Neuroprotective Effects of losartan and Captopril in an H2O2- Induced Neurotoxicity Model of Neuro-2A Cells

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Abstract

Background: Depression is one of the most common mental illnesses and knowledge about its pathophysiology will assist in smart treatment of depression. RAS is a hormonal system that regulates blood pressure and fluid balance. Role of brain RAS has been highlighted in many mental and neurological disorders. Many drugs that target this system, such as ACEIs and ARBs, have shown positive effects on improving depression in clinical studies and animal models.

Methods and results: Regarding the effectiveness RAS in depression, this study was conducted to compare the neuroprotective effects of ARB and ACEI drugs and common antidepressants on Neuro-2a cells. The cells were treated in the different concentrations of captopril, losartan, imipramine, and venlafaxine (1, 10, 50, 100 μM), after exposure to H_2O_2. Intracellular Ca^{2+} content, cell viability, SOD activity and ROS generation were measured in all groups. Our results show that cell viability of H_2O_2-treated cells was significantly increased in the presence of antihypertensive drugs. We observed a protective effect against ROS production in all drug groups in Neuro-2a cells. Losartan at all concentrations and captopril prevented cell damage caused by ROS. Cell death due to intracellular Ca^{2+}, was significantly reduced with all antidepressant. At low concentrations of losartan and captopril cell death due to intracellular $Ca^{2+}$was significantly reduced compared to the H_2O_2 group.

Conclusions: Antihypertensive drugs, especially losartan can have neuroprotective effects and if approved in animal models, it may be used in the future as an adjunct in psychiatric diseases such as depression.

1. Introduction

Depression is one of the most common mental illnesses with a not fully understood etiology that negatively affects one's feelings, thoughts, actions, and performance [1].

RAS, which regulates fluid balance in the body, also plays a remarkable role in the physiologic functions of the CNS. Recent studies have shown that central RAS contributes to the pathophysiology of disorders affecting CNS mainly by means of Ang II [2], the main effector of RAS. The potential function of Ang II has been implicated in the development of stress, depression and memory consolidation by binding to its main receptor, AT1R [3–5]. Ang II is produced from the conversion of Ang I by means of ACE [6, 7]. The beneficial effects of ARBs and ACEIs such as losartan and captopril in reducing depressive symptoms have been observed in animal models and patients [2, 8–10]. Captopril is a centrally acting ACEIs with mood elevating properties in patients with MDD. Captopril leads to a higher score of quality of life compared to enalapril (non-central ACEI) with equal antihypertensive effects [11–14].

ROS are among the key signaling molecules in Ang II–stimulated firing and activation of CNS neurons [15]. Increased generation of ROS is a result of the defective reduction of oxygen in the mitochondrial respiratory chain reactions or activation of oxidative enzymes such as the NADPH oxidase in the

cytoplasm of the cell [16]. Excessive generation of ROS in the brain is associated with induction of neuronal apoptosis and development of neuropsychiatric diseases such as depression [17, 18]. Accordingly, Ang II-mediated ROS generation is involved in the pathophysiology of depression. In fact, production of ROS in neuronal cells may be the resultant of binding of Ang II to AT1R [19, 20]. Following AT1R stimulation, NADPH-derived ROS generation is increased, leading to an increase in intracellular Ca^{2+} concentration [21, 22]. In response to increasing cytoplasmic Ca^{2+}, uptake of Ca^{2+} in mitochondria and mitochondrial ROS production are elevated as well [23]. On the other hand, mitochondrial ROS stimulates NADPH oxidases, thus a vicious cycle of ROS production is made in different parts of the cell [19, 24]. Massive induction of mitochondrial Ca2+ uptake by overproduction of ROS, leads to exacerbation of neurotoxicity (20, 21). A study of Neuro-2A, which express high levels of ATR1, showed that activation of Ang II-induced neurons and modulation of extracellular Ca^{2+} influx by voltage-sensitive channels is dependent on ROS production by NADPH oxidase [15, 25]. Lipid-rich tissue of the brain on one hand and it's high need for oxygen on the other hand makes it a very sensitive organ to excessive ROS production [26].

Protective effects of different classes of antidepressants such as SSRIs and TCAs, against oxidative damage is confirmed *in vitro* and *in vivo* [27, 28].

In this study, we aimed to investigate the possible neuroprotective mechanism of action of ARBs and ACE inhibitors and their association with oxidative stress and Ca2+ signaling. Additionally, neuroprotective roles of different classes of antidepressants were assessed in an H_2O_2-induced neurotoxicity model in Neuro-2a cells in order to provide a comparison with the neuroprotective roles of ARBs and ACE inhibitors.

