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**Mitochondrial dysfunction mediated by quinone oxidation products of dopamine:
Implications in dopamine cytotoxicity and pathogenesis of Parkinson's disease.**

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Abstract

The study has demonstrated that dopamine induces membrane depolarisation and a loss of phosphorylation capacity in dose-dependent manner in isolated rat brain mitochondria during extended in vitro incubation and the phenomena are not prevented by oxyradical scavengers or metal chelators. Dopamine effects on brain mitochondria are, however, markedly prevented by reduced glutathione and N-acetyl cysteine and promoted by tyrosinase present in the incubation medium. The results imply that quinone oxidation products of dopamine are involved in mitochondrial damage under this condition. When PC12 cells are exposed to dopamine in varying concentrations (100-400 μ M) for up to 24 h, a pronounced impairment of mitochondrial bio-energetic functions at several levels is observed along with a significant (nearly 40%) loss of cell viability with features of apoptotic nuclear changes and increased activities of caspase 3 and caspase 9 and all these effects of dopamine are remarkably prevented by N-acetyl cysteine. N-acetyl cysteine also blocks nearly completely the dopamine induced increase in reactive oxygen species production and the formation of quinoprotein adducts in mitochondrial fraction within PC12 cells and also the accumulation of quinone products in the culture medium. Clorgyline, an inhibitor of MAO-A, markedly decreases the formation of reactive oxygen species in PC12 cells upon dopamine exposure but has only mild protective actions against quinoprotein adduct formation, mitochondrial dysfunctions, cell death and caspase activation induced by dopamine. The results have indicated that quinone oxidation products and not reactive oxygen species are primarily involved in cytotoxic effects of dopamine and the mitochondrial impairment plays a central role in the latter process. The data have clear implications in the pathogenesis of Parkinson's disease.

Research highlights:

- Dopamine inhibits mitochondrial oxidative phosphorylation.
- Mitochondrial dysfunction is mediated by dopamine derived quinones.
- In PC12 cells dopamine induces apoptosis through mitochondrial impairment.
- Quinone scavengers can have neuroprotective potential in Parkinson's disease.

Key Words:

Dopamine, Parkinson's disease, Mitochondria, Quinones, Apoptosis, Oxidative stress.

1. Introduction

The dopaminergic neuronal death in Parkinson's disease (PD) may involve a complex interplay of genetic and extra-genetic mechanisms but mitochondrial dysfunction and oxidative stress are thought to be crucial in this process as indicated from studies in experimental models and analysis of post mortem brain samples of PD patients [1-4]. Experimental models of PD have utilized both animals and cultured cells exposed to various toxins like MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), 6-hydroxy dopamine or rotenone to induce neuronal death in which oxidative stress, mitochondrial impairment and apoptosis have been implicated [5-9]. Although these models are useful, MPTP, rotenone and 6-hydroxy dopamine are not endogenously available and as such it is not clear whether the proposed mechanisms of actions of these toxins really represent the in vivo scenario in sporadic PD. On the other hand endogenously available dopamine (DA) in nigral neurons can undergo autoxidation or enzyme catalyzed oxidation to produce reactive oxygen species (ROS) and an array of toxic quinones and both these groups of compounds have been implicated in the pathogenesis of PD [10-12]. The toxic effects of DA which have been extensively studied on cultured cells of neural origin or on isolated brain mitochondria or in animal models are, therefore, of physiological significance and such systems serve as useful tools to understand PD pathogenesis [13-19]. Dopamine induced apoptosis has been consistently shown in some of these models and post-mortem studies have also demonstrated the features of apoptosis in PD brains and thus these models have been validated to an extent in explaining PD pathogenesis or searching for new therapeutic targets [20-23]. However, many uncertainties exist as to the mediators and mechanisms of DA induced cell death in such models. Apart from mitochondrial impairment, the inactivation of proteasomal activity or the activation of nuclear factor- κ B (NF- κ B) or various mitogen-activated protein kinase (MAPK) pathways like p38 family of kinases or c-Jun N-terminal kinases (JNK) has also

been implicated in DA-induced cytotoxicity and likewise the involvement of autophagic and necrotic death instead of apoptosis has been suggested in several studies [24-31]. The relative contributions ROS and toxic quinones in DA mediated cytotoxicity also remain unresolved, although this information may be critical in developing or screening neuroprotective drugs for PD using such model systems.

Although many studies have demonstrated the importance of ROS in DA cytotoxicity in experimental models, the antioxidants like vitamin C and vitamin E and the enzymes like SOD and catalase have variable protective effects in such systems and the use of antioxidants have met with near complete failure as neuroprotective agents in PD in clinical trials [22, 26, 32]. On the other hand the role of toxic quinones in DA induced neuronal damage has been studied in a relatively few studies, although these reactive nucleophiles have been shown to damage various brain sub-cellular components including mitochondria and inactivate different enzymes during *in vitro* incubation [12, 19, 33-36]. The present study has been undertaken to critically review the damaging effects of DA on isolated mitochondria with regard to the involvement of ROS and toxic quinones as an extension of our earlier published work and further to examine if similar mechanisms are also operative when intact rat pheochromocytoma (PC12) cells are exposed to DA. In addition the consequences of DA effects on mitochondrial bioenergetics in the context of cell death and apoptosis have been worked out and agents that can block these processes have also been tested. The results of this study are therefore relevant not only in understanding the etiopathogenesis of PD but also in finding suitable neuroprotective agents against this disease condition.

2. Material and methods

2.1. Animals

Young albino rats of 'Charles-Foster' strain (5-6 months old, body weight 120-150g) kept on commercially available laboratory chow and water ad libitum were used in this study. The animals were maintained and used as per the regulations and guidelines of the 'Animal Ethical Committee' of the Institute.

2.2. Isolation of rat brain mitochondria and incubation with DA

Rat brain mitochondria were isolated by a method based on differential centrifugation with digitonin treatment as published earlier [16, 19]. The final mitochondrial pellet was suspended in isotonic buffer A (145 mM KCl, 50 mM sucrose, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM MgCl₂, 10 mM phosphate buffer, pH 7.4) and incubated immediately with or without DA (100-400 μ M) in the presence or absence of catalase (50 μ g/ml) or mannitol (20 mM) or dimethyl sulfoxide (DMSO, 20 mM) or sodium benzoate (20 mM) or diethylene triamine pentaacetate (DTPA, 0.1 mM) or N-acetylcysteine (NAC, 2.5 mM) or reduced glutathione (GSH, 2.5 mM) for up to 2 h in a total volume of 400 μ l. At the end of the incubation the mitochondria were pelleted out and washed with an excess of isotonic buffer A and finally resuspended in the same buffer. An aliquot of resuspended mitochondria was then used immediately for the measurement of transmembrane potential or phosphate utilization. For some experiments, a short term incubation of mitochondria with DA with or without tyrosinase (250 units/ml, Sigma Aldrich, USA) and other additions was carried out.

2.3. Measurement of transmembrane potential of rat brain mitochondria after DA exposure

The transmembrane potential in isolated mitochondrial suspension was measured using the mitochondrial membrane potential sensitive carbocyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbo-cyanine iodide, Alexis Biochemicals, USA) as described previously [37]. This lipophilic and cationic dye accumulates within mitochondria driven by the negative transmembrane potential and forms concentration dependent J-

aggregates. When excited at 490 nm, the monomeric form of the dye emits at 527 nm while the J-aggregates shows maximum emission at 590 nm [37, 38]. Briefly, an aliquot of mitochondria (control or DA-treated or DA-treated in the presence of other additions) was incubated at 37°C for 30 min in isotonic buffer A containing 10 mM pyruvate, 10 mM succinate and 1mM ADP in the presence of 5 μ M JC-1. At the end of the incubation, the dye loaded mitochondria were collected by centrifugation, washed extensively with isotonic buffer A to remove the excess dye from outside and then resuspended in the same buffer in appropriate dilution, followed by the measurement of strong red fluorescence of J-aggregates (λ_{ex} 490nm, λ_{em} 590nm) in a spectrofluorometer (FP 6300, JASCO International Co., Japan).

2.4. Measurement of phosphorylation capacity of rat brain mitochondria after DA treatment

The mitochondrial phosphate utilization was assayed following the method published earlier [39, 40]. Briefly, in a total volume of 500 μ l in a medium containing 125 mM KCl, 75 mM sucrose, 0.1 mM EGTA, 1 mM MgCl₂, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM phosphate, 0.3% BSA (bovine serum albumin), 0.5 mM ADP, 5 mM pyruvate, 10 mM succinate and 10 mM glucose was added an aliquot of 50 μ l mitochondrial suspension (control or DA-treated or DA-treated in the presence of other additions) containing 100-150 μ g of protein, followed by the immediate addition of 5 units of hexokinase (Sigma Aldrich, USA) and the incubation was continued at 37°C for 30 min. The incubation was terminated by the addition of 5% ice cold TCA (trichloroacetic acid) and the amount of inorganic phosphate estimated spectrophotometrically using a reaction based on the reduction of phosphomolybdate as described earlier [34]. A '0' min sample was also assayed for inorganic phosphate content where hexokinase addition was immediately followed by the treatment with 5% ice-cold TCA. The difference in inorganic phosphate content between 0 min and 30 min samples was calculated to estimate the phosphorylation

capacity of brain mitochondria. Glucose and ADP in the reaction mixture were used as a trap for ATP to maintain the level of ADP in the system as also to prevent the release of free inorganic phosphate from ATP by the action of various phosphatases.

