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Fasudil attenuated 6-OHDA cytotoxicity in PC12 cells through inhibition of JAK/STAT and apoptosis pathways

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Running title: The effect of fasudil in 6-OHDA induced cytotoxicity

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Abstract

Background: 6-Hydroxydopamine (6-OHDA) is widely used to induce neurotoxicity and investigate the mechanisms of Parkinson disease. 6-OHDA causes cell injury through various mechanisms including oxidative stress, inflammation and apoptosis. The selective Rho-kinase inhibitor, fasudil displays neuroprotective effects in several neurodegenerative disorders. The aim of this study was to assess the protective effect of fasudil in PC12 cytotoxicity induced by 6-OHDA.

Methods: PC12 cells were exposed to 5, 10, 25, and 50 µM of fasudil concentrations. After 24 h, the IC_{50} value of 6-OHDA (150 µM) was added. Twenty-four hours later, the viability of cells was evaluated via MTT assay and the formation of reactive oxygen species (ROS) was measured by the fluorimetric method. At the 50 µM concentration of fasudil, with or without 6-OHDA, the changes of protein levels including STAT3, p-STAT3, JAK2, p-JAK2, and caspase-3 were determined via western blotting.

Results: Our results showed that 6-OHDA increased the intracellular level of ROS, reduced cell viability, upregulated p-STAT3/STAT3 and p-JAK2/JAK2 ratios and significantly raised cleaved caspase-3 in comparison to control group. Furthermore, pretreatment of cells with fasudil (50 µM) for 24 h could reverse all changes induced by 6-OHDA.

Conclusion: 6-OHDA caused cytotoxicity in PC12 cells through inducing of oxidative stress and activating of JAK/STAT and apoptosis pathways, while pretreatment with fasudil exhibited protective effect on 6-OHDA-induced neurotoxicity via the inhibition of oxidative stress and prevention of these pathways.

Keywords: Apoptosis, Fasudil, 6-Hydroxydopamine, JAK/STAT, Neurotoxicity, Oxidative stress.

1. Introduction

6-hydroxydopamine (6-OHDA), as a neurotoxin agent, is commonly utilized to develop Parkinson disease (PD) in experimental models. It has a similar structure to dopamine and norepinephrine and enters to the neurons through catecholamine receptors of cytoplasmic membrane.[1] In dopaminergic neurons, 6-OHDA produces free radicals, induces oxidative stress and finally causes inflammation via disturbance of dopaminergic pathway.[2] Additionally, the aggregation of this compound in mitochondria can induce the inhibition of electron transport chain and the loss of mitochondrial membrane potential, resulting mitochondrial dysfunction and nerve cells death.[3,4] Moreover, free radicals produced by 6-OHDA damage DNA, cell skeletal structure and macromolecules like lipids and proteins, which leads to cell death and apoptosis of neurons.[5] Thus, the formation of free radicals as well as reactive oxygen species (ROS), mitochondrial dysfunction and apoptosis are the main mechanisms of 6-OHDA neurotoxicity.

JAK/STAT is the one of cell signaling pathways that involves in several biological processes such as cell proliferation, hematopoiesis, immune system regulation and apoptosis.[6] In CNS, this pathway mainly correlates to gene regulation during evolution, hormone secretion, inflammation or tumorigenesis.[7] It is reported that JAK/STAT pathway has a pathological importance in neuroinflammation diseases like PD and leads to active innate and adaptive immune responses in these disorders.[8] Therefore, the regulation of JAK/STAT pathway provides a suitable treatment option for Parkinson's patients.[8]

Fasudil, as a Rho-Kinase (ROCK) inhibitor, is a synthetic isoquinoline sulfonamide derivative that has an extensive range of pharmacological effects such as vasodilation and the protection of liver, kidney and brain.[9-12] It is shown that fasudil exerts its beneficial effects in various tissue damage including ischemic neuronal damage,[13] acute kidney injury,[14] myocardial infarction,[15] and diabetic peripheral neuropathy[16] through the regulation of different signaling pathways such as inflammation, oxidative stress and apoptosis. Moreover, in a mouse model of PD, fasudil protected dopaminergic neurons against MPTP-induced degeneration.[17]

Therefore, the aim of this research was to investigate whether fasudil had a protective effect against 6-OHDA induced cytotoxicity in PC12 cells and whether this effect is applied through JAK/STAT and apoptosis pathways.