2. Materials And Methods

2.1 Cell culture and experimental treatment

This study was conducted and approved at Shiraz University of Medical Sciences. In the present study, Neuro-2a cells were supplied by Pasteur Institute, Tehran, Iran. Neuro-2a cells were thawed and propagated in 25 ml and 100 ml sterile plastic flasks with DMEM (Dulbecco's Modified Eagle Medium) high glucose (Bio idea®) and supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Gibco®) and 1% penicillin/streptomycin (Bio Idea®).

The culture medium was replaced with fresh medium twice a week. The cells from 3rd passage were used for drug experiments. Cell line was maintained in a humidified atmosphere of 5% CO_2, 95% air at $37^{\circ C}$ until they reached a confluence of 80%. Cultured cells were trypsinized with trypsin/EDTA for 5 min and then plated at an appropriate density according to each experiment [29].

2.2. Drug treatment

Drugs and hydrogen peroxide (H_2O_2) were freshly prepared from stock solution prior to each experiment. All drugs except for imipramine and venlafaxine were purchased from Exir Company, Iran. Imipramine

and venlafaxine were purchased from Sobhan daroo, Iran. The 20000 µM stock solution of study drugs was prepared in double-distilled water and sterilized with a syringe filter. The stock solution of H_2O_2 was prepared in phosphate-buffered saline (PBS). After exposure to H_2O_2 1% for 30 minutes, the cells were incubated with different concentrations of venlafaxine (1, 10, 50, 100 µM), Imipramine (1, 10, 50, 100 µM), captopril (1, 10, 50, 100 µM) and losartan (1, 10, 50, 100 µM). The following experiments were carried out 24hrs after drug treatment of the cells with at least 3 repetitions for each sample [29].

2.3. Assay of cell viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted as a measure of cell viability. MTT, a tetrazolium salt, is cleaved to the insoluble formazan by succinate dehydrogenase, an active enzyme for the mitochondrial respiratory chain in living cells.

Neuro-2a cells were seeded in 96-well plates at a density of 1×10^4 cells/well incubated at 37°C, 5% CO_2. Cells were treated with 20µl H_2O_2 1% for 30 min. Drugs were then added to each well at the mentioned concentrations. After 24 hrs of incubation, MTT (5 mg/ml (1.13 µM) stock solution in PBS) was added to the plates with a final concentration of 0.45 mg/ml and incubated for 2 hrs at 37°C. The supernatants were discarded and 100µl of dimethyl sulfoxide (DMSO) was added and mixed to dissolve the formed formazan crystals. The absorbance was assessed with an ELISA microplate reader at 570 nm. Viability of Neuro-2a cells in each well was demonstrated as a percentage of treated to untreated control cells [30, 31].

2.4. Measurement of intracellular ROS using DCFH-DA assay

Determination of intracellular ROS, is a cell-based assay for measuring the concentration of hydroxyl, peroxyl, superoxide and other ROS activity within a cell. Dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to measure intracellular ROS production [32]. Neuro-2a cells were plated at a density of 1×10^4 cells/well in 96-well plates. Afterwards, 20µl of H_2O_2 was added to each cell, after 30 min of incubation at 37°C, 10 µl of drug samples (venlafaxine, Imipramine, captopril, losartan) (1, 10, 50, 100 µM) were added to the wells. After the treatment period, cells were collected, washed twice with PBS and stained with DCFH-DA at a final concentration of 20 µM for 30 min at 37°C. Then, cells were washed again with PBS for removing excess DCFH-DA. Fluorescence intensity was measured at excitation and emission wavelengths of 345 and 425 nm, respectively with a fluorimeter [33].

2.5. Measurement of SOD activity

SOD activity was determined using the nasdox® superoxide dismutase (SOD) activity assay kit (Navand Salamat®, Iran) according to manufacturer's protocol. Neuro-2a cells were seeded at a density of $1x10^6$ cells/ml in 6-well plates and cultured for 24 hrs. Then 200 µl H_2O_2 was added to each cell. After 30 min of incubation at 37°C, 100 µl of drug samples (venlafaxine, Imipramine, captopril, losartan) with mentioned concentrations were added to each well.

After 24 hrs of incubation, the cells were centrifuged (for 2 min at 800 rpm). Cell precipitates were washed with cold PBS, and the previous process was repeated. Lysing buffer was added, thereafter. After cooling in ice, the mixture was centrifuged at 12,000 × g for 5 min. Standard reagents were added to each well and after incubation, absorbance was read at 405 nm.

2.6. Bradford assay

Bradford was determined using the Nadford® protein assay kit (navand salamat®, Iran) according to manufacturer's protocol. Neuro-2a cells were seeded at a density of $1x10^6$ cells/ml in 6-well plates and cultured for 24 hrs. Then 200µl H_2O_2 added to each well. After 30 min incubation at 37°C, 100µl of drug sample (venlafaxine, Imipramine, captopril, losartan) were added to the wells.