2.5. PC 12 cell culture and treatment paradigm

Rat pheochromocytoma (PC12) cells (National Centre for Cell Sciences, Pune, India) were grown in RPMI 1640 medium (Gibco, Invitrogen, USA) containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin and 2.5 µg/ml amphotericin B (Gibco, Invitrogen, USA) in a humidified environment containing 5% CO₂ and 95% air at 37°C. Cells were maintained and grown in 25-cm² sterile tissue-culture flasks or 12-well plates and the experiments were conducted with varying concentrations of DA (100-400 µM) for up to 24 h with or without other additions like NAC (2.5 mM), MAO-A inhibitor clorgyline (10 µM) or TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, 1 mM) in the culture medium. N-acetyl cysteine or clorgyline or TEMPOL was added 1 h prior to DA addition. For some experiments involving fluorescence microscopy, cells were harvested on poly-L-lysine (5 mg/ml) coated glass coverslips.

2.6. Preparation of mitochondria from cultured PC12 cells

Mitochondria were isolated from cultured PC12 cells following the method of Cui et al. with some modifications [41]. Control or treated cells were thoroughly washed in phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄ and the cell pellet was resuspended in ice-cold cell-lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris, 0.1 mM PMSF (phenylmethylsulfonyl fluoride)), kept on ice for 10min and then homogenized thoroughly followed by immediate addition of 1/3rd volume of mitochondria stabilization buffer (1M sucrose, 35 mM EDTA, 50 mM Tris). The homogenate

was then centrifuged at 2000 g for 5 min at 4°C and the supernatant collected and centrifuged at 12000 g for 10 min at 4°C to obtain the mitochondrial pellet. Mitochondrial pellet was washed once in isotonic buffer B (145 mM KCl, 50 mM sucrose, 5 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 10 mM phosphate, pH 7.4) and finally resuspended in either 50 mM phosphate buffer, pH 7.4 or in 100 mM phosphate buffer, pH 7.4 for further experimentation.

2.7. Measurement of ROS production in PC12 cells after DA exposure

Intracellular generation of ROS in PC12 cells was measured by using ROS sensitive cell permeable fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Alexis Biochemicals, USA). The dye was converted intracellularly to DCFH₂ which in the presence of ROS was oxidized to DCF (2',7'-dichlorofluorescein) a highly fluorescent compound with λ_{ex} 475 nm and λ_{em} 525 nm [42]. Cells were incubated with varying concentrations of DA (100-400 μ M) with or without other additions for 12-24 h. After the incubation the cells were thoroughly washed in pre-warmed PBS and loaded with H₂DCF-DA (10 μ M) for 30 min at 37°C in the dark in Krebs–Ringer's buffer (130 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM MgCl₂, 1 mM CaCl₂, 20 mM HEPES). The dye loaded cells were washed twice with Krebs–Ringer's buffer and used for the measurement of DCF-fluorescence.

The production of H₂O₂ following DA exposure was also monitored by H₂O₂ specific fluorescent dye Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine), a fluorogenic substrate with very low background fluorescence, that reacts with H₂O₂ with a 1:1 stoichiometry to produce highly fluorescent resorufin [43]. In our study, the control and experimental cells were thoroughly washed in PBS and incubated with 50 μ M amplex red reagent and 1 U/ml horseradish peroxidase in pre-warmed Krebs–Ringer's buffer for 30 min in the dark at room temperature. After the incubation with amplex red, cells were pelleted down by centrifugation, and the fluorescence intensity of the supernatant was measured in a JASCO

FP-6300 spectrofluorometer at an excitation wavelength of 560 nm and an emission wavelength of 590 nm with appropriate subtraction of the background fluorescence.

2.8. Measurement of mitochondrial quinoprotein adduct formation in PC12 cells

Quinoprotein formation was measured by the NBT (nitroblue tetrazolium)/glycinate assay in the TCA-precipitated and delipidated mitochondrial protein from PC12 cells following an earlier published method [19, 44]. The blue-purple colour developed in the reaction mixture was suitably diluted and measured spectrophotometrically at 520 nm.

2.9. Measurement of quinones and melanin by dopamine in cultured PC12 cells

Quinone formation was monitored in the culture media following exposure of PC12 cells to DA for 24h by absorbance change at 480 nm keeping the appropriate blank [19, 45]. Melanin, the end product of the autoxidation of dopamine, was also monitored spectrophotometrically at 405 nm in the same cultured media [45].

2.10. Assessment of mitochondrial electron transport chain (ETC) function in PC12 cells after DA exposure

NADH-ferricyanide reductase (Complex I) assay was carried out in a reaction mixture (1 ml) containing 0.17 mM NADH, 0.6 mM ferricyanide and 0.1% (v/v) triton X-100 in 50 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of mitochondrial suspension (10-30 μ g) in the sample cuvette and the decrease in absorbance at 340 nm noted as described earlier [19, 46]. The enzyme activity was expressed as n moles of NADH oxidized/min/mg protein.

The activity of complex II-III (succinate-cytochrome c reductase) was assayed by following the succinate supported reduction of ferricytochrome c to ferrocytochrome c at 550 nm [46]. The sample cuvette contained 100 mM phosphate buffer, 2 mM succinate, 1 mM KCN, 0.3 mM EDTA and 1.2 mg/ml cytochrome c in a total volume of 1 ml. The reference cuvette

contained all the reagents in the same concentration except cytochrome c. The reaction was initiated by adding mitochondrial suspension (10-30 μg) in the sample cuvette. The increase in absorbance at 550 nm was monitored for a period of 3 min. The same assay was repeated with antimycin A (10 μM) to determine the inhibitor sensitive rate. The enzyme activity was then calculated by subtracting the antimycin A sensitive rate from overall rate and expressed as μ moles oxidized cytochrome c reduced/min/mg protein [46].

The activity of complex IV (cytochrome c oxidase) was assayed by following the oxidation of reduced cytochrome c (ferrocyanochrome c) at 550 nm [19, 46]. Reduced cytochrome (50 μM) in 10 mM phosphate buffer, pH 7.4 was added in each of two 1 ml cuvette. In the blank cuvette ferricyanide (1 mM) was added to oxidize ferrocyanochrome c and the reaction is initiated in the sample cuvette by addition of mitochondrial protein (10-30 μg). The rate of increase of absorbance at 550 nm was measured at room temperatures. The activity was calculated from first order rate constant taking into account the concentration of reduced cytochrome-c in the cuvette and the amount of mitochondrial protein added [19, 46].

2.11. Measurement of mitochondrial transmembrane potential in cultured PC 12 cells after DA exposure

Mitochondrial transmembrane potential in intact PC12 cells was measured by using JC-1 [38]. The monomeric form of the dye remains distributed in the cytosol, whereas the dye accumulates within mitochondria depending on the negative mitochondrial membrane potential to form J-aggregates. When excited at 490 nm, the monomeric dye emits at 527 nm whereas the J-aggregates emit at 590 nm. The ratio of fluorescence intensities at 590 nm and 535 nm measured within intact cells is considered indicative of mitochondrial membrane potential [47]. For experimental purpose, control or treated cells were washed twice with pre-warmed PBS, re-suspended with 1 ml of serum free media containing JC-1 (final concentration, 10 μM) and incubated for 30 min at 37°C in the dark. The dye-loaded cells

were washed twice with pre-warmed serum free media to remove the excess dye and then suspended in the same media in appropriate dilution, followed by the measurement of fluorescence intensities (λ_{ex} 490 nm, λ_{em} 535 nm and 590 nm) in a JASCO, FP 6300 spectrofluorometer.

2.12. Measurement of intracellular ATP concentration in cultured PC12 cells

The intracellular ATP content was measured in control and DA treated PC12 cells by using a commercial kit based on luciferin-luciferase reaction for ATP measurement (Sigma Aldrich, USA). Briefly, following incubation with DA for upto 24h, the treated and untreated control cells were washed with PBS to remove culture media and aliquots (100 μ l) of resuspended cells were disrupted by an ice-cold lysis-buffer containing 10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, pH 7.6 and then immediately used for ATP measurement using the ATP-assay mix provided by the kit [40]. The luminescence generated thereafter was measured immediately in a microplate luminometer (Biotek, ELX -800, USA). Appropriate blank reactions consisting of ATP-assay mix or lysis buffer without cells were run to measure background luminescence. The intracellular content of ATP was calculated from an ATP-standard curve generated by plotting the luminescence readings against known concentrations of ATP solutions provided in the kit and the values were expressed as n mol of ATP/mg of protein [40].

