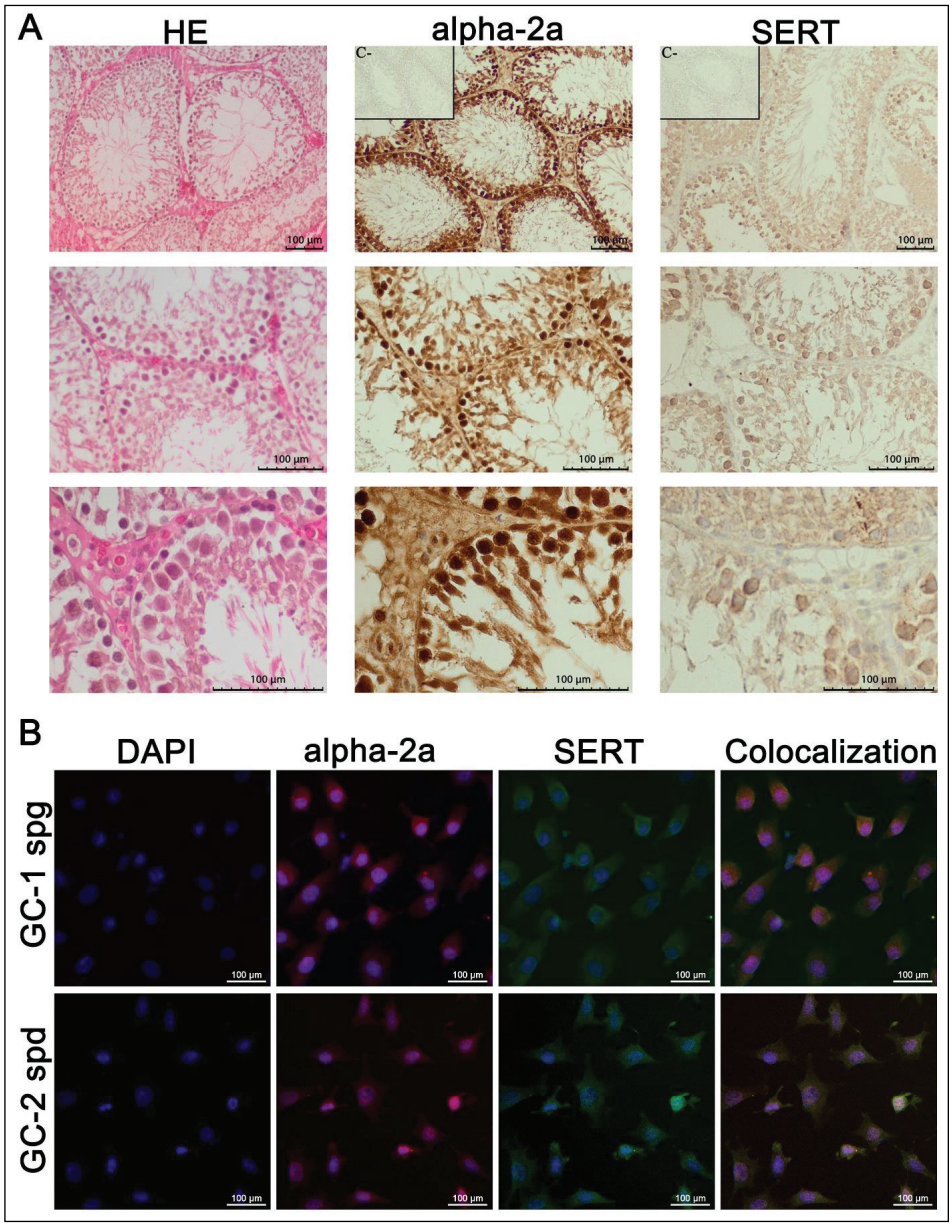

Fig. 1. The presence of alpha-2 adrenoceptor and serotonin transporter in rat testicular tissues and GC-1 spg and GC-2 spd cell lines. Immunohistochemical location of alpha-2-adrenergic receptors (alpha-2a) and serotonin protein transporters (SERT) in histological preparations of rat testis tissues. Magnification of the objective lens ×10, ×20, and ×40. Negative controls - primary antibody omitted (A). Immunofluorescent co-localization of alpha-2 adrenoceptor and SERT in spermatogenic cells (GC-1 spg and GC-2 spd) after 48- and 96-hour culture under control conditions. Magnification of the objective lens ×10 (B).

