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Agomelatine and Duloxetine Synergistically Modulates Apoptotic Pathway by Inhibiting Oxidative Stress Triggered Intracellular Calcium Entry in Neuronal PC12 Cells: Role of TRPM2 and Voltage-Gated Calcium Channels

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Abstract Calcium ion (Ca^{2+}) is one of the universal second messengers, which acts in a wide range of cellular processes. Results of recent studies indicated that ROS generated by depression leads to loss of endoplasmic reticulum-Ca^{2+} homeostasis, oxidative stress, and apoptosis. Agomelatine and duloxetine are novel antidepressant and antioxidant drugs and may reduce oxidative stress, apoptosis, and Ca^{2+} entry through TRPM2 and voltage-gated calcium channels. We tested the effects of agomelatine, duloxetine, and their combination on oxidative stress, Ca^{2+} influx, mitochondrial depolarization, apoptosis, and caspase values in the PC-12 neuronal cells. PC-12 neuronal cells were exposed in cell culture and exposed to appropriate non-toxic concentrations and incubation times for agomelatine were determined in the neurons by assessing cell viability. Then PC-12 cells were incubated with agomelatine and duloxetine for 24 h. Treatment of cultured PC-12 cells with agomelatine, duloxetine, and their combination results in a protection on apoptosis, caspase-3, caspase-9, mitochondrial membrane depolarization, cytosolic ROS production, glutathione peroxidase, reduced glutathione, and lipid peroxidation, values. Ca^{2+} entry through non-specific TRPM2 channel blocker (2-APB) and voltage-gated Ca^{2+} channel blockers (verapamil and diltiazem) was modulated by agomelatine and duloxetine. However, effects of duloxetine on the Ca^{2+} entry through TRPM2 channels were higher than in agomelatine. Results of current study suggest that the agomelatine and duloxetine are useful against apoptotic cell death and oxidative stress in PC-12 cells, which seem to be dependent on mitochondrial damage and increased levels of intracellular Ca^{2+} through activation of TRPM2 and voltage-gated Ca^{2+} channels.

Keywords Agomelatine · Duloxetine · Oxidative stress · Calcium signaling · Apoptosis · TRPM2 channels

Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+}
2-APB	2-Aminoethyl diphenylborinate
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
GSH	Glutathione
GSH-Px	Glutathione peroxidase
LP	Lipid peroxidation
MDD	Major depressive disorder
$NMDG^+$	*N*-Methyl-D-glucamine
ROS	Reactive oxygen species
TRP	Transient receptor potential
TRPM2	Transient receptor potential melastatin
VGCC	Voltage-gated calcium channel
H_2O_2	Hydrogen peroxide

Introduction

Major depressive disorder (MDD) influences nearly 15 % of population these days. Patients diagnosed with MDD

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have an increased onset risk of aging-related somatic diseases such as Alzheimer's disease, diabetes, heart disease, and cancer (Michel et al. 2012; Hovatta et al. 2010). Patients with MDD are well related with increased amount of reactive oxygen species (ROS). ROS are usually produced during physiological conditions and are tightly regulated by the intracellular mechanism to maintain a redox balance together with antioxidants (Herken et al. 2006). ROS have been shown to be critical to various biological events including apoptosis, signal transduction, development, and aging. Moreover, the excessive production of ROS has been implicated in numerous pathological alterations of the central nervous system, principally psychiatry (Eren et al. 2007; Altinkilic et al. 2010). The main role of antioxidants, such as glutathione peroxidase (GSH-Px), is to protect the organism from oxidative damage, taking key roles in detoxification of ROS products. Nowadays, there are increasing numbers of studies indicating that ROS-induced neuronal damage has an important role in the pathophysiology of depression, probably via membrane damage and decreasing activity of catalase, GSH-Px, glutathione reductase, and superoxide dismutase (SOD) levels, suggesting oxidative damage (Eren et al. 2007; Maes et al. 2000).