2. Materials and methods

2.1. Chemicals and reagents

Monohydrochloride fasudil was purchased from the Santa Cruze Biotechnology Company, USA. 6-OHDA, MTT reagent, DCF and penicillin-streptomycin (PS) were bought from Sigma (Germany). RPMI1640 medium was made a purchase from Bioidea Company (Iran). Dimethyl sulfoxide (DMSO) from Merck (Germany) and fetal bovine serum (FBS) from Gibco (USA) were obtained. Moreover, all of used antibodies were taken from Cell Signaling Company (USA).

2.2. Cell Culture

PC12, derived from rat pheochromocytoma cell line, was obtained from Pasteur Institute (Tehran, Iran). Complete culture media containing RPMI 1640, 10% heat-inactivated FBS, and 1% penicillin-streptomycin was used to cultivate the cells. Then, cells were maintained in 37 ºC incubator accompanied by 95% humidified atmosphere and 5% CO_2. The cells were passage at the confluence of 80%.[18]

2.3. MTT assay

To evaluate the cell viability by MTT assay and calculate the IC_{50} value (the inhibitory concentration of 50%), PC12 cells (4000 cells/well) were planted in microplate for 24 h. Then, the cells were exposed to various concentration of 6-OHDA (50, 100, 150, 200, 300 µM) and fasudil (5, 10, 25, 50, 100 µM) for 24 h and 48 h, respectively. When the exposure time is finished, the final concentration of MTT solution (0.5 mg/mL) was added to each well and the cells were incubated for 3 h at 37 ºC. The upper medium was then removed from each well, and DMSO (150 µl) was added to dissolve the purple formazan product. A microplate reader (Start Fax-2100, UK) was used to read the absorbance at 545 and 630 nm. Then, the proportion of viable cells and the IC_{50} value were calculated.[18]

In order to assess the fasudil effect on 6-OHDA-induced cytotoxicity, the various concentrations of fasudil (5, 10, 25, 50, 100 µM) were exposed to PC12 cells in 96-well microplate for 24 h and then 6-OHDA (150 µM; the concentration which determined based on the concentration assay of IC_{50}) was added. After 24 h, MTT solution (0.5 mg/mL) was added to each well and the cells were incubated for 3 h at 37 ºC. The upper medium was then removed from each well, and DMSO (150 µl) was added to dissolve the purple formazan product and the absorbance was read at 545 and 630 nm. The cell viability was represented as a percentage of the value in control cultures.

Measurement of intracellular ROS production

DCFH-DA reagent was applied to evaluate the amount of ROS. In this line, PC12 cells (4000 cells/well) were seeded in 96-well microplate for 24 h. At first, the cells were incubated by fasudil (5, 10, 25, 50, 100 μM) before exposed to 6-OHDA (150 μM) for 24 h. After removing the upper mediate, the final concentration of DCFH-DA (10 μM) was added to the cultured cells for 30 min in a darkness at 37 ºC in an incubator. After that, cells were washed with PBS and the fluorescence intensity of DCF was read at 485 and 538 nm for the excitation and emission wavelength, respectively by a microplate-reader.[19]

2.4. Western blot analysis

This test was used to investigate the changes in the protein levels including JAK2, P-JAK2, STAT3, P-STAT3 and caspase-3 in PC12 cells treated by 6-OHDA and fasudil. For this purpose, PC12 cells were exposed to fasudil (50 μM) for 24 h before 6-OHDA (150 μM). After 24 h, the cells were separated with trypsin and washed by PBS buffer. After centrifuge and removing supernatant liquid, lysis buffer containing 2 mM EDTA, 2 mM EGTA, 50 mM Tris-HCl, 0.2% W/V sodium deoxycholate, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, along with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) were added to the cells. The concentration of proteins in samples were measured based on Bradford protein assay with some modification.[20]