Nadford® reagent was prepared by diluting with double distilled water. After treatment and incubation for 24 hrs, cells were collected and the stabilizing solution was added. According to the protocol, 10µl of sample and 300µl of Nadford® reagent were mixed and incubated at $37^{\circ c}$ for 10 min. Absorbance was measured at 630 nm. Bradford standards were prepared and the standard curve was plotted. Relative protein concentrations were calculated from the standard curves and expressed as µg/ml.

2.7. Determination of intracellular Ca^{2+} concentration with flow cytometry:

Intercellular Ca^{2+} levels were determined by flow cytometry as described previously [34].

Neuro-2a cells were seeded at a density of $1x10^6$ cells/ml in 6-well plates and cultured for 24 hrs. One ml of H_2O_2 was added to each cell and incubated for 30 min at $37^{\circ C}$. After treatment with different concentrations of drug samples, the cells were exposed to 4 µl of Fluo-3-AM (final concentration of 4 µM) and pluronic acid 20% (final concentration of 5 µM) which were separately dissolved in DMSO and mixed before use with the ratio of 1:1. The cells were then washed twice with Ca^{2+}-free PBS.

After 30 min incubation in a dark environment at $37^{\circ C}$, all the micro tubes containing Fluo-3-AM were centrifuged at 500 rpm for 5 minutes. The supernatant was discarded and DMEM was added to each micro tube. A min prior to flow cytometry, 16.6 µl PI (propidium iodide) dissolved in PBS (final concentration of 5µM) was added to this solution. Analysis was performed by FACS® Calibur flow cytometer (BD Bioscience, USA) at the excitation wavelength, 506 nm, and the emission wavelength, 525 nm. The results were analyzed using FlowJo version 7.6 software.

2.8. Data analysis

Statistical analysis was performed using GraphPad prism software package version 8.0.2.263 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data are expressed as mean ± SD. Parametric data were analyzed using one-way ANOVA. As the post-hoc test Bonferroni multiple comparison was used. Non parametric data were analyzed using Kruskal–Wallis and Dunn's as the post-hoc multiple comparison test was used. P-value <0.05 was considered to be statistically significant.

3. Results

Comparison of cell viability in different study groups is demonstrated in Fig. 1. As shown captopril, venlafaxine, imipramine, and losartan significantly reduced H_2O_2-induced toxicity in Neuro-2a cells at all concentrations (P< 0.0001).

Fig.2 represents the percentage of ROS generation in different study groups. The level of ROS generation in H2O2-treated Neuro-2a cells in different concentrations of venlafaxine (1 μM, p<0.001 and 10 μM, p<0.0001), and captopril (10 μM, p<0.0001 and 50μM, p<0.005) was significantly increased compared to the control group.

ROS generation was significantly reduced in groups treated with all concentrations of venlafaxine, imipramine and losartan (P<0.05) vs the H_2O_2 group. Also a significant decrease in ROS level was observed at concentrations of 1, 100 μM and 50 μM of captopril compared to the H_2O_2 group (P<0.0001).

SOD activity in different study groups are presented in Fig.3. The level of SOD activity was significantly differed in all concentrations of venlafaxine and 50 μM of captopril (p<0.005) from the H_2O_2 group. Significant difference in SOD enzyme activity was observed at concentrations of 100 μM of captopril and 1 μM of losartan compared to the control group (P <0.05).

As shown in Fig.4., all concentrations of imipramine along with 1 and 50 μM of venlafaxine and 1 μM of losartan (p<0.0001) showed a significant reduction in the percentage of dead cells with high intracellular calcium compared to the control group. There was a significant difference between the percentage of dead cells with high intracellular calcium in the presence of all concentrations of venlafaxine, and imipramine compared to H_2O_2-treated cells alone (P<0.0001).

Discussion

Depression is one of the most common mental illnesses that may remarkably affect one's quality of life. Knowledge of the pathophysiology will assist in smart treatment of depression [1]. In the clinical setting, elements of RAS are known as blood pressure regulators. In addition to its "classical" role, RAS exists locally in various tissues, including the brain [35]. Binding of Ang II to its main receptors, AT1 and AT2, is accountable for most of RAS function in the brain [15, 36]. The potential harmful effects of Ang II in the brain are often due to its binding to the AT1 receptor. RAS plays an important role in balancing the inflammatory and anti-inflammatory pathways through its two receptors in the brain [37, 38]. Recent studies have also shown that increased RAS activity increases the risk of depression by increasing AT1 receptor response to angiotensin II [37, 39, 40]. This stimulation leads to the release of inflammatory mediators through various intracellular mechanisms in the CNS and ultimately causes oxidative stress [7, 41].