Agomelatine (AGOM) is a newly discovered and clinically effective antidepressant drug (De Berardis et al. 2013) with melatonergic (MT1/MT2) agonist and 5-HT (2C) receptor antagonist properties, commonly used to treat depressive disorders (Srinivasan et al. 2012). It has been previously mentioned its trophic effects; AGOM has also regulatory activity of signal transduction pathways, such as extracellular kinases synaptic plasticity, as a response to stress (Manji et al. 2003; Duman et al. 1999). Duloxetine (DULOX) is an effective serotonin and norepinephrine reuptake inhibitor in the central nervous system (CNS) widely available to use for three main aims, including major depressive disorders, though it has low affinity for neuronal receptors.Until now, there has been no article on actual knowledge about AGOM and DULOX efficacy on intracellular calcium signaling, mitochondrial function, and caspase activation cascades.

As a cation, calcium ion (Ca^{2+}) is well known for its role as a crucial second messenger in modulating many cellular physiological functions, including cell survival and signal transduction. Ca^{2+} overload is detrimental to cellular function and may pose as an important cause of cellular oxidative stress generation and apoptosis (Uguz et al. 2009; Uguz and Naziroglu 2012). If the intracellular ROS amounts increase, then the cell will be depolarized and more cation will flow from the outside to the inside of the cell which will end in cell death (Naziroglu 2009). Transient receptor melastatin 2 (TRPM2) is non-selective cation channel and is a member of transient receptor potential (TRP) family of (Naziroglu 2007). The channel displays a wide expression profile in neuronal cells, such as brain and dorsal root ganglion (DRG) (Naziroglu 2007; Staaf et al. 2010). The principal activator of TRPM2 is ADP-ribose (ADPR) which binds to a special domain located at the C-terminus of the channel (Fonfria et al. 2005). The channel is also stimulated by oxidative stress and extracellular application of hydrogen peroxide (H_2O_2) (Naziroglu and Luckhoff 2008a, b). It is well known that oxidative stress and Ca^{2+} entry through cation channels have important roles on pathophysiology of depression in humans although antidepressant induced modulator role on Ca^{2+} entry and cation channels the in neuronal cells (Gao et al. 2010; Wang et al. 2006). The result of recent studies indicated importance of oxidative stress-dependent activation of TRPM2 cation channels in depression (Xie et al. 2011). However, there is no report on effects of AGOM and DULOX on the TRPM2 cation channels in neuronal cells.

AGOM and DULOX may reduce oxidative stress, apoptosis and Ca^{2+} entry through modulation of TRPM2 and voltage-gated calcium channel (VGCC) in the PC12 neuronal cells. We aimed to test the effects of AGOM, DULOX, and their combination on Ca^{2+} influx, oxidative stress, mitochondrial depolarization, apoptosis, and caspase values in the PC-12 neuronal cells.

Materials and Methods

Chemicals

Fura-2/AM was obtained from Calbiochem (Darmstadt, Germany). RPMI-1640 medium, reduced glutathione (GSH), *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (ACDEVD-AMC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[(3chomalidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and dithiothreitol (DTT), Trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine-123 (DHR 123) was purchased from Molecular Probes (Eugene, OR, USA). *N*-Acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) was purchased from Bachem (Bubendorf, Switzerland). All other reagents were of analytical grade.

Preparation of PC 12 Cells

The PC12, a rat pheochromocytoma-derived cell line was selected as the neuronal model and cultured in RPMI-1640 medium. The cell line was originally obtained from DSMZ Cell Lines Bank (Germany). Before treatment, PC12 cells were placed in poly-D-lysine-coated cell culture flask at a density of $1 \times 10^5/cm^2$ and were allowed to attach for 24 h in RPMI-1640 medium supplemented with 10 % horse

serum, 5 % fetal bovine serum, 4 mM glutamine, and a mixture of 1 % of 100 IU 7 ml of penicillin/streptomycin/L-glutamine as outlined in protocols by the supplier. Cells were incubated at 37 °C in humid, 5 % CO_2, and 95 % air environment (Altinkilic et al. 2010). When the cells reached to the 80 % of confluent, they were dislodged from the flak surface by trypsin-EDTA and dispersed through a 22-gauge needle. Differentiation was induced by the addition of (NGF) to the cultures (50 ng/ml) for 5 days.