Afterward, the separation of proteins were done by means of SDS-PAGE electrophoresis technique and transferred to a PVDF membrane. Blocking step was done on PVDF membranes applying by 5% skimmed milk or 5% BSA (Bovine Serum Albumin) for non-phosphorylated or phosphorylated proteins, respectively for 2 h at room temperature. Thereupon, membranes were incubated with appropriate primary rabbit monoclonal antibodies against caspase-3 (Cell Signaling, #9665), STAT3 (Cell Signaling, #12640), P-STAT3 (Cell Signaling, #9145), JAK2 (Cell Signaling, #3230), P-JAK2 (Cell Signaling, #3776) and mouse monoclonal antibody against β-actin (Cell Signaling, #3700). After membranes washing, they were incubated with anti-rabbit (Cell Signaling, #7074) or anti-mouse (Cell Signaling, #7076) IgG antibody labeled with horseradish peroxidase as secondary antibodies. To visualize the protein bands, enhanced chemiluminescence was used by Alliance 4.7 Gel doc (UK) and all of bonds were analyzed by UVtec software (UK). Then, the protein expression levels were normalized with their corresponding β-actin level.

2.5. Statistics analysis

All data analyzed by means of GraphPad Prism software (version 8). All results are exhibited as means ± SD. The comparison among different groups were done by one-way analysis of variance (ANOVA) and Tukey–Kramer post hoc statistical test. $P<0.05$ was considered as statistically significant levels.

3. Results

3.1. 6-OHDA effect in PC12 cell viability

The cells were treated 24 h by various concentrations of 6-OHDA (50, 100, 150, 200, 300 µM). The result of MTT assay showed that the exposure to all 6-OHDA concentrations decreased cell viability compared to control (Figure 1A). Additionally, the calculated IC_{50} for 6-OHDA after 24 h exposure was 150 ± 26.46 µM in PC12 cells.

3.2. Fasudil effect in PC12 cell viability

Figure 1B shows that the exposing PC12 cells to fasudil at doses of 5, 10, 25, and 50 µM for 24 h had no effect on cell viability as compared to untreated cells, while 100 µM concentration of fasudil could significantly reduce the viability of cells compared with control group ($P<0.01$).

3.3. Fasudil effect on 6-OHDA-induced cytotoxicity in PC12 cells

To investigate the fasudil protective effect on cytotoxicity by 6-OHDA, the cells were treated 24 h by various concentrations of fasudil (5-50 µM) before 24 h incubated by 6-OHDA (150 µM). Figure 1C shows that the 6-OHDA exposure resulted in cell viability reduction compared to control group, while the pretreatment of cells with 50 µM fasudil markedly elevated cell viability compared with 6-OHDA exposure group ($P<0.05$). The pretreatment of cells with other concentrations of fasudil had no significant effect on PC12 cell viability compared with 6-OHDA group (Figure 1C).

3.4. Fasudil effect on ROS production by 6-OHDA in PC12 cells

The result showed that after 24 h exposure to 6-OHDA (150 µM), the production of intracellular ROS increased in comparison to control ($P<0.001$). Remarkably, the fasudil pretreatment at 5, 10, 25 and 50 µM concentrations could reduce the intracellular ROS generation compared with 6-OHDA group (Figure 2).

3.5. Fasudil and 6-OHDA effects on JAK2 and STAT3 proteins levels in PC12 cells

The PC12 cells treatment by 6-OHDA enhanced P-JAK2/JAK2 and P-STAT3/STAT3 ratios in comparison to untreated cells ($P<0.05$ and $P<0.01$, respectively), whereas the fasudil pretreatment reduced these ratios as compared with 6-OHDA treated cells ($P<0.01$) (Figure 3).

3.6. Fasudil and 6-OHDA effects on caspase-3 protein level in PC12 cell

As shown in figure 4, the PC12 cells treatment with 6-OHDA markedly enhanced the levels of caspase-3 cleaved compared with control ($P<0.01$), while pretreatment of the cells with fasudil could decrease the cleaved caspase-3 level in PC12 cells compared to 6-OHDA group ($P<0.05$). In addition, the results demonstrated no significant differences between groups in the level of pro caspase-3.

4. Discussion

The purpose of this study was to evaluate the fasudil protective effect on 6-OHDA-induced cytotoxicity through JAK/STAT and apoptosis pathways. The results showed that 6-OHDA exposure reduced cell viability, induced oxidative stress and caused cell death through the enhancement of apoptosis and the phosphorylated form of JAK2 and STAT3 proteins in PC12 cells. The fasudil pretreatment reversed the 6-OHDA-induced alterations in oxidative stress, JAK/STAT and apoptosis proteins.