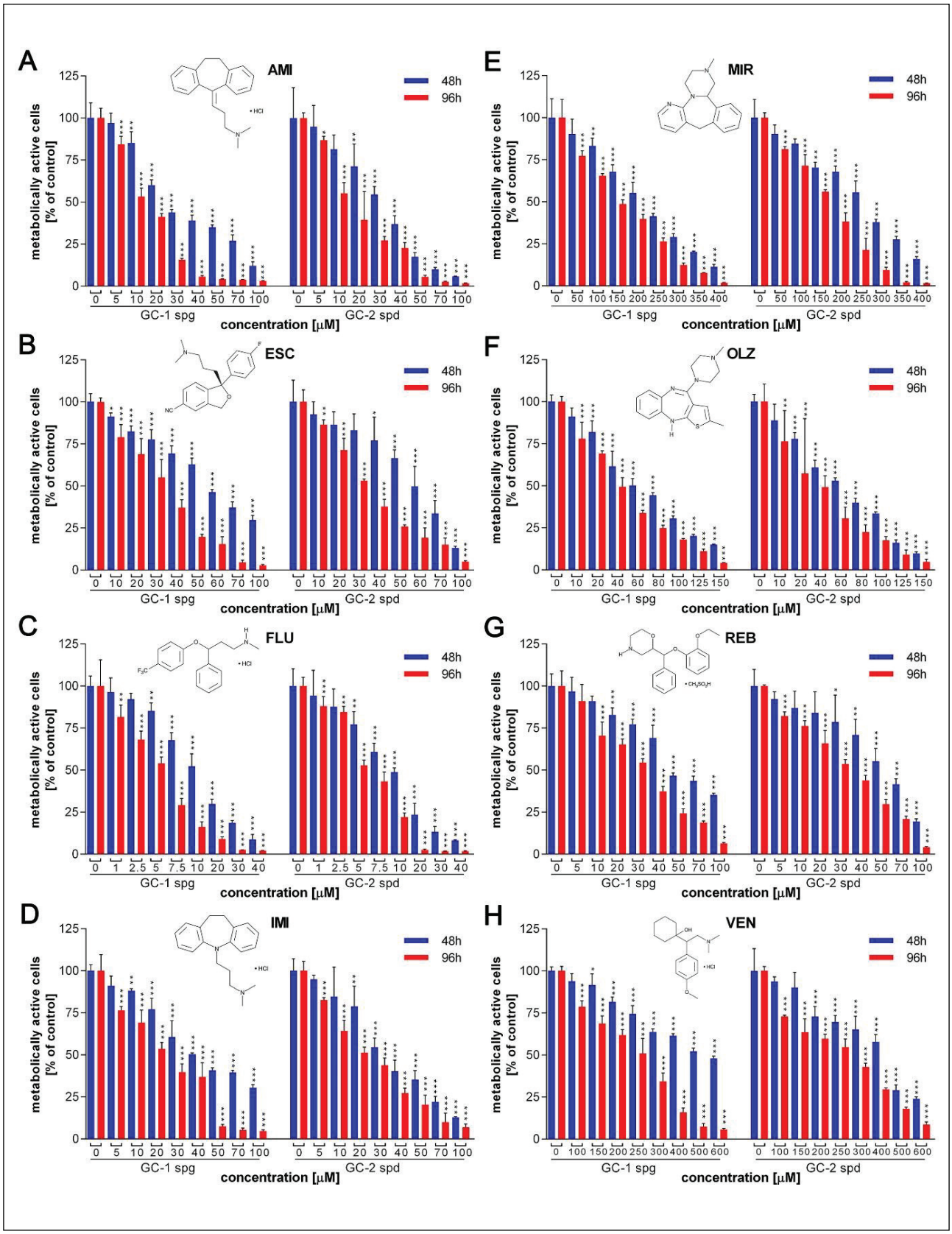

Fig. 2. Antidepressant treatment-mediated changes in metabolic activity of spermatogenic cells. GC-1 spg and GC-2 spd cells were treated with antidepressants (A): amitriptyline (AMI); (B): escitalopram (ESC); (C): fluoxetine (FLU); (D): imipramine (IMI); (E): mirtazapine (MIR); (F): olanzapine (OLZ); (G): reboxetine (REB); and (H): venlafaxine (VEN) in a wide range of concentrations, incubated for 48 and 96 h, and MTT test was performed. Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. p-values < 0.05 were considered statistically significant. Asterisks (*) indicate the comparison between CTRL (non-treated) and antidepressants-treated cells. Bars indicate SD, n = 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication - no statistical significance.

with DAPI at a final concentration of 1 µg/ml (Sigma, Saint Louis, MO, USA). Fluorescent images were taken with an InCell Analyzer 2000 (GE Healthcare, Chicago, IL, USA).

Statistical data analysis

The results are presented as the mean ± standard deviation of at least three biological replicates and three technically independent experiments (n = 3). Statistical differences were determined using a one-way analysis of variance (ANOVA) followed by Dunnett's posttest (treatments vs. control). A statistical summary of results and calculations was prepared in GraphPad Prism. A p-value of < 0.05 was considered as statistically significant (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, no indication - no statistical significance). (*) indicates a comparison between CTRL (non-treated) and antidepressants-treated cells. All presented photos were not subjected to any image processing and represent raw data. Adobe Photoshop CC was used to design figures.

RESULTS

Alpha-2a adrenoreceptor and serotonin transporter as receptors mediating interactions of cells with drugs

Since the interactions on the line antidepressants-spermatogonia/spermatocytes are a bit controversial, we decided to confirm the presence of receptors involved in antidepressants signaling between and/or within cells and thus regulating cellular biochemical processes. Therefore, the expression of alpha-2a receptors, as well as serotonin transporters (SERT), was verified in testicular tissues of male rats and cell lines used in further experiments. Standard hematoxylin and eosin staining confirmed the regular histological structure of rat testicular tissues and seminiferous tubules with no abnormalities. Moreover, we observed the presence of both, alpha-2a receptors as well as serotonin transporters, in the analyzed rat testicular tissue samples. The localization of these two types of receptors was established as membrane-located (*Fig. 1A*). We also confirmed the presence of both receptors in mouse type B spermatogonia (GC-1 spg) and spermatocytes (GC-2 spd) *in vitro* by immunofluorescence (*Fig. 1B*).

Cytotoxic effects of antidepressant treatment

Available literature does not provide comprehensive data on the effects of antidepressants on spermatogonia/spermatocytes. Thus, at the beginning of the project, we decided to perform titration studies to select one dose of each antidepressant for further studies. We targeted concentration resulting in the inhibition of cell metabolic activity and proliferative capacity to approximately 50% after 96 hours of exposure. In general, using the MTT test, we confirmed a dose-dependent decrease in cellular viability over a wide range of concentrations tested, and the observed changes were even more pronounced with longer incubation time. Moreover, the reported results were consistent for both cell lines tested. As said, the following doses for all further experiments: AMI 10 µM (*Fig. 2A*), ESC 30 µM (*Fig. 2B*), FLU 5 µM (*Fig. 2C*), IMI 20 µM (*Fig. 2D*), MIR 150 µM (*Fig. 2E*), OLZ 40 µM (*Fig. 2F*), REB 30 µM (*Fig. 2G*), VEN 250 µM (*Fig. 2H*) were selected.