Increased levels of ROS and intracellular calcium in Ang II-stimulated Neuro-2A cells has been repeatedly reported [15, 21, 42, 43]. On the other hand, there is some evidence that ROS can also induce Ang-II activity. The mitogen-activated protein kinase (MAPK), an important regulator of signal transduction pathways, is independently activated by Ang-II and ROS. Increased MAPK leads to further ROS formation through NADPH oxidase activation. As a result of MAPK activation, nuclear transcriptional factors are activated that modulate the expression of tyrosine AT1 receptors. Indeed, Ang-II and ROS enhance each other's activities in a variety of ways. Finally, all of the pathways generally lead to an increase in the intracellular calcium concentration that causes neuronal death and an inflammatory response [15, 44-48].

It has been seen that most antihypertensive drugs improve the symptoms of depression as well as brain damage in cardiovascular patients [49-52]. The first evidence of a possible role for RAS in depression was reported following the improvement of depressive symptoms in captopril-treated hypertensive patients [53, 54]. In many experiments, captopril has a similar healing effect to imipramine on depressed rat model induced by foot shock [10]. On the other hand, many findings show that some antidepressants can have some positive effects by inhibiting the brain RAS [55, 56].

In the present study, captopril-treated Neuro-2a cells in the presence of H_2O_2 showed a significant difference in viability comparing with the H_2O_2 and control groups. No cytotoxic effects were observed at any of the captopril concentrations. The result showed that exposure of Neuro-2a cells to H_2O_2 significantly decreased cell viability, and captopril can protect the cells from H_2O_2-induced cytotoxicity. Captopril also significantly reduced the accumulation of intracellular ROS, which may be the reason for the significant difference in cell viability compared to H_2O_2 -treated cells. The greatest effect was observed at a concentration of 100 μM. At all concentrations except for 10 μM, captopril significantly decreased ROS level in the H_2O_2-treated cells compared to the H_2O_2 alone but not in a dose-dependent manner. Due to the increased oxidative stress of the cells at a concentration of 50 μM, the activity of the SOD enzyme was expected to increase to prevent cell necrosis, which was consistent with our observations. However, the percentage of dead cells with high intracellular calcium in captopril-treated cells at all concentrations except 10 μM was significantly different from the H_2O_2-treated cells. However, cell viability has improved at this concentration of captopril. According to the improvement of cell viability, ROS generation and the percentage of dead cells with high intracellular calcium, especially in the concentration of 100 μM, the neuroprotective effects of captopril may be related to the inhibition of the RAS system in Neuro-2a cells. But at lower concentrations, the increased viability of H_2O_2-treated cells may be dependent on SOD activity. These results are in line with previous report that studied the neuroprotective effects of captopril in high glucose-induced toxicity in PC12 cells [57].

At different concentrations of losartan, we observed a good cell viability in H_2O_2-treated Neuro-2a cells. However, in none of the losartan concentrations there was a significant difference in SOD activity compared to the H_2O_2 group. Losartan along with imipramine were among the drugs that captured ROS the most compared to the H_2O_2-treated group. Percentage of dead cells with intracellular calcium indicated that higher concentrations of losartan was associated with death due to high intra cellular

calcium and at the concentration of 1 μM it can prevent cell death due to high calcium. According to the results, we concluded that losartan at lower concentrations such as 1 μM, plays a neuroprotective role by reducing mitochondrial oxidative stress and thus reducing intracellular calcium levels. While at higher concentrations, losartan showed its neuroprotective effects by decreasing ROS activity through the inhibition of the RAS system. Various studies have shown that ARBs such as losartan have protective effects in some tissues following various injuries [58]. Losartan has shown protective properties against the neuronal damage caused by oxidative stress [59-61]. Also, in line with the results of our study, losartan inhibits oxidative stress-induced neurotoxicity in animal models of Parkinson's disease and also in cell lines such as PC12 [57, 62].

Venlafaxine is known as a serotonin-norepinephrine reuptake inhibitor (SNRI) that is widely used in the treatment of MDD, anxiety, and neuropathy [63]. Venlafaxine has neuroprotective effects due to its anti-inflammatory activities [64, 65]. Based on our results, the highest cell viability belonged to 100 μM of venlafaxine. The activity of the SOD enzyme in all concentrations of venlafaxine was significantly higher in H_2O_2-treated group. ROS levels were significantly reduced in venlafaxine-treated cells in different concentrations compared with the H_2O_2 group. Also, at the concentration of 50 μM, treatment with venlafaxine caused the lowest percentage of dead cells with high calcium compared to all other groups treated with other drugs and even the control group. According to the results, it appears that venlafaxine increases the activity of SOD enzyme and also prevents the accumulation of ROS and calcium and thus protects Neuro-2a cells against damage. Considering other neuronal cell lines, venlafaxine entail its neuroprotective effects in PC12 cells through an unclear mechanism [66]. It also suppresses oxidative stress in human cerebrovascular microvascular endothelial cells (HBMECs) damaged by methylglyoxal by reducing intracellular ROS[67].