2.13. Cell viability assay by trypan blue

An equal volume of trypan blue solution (0.4% in PBS) was added to the cell suspension for 5 min and the number of stained and unstained cells was counted. Typically, 250-300 cells were counted in each group with a Neubauer counting chamber by an observer blind to experimental history [47]. The cell death was expressed as the percentage of trypan blue positive cells in the total population of both stained and unstained cells.

2.14. Assessment of nuclear morphology of PC12 cells by Hoechst 33258 staining

For morphological assessment of apoptosis, a nuclear-chromatin binding cell permeable dye Hoechst 33258 (2-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5-bis-1H-benzimidazole trihydrochloride pentahydrate, Sigma Aldrich, USA) was used [48]. In brief, PC12 cells were grown in the absence or presence of DA (400 μ M) for 24 h on poly-L-lysine coated glass coverslips. The cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min and then exposed to 1 μ g/ml Hoechst 33258 dye for 30 min at 37°C. The cells were then rinsed twice with PBS, mounted on thin microscope slides with mounting media containing 50% glycerol and 50% PBS and visualized under a fluorescence microscope (Carl Zeiss, Germany).

2.15. Measurement of caspase 3 and caspase 9 activity in PC12 cells

The caspase 3 activity in control and DA treated PC12 cells was measured using a commercial kit (Promega, USA) which utilized a luminogenic caspase-3 substrate (DEVD-aminoluciferin) and luciferase. The cells were washed with PBS and aliquots (100 μ l) of resuspended cells were taken in 96 well plates. Equal volume of freshly prepared caspase 3 reagent containing caspase 3 assay buffer and luminogenic caspase 3 substrate were added to each well, mixed briefly by orbital shaking and incubated at 37°C for 45 min in the dark. The luminescence was measured in a microplate luminometer (Biotek, ELX -800, USA), keeping appropriate blanks. Caspase 3 activity was expressed as relative luminescence units/mg of protein.

The caspase 9 activity was also measured in a similar way using a commercial kit (Caspase-Glo® 9 Assay kit, Promega, USA) with a luminogenic caspase-9 substrate (LEHD-aminoluciferin).

2.16. Protein estimation

The protein was estimated after solubilizing the membranes in 1% SDS by a modification of Lowry's method [49].

3. Results

3.1. Alteration of mitochondrial transmembrane potential and phosphorylation capacity by DA during extended incubation

Results presented in Fig. 1A show a dose-dependent loss of brain mitochondrial membrane potential as indicated by the decrease in mitochondrial membrane potential sensitive JC-1 fluorescence following an extended incubation with DA (100-400 μ M) for 2 h with nearly 65% decrease in fluorescence with 400 μ M of DA. Further, DA induced loss of rat brain mitochondrial membrane potential was not prevented by hydroxyl radical scavengers like mannitol (20 mM) or DMSO (20 mM) or the antioxidant enzyme catalase (50 μ g/ml) or the metal chelator DTPA (Fig. 1B). On the other hand, thiol compounds like GSH (2.5 mM) and NAC (2.5 mM) prevented nearly completely the DA-mediated loss of mitochondrial membrane potential (Fig. 1B). Mannitol, DMSO, DTPA, catalase, GSH and NAC individually at concentrations used in this study did not have any detrimental effect on mitochondrial membrane potential in the absence of DA (results not shown).

The phosphorylation capacity of rat brain mitochondria as measured by inorganic phosphate utilization was dramatically reduced after incubation for 2 h with DA (100-400 μ M) in a dose-dependent manner. In the presence of 400 μ M DA, phosphorylation capacity of mitochondria after incubation for 2 h was reduced by more than 90 percent (Fig. 2A). Such DA - mediated loss of mitochondrial phosphorylation capacity was not prevented by radical scavengers like mannitol (20 mM) or benzoate (20 mM) or the antioxidant enzyme catalase (50 μ g/ml) or the metal-chelator DTPA (0.1 mM) as presented in Fig. 2B. However, GSH (2.5

mM) or NAC (2.5 mM) provided very significant (~ 80%) protection against DA effect on mitochondrial phosphorylation capacity. Catalase, DTPA, mannitol, benzoate, GSH or NAC when added individually in the absence of DA did not affect mitochondrial phosphorylation but DMSO (20mM) had a distinct inhibitory effect (results not shown) which prompted us to use benzoate as a radical scavenger in stead of DMSO for this set of assays.

3.2. Effect of DA on mitochondrial membrane potential and mitochondrial phosphorylation capacity during short term incubation

When rat brain mitochondria were incubated with DA (400 μ M) for a brief period (up to 15 min), no significant decrease in transmembrane potential was noticed, while phosphorylation capacity was diminished marginally (~10%, Fig. 3). However, when tyrosinase (250 units/ml) was added in the incubation mixture containing brain mitochondria and DA, a striking loss of mitochondrial transmembrane potential and phosphorylation capacity was observed within 15 min and these effects were also prevented nearly completely by GSH (2.5 mM) present in the incubation mixture (Fig. 3). Tyrosinase alone showed no detrimental effect on mitochondrial membrane potential or on mitochondrial phosphorylation in the absence of DA (data not shown).

3.3 ROS production in PC12 cells following DA exposure

When PC12 cells were exposed to varying concentrations of DA (100-400 μ M), a dose-dependent increase in ROS production was noticed at 24 h as measured by using the ROS-reacting fluorescent dye DCF-DA (Fig. 4A). The increase in ROS production was also observed, but to a lesser extent, at 12 h after DA exposure (data not shown). N-acetylcysteine (2.5 mM) or MAO-A inhibitor clorgyline (10 μ M) prevented DA induced ROS production by nearly 85% and 75% respectively (Fig. 4A). However, co-incubation with cell-

permeable radical scavenger TEMPOL (1 mM) provided only a marginal protection against DA induced ROS production (Fig. 4A).

Using the amplex red method for the measurement of H_2O_2 , similar results were obtained with a significant increase in H_2O_2 production by PC12 cells at 24 h after DA exposure which was very significantly and nearly to the same extent inhibited by co-incubation either with NAC (2.5 mM) or clorgyline (10 μ M) (data not shown).

3.4. *Quinones and quinoprotein adducts during DA exposure of PC 12 cells*

When PC12 cells were incubated with DA (400 μ M) for 24 h in RPMI medium, oxidation products of DA like quinones and melanin accumulated in the medium and the process was nearly completely prevented by quinone scavengers like NAC and GSH (Table 1). Under similar conditions of incubation DA caused a significant increase in quinoprotein adduct formation in mitochondrial fraction after 24 h treatment in a dose-dependent manner. N-acetyl cysteine (2.5 mM) almost completely (~85%) prevented DA induced quinoprotein adduct formation while GSH (2.5mM) only partially (~40%) prevented the latter process (Fig. 4B). Surprisingly, clorgyline (10 μ M) also prevented the DA mediated mitochondrial quinoprotein adduct formation in PC12 cells marginally (~15%), but TEMPOL did not show any effect in this regard (Fig. 4B).

3.5. *Effect of DA on mitochondrial bioenergetics in PC12 cells*

Following 24 h of DA exposure, a significant dose-dependent (100-400 μ M) decrease in the activities of mitochondrial respiratory chain complexes (complex I, complex II-III and complex IV) was noticed (Fig. 5A, B and C). The extent of inhibition of complex I (67%) and complex IV (65%) by DA (400 μ M) was found to be reasonably similar while complex II-III was much less affected by DA treatment (Fig. 5). The extent of DA (400 μ M) effect on mitochondrial ETC complexes was also time-dependent upto 24 h (data not shown). The thiol

antioxidant NAC (2.5 mM) provided a very significant protection against DA induced inhibition of mitochondrial respiratory complexes blocking the DA effects on complex I, complex II-III and complex IV by approximately 80%, 75% and 85% respectively (Fig. 5). However, MAO-A inhibitor clorgyline (10 μ M) was only partially effective in preventing the effects of DA on respiratory complexes in PC12 cells (Fig. 5).

Following 24 h of exposure to DA (100-400 μ M) a significant dose-dependent decrease in mitochondrial transmembrane potential was also observed in PC12 cells as indicated by the decrease in the ratio of JC-1 emission fluorescence at 590 nm and at 527 nm (Fig. 6A). N-acetyl cysteine provided a very significant protection (90%) against DA induced mitochondrial membrane depolarization but clorgyline was only partially effective (~26%) in this respect (Fig. 6A). When added in the absence of DA in the culture media with PC12 cells, NAC or clorgyline did not show any noticeable effect on mitochondrial complex activities and mitochondrial transmembrane potential after 24 h of incubation (results not shown). The impairment of mitochondrial oxidative phosphorylation process following 12 h or 24 h exposure was also evident from a decreased basal ATP content in PC12 cells (Fig. 6B).

3.6. Effect of DA exposure on PC12 cell viability: apoptosis and caspase activation

The exposure of PC12 cells to DA (100-400 μ M) for 24 h led to a significant loss of cell viability in a dose-dependent manner (Fig. 7A). Cell death occurred to the extent of nearly 40% after 24 h exposure to 400 μ M of DA (Fig. 7A). Concurrent incubation with thiol-antioxidant NAC (2.5 mM) or MAO-A inhibitor clorgyline (10 μ M) resulted in nearly 80% and 28% protection respectively against DA induced cell death (Fig. 7B). N-acetyl cysteine or clorgyline when added in the absence of DA in the culture media with PC12 cells for 24 h did not show detrimental effect on the viability of PC12 cells (results not shown).