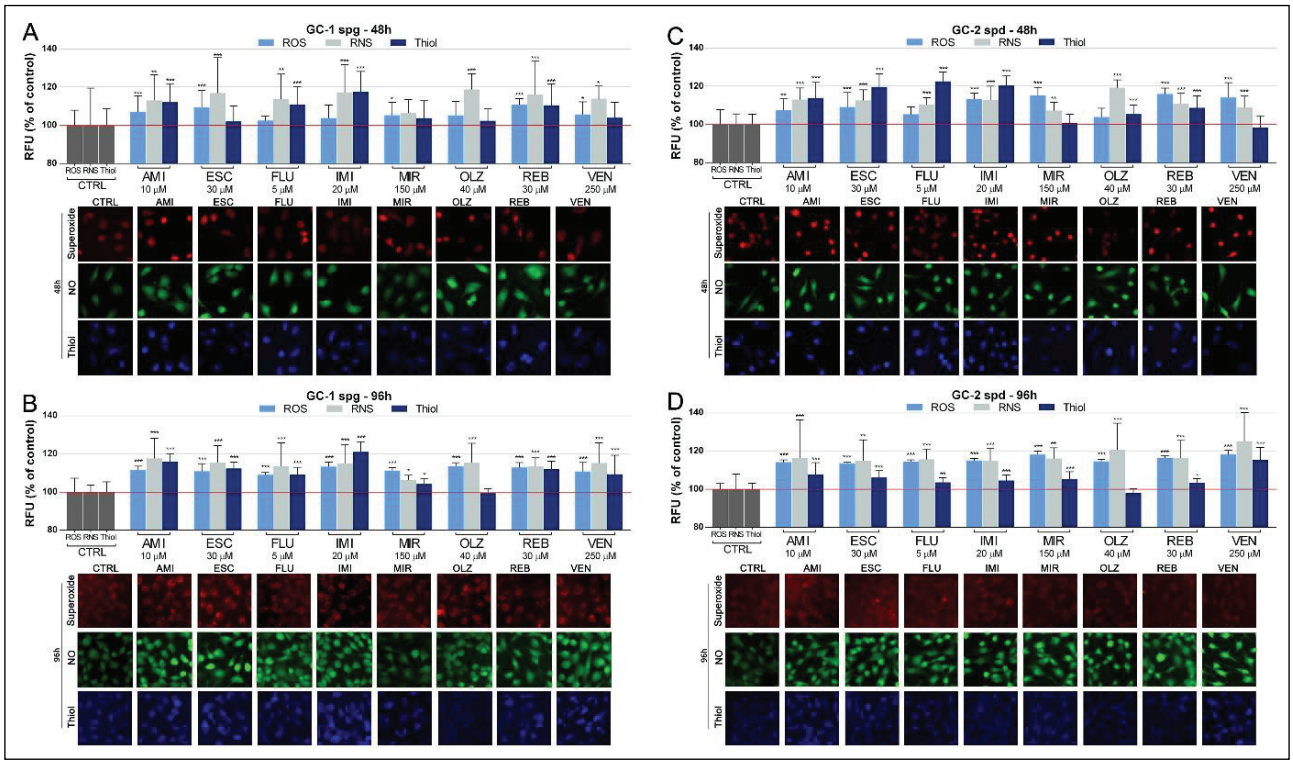

Fig. 3. Antidepressants modulate redox balance. GC-1 spg and GC-2 spd cells were treated with antidepressants, incubated for 48 and 96 h, and ROS, RNS, Thiol levels were controlled (A-D). Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. p-values < 0.05 were considered statistically significant. Asterisks (*) indicate the comparison between CTRL (non-treated) and antidepressants-treated cells. Bars indicate SD, n = 3, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, no indication - no statistical significance. Magnification of the objective lens ×10.