Imipramine, a tricyclic antidepressant (TCA) known as an effective drug in treating depressive disorders, neurodegenerative diseases, and other mental disorders [68]. Treatment with antidepressants such as imipramine has shown to increase BDNF [69]. Many results also confirm that imipramine reduces the expression of inflammatory cytokines and has neuroprotective and anti-inflammatory effects [70-74]. In our study, no cytotoxic effect was observed in any of the concentrations of imipramine. Imipramine and losartan showed the lowest levels of ROS at different concentrations compared to other drugs. The level of ROS in cells treated with concentrations of 40, 50, and 100 μM of imipramine was almost the same as the control group. Cell death due to incline in intracellular calcium was significantly lower in all concentrations of imipramine compared to both the control and the H_2O_2-treated groups. Considering the neuroprotective effects of imipramine in most studies on neurons [71, 73, 75, 76] and our results, it seems that imipramine is involved in the protection and survival of neurons by inhibiting the production and accumulation of ROS and associated downstream pathways such as MAPK/extracellular-regulated kinase (ERK) in neural stem cells (NSCs) [73]. In SH-SY5Y cells, imipramine plays its neuroprotective effects against H_2O_2-induced cell death by increasing the expression of the anti-apoptotic protein, Bcl-2 [77]. Additionally, imipramine inhibits LPS-induced toxicity in oligodendrocyte cell line, OLN-93 by reducing the levels of ROS[78].

In summary, the findings of the present study demonstrated that imipramine, venlafaxine, as well as captopril and losartan, reduce H2O2-induced oxidative stress in Neuro-2a cells and may exert their protective effects against neuronal cell death by inhibition RAS. Losartan and captopril at low concentrations protect cell death due to elevation of intracellular calcium.

Based on the results, we can conclude that losartan and captopril have the ability to produce neuroprotective effects. Therefore, if approved in animal models, it may be used in the future as an adjunct in psychiatric diseases such as depression.

Abbreviations

RAS: Renin-angiotensin system, ARBs: Ang II receptor blockers, ACEIs: ACE inhibitors, SOD: Superoxide dismutase, ROS: Reactive oxygen species, CNS: Central nervous system, Ang II: Angiotensin II, AT1R: Ang II type 1 receptor, ACE: Angiotensin converting enzyme, Neuro-2A: Neuroblastoma cells, SSRIs: Selective serotonin reuptake inhibitors and TCAs: Tricyclic antidepressants

Declarations

Statements & Declarations

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Conflict of interest:

The authors declare no conflict of interest.

Compliance with Ethical Standards:

