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Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity: implications for Parkinson's disease

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Running Title: GSH decrease leads to selective complex I inhibition

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SUMMARY **Oxidative stress appears to play an important role in degeneration of dopaminergic neurons of the substantia nigra (SN) associated with Parkinson's disease (PD). The SN of early PD patients have dramatically decreased levels of the thiol tripeptide glutathione (GSH). GSH plays multiple roles in the nervous system both as an antioxidant and a redox modulator. We have generated dopaminergic PC12 cell lines in which levels of GSH can be inducibly down-regulated via doxycycline (dox) induction of antisense messages against both the heavy and light subunits of gamma glutamyl cysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione synthesis. Down-regulation of GCS results in reduction in mitochondrial GSH levels, increased oxidative stress, and decreased mitochondrial function. Interestingly, decreases in mitochondrial activities in GSH-depleted PC12 cells appears to be due to a selective inhibition of complex I activity as a result of thiol oxidation. These results suggest that the early observed GSH losses in the SN may be directly responsible for the noted decreases in complex I activity and the subsequent mitochondrial dysfunction which ultimately leads to dopaminergic cell death associated with PD.**

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Parkinson's disease (PD) *involves degeneration of dopaminergic neurons of the substantia nigra (SN). Oxidative stress appears to play a major role in the death of these neurons. Dopaminergic neurons are believed to be especially prone to oxidative stress due to the potential for dopamine oxidation to occur either through auto-oxidation or via metabolism by the enzyme monoamine oxidase (MAO). Oxidation of dopamine produces reactive oxygen species (ROS) including hydrogen peroxide (H2O2). H2O2 can react with ferrous (Fe^{2+}) iron to produce hydroxyl radicals (OH·) which can damage nearby proteins, nucleic acids and membrane phospholipids[1]. Iron levels are increased in the SN of PD patients along with elevations in various indices of oxidative damage[2-4].

PD is also characterized by decreases in SN levels of the thiol antioxidant glutathione (GSH). Although GSH is not the only antioxidant molecule reported to be altered in PD, the magnitude of GSH depletion appears to parallel disease severity and it is the earliest known indicator of oxidative stress in presymptomatic PD, preceding decreases in both mitochondrial complex I activity and dopamine levels[5,6]. Nigral neurons contain GSH and levels are reduced in the PD brain[7].

GSH is synthesized by a two-step reaction involving the enzymes γ-GCS and glutathione synthetase. γ-GCS is the rate-limiting enzyme in this process and brain GSH appears to primarily arise through synthesis from its constituent amino acids via this enzyme[8]. γ-GCS is a

* Abbreviations: PD, Parkinson's disease; SN, substantia nigra; GSH, Glutathione; γ -GCS, gamma glutamyl cysteine synthetase; dox, doxycycline.

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dimer composed of a heavy catalytic subunit and a light regulatory subunit[9].

GSH is synthesized in the cytosol and transported into the mitochondria via an energy-dependent transporter[8]. The mitochondria contains GSH peroxidase and all other components necessary for detoxification of hydroperoxides but no catalase. Decreases in GSH availability in the brain therefore is believed to promote mitochondrial damage via increased ROS[10]. Mitochondrial dysfunction appears to play a role in the neurodegeneration associated with PD[1].

We have created permanent PC12 cell lines in which GSH can be inducibly down-regulated by expressing antisense rat γ-GCS heavy and light subunit cDNAs in a reverse doxycycline (dox)-inducible system making decreases in GSH levels drug-dependent. These cells were used to explore the affects of lowering GSH levels on mitochondrial function in dopaminergic cells as a model for PD.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture supplies were obtained from Gibco. All other chemicals were obtained from Sigma unless otherwise stated.

Creation of dox-inducible anti γ-GCS PC12 cell lines. Dox inducible anti γ-GCS lines (anti γ-GCS) were produced by transfecting PC12 cells with pUHD172-1neo regulatory plasmid using lipofectamine reagent® (Gibco)(Fig. 1). Cells were grown for 15 days in media containing 1mg/ml geneticin (G418). Resulting drug-resistant colonies were transiently transfected with response plasmid containing lacZ (pBIG, Clontech) and treated with either 0 or 25 ug/ml dox followed by X-Gal staining to identify rTta lines with low basal expression and high dox inducibility (data not shown). These lines were stably transfected with response plasmid (pBI, Clontech) containing antisense heavy and light rat γ-GCS cDNAs cloned via RT-PCR from adult rat brain tissue using primers generated from previously published sequences[9,11]. Cells

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were grown in media containing 1mg/ml G418 and 200ug/ml hygromycin for 15 days and drug-resistant colonies selected. Experiments were run on two to three separate anti γ-GCS cell lines using cells containing rTta alone as a negative control.