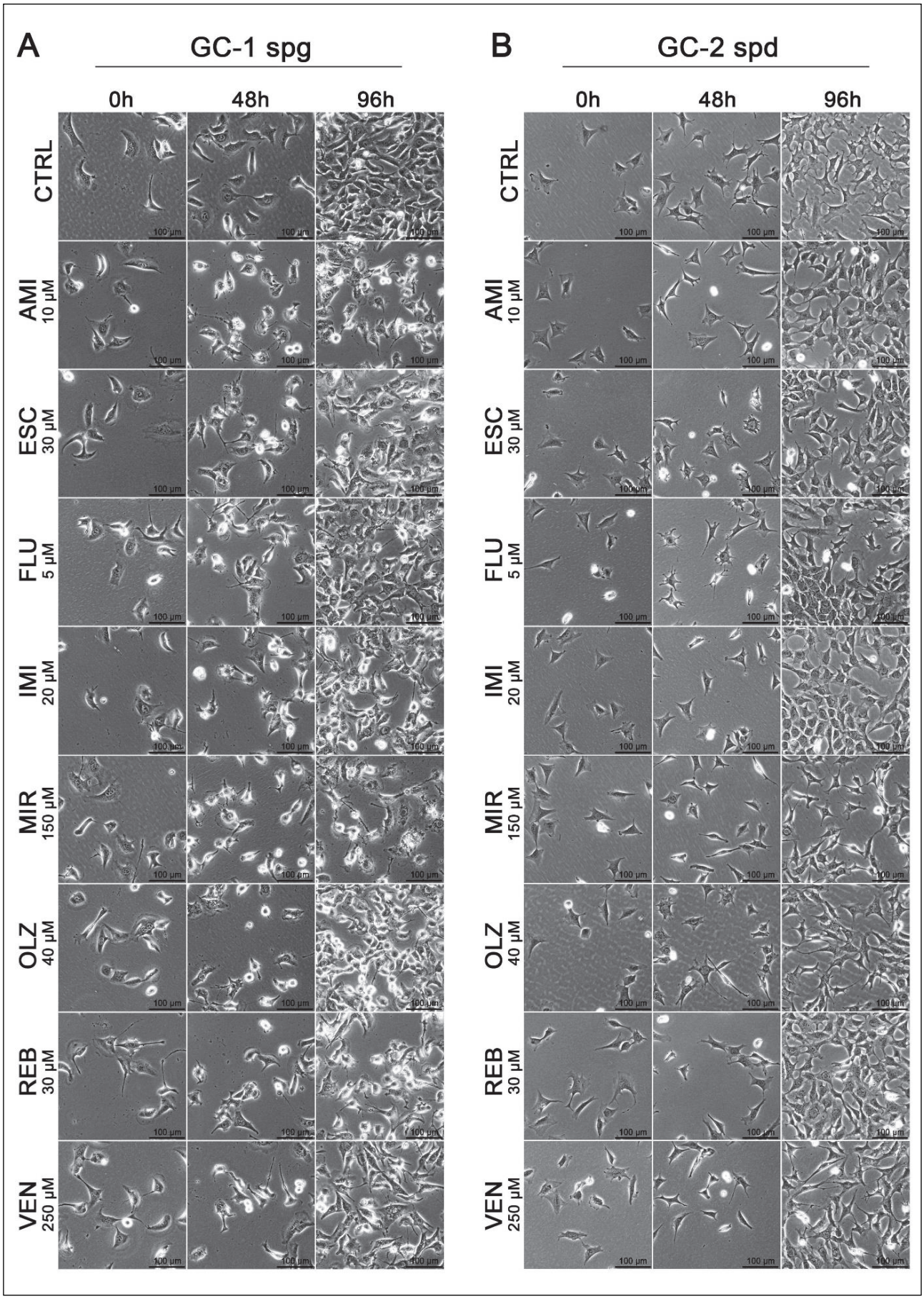

Fig. 4. Antidepressants modulate spermatogenic cell morphology *in vitro*. Morphological changes in GC-1 spg (A) and GC-2 spd (B) cells treated with antidepressants for 48 and 96 hours (10 × magnification).

Antidepressants affect intracellular redox status

Oxidative stress is considered as a central player in the reprogramming of cellular metabolic activity. What's more, when prolonged it can lead to observed in this study decreases in metabolic activity. Finally, next to redox imbalance, free radical scavengers also contribute to the impairment of cellular repair and adaptation mechanisms. Thus, in the next part of this study, we assessed the parameters of redox balance in antidepressants-treated spermatogenic cell systems. We used fluorescent probes to measure the level of superoxide, nitric oxide and reduced glutathione and observed an increase in the reactive oxygen (ROS, superoxide) and nitrogen species (RNS, nitric oxide) production after 48 h, except ROS for FLU and OLZ (GC-1 spg and GC-2 spd line) and RNS for MIR (GC-1 spg line). Observed upregulations were even more pronounced after 96 hours of exposure for all experimental sets compared to the control conditions. Thus, the results indicate the initiation of oxidative and nitrosative stress, which in turn can negatively affect cellular structures. Interestingly, the GC-2 spd cells were characterized by higher levels of relative fluorescence reflecting ROS/RNS than GC-1 spg cells.

Next, we evaluated whether the continued stress triggers mechanisms responsible for free radicals scavenging. Indeed, we noted the activation of glutathione reduction, an intracellular enzymatic cell protection mechanism. The results were consistent for both times of incubation. For GC-1 spg cells, the lowest level was observed for ESC, MIR, OLZ (48 h), and OLZ (96 h). In turn, in the case of GC-2 spd cells, the most pronounced effect was reported for MIR, VEN (48 h), and OLZ (96 h) (*Fig. 3A-3D*).

Antidepressant drugs modulate spermatogenic cells morphology in vitro

Various changes in redox homeostasis and mitochondrial dysfunctions may contribute to cellular morphology abnormalities. Besides, morphological changes may be another marker of toxicity. Thus, we evaluated the morphological defects after treatment with different antidepressants. As expected, toxic doses resulted in the first visible changes after the first 48 hours of treatment and progressed over time. In general, antidepressants-treated GC-1 spg cells were characterized by a nucleus with irregular shape and condensed cytoplasm, and the effect was followed by the loss of adhesion to the culture flask (*Fig. 4A*). In turn, GC-2 spd cells maintained the normal morphological shape in most experimental sets and regardless of the incubation time (*Fig. 4B*).

Quantification of cell division-related genes

Few reports provide evidence of the genotoxic effects of drugs that can induce mitotic and meiosis disorders, as well as