6-OHDA is one of the neurotoxins that induce neurotoxicity in diverse models of PD. Oxidative stress as well as apoptosis are considered as main mechanisms of neurotoxicity by 6-OHDA in both *in vitro* and *in vivo* models.[21-23] In this regard, 6-OHDA diminished cell viability, increased ROS and induced cell death via the enhancement of apoptosis proteins such as Bax and caspase-3 in PC12 cells.[22-24] The neurotoxicity induced by 6-OHDA attenuated by medicinal plants or synthetic compounds which have a potent antioxidant property.[22-24] For instance, carvacrol is a monoterpenic phenol and main constituents of many aromatic plants that could increase cell viability and reduce intracellular lipid peroxidation and ROS as well as apoptosis in PC12 cells.[25] Moreover, 6-OHDA induced mitochondrial dysfunction, ROS production, and p38 MAPK activation and augmented the level of cleaved caspase-9 and caspase-3 in SH-SY5Y cells.[26] Similar result of 6-OHDA neurotoxicity was reported by Hanrott et al.[27] in which 6-OHDA initiated mitochondrial dysfunction, oxidative stress and apoptosis though the caspases-3 and caspase-7 activating in PC12 cells.

Our results indicated that the exposure to 6-OHDA significantly reduced cell viability and raised ROS production in PC12 cells. In addition, 6-OHDA could induce apoptosis pathway through the cleaved caspase-3 elevation in PC12 cells. Furthermore, fasudil concentration was able to prevent cytotoxicity induced by 6-OHDA. The ROS production as well as caspase-3 expression were decreased following pretreatment with fasudil in PC12 cell. Kianfar et al.[18] reported the fasudil protective effect in acrylamide-induced toxicity in PC12 cells through the decreased ROS production and downregulation of apoptosis proteins such as Bax/Bcl2 ratio, caspase-3 and caspase-9. Zhang et al.[28] reported that fasudil attenuated myocardial infarction size and apoptosis in cardiomyocytes and diminished the p-JAK level in ischemia/reperfusion injury model of rat heart. Besides, fasudil exhibited the protective effect in ischemic neuronal damage by increasing ROCK activity and inhibition of cell death.[13] Also, Song et al.[29] demonstrated that fasudil could protect the rat retina against I/R injury through the reduction of Bax/Bcl-2 mRNA ratio, the caspase-3 level and inducible nitric oxide synthase (iNOS) expression. In addition, fasudil mesylate can be effectively improved oxidative stress-induced neuronal damage through the decrease of ROS accumulation as well as downregulation of Bax/Bcl-2 ratio in PC12 cells.[30]

JAK/STAT, as an important intracellular signaling pathway, has an essential role in different biological processes such as cell proliferation and differentiation, immune response, apoptosis and inflammation.[6,7] The role of JAK/STAT pathway has been reported in nueroinflammation diseases and several models of PD. For instance, the administration of AZD1480, a JAK1/JAK2 inhibitor, suppressed JAK, STAT1 and STAT3 activation in microglia and macrophages cells and prevented the degeneration of dopaminergic neurons in rats treated by α-synuclein as a model of PD.[31] Wang et al.[32] reported that the phosphorylation of JAK2 and STAT3 significantly elevated in LPS-treated microglia BV2 cells. Moreover, the neurotoxin MPP^+ treatment increased the expression level of STAT1 as well as STAT1 phosphorylation and induced apoptosis in cerebellar granule neurons cells.[33] In this regard, the type I interferons (IFN-α and IFN-β) treatment induced neurotoxicity in mouse primary neurons and SH-SY5Y neuroblastoma cells. In this study, the apoptotic cell death by cleavage of caspase-3, caspase-9, caspase-7 and cytochrome C release as well as STAT1 and STAT3 phosphorylation were considered as main mechanisms of IFNs. Interestingly, the effects of IFNs reversed by pyridone 6 as a JAK inhibitor.[34] In this matter, our results exhibited that in PC12 cells, the 6-OHDA exposure markedly enhanced the phosphorylation levels of JAK2 and STAT3.