Measurement of γ-GCS heavy and light subunit protein levels by Western blot analysis. Western blot analysis of cell extracts from anti γ-GCS and rTta cells grown in the presence of increasing concentrations of dox was performed as previously described[12].

Gamma glutamyl cysteine synthetase activity. Cell homogenates resuspended in 100 mM Tris-Cl, pH 8.0 were used to assay γ-GCS activity levels as previously described[11]. Assays were run in the absence of alpha-aminobutyrate as blank. γ-GCS values were normalized per protein using reagent from BioRad.

Glutathione (GSH) and glutathione disulfide (GSSG) levels in whole cells and mitochondria. Cellular GSH and GSSG levels were measured by the method of Griffith (1980). Total GSH and GSSG was measured after addition of 5'-5' dithiobis(2-nitrobenzoic acid), 3 carboxy-4 nitrophenyl disulfide (DTNB) at an absorbance of 412 nm[13]. GSSG was selectively measured after assaying samples in which GSH is masked by pretreatment with 2-vinylpyridine. The difference between the two values gives the GSH levels in the cells. For measurement of mitochondrial GSH and GSSG levels, high quality mitochondria were isolated from anti γ-GCS and control cells as previously described[14]. All values were normalized per protein and/or citrate synthetase activity.

Cell viability and growth. Dying vs. live cells were visualized by incubating cells for 5 min in 0.2% trypan blue in sterile PBS at room temp and noting the presence of any blue (nonviable) cells vs. phase bright cells under the light microscope (n=10 fields examined per cell type).

Measurement of oxidative stress via DCF fluorescence in whole cells and mitochondria. Cells or isolated mitochondria were washed with PBS and resuspended in 20 uM 2'-7' dichlorofluorescein diacetate (DCFDA, Molecular Probes,) for 30 min, 37^{o} C. Cells were centrifuged at 12,000 x g in a microfuge and pellets resuspended in 50 uM digitonin for 20 min, room temperature. Addition of digitonin alone did not contribute to DCF fluorescence.

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Following centrifugation, fluorescence was monitored in the supernatant in a Turner fluorimeter at an excitation wavelength of 488 nm and emission wavelength of 525 nm. DCF values were normalized per protein.

Cellular ATP levels. Cells were harvested in cold 1:1 mix of 12% TCA and 0.2 M sodium citrate, pH 7.0, centrifuged, and the supernatant used for assay of total cellular ATP levels by the method of Mattson et al.[15]. ATP values were normalized per protein.

Pyruvate-dependent MTT reduction in isolated mitochondria. MTT reduction in isolated mitochondria was measured using the method of Berridge and Tan[16]. MTT values were normalized per protein and reported as percent control[17].

Mitochondrial respiration. Oxygen consumption was monitored in isolated mitochondria using a biological oxygen monitor (YSI Model 5300, YSI Inc.). Rate of oxygen consumption was measured after addition of 7 mM pyruvate/malate and 125 nmol ADP as previously described[17,18].

Activities of mitochondrial complexes. Measurement of all mitochondrial enzyme complex activities were done according to the method of Trounce et al.[14]. Isolated mitochondria were preincubated in either the presence or absence of the thiol reductant DTT (4 mM, 35 min) prior to assay.

Immunoprecipitation. Immunoprecipitation was performed on isolated mitochondrial fractions under non-denaturing conditions with an antibody generated against the ND1 subunit of NADH dehydrogenase using the IMMUNOcatcher kit from CytoSignal Research Products (Irvine, CA) according the manufacturer's instructions. The ND1 polyclonal antibody was raised against a synthetic peptide representing amino acid residues 33-43 of the ND1 subunit of complex I[19]. Western blot analysis was performed as previously described to confirm presence of immunoprecipitated protein.

Measurement of reduced protein sulfhydryl residues. Amounts of reduced sulfhydryl groups

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were measured in both whole mitochondrial and immunoprecipitated NADH dehydrogenase subunit-containing fractions by the method of Habeeb[20]. Samples treated with 50 mM N-ethylmaleimide (NEM) were used as a blank**.**

Statistical analysis. Biochemical data are given as mean +/- standard deviation and significance testing was performed using ANOVA.