Following DA (400 μ M) treatment for 24 h, typical apoptotic nuclear morphology e.g. condensed and fragmented nuclei with several discrete chromatin bodies, appeared in PC12 cells with respect to the untreated control cells (Fig. 8A and B). The activities of caspase 3 and caspase 9 were also evaluated in DA-treated PC12 cells to further substantiate the involvement of apoptosis in cell death. A dose-dependent increase in caspase 3 and caspase 9 activities was noticed in PC12 cells after 24 h of DA exposure (Fig. 9A and B). The caspase-3 activity was increased by \sim 3.4 folds and caspase-9 activity by \sim 2.8 folds at 24 h after DA (400 μ M) treatment. Concurrent presence of NAC (2.5mM) in the medium markedly prevented DA induced activation of caspase 3 and caspase 9, while clorgyline (10 μ M) was only partially effective in this respect (Fig. 9A and B).

Discussion:

4.1. DA impairs oxidative phosphorylation in isolated rat brain mitochondria

The effects of DA on isolated mitochondria have been widely reported indicating alterations in mitochondrial respiration, activities of ETC complexes, mitochondrial membrane potential and activation state of permeability transition pore [16, 18, 45, 50-52]. Most studies have used a short-term exposure of mitochondria to DA (5-15 min) followed by the measurement of mitochondrial functional parameters but the results have been somewhat inconsistent. A significant inhibition of mitochondrial state III respiration by DA (100 μ M) has been reported by several groups while others have failed to observe such a change at 70 μ M DA concentration [16, 18, 53]. Calcium induced mitochondrial membrane swelling indicative of opening of permeability transition pore has been variously reported to be increased, decreased or unaltered by DA from various groups under comparable incubation conditions [16, 45, 51, 53]. Likewise, DA has been reported to produce a substantial loss of

mitochondrial membrane potential or to have no effect during short-term incubation [45, 51, 53]. Further, the effects of DA on mitochondrial respiratory chain activity after short-term exposure as measured by direct enzyme assays in lysed mitochondrial preparation have been equivocal in several different studies [50, 52-54]. Apart from such inconsistencies, a short-term exposure is clearly unsuitable for identification of the toxic actions of quinones on mitochondria, since quinone formation from DA during *in vitro* incubation is very slow. Our earlier study, however, has clearly demonstrated that during extended incubation (2 h) DA produces a very significant inhibition of mitochondrial ETC complexes in a dose-dependent manner which is mediated by toxic quinones instead of ROS [19]. In the present study we have observed that DA further causes mitochondrial membrane depolarisation and loss of phosphorylation capacity during extended *in vitro* incubation in a dose-dependent manner which are not prevented by radical-scavengers, catalase or metal-chelators indicating that ROS are not involved in this process (Fig. 1 and 2). On the other hand, GSH and NAC prevent DA effects nearly completely presumably through their quinone scavenging functions (Fig. 1 and 2). The involvement of toxic quinones in DA induced mitochondrial membrane depolarization and inhibition of phosphorylation capacity has been confirmed from our short-term experiments with tyrosinase which rapidly converts DA to quinone products without producing ROS. Tyrosinase causes a dramatic potentiation of DA effects on mitochondria during 15 min exposure (Fig. 3). The present results are, therefore, in complete agreement with our earlier published data where quinone-dependent inhibition of brain mitochondrial ETC activity is demonstrated during extended incubation [19, 55]. It is likely that DA induced ETC inhibition is the primary event that leads to loss of mitochondrial membrane potential with consequent loss of mitochondrial phosphorylation presumably through uncoupling phenomenon. It may be interesting to point out here that the degree of inhibition of phosphorylation is much greater than the loss of mitochondrial membrane potential at each

concentration of DA (Fig. 1 and 2). This indicates that the loss of mitochondrial transmembrane potential only partly accounts for the inhibition of phosphorylation capacity which in addition may be further affected by other mechanisms including DA-mediated damage to complex V and phosphate transport system. Other studies have also revealed the toxic actions of DA derived quinones on mitochondrial permeability transition and state IV respiration and swelling and disruption of outer and inner mitochondrial membranes with associated collapse of mitochondrial membrane potential [16, 56]. The effects of DA - derived quinones on mitochondrial proteins have been extensively analyzed in recent publications using 2D-electrophoresis, image analysis, autoradiographic determination or mass spectrometric analysis revealing a series of proteins involved in mitochondrial structure maintenance, transport and oxidative phosphorylation which are damaged by quinones and such results strongly support our present findings [35, 36]. It has been further shown that tetrahydrobiopterin facilitates the formation of quinone products from DA which in turn lead to dopaminergic neuronal damage through inhibition of mitochondrial ETC activity, membrane depolarization and the release of cytochrome c [57]. Another recent study has highlighted the toxic effects of DA derived quinone products on isolated respiring mitochondria through interaction with NADH and GSH pool [58].

4.2. DA causes mitochondrial dysfunctions in PC12 cells: role of ROS and toxic quinones

Several studies have demonstrated that DA added to the medium causes apoptotic death of PC12 cells or other cell lines or primary culture of neurons, but the importance of mitochondrial bioenergetic failure in this context has not been clearly worked out [13, 20, 26, 59, 60]. The results of DA effects on isolated brain mitochondria prompted us to investigate if similar quinone dependent mitochondrial impairment is also operative when intact PC12 cells are exposed to varying concentrations of DA for 24 h and to further explore the consequences of such DA exposure on cell viability and apoptosis. When added to the culture

medium, DA undergoes both extracellular and intracellular oxidation to produce ROS and toxic quinones. The extracellular oxidation is essentially autoxidation, while both autoxidation and enzymatic oxidation take place in the intracellular compartment. Within PC12 cells DA can be acted upon by MAO to produce DOPAC (3,4-dihydroxyphenylacetic acid) and H_2O_2 , while autoxidation leads to the formation of reactive quinones and superoxide radicals which can further give rise to H_2O_2 and other active oxygen species. Moreover, within the intracellular compartment DA can be oxidized by the actions of various enzymes like tyrosinase, prostaglandin H synthase, lipoxygenase and some uncharacterized enzyme activities to produce toxic quinones [55, 61-63].

In the present study the increased ROS production in PC12 cells after DA exposure is significantly prevented by MAO-A inhibitor clorgyline (Fig. 4A) indicating clearly that H_2O_2 formation through MAO-catalyzed oxidation of DA is the predominant mechanism of ROS production within PC12 cells under this condition. This is further verified by amplex red assay which specifically measures H_2O_2 concentrations and DA exposed cells have been seen to produce more H_2O_2 in our study (data not shown). In this study, we have used only clorgyline instead of other MAO inhibitors, since MAO-A is predominantly expressed in PC12 cells [64]. The cell-permeable nitroxide spin-trapping antioxidant TEMPOL is known to scavenge several species of reactive radicals especially superoxide radicals and also hydroxyl and carbon-centred radicals and it can also inhibit Fenton's reaction [65-68]. In our experimental system it provides only mild protection against H_2O_2 accumulation in PC12 cells after DA exposure presumably because TEMPOL does not remove H_2O_2 directly and can in fact generate H_2O_2 while scavenging superoxide radicals by dismutation reaction [65, 69]. Apart from ROS formation within PC12 cells after DA exposure for 24 h, a considerable increase in quinoprotein adducts in the mitochondrial fraction of the former suggests that toxic quinones could be of critical importance in DA toxicity. N-acetyl cysteine is a potent

scavenger of both ROS and toxic quinones [70] and expectedly prevents both the increased intracellular accumulation of ROS and formation of quinoprotein adducts in mitochondria in DA exposed PC12 cells (Fig. 4). It has been shown earlier that clorgyline independent of its inhibitory action on MAO-A can inactivate some uncharacterized mitochondrial enzyme converting DA to quinone products and thus can decrease the formation of quinoprotein adducts in mitochondria co-incubated with DA [19, 55]. This mechanism probably explains our present observation that even clorgyline partially prevents quinoprotein adduct formation in mitochondrial fraction of DA exposed PC12 cells. The actions of clorgyline and NAC have provided us with important clues to reveal the contributions of ROS and toxic quinones to the damaging effects of DA on mitochondria within intact PC12 cells. In DA exposed PC12 cells, there occurs a marked failure of mitochondrial functions with loss of transmembrane potential, and inhibition of ETC complexes at various levels (Fig. 5 and 6). The alterations in mitochondrial functions after DA exposure is only slightly protected by clorgyline which, however, prevents the increased formation of intracellular ROS from DA very considerably and thus this observation rules out any significant involvement of ROS in mediating DA effects on mitochondria of intact PC12 cells. N-acetyl cysteine on the other hand nearly completely protects mitochondria of PC12 cells from the deleterious actions of DA, which can be ascribed to its quinone scavenging actions and ability to prevent quinoprotein adduct formation. The fact that clorgyline also partially prevents the formation of quinoprotein adducts (Fig. 4B), probably explains the ability of the former to confer some protection against DA induced mitochondrial inactivation under our experimental conditions. The involvement of toxic quinones in DA mediated mitochondrial impairment in intact PC12 cells is in clear agreement with our results obtained with isolated rat brain mitochondria in the present study as also those in earlier studies [19, 55]. In contrast to our present results, direct uptake of DA in mitochondria followed by inhibition of complex I without any involvement

of oxidative catabolites of DA in intact SHSY5Y cells has also been reported [52]. Likewise ROS mediated damage to mitochondria after exposure of cells to DOPA or DA has also been reported [17, 42]. However, the present study has clearly brought out the role of toxic quinones in triggering mitochondrial damage in intact PC12 cells and this is well supported by some proteomic based recent studies which have identified several mitochondrial proteins modified by DA derived quinones in SHSY5Y cells or rat brain mitochondria [35, 36].