Study Groups

PC12 cells were divided into four groups as follows:

Group I was the control group, and PC12 cells were incubated (37 °C and 5 % CO2) for 24 h with normal medium.
Group II was the agomelatine-treated group. The PC12 cells were incubated with agomelatine according to MTT results (20 μM) for 24 h.
Group III was the duloxetine group. The PC12 cells were incubated with duloxetine (10 μM) for 24 h as indicted before (Prickaerts et al. 2012).
Group IV was the combination group of agomelatine and duloxetine. The PC12 cells were incubated with agomelatine (20 μM) for 24 h and with duloxetine (10 μM) for 24 h

Cell Viability (MTT) Assay

Cell viability was evaluated by the MTT assay based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product as described in own study (Uguz and Naziroglu 2012). Optical density was measured in a spectrophotometer at 490 and 650 nm and presented as the fold increase over the pretreatment level (experimental/control).

Lipid Peroxidation, Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-Px), and Protein Assay

Lipid peroxidation levels in the PC12 neurons were measured with the thiobarbituric acid reaction by the method of (Placer et al. 1966). A homogenate of PC12 neurons was prepared in 50 mM phosphate buffer (pH 7.4). The amount of thiobarbituric acid reactive substances such as malondialdehyde was measured by the reaction with thiobarbituric acid at 532 nm (UV-1800, Schimadzu, Kyoto, Japan). The values of lipid peroxidation in the neurons were expressed as μmol/g protein.

The GSH content of the PC12 neurons was measured at 412 nm using the method of Sedlak and Lindsay as described in our previous study (Uguz and Naziroglu 2012). GSH-Px activities of the PC12 neurons were measured by the spectrophotometer at 37 °C and 412 nm according to the method of (Lawrence and Burk 1976). The protein content in the PC12 neurons was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Measurement of Intracellular Reactive Oxygen Species (ROS) Measurement

Rhodamine 123 (Rh 123) is a non-fluorescent, non-charged dye that easily penetrates cell membranes. Once inside the cell, DHR 123 becomes fluorescent upon oxidation to rhodamine 123 (Rh 123), fluorescence being proportional to ROS generation (Rothe et al. 1988). In brief, the PC12 neurons (10^3 cells/ml) were washed with serum-free RPMI-1640 medium and incubated with 0.02 mM DHR 123 at 37 °C for 25 min (Uguz et al 2012). Cells were then washed in PBS. The fluorescence intensity of Rh 123 was measured in an automatic microplate reader (Infinite pro200; Tecan Austria GmbH, Groedig, Austria). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in triplicate. The data are presented as fold increase over the pretreatment level (experimental/control).

Measurement of Intracellular Calcium ($[Ca^{2+}]_i$) Concentration

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent indicator Fura-2-AM. For this purpose, cells were trypsin digested, allowed to sediment, re-suspended in HEPES-buffered medium, consisting of 20 mM Hepes (7.4), 10 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose and 0.1 % bovine serum albumin (fatty acid free), and then loaded with 5 μM fura-2/AM for 45 min. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspensions at 37 °C using a spectrofluorometer (Carry Eclipse, Varian Inc, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2-AM 340/380 nm fluorescence ratio and were calibrated according to the method of (Grynkiewicz et al. 1985).

Ca^{2+} release in the PC12 cells was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 120 s after addition of H_2O_2 (0.1 mM). Ca^{2+} release is expressed in nanomolar quantities by taking a sample every second as previously described (Korkmaz et al. 2011). In some experiments, the PC-12 neuronal cells were incubated with 2-APB (0.1 mM and 30 min), verapamil + diltiazem (0.01 mM and 30 min) before measurement of $[Ca^{2+}]_i$ concentration.

Apoptosis Assay

The APOPercentage™ assay (Biocolor Ltd., Belfast, Northern Ireland) was performed according to the instructions provided by Biocolor Ltd. and elsewhere (Beales and Ogunwobi 2010). The APOPercentage™ assay is a dye-uptake assay, which stains only the apoptotic cells with a red dye. When the membrane of apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometer (Li et al. 2010).