It seems that there is an interplay between Rho-kinase and JAK/STAT signaling pathways. Loucks et al.[35] mentioned that JAK/STAT is a pro-apoptotic pathway, which regulates the downstream of Rho family in cerebellar granule neurons. Moreover, the activation of Rho family indirectly stimulates the STAT transcriptional activity as well as STAT3 phosphorylation in cytosolic and nuclear fractions through the enhancement of JAK2 phosphorylation in HEK293 cells.[36] Besides, Rho-kinase inhibitor could prevent STAT3 phosphorylation and activation.[36] Interestingly, Jaffe et al.[37] demonstrated that the RhoA activation by leptin was inhibited following the treatment of human colonic epithelial cell line with JAK2 inhibitor (AG490). Furthermore, it has been demonstrated that CPL409116, as a novel dual JAK/ROCK inhibitor, could block the STAT1 and STAT3 phosphorylation and reduce pro-inflammatory cytokines expression in systemic lupus erythematosus.[38] These evidence suggest that Rho-kinase pathway may be an effective regulator in the JAK2/STAT3 pathway activation and inhibition. In this regard, our results showed that the fasudil pretreatment declined the phosphorylation levels of JAK2 and STAT3 and decreased P-JAK2/JAK2 and P-STAT3/STAT3 ratios in PC12 cells treated with 6-OHDA.

5. Conclusion

In summary, present study exhibited that 6-OHDA induced cytotoxicity in PC12 cells via increased oxidative stress and the activation of JAK/STAT and apoptosis pathways. On the other hand, the Rho-kinase inhibitor, fasudil, displayed a neuroprotective effect against 6-OHDA in PC12 cells through the inhibition of oxidative stress and suppression of JAK/STAT and apoptosis pathways, which are important mechanisms of 6-OHDA neurotoxicity. However, more experimental and clinical studies are suggested to investigate the role of fasudil in people who are suffering from PD.

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8. Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

9. Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Seyyed Masoud Hosseini and Samira Barangi. The first draft of the manuscript was written by Samira Barangi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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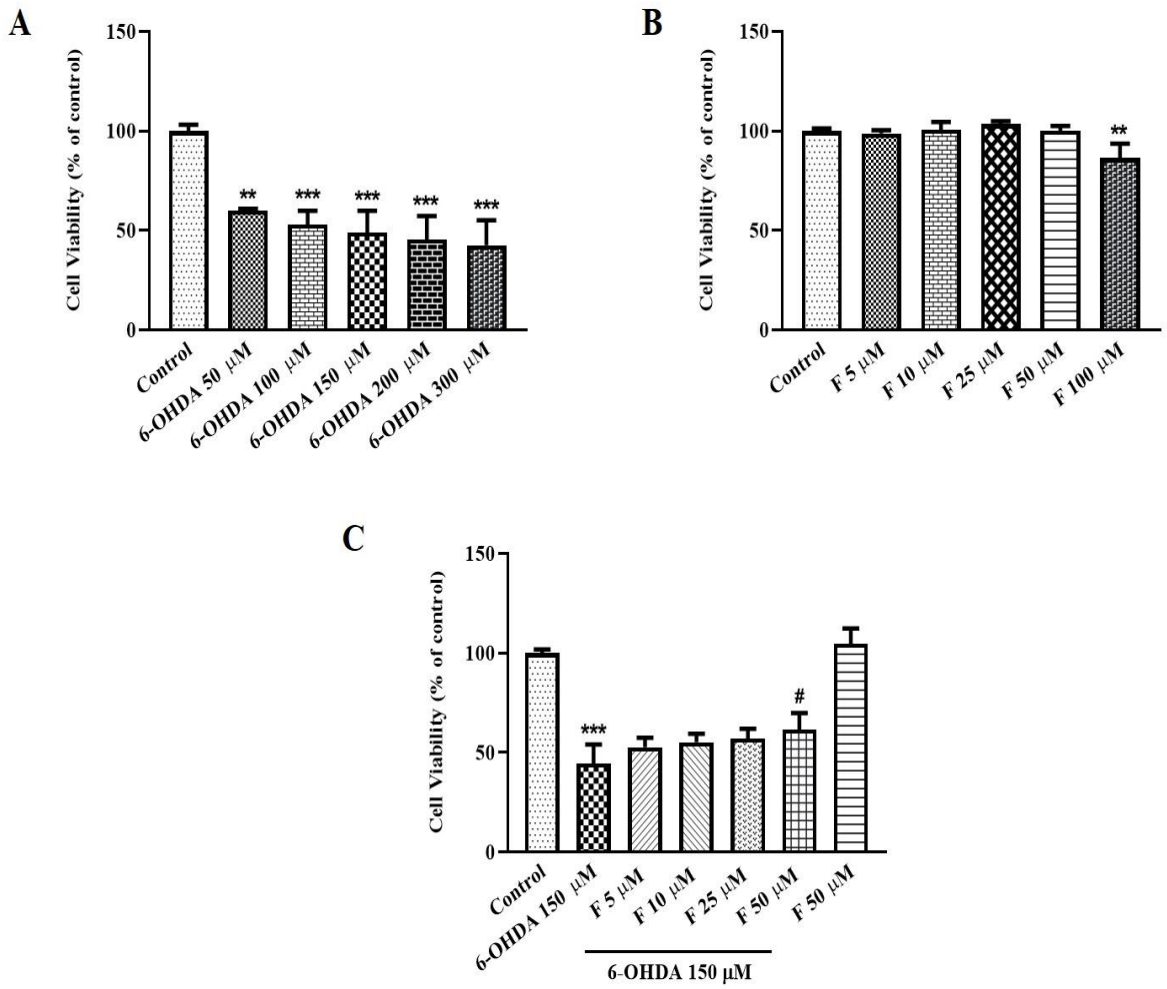