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

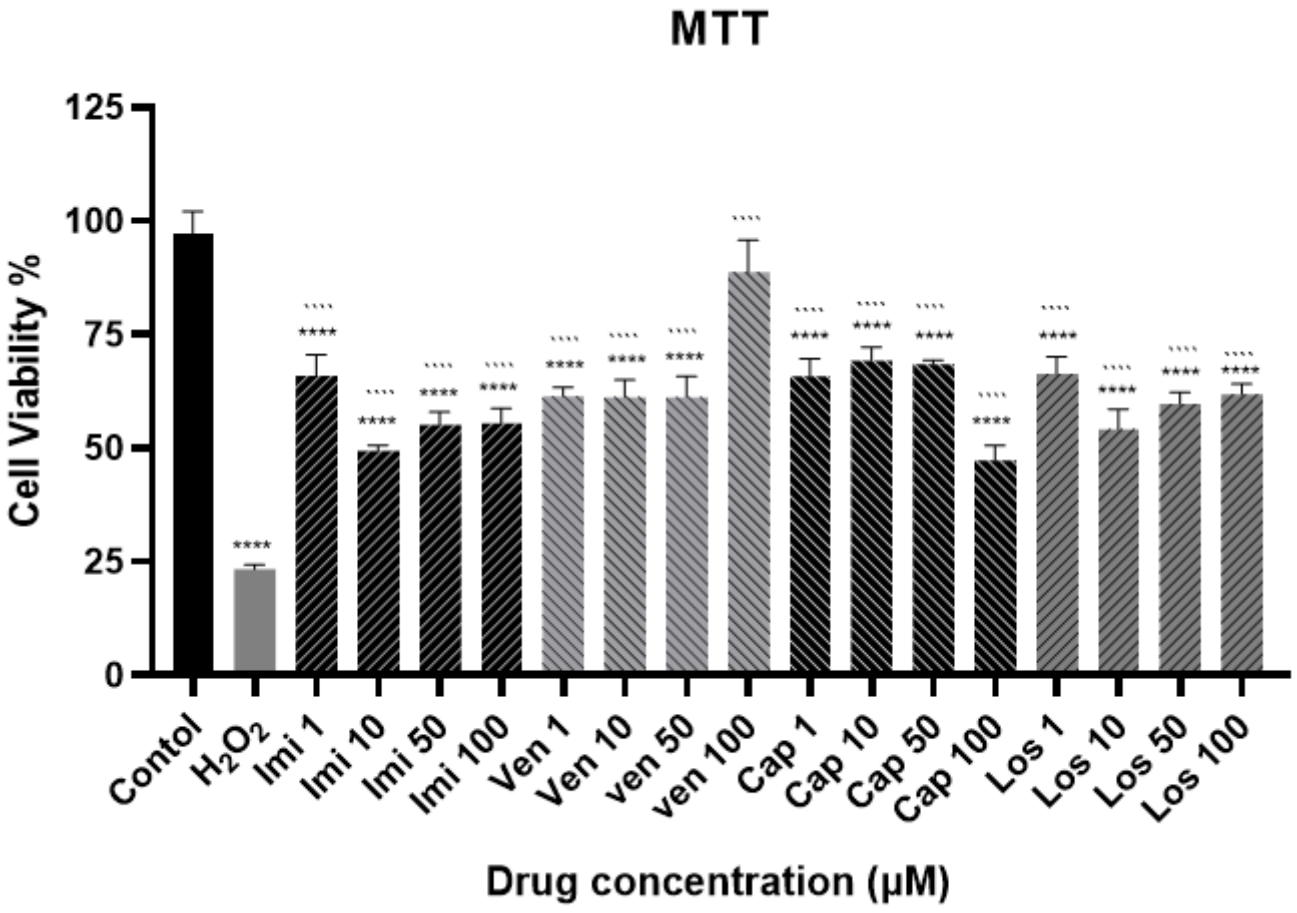

Figure 1

Cell viability of the H_2O_2-treated Neuro-2a after drugs treatment in the different groups.