Assay for Caspase-3 and -9 Activities

The determination of caspase-3 and caspase-9 activities was based on a method previously reported (Uguz et al. 2009) with minor modifications. Stimulated or resting cells were washed once with PBS. After centrifugation, cells were re-suspended in PBS at a concentration of 10^7 cells/ml. Fifteen microliters of the cell suspension was added to a microplate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer that was composed of 100 mm HEPES, pH 7.25, 10 % sucrose, 0.1 % CHAPS, 5 mm DTT, 0.001 % NP40 and 40 ml of caspase-3 substrate (AC-DEVD-AMC) or 0.1 m MES hydrate, pH 6.5, 10 % PEG, 0.1 % CHAPS, 5 mm DTT, 0.001 % NP40, and 0.1 mM of caspase-9 substrate (AC-LEHD-AMC). Substrate cleavage was measured with the microplate reader (Infinite pro200 Tecan, Austria) with excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as fold increase over the pretreatment level (experimental/control).

Measurement of Mitochondrial Membrane Potential

Cells were incubated with 1 ml JC-1 for 15 min at 37 °C as previously described (Uguz et al. 2012). Fluorescence changes were analyzed using a fluorescence spectrophotometer (RF-5301-PC; Shimadzu, Kyoto, Japan). Treatments were carried out in triplicate. Data are presented as emission ratios (590/535). Changes in mitochondrial membrane potential were quantitated as the integral of the decrease in JC-1 fluorescence ratio.

Statistical Analyses

All results were expressed as mean ± SD. Significant values in three groups were assessed with an unpaired Mann–Whitney *U* test. Data were analyzed using the SPSS statistical program (version 17.0 software, SPSS Inc. Chicago, Illinois, USA). *P* values of less than 0.05 were regarded as significant (Fig. 1).

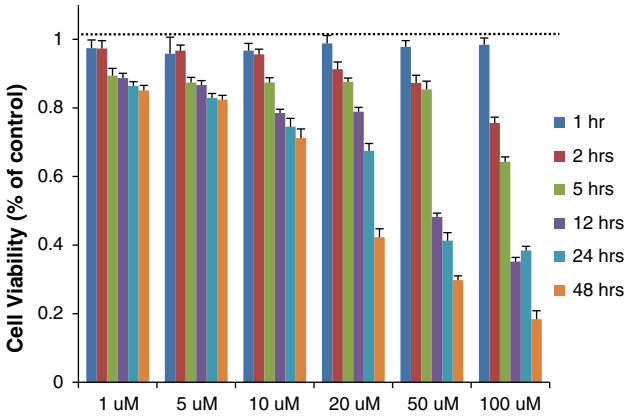

Fig. 1 Effect of agomelatine on PC-12 cells viability (MTT). Cells were incubated with the increasing concentrations of agomelatine (1–100 μM) for various times (1–24 h). The moderate dose of agomelatine was determined at 20 μM concentration and 24 h after incubation ($P < 0.001$) ($n = 6$ for each)

Results

Effects of AGOM, DULOX, and Their Combination on H_2O_2-Induced $[Ca^{2+}]_i$ Concentration in PC-12 Neuronal Cells

Effects of AGOM, DULOX, and their combination on $[Ca^{2+}]_i$ release in the PC-12 cells are shown in Figs. 2 and 3, respectively. The $[Ca^{2+}]_i$ release values were significantly ($P < 0.001$) lower in AGOM and DULOX than in control. 2-APB is a non-specific TRPM2 channel antagonist, though verapamil and diltiazem are voltage-gated calcium channel blockers. In some groups, the neurons were incubated by 2-APB and verapamil + diltiazem before H_2O_2 stimulation. The $[Ca^{2+}]_i$ concentrations were significantly ($P < 0.001$) lower in 2-APB and verapamil + diltiazem group than AGOM and DULOX groups.