Fig. 1. The effect of 6-OHDA (50-300 µM; A), fasudil (5-100 µM; B), and the effect of fasudil (F) on 6-OHDA-induced cytotoxicity (C) on PC12 cells. Cell viability was evaluated by MTT test. Data are expressed as means ± SD from three independent experiments. Data were analyzed by one-way ANOVA following Tukey-kramer post test for multiple comparisons. $^{**}P<0.01$ and $^{***}P<0.001$ vs. control group, $^{\#}P<0.05$ vs. 6-OHDA treated group.

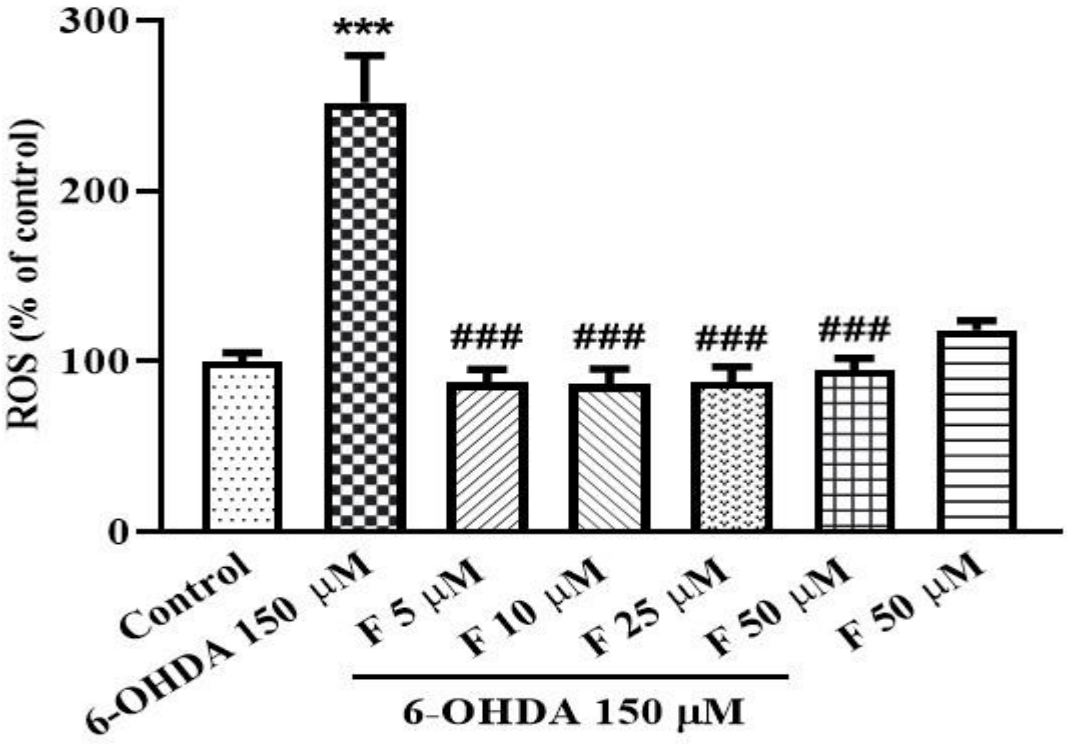

Fig. 2. The effect of fasudil (F) on 6-OHDA-induced ROS production in PC12 cells. ROS generation was determined using the DCFH-DA reagent. Data are expressed as means ± SD from three independent experiments. Data were analyzed by one-way ANOVA following Tukey-kramer post test for multiple comparisons. $^{***}P<0.001$ vs. control group, $^{###}P<0.001$ vs. 6-OHDA group.