Ven: Venlafaxine, Cap: Captopril, Imi: Imipramine, Los: Losartan

****p<0.0001 vs. control; $p < 0.05$, $p < 0.005$, p<0.001 & p<0.0001 vs. H_2O_2

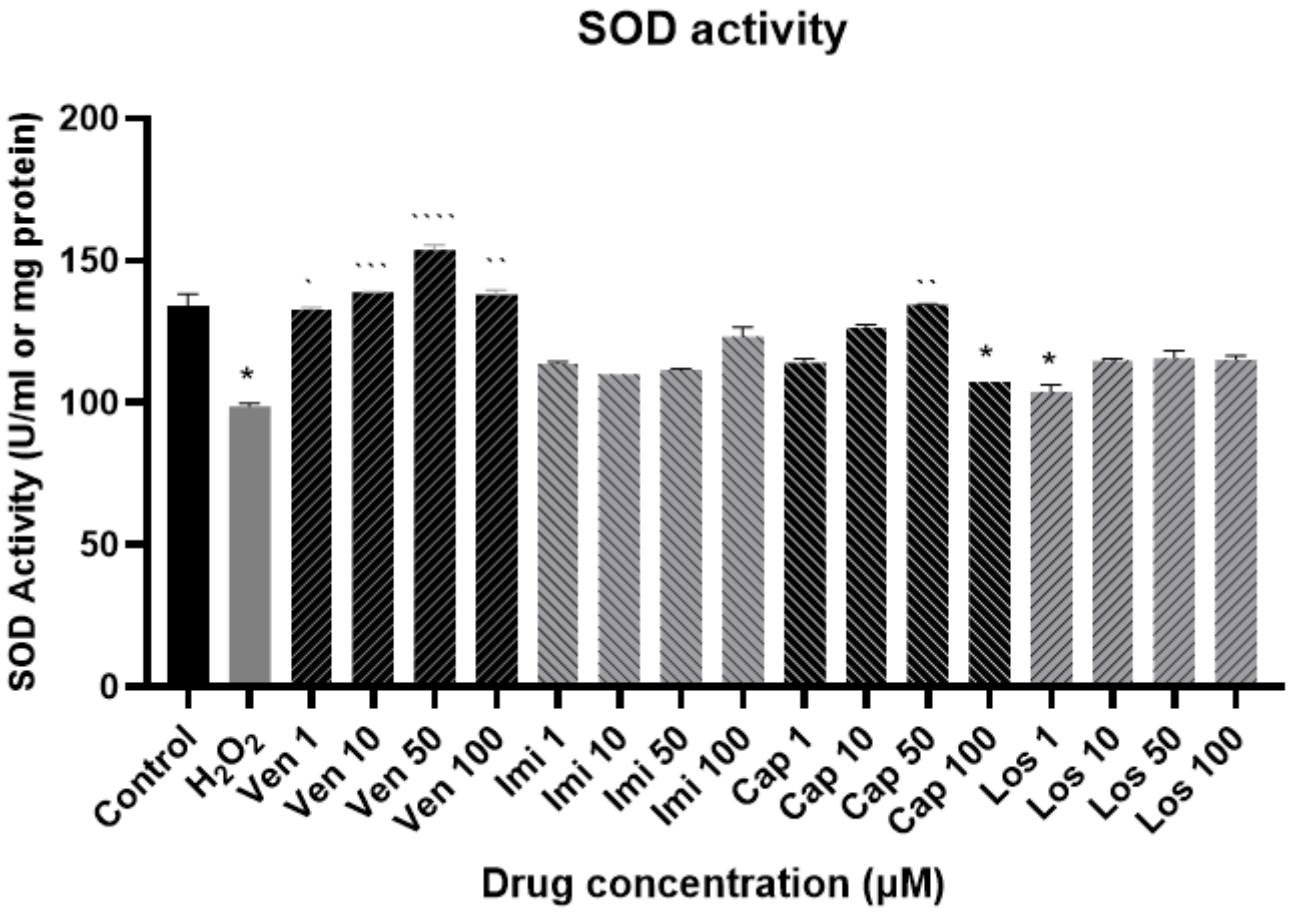

Figure 2

SOD activity of the H_2O_2-treated Neuro-2a cells after drugs treatment in the different groups.

Ven: Venlafaxine, Cap: Captopril, Imi: Imipramine, Los: Losartan

*p<0.05 vs. control; $p < 0.05$, $p < 0.005$, p<0.001 & p<0.0001 vs. H_2O_2

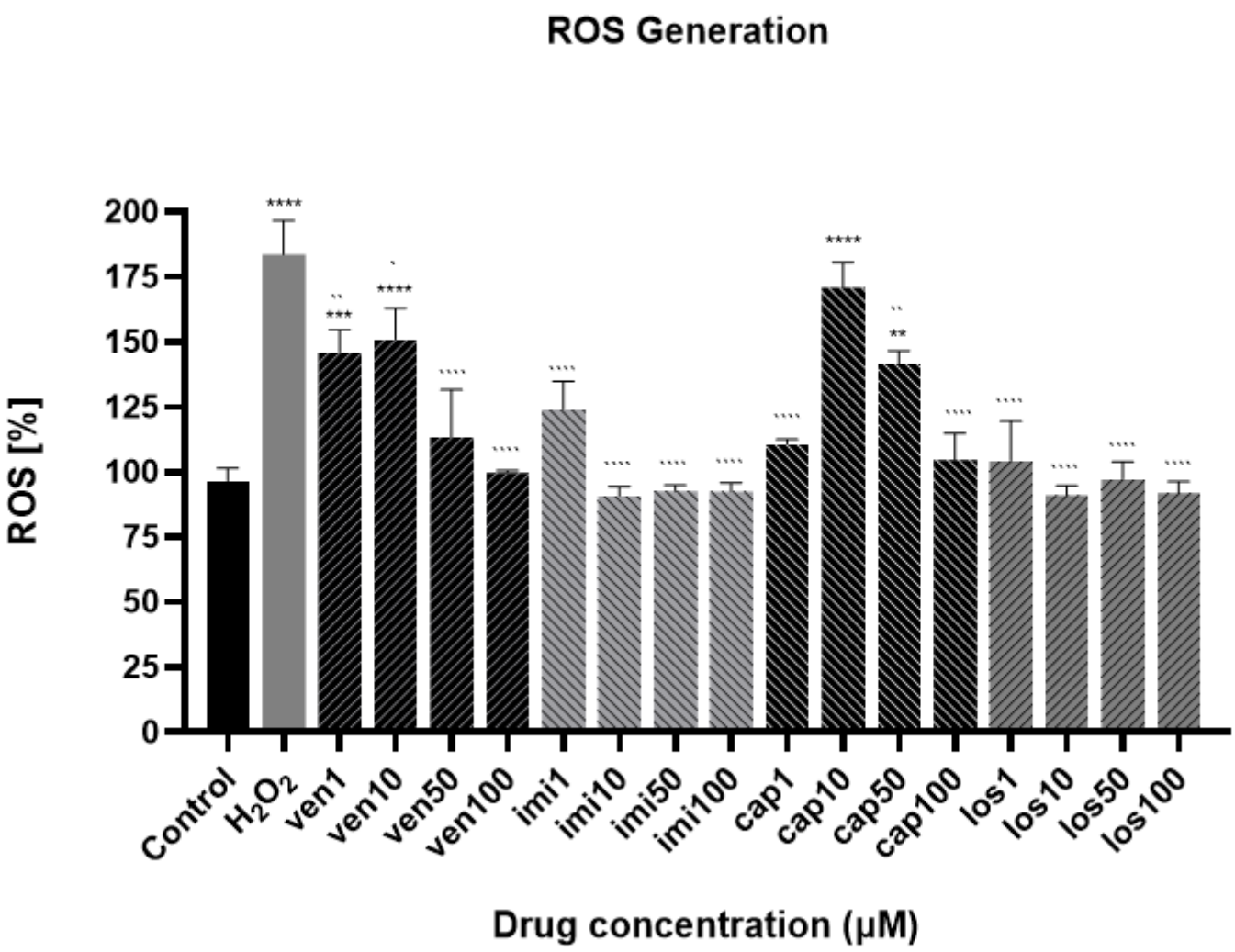

Figure 3

Percentage of ROS generation of the H_2O_2-treated Neuro-2a cells after drugs treatment in the different groups.