RESULTS

Generation of PC12 cells with inducibly reduced GSH levels. Using a reverse doxycycline inducible system, we have generated PC12 cell lines in which activity levels of γ-GCS, the rate-limiting enzyme in GSH synthesis, can be inducibly down-regulated via addition of dox (Fig.1). Western blot analysis of anti γ-GCS cells demonstrated that increasing concentrations of dox resulted in a dose-dependent decrease in levels of both heavy and light subunit γ-GCS protein in these cells (Fig. 2A and B). No corresponding decrease in either heavy or light γ-GCS subunit protein was seen in rTta control cells treated with dox at the same concentrations (data not shown). Levels of γ-GCS activity were also found to decrease in antisense γ-GCS containing cell lines in a dose-dependent manner following treatment with dox (Fig. 3A). In contrast, control rTta cells showed no significant changes in γ-GCS activity. Dox-dependent decreases in γ-GCS levels were found to vary proportionally with time up to at least 72 hours following initial drug induction while no change was observed in γ-GCS enzyme levels in the rTta control cells (Fig 3B). Cells containing only rTta plasmid showed no significant changes in γ-GCS levels for cultures incubated for the same time periods.

The dependence of the levels of total cellular glutathione (GSH and its oxidized form, GSSG) on dox concentration were seen to parallel γ-GCS activities, decreasing with decreasing enzyme activity (Fig.3C). Treatment with 25 ug/ml dox for 24 hours caused a decrease in GSH levels of approximately 50% in the antisense γ-GCS cell lines, a similar decrease to that observed in the SN of early PD brains[2,5,21-23]. This dosage was used for all subsequent studies unless

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otherwise noted.

Acute decreases in GSH levels had no effect on cell viability or growth as measured by trypan blue staining (data not shown); only rare blue cells were noted in either the rTta or antisense γ-GCS cells following a 24 hr treatment with 25 μg/ml dox.

Reduced GSH levels results in increased oxidative stress. As GSH is an important redox regulator, cellular levels of ROS and related species were estimated to examine the effects of reduced GSH levels on production of oxidative stress (Fig. 4). Lowering GSH levels was found to cause a significant increase in both cellular and mitochondrial ROS values in the anti γ-GCS cell lines. rTta lines showed no change (data not shown).

Reduced GSH levels results in decreases in mitochondrial function due to specific inhibition of complex I via thiol oxidation. GSH is known to be synthesized in the cytoplasm and to enter the mitochondria via an energy-dependent transport-mediated process[8]. Decreases in cellular GSH in the anti γ-GCS cells following dox induction resulted in a significant decrease in both mitochondrial GSH and GSSG levels (Fig. 3D).

The effect of decreased mitochondrial GSH levels on mitochondrial performance was quantified by measurement of cellular ATP levels (Fig.5A) as well as mitochondrial pyruvate-dependent MTT reduction (Fig. 5B) and oxygen consumption (Fig. 5C and D). All were found to be significantly reduced after treatment with dox. These results suggest that decreasing GSH levels has a profound affect on mitochondrial function.

PD appears to involve selective decreases in mitochondrial complex I activity[1,6,24,25]. MTT reduction and state 3 respiration assayed using the complex I substrate pyruvate were both found to be decreased upon GSH depletion of PC12. To see whether the effects of GSH depletion were specific to complex I or more general, we compared the activies of complex I vs. complexes II-III and IV in isolated mitochondria from dox-treated vs. untreated cells. Lowering of GSH levels in these cells resulted in a significant decrease in complex I activity but interestingly no significant losses were seen in either complex II-III or IV (Fig. 6). Addition of the thiol reducing agent dithiothreitol (DTT) was found to restore complex I activity to levels comparable with

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those found in corresponding controls, indicating that the inhibition of enzyme activity is likely due to oxidation of sulfhydryl groups within the enzyme complex. DTT had no affect on either complex I activity alone, or on II-III and IV activities with or without dox addition.

Levels of reduced protein sulfhydryl residues were decreased in a dox-dependent manner in mitochondria isolated from GSH-depleted cells suggesting that decreases in cellular GSH levels results in increased thiol oxidation of mitochondrial proteins (Fig. 7A).

Non-denaturing immunoprecipitation performed using antibodies against NADH dehydrogenase (ND1 subunit), the active enzymatic component of complex I, followed by subsequent measurement of reduced protein sulfhydryls in the immunoprecipitated fractions revealed a significant decrease in their levels in this protein following GSH depletion (Fig. 7C). This decrease in reduced thiol groups was in the absence of any GSH depletion-mediated loss in immunoprecipitated protein levels (Fig. 7B).