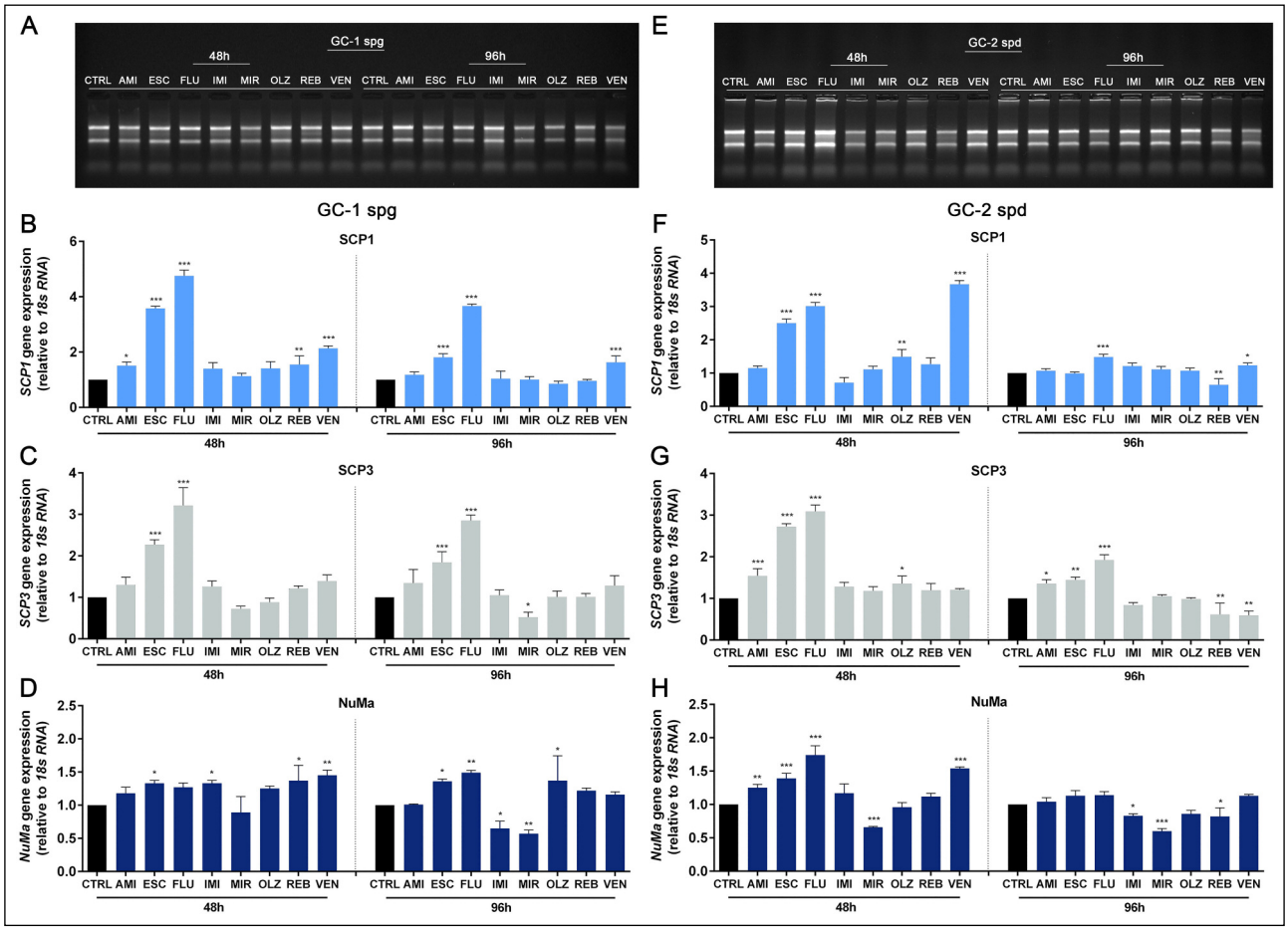

Fig. 5. Antidepressants modulate the expression of key genes involved in a cell division. GC-1 spg and GC-2 spd cells were treated with antidepressants for 48 and 96 h. Agarose Gel Electrophoresis - 18S and 28S ribosomal RNA bands of the intact RNA samples GC-1 spg (A) and GC-2 spd (E). Gene expression profiles of *SCP1* (B, F), *SCP3* (C, G), *NuMa* (D, H) relatively to the *18S rRNA*. Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. p-values < 0.05 were considered statistically significant. Asterisks (*) indicate the comparison between CTRL (non-treated) and antidepressants-treated cells. Bars indicate SD, n = 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication - no statistical significance.

cause chromosomal aberrations. Here, we also observed statistically significant changes in the levels of several gene expressions after 48 and 96 h incubations. For the *SCP1* and *SCP3* genes, the highest level of expression was noted for ESC, FLU, and VEN (both cell lines tested). In turn, the lowest level of expression was observed for MIR (*Fig. 5A-5D*). Changes in the *NuMa* gene expression were not as significant as in the case of the previous two genes; however, we noted clear differences especially for GC-2 spd cell lines. In addition, the highest increases for ESC, FLU, VEN (only GC-2 spd) and a decrease for MIR (both cell lines) were noted. Interestingly, after 96 hours of treatment a decrease in *NuMa* expression for IMI was discovered (*Fig. 5E-5H*).

Activation of adaptation pathways

In response to cellular damage, many proteins and different intracellular signaling pathways can be activated (*Figs. 6A* and *7A*). Continuing the above, short-term incubation (48 h) activated the NF-κB transcription factor and FGF2 fibroblast growth factor. The highest level of NF-κB synthesis was observed for GC-1 spg cells exposed to FLU, IMI, REB, and VEN (*Fig. 6B*). The GC-2 spd cells were also characterized by a slight increase; however, some of the results were not statistically significant (e.g. NF-κB, 48 h) (*Fig. 7B*). Next, an increase in the level of FGF2 for IMI, MIR, OLZ (GC-1 spg, and GC-2 spd) was noted (*Figs. 6C* and *7C*). Interestingly, longer drug exposure resulted in the up-regulation of factors tested in most experimental sets for both cell lines. We found that antidepressants increased the synthesis of SCP1, SCP3, NuMa, calnexin in all experimental sets of the GC-1 spg line except OLZ (SCP1) and 48 h exposure (*Fig. 6D*, *6E*, *6H* and *6I*). A similar observation was done for GC-2 spd cells (*Fig. 7D, 7E*, *7H* and *7I*). Further, the long-term exposure led to a decrease in all mentioned protein levels in most of the sets tested. Moreover, the results of the protein synthesis levels are supported by the