The contributions of extracellularly generated quinones and those produced intracellularly from DA have also been compared in this study in the context of DA cytotoxicity by observing the effects of potent quinone scavengers like the cell-permeable NAC and the cell-impermeable GSH on generation of quinones and melanin in the medium and quinoprotein adduct formation in the mitochondrial fraction of the cells (Table 1). Our results clearly demonstrate that quinone products including melanin accumulate after DA addition in the medium outside the cells and the process is completely blocked by both GSH and NAC (Table 1). On the other hand, the formation of quinoprotein adducts in the mitochondrial fraction of PC12 cells upon DA exposure is markedly prevented by NAC but only partially by GSH (Table 1). Thus quinoprotein adduct formation in mitochondria and concomitant impairment of mitochondrial functions may result partly from the entry of reactive quinones from the external medium but also substantially may be caused by intracellularly generated quinones after DA is taken up by the cells. Earlier studies using DA transporter blocker nomifensine have also suggested the important role of intracellular oxidation of DA in its cytotoxicity [27, 28].

4.3. DA induces cell death and apoptosis in PC12 cells through quinone oxidation products

Since mitochondrial impairment has been linked with several damage pathways like apoptosis, necrosis and autophagy, [31], we have investigated the effects of DA exposure on the viability of PC12 cells. In our study, a significant and progressive loss of cell viability has

been observed after DA exposure with activation of caspase 3 and caspase 9 and nuclear morphology characteristic of apoptosis as observed after Hoechst staining (Fig. 7, 8 and 9). The loss of cell viability and activation of caspases following DA exposure are partially protected by clorgyline but very conspicuously by NAC (Fig. 7 and 9). The effects of clorgyline and NAC on DA induced cell death are thus remarkably similar to their protective actions against mitochondrial impairment caused by DA. It appears that quinone mediated mitochondrial damage after DA exposure acts as a trigger to initiate cell death by apoptosis in PC12 cells and NAC provides a dramatic protective effect through its quinone-scavenging property. Several earlier studies have shown DA or L-DOPA induced death of various catecholaminergic cells where DA derived ROS have been implicated in the damage process [20, 42, 59, 60]. However, in such studies thiol antioxidant NAC or GSH has been shown consistently to produce significant protective action while various antioxidants have produced variable results [20, 26, 59, 60, 71, 72]. It is therefore conceivable that the protective action of NAC against DA cytotoxicity is unrelated to its antioxidant property and may be explained by its quinone scavenging property as shown here as also in our earlier published study [70]. The importance of toxic quinones in DA cytotoxicity is further supported by earlier studies where human SK-N-MC neuroblastoma cells stably transfected with NAD(P)H quinone oxidoreductase 1 (NQO1) have shown to be highly protective against DA mediated cell death [73]. Although mitochondrial dysfunction has been highlighted as the primary cause for DA induced cell death in this study, the involvement of parallel damage pathways by toxic quinones cannot be ruled out. In particular, after DA exposure, the activation of NF- κ B, or multiple MAP kinase pathways or proteasomal dysfunction or the over expression of α -synuclein could be important in the context of cell death [24-27, 74]. Since mitochondrial dysfunction in our experimental model has led to ATP depletion, it seems a little paradoxical that apoptotic caspase activation has occurred in such energetically compromised PC12 cells.

From the data presented in Fig. 6B and Fig. 9, it appears that apoptotic caspase activation in PC12 cells starts within 6 h of DA exposure when the basal ATP concentration in PC12 cells is still maintained.

Dopamine induced cytotoxicity has been suggested to involve apoptosis, necrosis and autophagy in different experimental models and in all three forms of cellular death mitochondrial dysfunction plays a central role [31]. The present study showing the various forms of mitochondrial dysfunction both in isolated mitochondria and in intact PC12 cells through DA derived quinone products is, therefore, extremely significant. However, the cross-talk among the damage pathways and mitochondrial dysfunction needs to be investigated thoroughly and it is further important to compare these results from experimental model systems with post mortem data so as to get a clear view of the in vivo pathology of the disease. More importantly from the point of view of drug development it is important to identify the initial toxic species triggering these interdependent damage pathways within dopaminergic neurons. The importance of toxic quinones in DA cytotoxicity as has been highlighted here implies that scavengers of quinones rather than antioxidants could be potential neuroprotective agents in PD and compounds like NAC can serve as parent molecules from which more potent neuroprotective molecules can be developed in PD.

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Figure Captions

Fig. 1. Loss of rat brain mitochondrial membrane potential after DA exposure. Rat brain mitochondria were incubated without (control) or (A) with varying concentrations of DA (100-400 μ M) or (B) with DA (400 μ M) in the presence or absence of GSH (2.5 mM) or NAC (2.5 mM) or mannitol (MAN, 20 mM) or DMSO (20 mM) or DTPA (0.1 mM) or catalase (CAT, 50 μ g/ml) for 2 h at 37°C and mitochondrial membrane potential was measured using JC-1. Values are the means \pm SEM of eight observations. * $p < 0.001$ vs. control, Ψ $p < 0.001$ vs. DA (400 μ M); Student's 't' test, paired.

Fig. 2. Inhibition of mitochondrial phosphorylation capacity after in vitro incubation with DA. Rat brain mitochondria were incubated without (control) or (A) with varying concentrations of DA (100-400 μ M) or (B) with DA (400 μ M) in the presence or absence of GSH (2.5 mM) or NAC (2.5 mM) or catalase (CAT, 50 μ g/ml) or mannitol (MAN, 20 mM) or benzoate (BNZ, 20 mM) or DTPA (0.1 mM) for 2 h at 37°C and phosphorylation capacity was measured by as inorganic phosphate utilization as detailed in Material and methods. The

values are the means \pm SEM of eight observations. * $p < 0.001$ vs. control, $\Psi p < 0.001$ vs. DA(400 μ M); Student's 't' test, paired.

Fig. 3. Tyrosinase potentiates DA induced loss of mitochondrial membrane potential and phosphorylation capacity during short-term incubation. Aliquots of rat brain mitochondrial preparations were incubated without (control) or with DA(400 μ M) or DA(400 μ M) plus tyrosinase (TYR, 250 U/ml) in the presence and absence of GSH (2.5 mM) for 15 min at 37°C followed by the measurement of mitochondrial membrane potential (A) and mitochondrial phosphorylation capacity (B) as described in Material and methods. Values are the means \pm SEM of six observations. Statistical significance was calculated by Student's 't' test, paired, $\blacklozenge p < 0.05$ vs. control, * $p < 0.001$ vs. DA, $\Psi p < 0.001$ vs. DA + TYR.

Fig. 4. ROS generation and formation of mitochondrial quinoprotein adducts in PC12 cells after DA exposure. PC12 cells were incubated for 24 h without (control) or with varying concentrations of DA (100 - 400 μ M) or with 400 μ M DA in the presence of other additions like NAC (2.5 mM) or GSH (2.5 mM) or clorgyline (CLOR, 10 μ M) or TEMPOL (TEMP, 1 mM) as described in Material and methods. Intracellular ROS generation was measured using H₂DCFDA (A) while quinoprotein adduct formation in mitochondrial fraction was determined spectrophotometrically (B). Each value is expressed as the mean \pm SEM of five observations. $\blacklozenge p < 0.01$; * $p < 0.001$ vs. respective controls; $\Phi p < 0.05$ and $\Psi p < 0.001$ vs. DA (400 μ M), Student's 't' test, paired.

Fig. 5. Inhibition of mitochondrial respiratory chain complexes in PC12 cells following DA exposure. PC12 cells were incubated alone (control) or with varying concentrations of DA (100 - 400 μ M) or with 400 μ M DA in the presence of NAC (2.5 mM) or clorgyline (CLOR, 10 μ M) for 24 h as described in the text. Mitochondria were isolated from the cells and activities of complex I (A), complex II-III (B) and complex IV (C) measured as described in

Material and methods. The values are the means \pm SEM of six observations. Statistical significance was calculated by Student's 't' test, paired. \blacklozenge $p < 0.05$, $**$ $p < 0.01$; $*$ $p < 0.001$ vs. respective controls; λ $p < 0.05$; Φ $p < 0.01$; Ψ $p < 0.001$ vs. DA (400 μ M).