DISCUSSION

Early depletions in nigral GSH levels observed in the Parkinsonian brain are not explainable by increased oxidation of GSH to GSSG. GSH losses have been suggested to be due to increased activity of the enzyme γ-glutamyltranspeptidase resulting in increased removal of both GSH and GSSG from cells although this has yet to be definitely proven[26]. To explore the effects of a depletion in GSH on dopaminergic cells like those in the SN, we constructed a model in which levels of the rate-limiting enzyme in glutathione synthesis, γGCS, is inducibly depleted. Although GCS activity levels do not appear to be specifically impaired in sporadic cases of PD[26], the net effect of our genetic manipulation mimics that which is seen in the Parkinsonian brain, i.e. a decrease in GSH levels without correponding increases in GSSG levels. Using this model we have demonstrated that lowering GSH levels in PC12 appears to elicit a selective inhibition of mitochondrial complex I activity leading to decreased mitochondrial function. Decreased

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mitochondrial activities following glutathione depletion in PC12 includes a loss in cellular ATP levels. Although acute depletion of GSH appeared to have no effect on overall cell viability or growth after 24 hrs, ATP is required for various cellular activities including the synthesis of GSH itself and therefore prolonged decreases in ATP levels would be expected to eventually result in decreased cell viability such as seen in PD.

Selective reductions in GSH levels which precede losses in mitochondrial complex I activity have been reported to occur not only in Parkinson's disease but also in toxin models associated with it such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[21, 27-29]. Whether the inhibition of complex I activity and subsequent decreases in ATP following MPTP administration can be totally accounted for by decreases in GSH levels is unclear as complex I also appears to be inhibited by direct interaction with MPP^+ formed during MAO-B-mediated oxidation of MPTP[30]. However decreasing GSH levels by treatment with the pharmacological agent buthionine sulfoxamine (BSO) has been shown to potentiate the neuodegenerative effects of MPTP in the SN and to alone cause sublethal damage to the nigrostriatal system[31,32].

Our studies suggest that complex I activity is especially susceptible to decreases in GSH levels. Previous studies have shown that complex I activity in bovine heart submitochondrial particles is particularly effected by oxidative stress and that ROS generated by the hypoxanthine-xanthine system selectively affects utilization of oxygen by NAD-linked substrates in rat brain mitochondria[25, 33]. Complex I is in fact considered to be one of the most severely affected by age-related increases in oxidative stress[34]. In synaptic mitochondria, complex I exerts a major control over oxidative phosphorylation such that at 25% inhibition, energy metabolism is

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disturbed resulting in decreased ATP synthesis; complex III and IV inhibition in the range of 70-80% are required to exert similar effects[35].

GSH is known to protect proteins from oxidation by conjugating with oxidized thiol groups to form protein-SS-GSH mixed disulfides which can then be re-reduced to protein and GSH by glutathione reductase, thioredoxin, or protein disulfide isomerase[36,37]. GSH is the major cellular component involved in maintaining protein sulfhydryl groups in their reduced state and much emphasis has been placed lately on its role in redox regulation as a mechanism for controlling activities of various thiol-dependent enzymes including those involved in metabolic regulation (for review, see 38, 39). Furthermore, a recent study by Sriram et al.[29] demonstrated that administration of the excitotoxic compound L-β-N-oxalylamino-L-alanine (L- BOAA) in vitro and in vivo resulted in depletion of GSH leading to inactivation of mitochondrial complex I activity via thiol oxidation. This appears to be the cause for the characteristic mitochondrial dysfunction and subsequent corticospinal neurodegeneration mediated by this neurotoxin; both could be prevented by treatment with antioxidant thiol agents. Previous experiments by Cohen et al.[17] suggest that oxidation of protein sulfhydryl residues can result in inhibition of mitochondrial electron transport when the complex I substrate pyruvate is used as the electron donor. Activity of NADH dehydrogenase, the enzymatic component of complex I, appears to be thiol-regulated[40,41]. Our data demonstrates that not only is the decrease in NADH dehydrogenase activity elicited by GSH depletion of dopamine-containing cells restored by treatment with the thiol-reducing agent DTT, but also that lowering levels of GSH results in the biochemical oxidation of protein sulfhydryl groups contained within this enzyme. Taken

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together, this data suggests that GSH depletion may lead to oxidation of protein sulfhydryl residues in the enzyme important for its function resulting in profound effects on subsequent mitochondrial performance. Total glutathione depletion (GSH+GSSG) cannot cause oxidation of a sulfhydryl itself, but lowering glutathione levels can cause a rise in steady-state levels of H_20_2 (as we see in our cells) which in turn can result in oxidation of vicinal dithiols to a dithiol. The reversibility in the loss of complex I activity with DTT suggests that this is likely a mixed disulfide or intramolecular disulfide formed from vicinal thiols rather than the formation of a sulfinic ($-S0_2H$) or sulfonic ($S0_3H$) acid. Mixed disulfides can be formed through a sulfenic (-SOH) intermediate produced by reaction of H_20_2 with a thiolate (-S-), followed by reaction with GSH. Intramolecular disulfides can be formed by the same route where the vicinal thiol displaces the GS- or by direct reaction of the vicinal thiol with an –SOH intermediate.