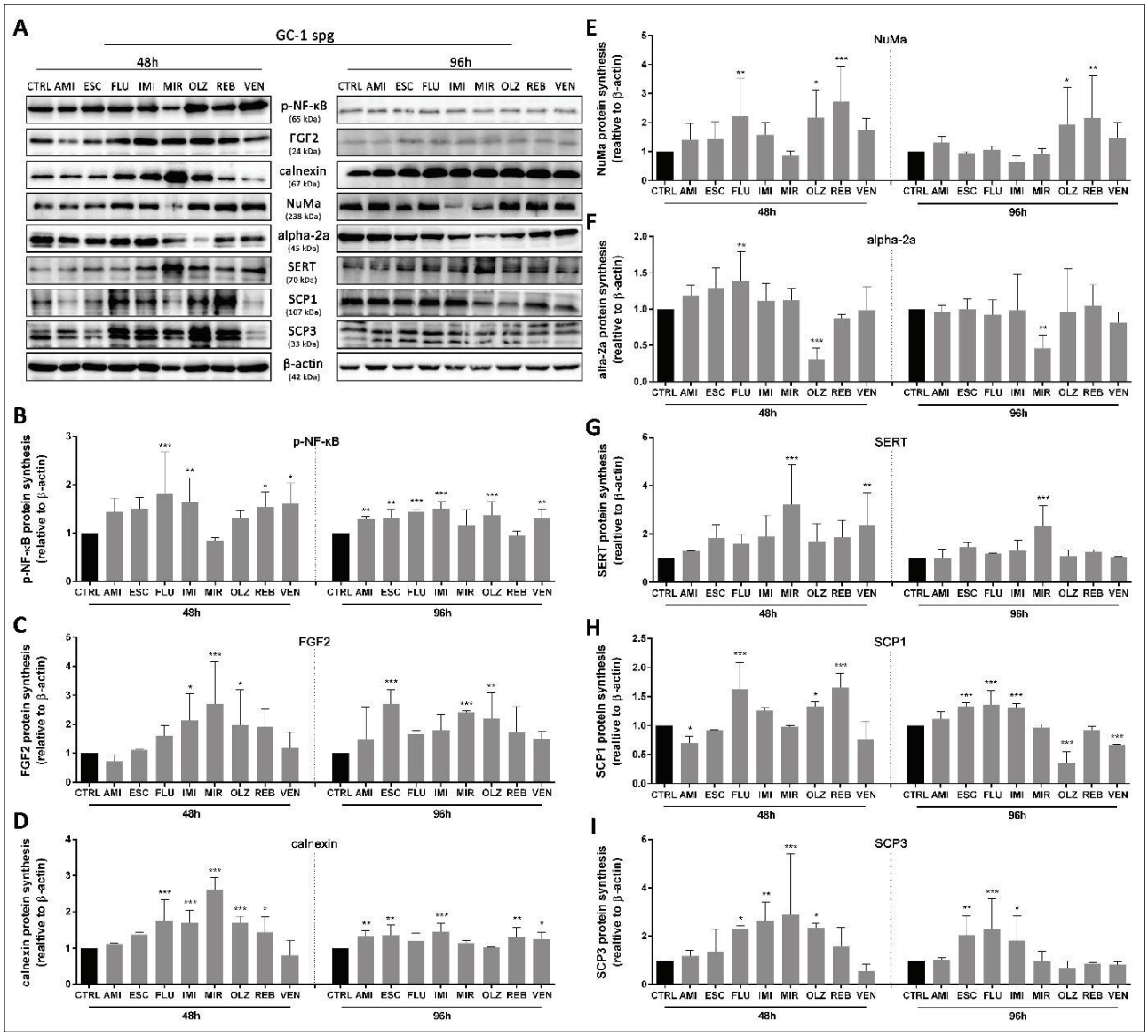

Fig. 6. Antidepressants-mediated effects on GC-1 spg cellular protein pathways. GC-1 spg cells were treated with antidepressants for 48 and 96 h then, the expression level of proteins of interest was controlled. Representative Western blots are shown (A). Proteins expression was evaluated: p-NF-κB (B), FGF2 (C), calnexin (D), NuMa (E), alpha-2a (F), SERT (G), SCP1 (H) and SCP3 (I). Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. p-values < 0.05 were considered statistically significant. Bars indicate SD, n= 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication - no statistical significance.

earlier studies on gene expression. Stimulation of alpha-2a receptors, as well as serotonin transporters (SERT), initiates a signaling cascade that may involve cellular biochemical processes regulation. In comparison to control, samples submitted to antidepressants treatment presented an increase in alpha-2a and SERT synthesis in most experimental sets. Moreover, GC-2 spd cells were characterized by more significant upregulation mainly after 96 h exposure (*Figs. 6F*, *6G*, *7F* and *7G*).

DISCUSSION

Previous studies demonstrated that antidepressant treatment initiates adaptation mechanisms that allow neuronal cells to adjust to stress conditions. In contrast, the data concerning the effect of antidepressants on the male reproductive system is limited to a few studies. Interestingly, the literature data indicate that reproductive cells appear to be surprisingly resistant to factors inducing DNA damage. This characteristic makes them useful for a better understanding of the side effects of drugs, including antidepressants with different mechanisms of action.

The potential side effects of antidepressants tested in this study may be dependent on several factors. Extremely important is the presence of the specific membrane-bound receptors involved in the antidepressant-mediated cellular response. Here, we confirmed the presence of alpha-2a receptors and serotonin protein transporters in rat testicular tissues as well as in the GC-1 spg and GC-2 spd cells what is in agreement with others (19-22). Continuing experiments, our results indicated a dose- and time-dependent decrease in cell proliferation rate and metabolic activity. Others also reported dose-dependent abnormalities in germ cells (23, 24) and significant, but reversible inhibition of spermatogenesis (12, 25-27). Besides, our observations are consistent with earlier studies based on various cell lines and