Effects of AGOM, DULOX, and Their Combinations on GSH-Px, GSH, and Lipid Peroxidation Values in PC-12 Neuronal Cells

Effects of AGOM and DULOX (20 and 10 μM for 24 h incubation, respectively) and their combination on GSH-Px, GSH, and lipid peroxidation values in PC-12 cells are shown in Table 1. Thiols, including GSH, are thought to play a pivotal role in protecting cells against ROS. A GSH level is one of the main markers which are used as an important biomarker for oxidative damage (Ozgul and Naziroglu 2012). Our data are consistent with that view, in that following incubation with AGOM, lipid peroxidation levels were significantly ($P < 0.05$) lower in the AGOM and DULOX group than in control. Lipid peroxidation levels were further ($P < 0.001$) decreased by their

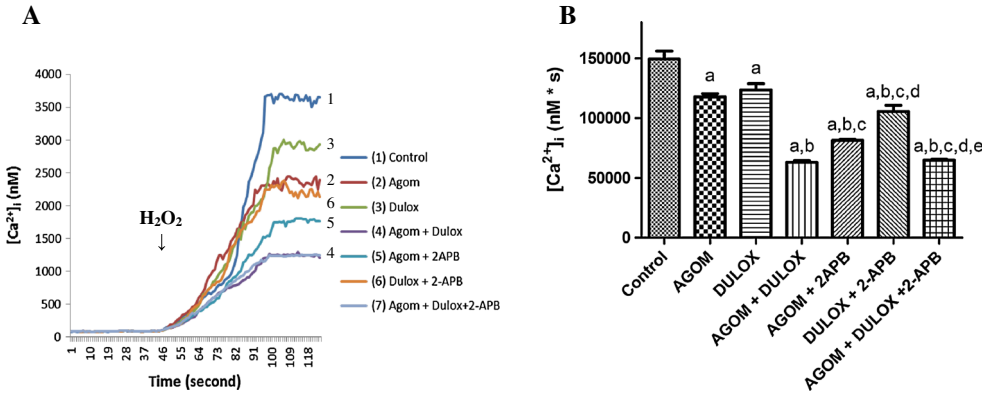

Fig. 2 **a** Calcium release through TRPM channels from PC-12 cells exposed to agomelatine (AGOM), DULOX, and their combination stimulated with H_2O_2. The cells were incubated by non-specific TRPM2 cation channel, 2-APB. Original time course chart recordings showing $[Ca^{2+}]_i$ transients in PC-12 cells. **b** *Bar charts* showing mean ± SD data for cytosolic Ca^{2+} ($[Ca^{2+}]_i$) concentration from H_2O_2-stimulated PC-12 cells ($n = 6$ for each). $^{a}P < 0.001$ versus control group, $^{b}P < 0.001$ versus AGOM and DULOX groups, $^{c}P < 0.001$ versus AGOM and DULOX groups, $^{d}P < 0.001$ versus AGOM + 2-APB group, $^{e}P < 0.001$ versus DULOX + 2-APB group