Fig. 6. Mitochondrial dysfunction in intact PC12 cells after DA exposure. (A) PC12 cells were treated without (control) or with DA in varying concentrations (100 - 400 μ M) or with 400 μ M DA in the presence of NAC (2.5mM) or clorgyline (CLOR, 10 μ M) for 24 h as detailed in Material and methods and the mitochondrial transmembrane potential in intact PC12 cells was measured by using JC-1. Each value represents the mean \pm SEM of six observations. (B) Intracellular ATP content was measured in PC12 cells treated without (control) or with DA (400 μ M) for varying periods of time (6-24 h) as described in Material and methods. Values (expressed as percentage of control) are the means \pm SEM of four observations. $**$ $p < 0.01$, $*$ $p < 0.001$ vs. respective controls : Φ $p < 0.05$, and Ψ $p < 0.001$ vs. control + DA(400 μ M). Student's 't' test, paired.

Fig. 7. Effect of DA on cell viability. PC12 cells were treated with DA (400 μ M) for varying periods of time (6-24 h) (A) or with varying concentrations (100-400 μ M) of DA and with 400 μ M DA in the presence of NAC (2.5mM) or clorgyline (CLOR, 10 μ M) for 24 h (B) as mentioned in the text. At the end of the incubation, the degree of cell death was assessed by trypan blue dye exclusion method. Each value is expressed as the mean \pm SEM of ten observations. \blacklozenge $p < 0.05$, $*$ $p < 0.01$ vs. control : Φ $p < 0.05$, Ψ $p < 0.01$, vs. DA (400 μ M), Student's 't' test, paired.

Fig. 8. Induction of apoptosis in cultured PC12 cells by DA. PC12 cells were treated without (A) or with DA (400 μ M) for 24h (B) followed by nuclear staining with Hoechst 33258 as described in the Material and methods. The figures are representatives of a set of identical

experiments repeated four times. Condensed and fragmented nuclei typical of apoptosis are indicated by arrowheads.

Fig. 9. Caspase activation in cultured PC12 cells by DA. PC12 cells were treated without (control) or with DA (400 μ M) for varying periods of time (6-24 h) or with 400 μ M DA in the presence of NAC (2.5 mM) or clorgyline (CLOR, 10 μ M) for 24 h. Caspase 3 (A) and caspase 9 (B) activities were measured by luminometric method as explained in the Material and methods. Each value is expressed as the mean \pm SEM of five observations. \blacklozenge $p < 0.05$, $\ast p < 0.001$ vs. control; $\Phi p < 0.01$, $\Psi p < 0.001$ vs. DA (400 μ M), Student's 't' test, paired.

Table 1

Accumulation of quinones and melanin in the medium during DA exposure of PC12 cells.

Incubation condition	Quinone (A_{480} / ml)	Melanin (A_{405} / ml)
Control	0.008±0.001	0.024±0.003
DA	0.247±0.018*	0.350±0.017*
DA+ NAC	0.033±0.005♦	0.013±0.002♦
DA+GSH	0.031±0.008♦	0.057±0.003♦

PC12 cells were incubated for 24h without (control) or with DA (400 μ M) in the presence or absence of NAC (2.5 mM) or GSH (2.5 mM). After the incubation quinone and melanin formation in the medium were measured spectrophotometrically as described in Material and methods. Each value is expressed as the mean \pm SEM of four observations. * $p < 0.001$ vs. respective controls; ♦ $p < 0.001$ vs. DA (400 μ M), Student's 't' test, paired.