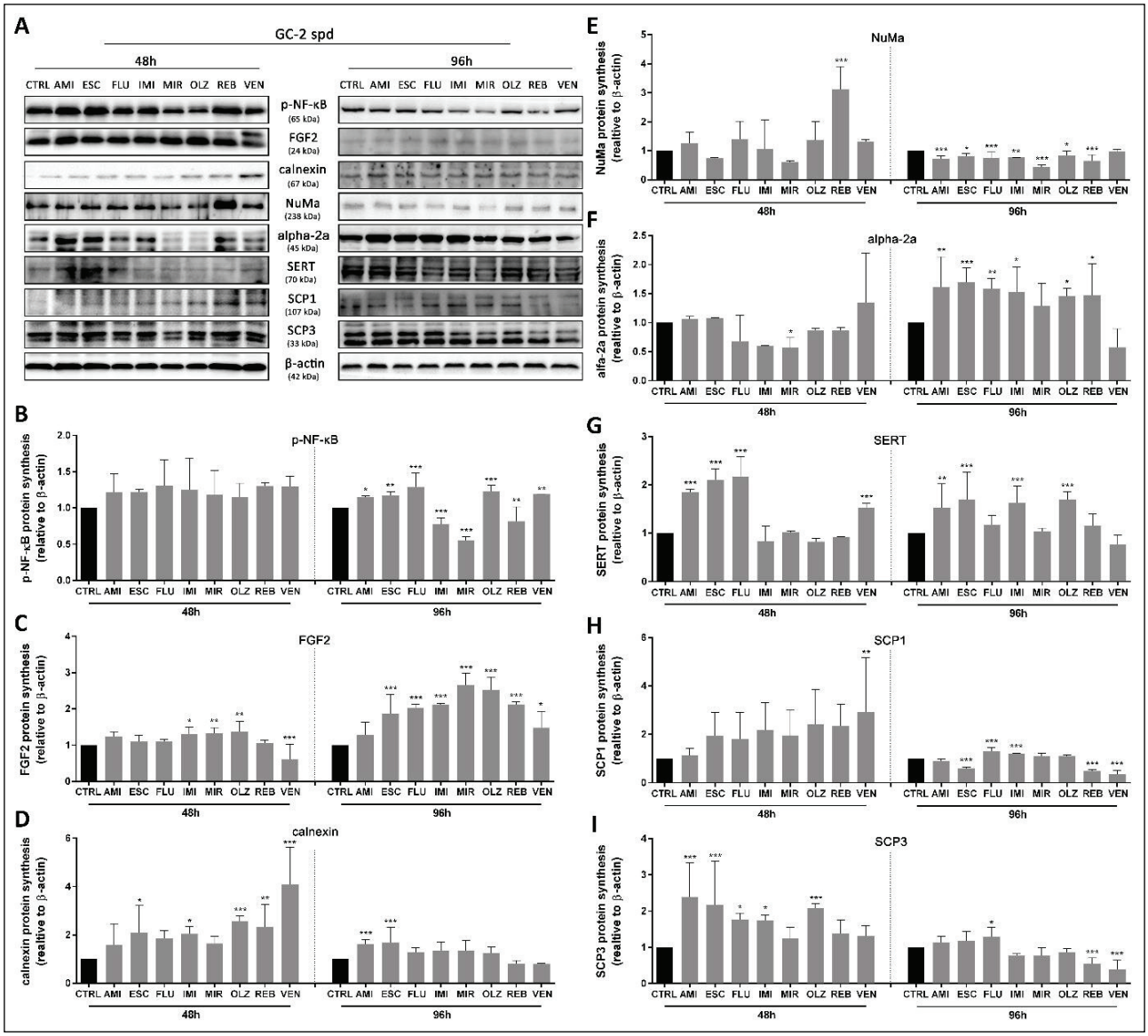

Fig. 7. Antidepressants-mediated effect on GC-2 spd cellular protein pathways. GC-2 spd cells were treated with antidepressants for 48 and 96 h then, the expression level of proteins of interest was controlled. Representative Western blots are shown (A). Proteins expression was evaluated: p-NF-κB (B), FGF2 (C), calnexin (D), NuMa (E), alpha-2a (F), SERT (G), SCP1 (H) and SCP3 (I) was evaluated. Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. p-values < 0.05 were considered statistically significant. Bars indicate SD, n = 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication - no statistical significance.

animal models (5). Furthermore, the inhibition of antidepressants-mediated cell proliferation in spermatogenic cells was associated with enhanced synthesis of FGF2. The explanation of this phenomenon may be the fact that the FGF2 factor regulates a wide spectrum of biological functions, including survival, migration, proliferation, and cell differentiation (28). Also, recent studies indicated that FGF signaling is crucial for spermatogenesis regulation of FGF2 isoforms may also participate in the first stages of spermatogenesis. However, the authors point out that the mechanism of FGF function in the testes is not very clear and requires further explanation (29, 30). The decrease in cell viability can be associated with redox imbalance as observed in this study. It is believed that testicular tissue and sperm cells are very sensitive to perturbations in intracellular ROS/RNS levels and lipid peroxidation (31-33). Data indicate that oxidative stress may be a consequence of antidepressant treatment and cause damage to all cellular components *in vitro*. Some drugs can additionally lead to sperm cell DNA damage by altering the redox balance (34-36) and ROS/RNS up-regulation may cause a decrease in the amount of unsaturated fatty acids in sperm cell membranes. Consequently, the acrosomal reaction is disturbed and the motility, as well as sperm count, decreased. More importantly, ROS and RNS inactivate several key enzymes associated with sperm antioxidant defense and indirectly entail testicular dysfunction, reduced gonadotropin secretion, and abnormal sperm parameters (31, 37-39). More and more evidence suggests that some antidepressants may also cause changes in the cellular antioxidant capacity and here, we noted an increase in thiol pool levels due to the drug treatment. Only a few reports confirm our results and provide experimental evidence for changes in glutathione levels in antidepressant-treated systems (animal models) (40, 41). Another important molecular mechanism involved in the cell protection and regulation of the ROS level is the NF-κB transcription factor. NF-κB plays an important role in cell development, growth, survival, and proliferation (42). Other factors such as Bcl-2, BDNF, CREB or BAG-1 can also protect the cells and their organelles. Importantly, some antidepressants (vortioxetine) or substances used in depression treatment (lithium carbonate) can affect cells in a neuroprotective manner. Consequently, the inhibition of apoptosis could be caused by an increase in the expression of these factors (43, 44). It cannot be ignored that NF-κB is involved in many pathological conditions (42). Here, we observed the activation of this defense mechanism against free radicals under stress conditions of antidepressant exposure. In addition, a decrease in cell viability observed earlier could be also associated with the selective activation of ERK kinase, the biosynthesis of the Egr-1 transcription factor, and the increase in the transcriptional activity of NF-κB (45). Some reports suggest the correlation of NF-κB with the regulation of genes involved in the course of spermatogenesis and sperm development (46, 47). Further, in this study, we observed numerous morphological abnormalities correlated with increasing incubation time, as another consequence of disturbed oxidative homeostasis. Although limited, literature data provide evidence regarding abnormal sperm morphology in patients treated for depressive disorders (12). Other observations concerning semen parameters reflect the results of animal studies, which suggest that antidepressants may negatively affect spermatogenesis (48). To control this parameter, in the next part of this study, we attempted to determine whether antidepressants regulate the level of genes expression that play a key role in the meiotic division. Meiosis disorders are a critical point in the formation of haploid gametes. Here, we provide for the first time, to our knowledge, the evidence that antidepressant treatment modulates the expression of synaptonemal complex genes and the *NuMa* gene required for spindle formation. Notably, this effect was maintained for a longer incubation time only in the case of GC-1 spg cells. Until now it has been only hypothesized that anti-cancer drugs affect the mitotic spindle assembly process. Indeed, preliminary studies suggested abnormal centrosome organization and distribution of genetic material among the daughter cells (49, 50). Moreover, the results of mRNA expression levels are generally reflected in the protein synthesis data. However, it is obvions that the gene expression does not always correspond to the level of protein synthesis due to the processes of post-transcriptional, translational, and protein degradation regulation (51). Therefore, as proteins are considered as executive products, we will focus on the level of protein synthesis. Incorrect synthesis of these proteins as a result of antidepressant treatment may contribute to genomic instability associated with chromosomal number or structure abnormalities (23, 52, 53). In this study, we observed increased