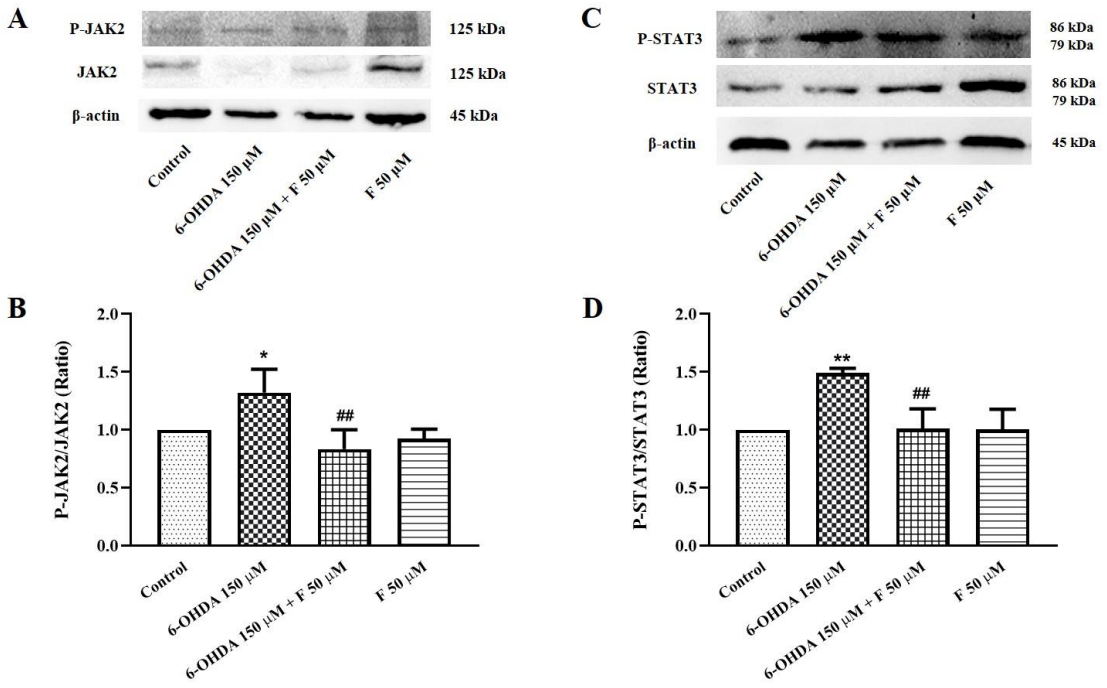

Fig. 3. The effect of fasudil (F) and 6-OHDA on protein level of JAK2, phosphor (P)-JAK2, STAT3 and phosphor (P)-STAT3 proteins in PC12 cells. The western blot bands (A and C), the bars (B and D) which exhibiting the densitometry analysis of blots for P-JAK2/JAK2 and P-STAT3/STAT3 ratio. Equal loading of proteins is illustrated by β-actin bands. Data are expressed as means ± SD from three independent experiments. Data were analyzed by one-way ANOVA following Tukey-kramer post test for multiple comparisons. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control group, $^{\#\#}P<0.01$ vs. 6-OHDA group.