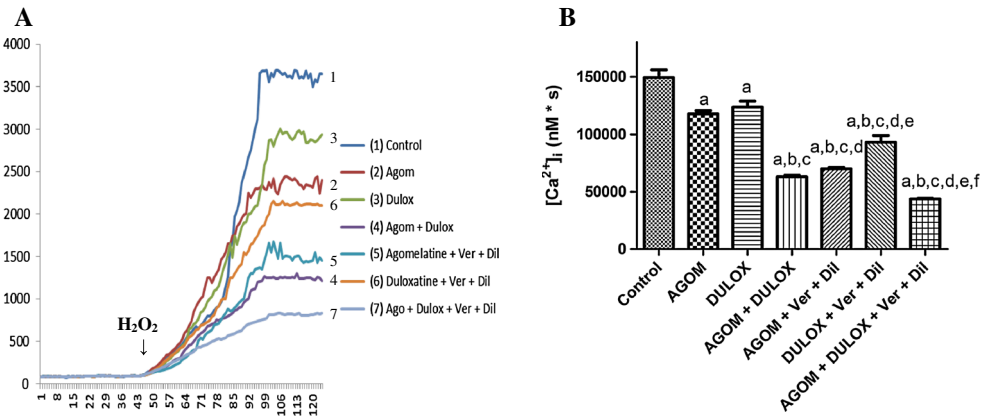

Fig. 3 **a** Calcium release from PC-12 cells exposed to AGOM and DULOX following stimulation with H_2O_2. Original time course chart recordings showing $[Ca^{2+}]_i$ transients in PC-12 cells. **b** *Bar charts* showing mean ± SD data for $[Ca^{2+}]_i$ from H_2O_2-stimulated PC-12 cells ($n = 6$ for each), $^{*}P < 0.001$ for PC-12 cells compared to control. Note the significant elevation in $[Ca^{2+}]_i$ for PC-12 cells compared to control. $^{a}P < 0.001$ versus control group, $^{b}P < 0.001$ versus AGOM group, $^{c}P < 0.001$ versus DULOX group, $^{d}P < 0.001$ versus AGOM + DULOX group, $^{e}P < 0.001$ versus AGOM + Ver + Dil, $^{f}P < 0.001$ versus DULOX +Ver + Dil

incubation and lipid peroxidation levels were significantly ($P < 0.01$) lower in the combination group than in the control group. GSH-Px and GSH values were significantly ($P < 0.001$) higher in the AGOM group than in the control. Hence, we observed antioxidant role of AGOM and DULOX in the neurons (Fig. 4).

Effects of AGOM and DULOX on Apoptosis Levels, Intracellular ROS Production, Mitochondrial Depolarization Levels, Caspase-3 and -9 Values in the PC-12 Neuronal Cells

Apoptosis levels in the four groups are shown in Table 1. The apoptosis levels decreased significantly ($P < 0.05$) in three groups compared to the control. Moreover, the combination group had lower apoptosis levels than AGOM and DULOX.

Intracellular ROS production values in the four groups are shown in Table 1. The ROS production values were significantly lower in three groups ($P < 0.05$) than in control. AGOM and DULOX combination did not change the ROS levels.

Mitochondrial membrane depolarization levels in the four groups are shown in Table 1. The mitochondrial membrane depolarization levels decreased significantly ($P < 0.05$) in three groups as compared to the control. Moreover, the combination group had lower apoptosis levels than AGOM and DULOX ($P < 0.001$).

Caspase-3 and caspase-9 activities in the four groups are shown in Table 1. The enzyme activities changed significantly ($P < 0.05$) in three groups compared to the control.

Table 1 Effects of agomelatine (AGOM), duloxetine (DULOX), and their combinations on lipid peroxidation (LP), glutathione peroxidase (GSH-Px), reduced glutathione (GSH), apoptosis, caspase-3, caspase-9, and cytosolic ROS production values in PC-12 neuronal cells

Groups/parameters	Control	AGOM	DULOX	AGOM + DULOX
LP (μmol/g protein)	13.65 ± 1.78	8.93 ± 0.59[a]	8.14 ± 1.33[a]	7.88 ± 1.63[b, c]
GSH (μmol/g protein)	2.39 ± 0.16	3.42 ± 0.36[a]	3.16 ± 0.19[a]	3.14 ± 0.21[a]
GSH-Px (IU/g protein)	8.25 ± 0.69	10.70 ± 0.69[a]	10.12 ± 0.82[a]	10.81 ± 0.39[a]
Apoptosis (absorbance)	0.214 ± 0.01	0.142 ± 0.01[a]	0.148 ± 0.01[a]	0.125 ± 0.03[a]
Caspase-3 (O.D.)	73,588 ± 7,365	60,761 ± 5067[a]	61,311 ± 7,623[a]	67,233 ± 6,696[b, c]
Caspase-9 (O.D.)	70,022 ± 4,015	52,413 ± 4,073[a]	61,668 ± 6,572[a, b]	60,348 ± 5,207[a,b]
ROS (O.D.)	39,185 ± 3,551	30,772 ± 3595[a]	30,802 ± 3,816[a]	30,136 ± 4,260[a]