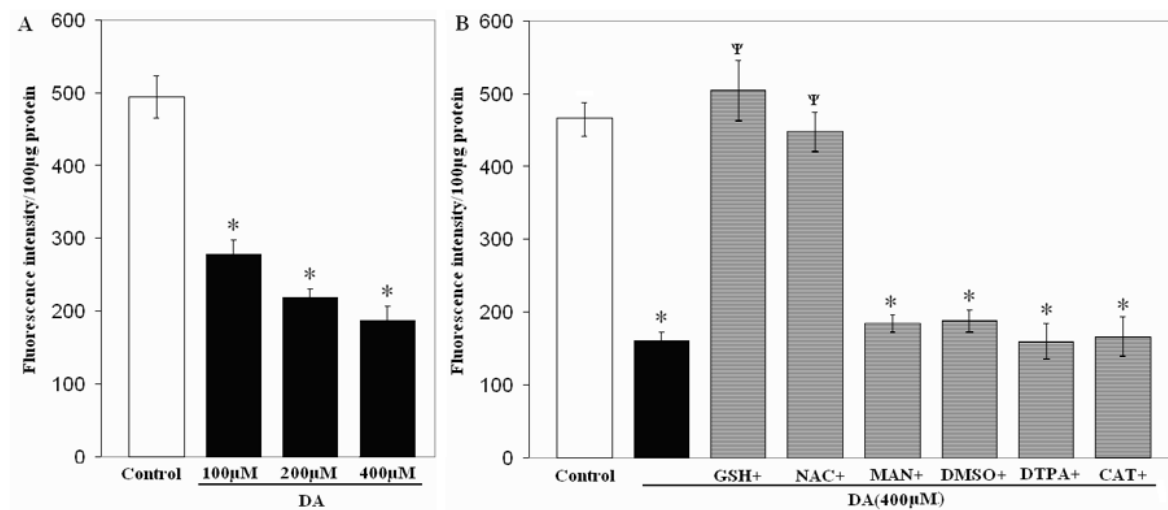


Fig. 1

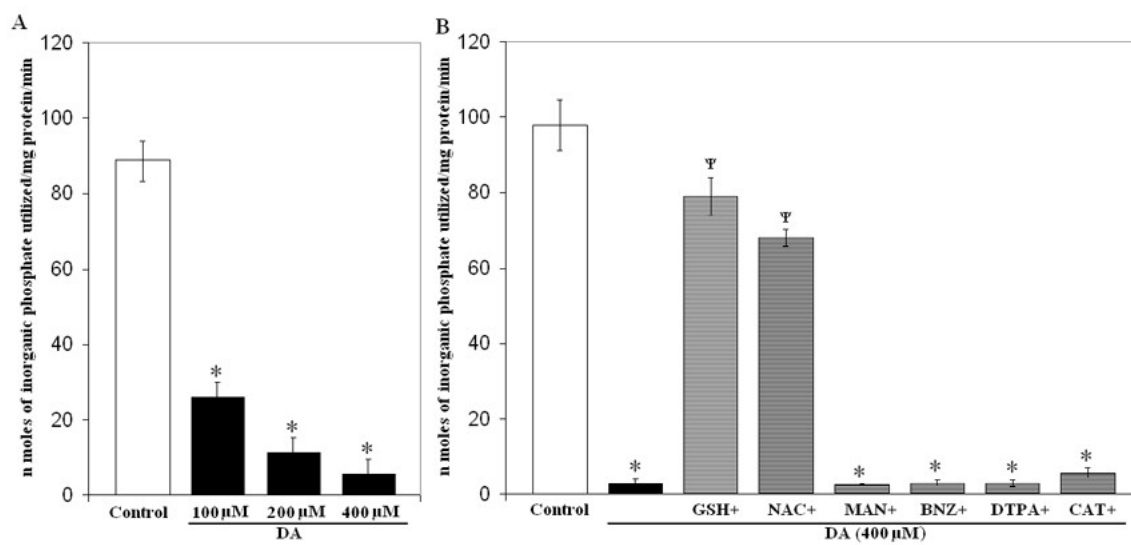


Fig. 2

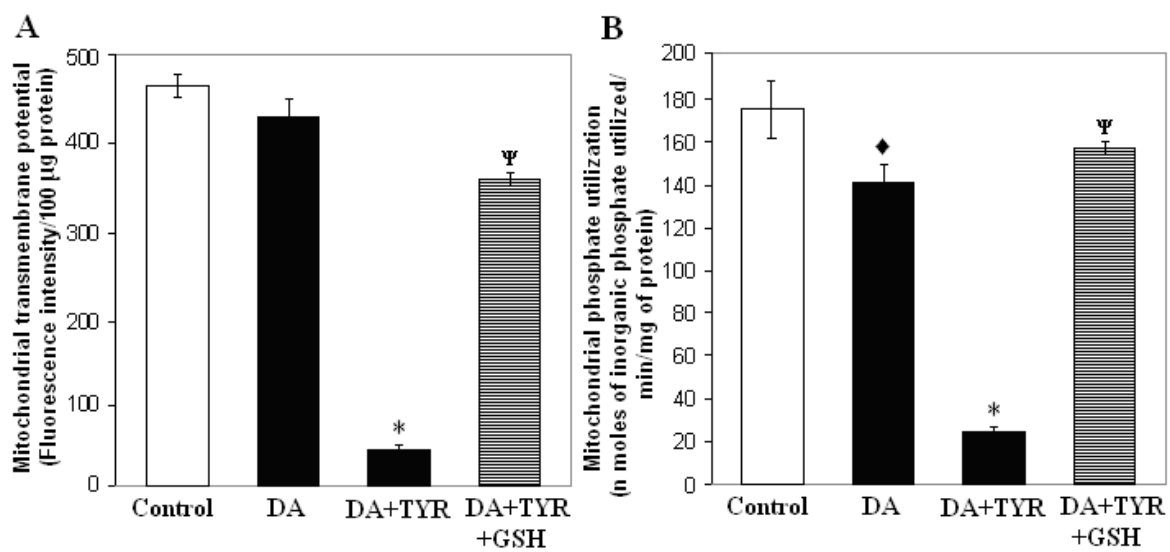


Fig. 3

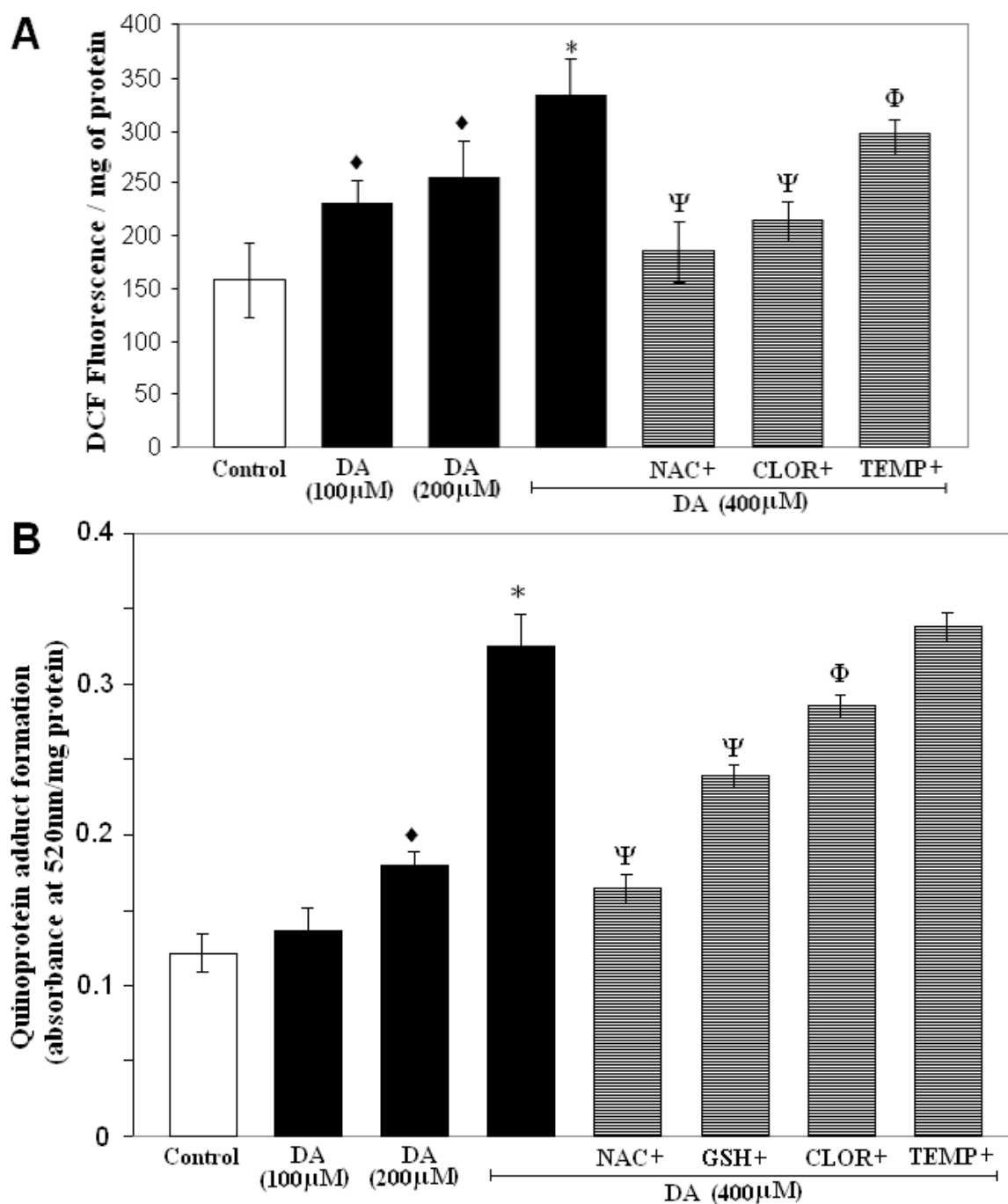


Fig. 4

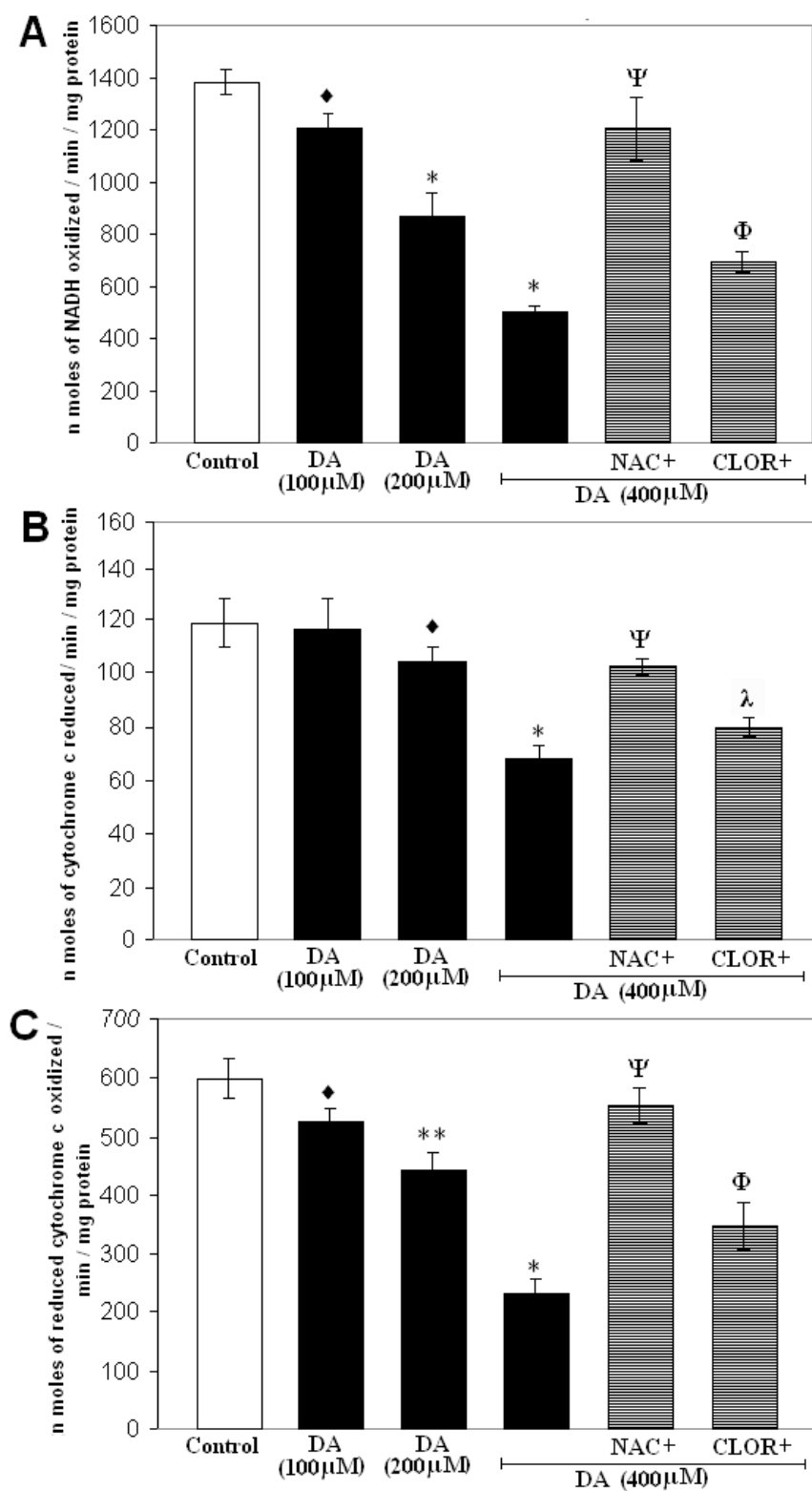


Fig. 5

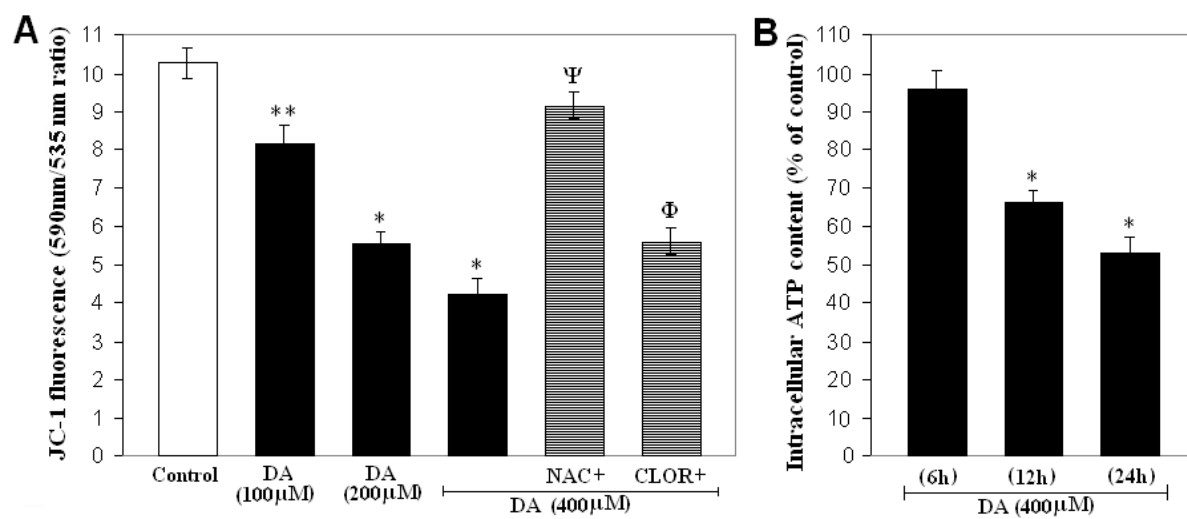


Fig. 6

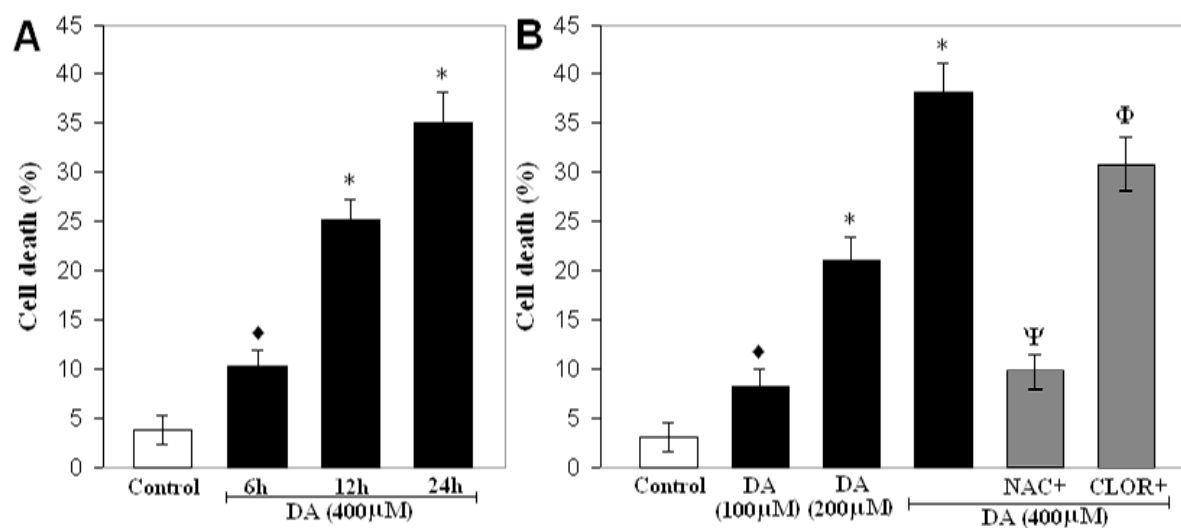


Fig. 7

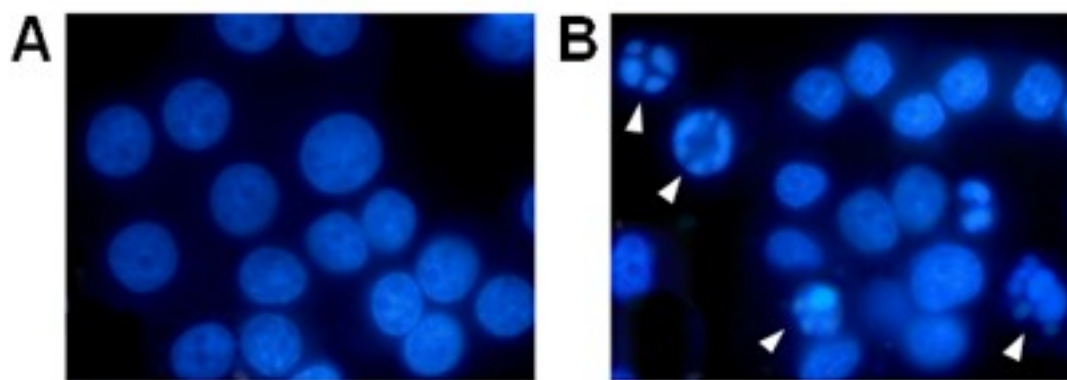