Ven: Venlafaxine, Cap: Captopril, Imi: Imipramine, Los: Losartan

**p<0.005, ***p<0.001 & ****p<0.0001 vs. control; $p < 0.05$, $p < 0.005$&`p<0.0001 vs. H_2O_2

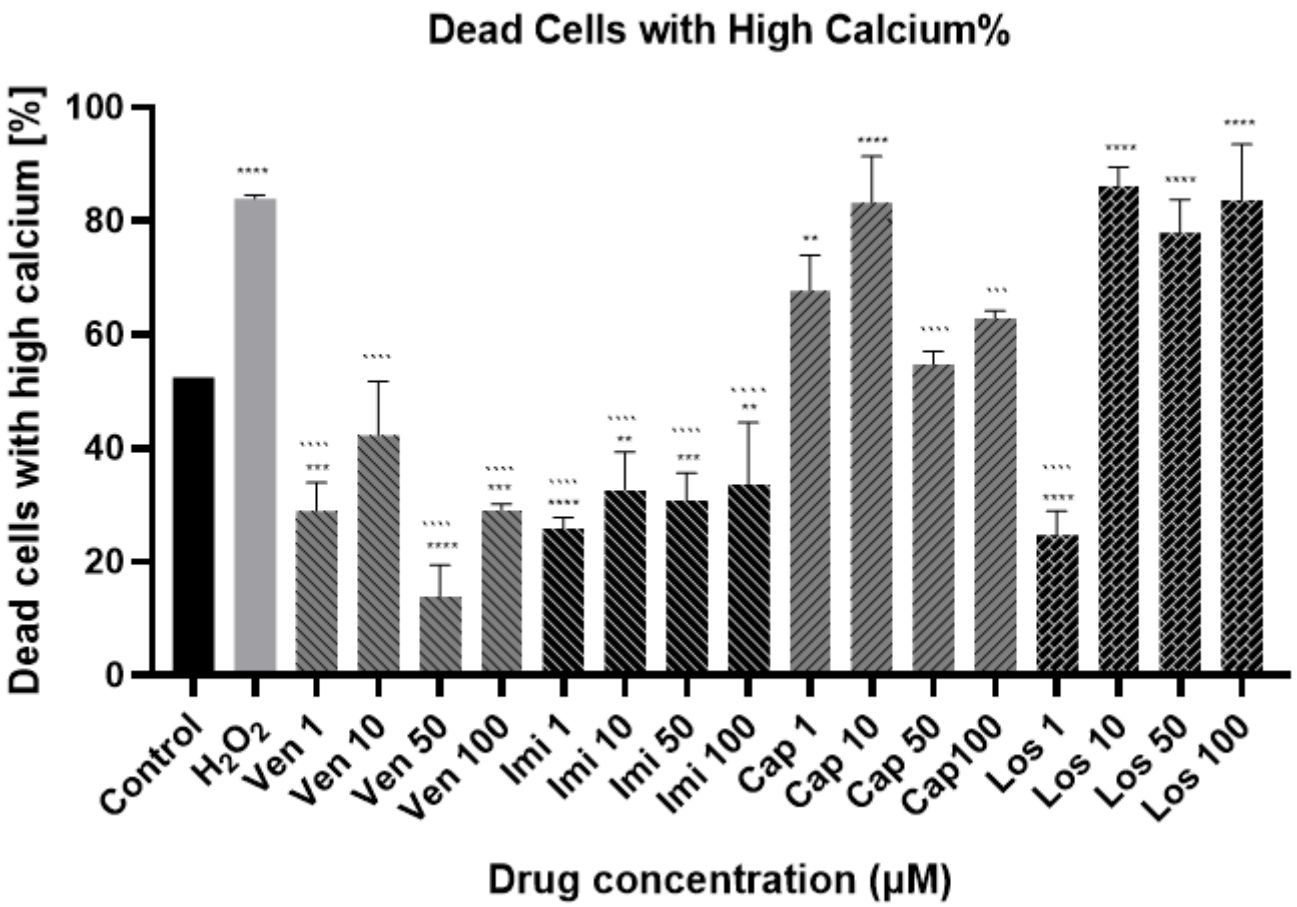

Figure 4

Percentage of dead cells with high intracellular calcium in Neuro-2a cells treated with H_2O_2 after drugs treatment in the different groups.

Ven: Venlafaxine, Cap: Captopril, Imi: Imipramine, Los: Losartan

*p<0.05, ***p<0.001 & ****p<0.0001 vs. control; $p < 0.05$, p<0.001 & p<0.0001 vs. H_2O_2