$n = 8$, mean ± SD, *O.D.* optical density

[a] $P < 0.05$ versus control group

[b] $P < 0.05$ versus AGOM group

[c] $P < 0.05$ versus DULOX group

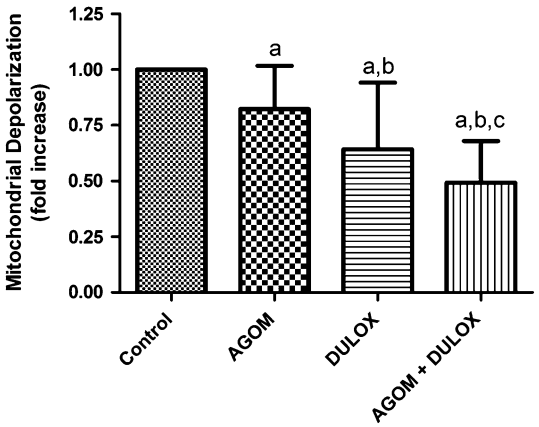

Fig. 4 Mitochondrial depolarization levels in PC-12 cells exposed to agomelatine, duloxetine, and their combination. PC-12 cells were incubated with agomelatine and duloxetine. Agomelatine and duloxetine decreased levels of mitochondrial depolarization. But, their combination is more effective than use as single ($n = 6$ for each). [a]$P < 0.05$ versus control group, [b]$P < 0.05$ versus AGOM group, [c]$P < 0.05$ versus DULOX group

Discussion

There are increasing numbers of literature on the functional effects of oxidative redox variation on new therapeutic agents for psychiatric diseases. However, so far few studies have investigated the effects of them on intracellular signaling cascades such as $[Ca^{2+}]_i$ signaling or caspase activations in cell culture model. In this paper, we put importance on the contribution of two antidepressant drugs' effects on intracellular antioxidant enzymes and Ca^{2+} signaling pathways with apoptotic molecules and their relations with ROS levels. AGOM's action on dendritic neurogenesis in different animal models of depression is also recognized in another study (Srinivasan et al. 2012). DULOX, as mentioned above, has low affinity to neural receptors and also to ion channel binding sites and other neurotransmitter transporters (Carter and McCormack 2009). For this aims, we aimed to combine two newly discovered antidepressant drugs.

The major findings determined in the current study are the significant and marked changes in apoptosis, mitochondrial depolarization, and $[Ca^{2+}]_i$ levels in neuronal PC-12 cells treated with AGOM and DULOX compared to controls. Although it is commonly confirmed that $[Ca^{2+}]_i$ signaling and deoxyribonucleic acid (DNA) damage might be the main reason for triggering the apoptosis, caspase 3 activation also plays a key role in the steering of the apoptosis (Qi et al. 2002; Pang and Hong 2010). As a second messenger, Ca^{2+} acts in many cellular processes. Special for Ca^{2+} cations, the concentration between inside and outside of cell states great differences. Any unwanted changes in $[Ca^{2+}]_i$ levels will provide a receptor dependent signal for early cell death pathway (Qi et al. 2002). If $[Ca^{2+}]_i$ levels are elevated for a continuous period, cells are induced to undergo apoptosis (Berliocchi et al. 2005). In the current study, we observed that AGOM and DULOX reduced $[Ca^{2+}]_i$ entry in the neuronal cells. When PC-12 neuronal cells exposed to non-specific TRPM2 channel antagonist (2-APB) and voltage-gated calcium channel blockers (verapamil + diltiazem) the $[Ca^{2+}]_i$ levels were significantly decreased. Some scientists even advocated that increased neuronal cell death may contribute to the pathogenesis of depression (Duman et al. 1999). In the current study, we observed increased levels of $[Ca^{2+}]_i$, ROS, caspase-3 and -9 values, though GSH and GSH-Px values were significantly decreased. BDNF is an effective regulator of neuronal organization, structure and function (Bramham and Messaoudi 2005). To our knowledge, there is no result on the values in literature, but (Amaral and Pozzo-Miller 2007) reported that brain-derived neurotropic factor (BDNF) exposure to CA1 pyramidal neurons evokes