Fig. 8

ACCEPTED MANUSCRIPT

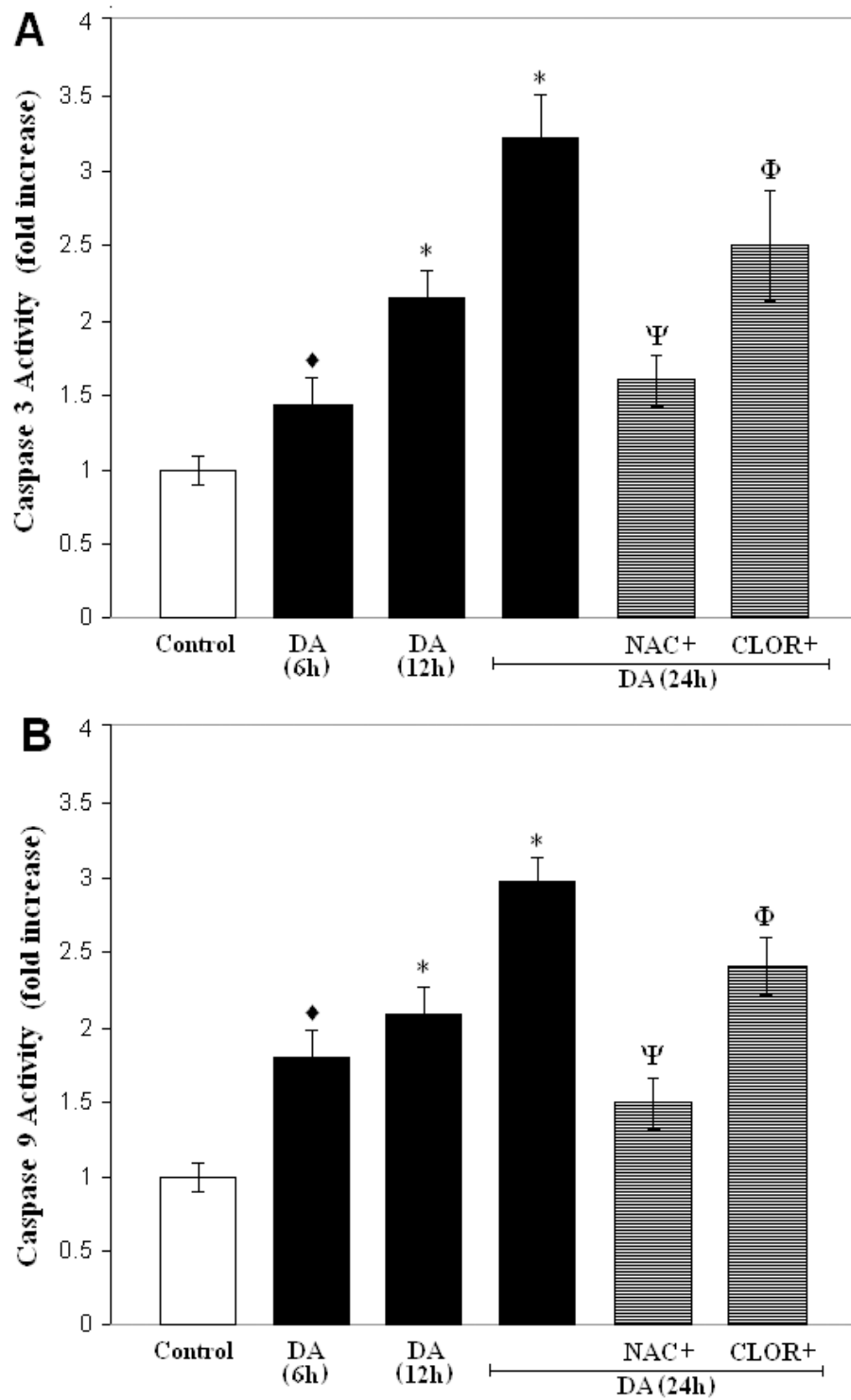


Fig. 9

Research highlights:

- Dopamine inhibits mitochondrial oxidative phosphorylation.
- Mitochondrial dysfunction is mediated by dopamine derived quinones.
- In PC12 cells dopamine induces apoptosis through mitochondrial impairment.
- Quinone scavengers can have neuroprotective potential in Parkinson's disease.