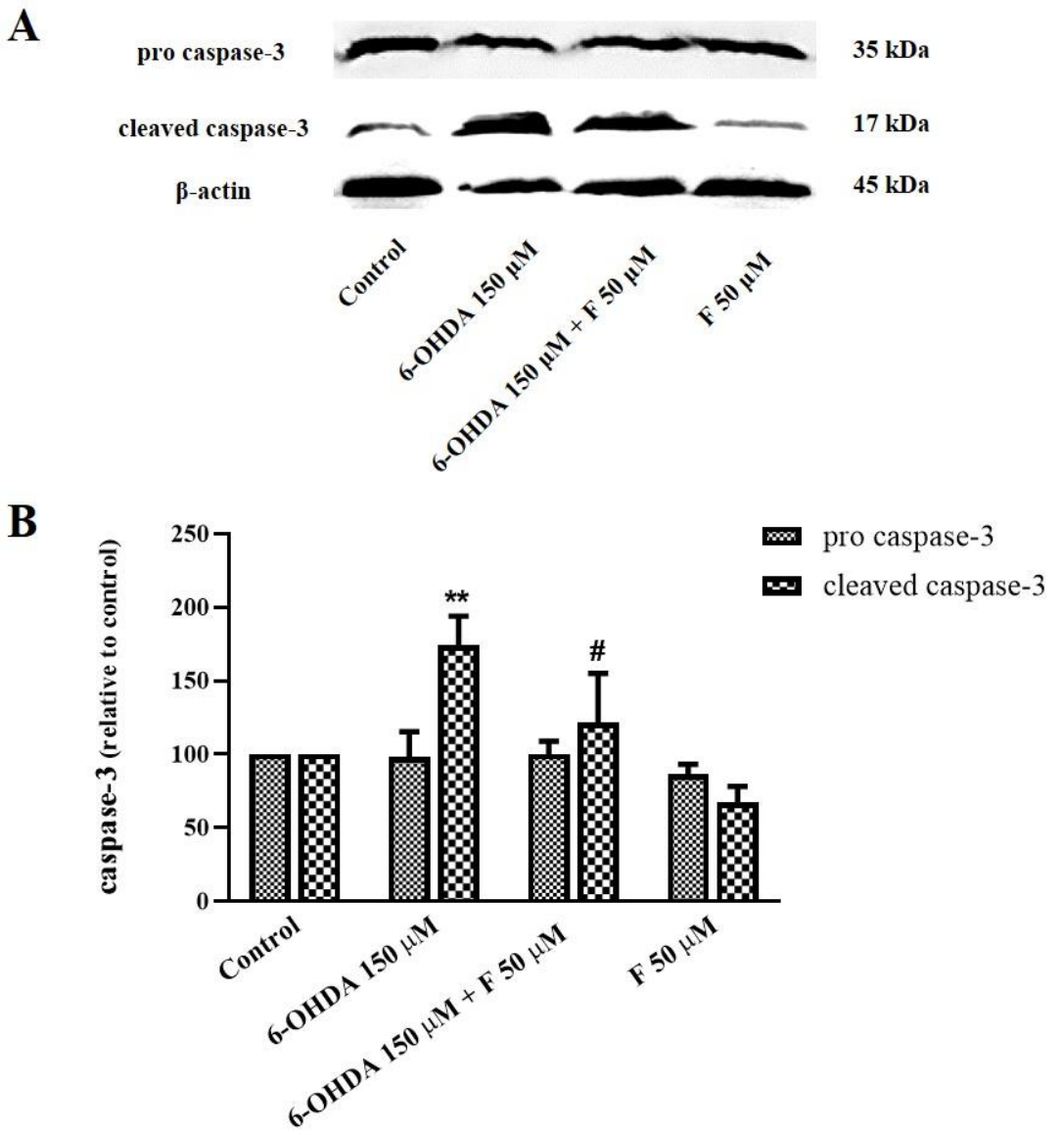

Fig. 4. The effect of fasudil (F) and 6-OHDA on protein level of caspase-3 (pro and cleaved) protein in PC12 cells. The western blot bands (A), the bars (B) which exhibiting the densitometry analysis of bands for the level of caspase-3 (pro and cleaved) protein. Equal loading of proteins is illustrated by β-actin bands. Data are expressed as means ± SD from three independent experiments. Data were analyzed by one-way ANOVA following Tukey-kramer post test for multiple comparisons. $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$ vs. 6-OHDA group.