transient elevations in $[Ca^{2+}]_i$ concentrations which are independent from membrane depolarization. This result supports our finding with the effects of both two drugs' regulatory effects on BDNF mechanism. Moreover, (Finkbeiner et al. 1997) and (Li et al. 1998; Liu et al. 2008) reported that BDNF-induced somatic Ca^{2+} elevations in cultured neurons were reduced, but not entirely blocked. Our results, which we obtained from cultured PC12 cells, are also overlapping with the results mentioned above. All these results suggest that both Ca^{2+} influx and mobilization from intracellular stores contribute to the responses. Some basic structures of these Ca^{2+} signals might be due to effects of capacitative Ca^{2+} entry (Putney 2003), a mechanism assumed to be intermediated by some members of the TRPC channel subfamily in neurons (Birnbaumer et al. 1996; Clapham 2003; Mikoshiba 1997).

The other potential target of antidepressant drug regulation is the intracellular antioxidant enzymes (Su et al. 2012). Glutathione peroxidase detoxifies hydrogen peroxide (H_2O_2) to water, though GSH has inhibitor role on free oxygen radicals. Therefore, ROS can be secondarily assessed by measurement of some antioxidants such as reduced glutathione (GSSG), GSH-Px. The endogenous antioxidant enzymes mainly function to reduce the oxidative stress levels of a cell and try to scavenge free radicals to prevent cell damage, neuronal death or premature aging (Bains and Shaw 1997). Various protective factors have been shown for GSH and GSH-Px (Naziroglu 2009). Furthermore, in vivo studies have demonstrated that upregulation of this enzyme is associated with neuroprotective capabilities in toxicity whereas its down regulation induces apoptosis of cultured DRG neurons and PC 12 neuronal cells (Altinkilic et al. 2010; Naziroglu et al. 2013). An increase in intracellular ROS has been reported as a nominee to trigger the initiation of apoptotic events (Castedo et al. 1996). Our findings also demonstrate these findings. Our results indicate that caspase-3 activation caused by the caspase-9 activation probably due to an increase in ROS generation. The elevated ROS levels trigger the caspase-3 and -9 activation, respectively. We provide satisfied results supporting that ROS generation might be important in increased absorbance levels of apoptosis determination kit. Since ROS products are generated in mitochondria by uncoupling of electron transport chain mechanism, as a step of apoptosis intrinsic pathway, ROS are able to induce oxidative damage of cell which then leads to apoptosis. We also assessed the effect of AGOM, DULOX, and their combinations on the mitochondrial membrane potentials. As shown in Table 1, treatment of cells for 24 h with AGOM, DULOX and their combination decreased the production of intracellular ROS levels, as revealed by the alteration in Rh-123 fluorescence. Our results overlap with another study. Aguiar et al. (2013) determined significant decrease in the lipid peroxidation levels in AGOM treated rat brains. We also demonstrate that the presence of these two antidepressant drugs was able to decrease caspase activation. The influence of these two drugs on maturation and cell survival was accompanied by a selective increase in the levels of BDNF (Warner-Schmidt and Duman 2007). In general, these growth factors improve adult neurogenesis and present pivotal antidepressant properties. Additionally, Soumier et al. (2009) demonstrated that agomelatine treatment accelerates all stages of neurogenesis.

In conclusion, the results of the present study point out that AGOM and DULOX have antioxidant activity. Their combinations are more effective against apoptosis and mitochondrial depolarization levels. Hence, we concluded that AGOM and DULOX show their effects as a regulator for intracellular signaling pathways. Although this mechanism needs further detailed studies, our findings provide some evidences for an important role of $[Ca^{2+}]_i$ and caspase 3 and 9 activations in the regulation of AGOM and DULOX. Our study is one of the interesting studies regarding the effects of antidepressant drugs on apoptosis. A new concept of their action has been suggested, treatments called as antidepressant drugs may help in improving the neuronal networks by regulating intracellular signaling pathways, protecting them against apoptosis.

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Conflict of interest None of the authors has any conflicts to disclose. All authors approved the final manuscript.

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