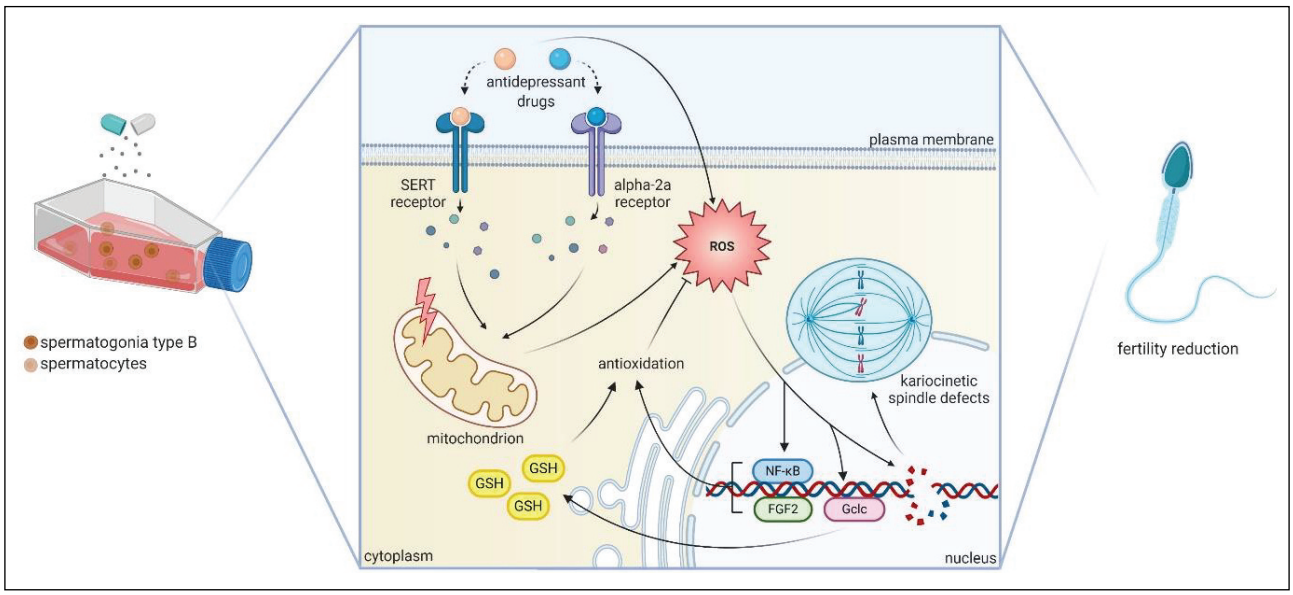

Fig. 8. Proposed mechanism underlying the reprotoxicity of antidepressants.

expression and synthesis of SCP1 and SCP3, which confirms that cells initiate adaptation mechanism in order to preserve reproductive function (54). In turn, the NuMA protein plays a significant role in the organization of cell division and is strictly dependent on the cell cycle phase, and its distribution is regulated by phosphorylation and dephosphorylation (55). Besides, the NuMa protein is also in direct interaction with tubulin during cell division (56). The consequence of antidepressants-mediated changes in the levels of NuMa may be associated with microtubule polymerization and depolymerization disorders, and thus may lead to the impairment of the spindle apparatus function. This point of view finds its further support by the observation of the antidepressants-mediated modulated levels of calnexin. Calnexin is considered as an endoplasmic reticulum marker (ER) and ER concentrates around the poles of the spindle apparatus by associating with astral microtubules to ensure proper partitioning to progeny cells (57). Thus, our results suggest that antidepressants lead to defects in spindle formation and improper organelle segregation. Overall, the GC-2 spd cells appear to be more resistant to antidepressants. The explanation for such results may be mainly due to different stages of development of germ cells tested.

At this point it is crucial to mention the several limitations of this study. The study was done on spermatogenic cells and presents the mechanistic data limited to the *in vitro* system. The testicular tissues are known for their extreme complexity and thus the obtained data needs to be further confirmed in *in vivo* animal systems. However, the presented results are important molecular data pointing directly on the exact mechanism associated with reprotoxic potential of antidepressants commonly used in clinical practice.

In summary, most antidepressants are characterized by a delayed onset of therapeutic effects. It is important to note that long-term treatment is often associated with significant side effects. Only a few reports indicate that this may result in distant modulations of various vital functions, including reproductive processes. Here, we provide the molecular mechanism of cytotoxic effect towards reproductive cells. The mechanism is mediated by disturbances in the redox balance (increased production of ROS and RNS), failure of enzymatic and non-enzymatic cell protection mechanisms (glutathione system, NF-κB and FGF2-mediated pathways), and impairment of mitochondrial functions. In addition, we provide for the first time, to our knowledge, evidence that antidepressant treatment may contribute to spindle apparatus assembly defects and organelle distribution during cell division *in vitro* (alterations in the levels of SCP1, SCP3, NuMa and calnexin protein levels). Thus, this study sheds new light on the pathomechanisms of antidepressant action and their associated toxicity towards the reproductive system (*Fig. 8*).

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