Based on our data, we propose that the early depletion of GSH in dopaminergic neurons of the SN in the PD brain may be responsible for selective inhibition of complex I activity and concomitant loss of mitochondrial function which have been associated with the neurodegeneration characteristic of the disease. A recent pilot study examining the effects of GSH administration in a small group of untreated PD patients report that daily intravenous delivery of the tripeptide for the period of a month resulted in a significant improvement in disability[42]. Whether such treatment is effective in actually altering brain levels of GSH and having lasting effects which can act to retard the progess of the disease is unclear, however our data suggests that maintaining thiol homeostasis may be critical for protecting dopaminergic neurons of the SN against neurodegeneration and that thiol reductants may be therapeutic in this

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disorder.

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*Abbreviations: PD, Parkinson's disease; SN, substantia nigra; GSH, Glutathione; GCS, glutamyl cysteine synthetase; dox, doxycycline.

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FIGURE LEGENDS

FIG. 1. **Schematic of the dox-inducible anti γ-GCS system**. The regulatory system used to generate dox-inducible GSH depletion in PC12 consists of: 1) a "regulatory plasmid" containing a reverse transcriptional transactivator (rTta) protein composed of reverse tet repressor (rTet-R) fused to a herpes simplex virus (HSV) VP16 transcription activation domain which is constitutively expressed from a cytomegalovirus (pCMV) immediate early promoter and 2) a "response plasmid" in which γ-GCS antisense heavy and antisense light subunit cDNAs were placed in separate multiple cloning sites on either side of a tet response element (TRE). The TRE consists of seven tandem tet operator sequences (Tet-O) fused to two minimal CMV promoters in opposite directions. The resulting rTta protein produced from the regulatory plasmid only binds and activates expression of the antisense γ-GCS light and heavy subunit mRNAs in the presence of tetracycline or its lipophilic derivatives, eg dox. Anti γ-GCS cells containing both plasmids were selected on the basis of resistance to both neomycin and hygromycin; rTta control cells were selected on the basis of resistance to neomycin alone.

FIG. 2. **Western blot analysis of γ-GCS heavy and light subunit protein levels in dox-treated anti γ-GCS cell lines.** (A) Heavy subunit protein levels after growth of cells in dox concentrations of 0, 20, 40, 60, and 80 ug/ml for 24 hours. (B) Light subunit protein levels after growth of cells in dox concentrations of 0, 20, 40, 60, and 80 µg/ml for 24 hours. In contrast, rTta cells showed no change in γ-GCS protein levels following dox addition (data not shown).

FIG. 3. **Measurement of GCS activity and cellular and mitochondrial glutathione levels in dox-treated anti γ-GCS cells.** (A) Dox concentration curve of γ-GCS activity in anti γ-GCS vs. control rTta cells. Cells were treated with 0, 20, 40, and 60 ug/ml dox for 24 hrs (100% value is equivalent to 2.5 +/- 0.3 nmol/min/mg protein) (B) Time curve of γ-GCS activity in antisense γ-GCS vs. rTta control cells. Cells were treated with 25 ug/ml dox and assayed for γ-GCS activity

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after 24, 48 and 72 hrs (100% value, 2.5 +/- 0.2 nmol/min/mg protein). (C) Dox concentration curve of GSSG and GSH levels in anti γ-GCS vs. control rTta cells. Cells were treated for 24 hrs. in 0, 20, 40, and 60 ug/ml dox (100% GSH value, 20.0 +/- 2.0 nmol/mg protein, 100% GSSG value, 0.8 +/- 0.04 nmol/mg protein). * p< 0.01 vs. control at 0 μg/ml. (D) Mitochondrial glutathione levels in anti γ-GCS cells treated for 24 hours in 0 vs. 25 ug/ml dox (100% GSH value, 3.2 +/- 0.4 nmol/mg protein, 100% GSSG value, 0.12 +/- 0.03 nmol/mg protein). * p < 0.01 vs. control at 0 ug/ml.

FIG. 4. **Effects of glutathione depletion in PC12 on cellular and mitochondrial ROS levels.** Estimation of whole cell and mitochondrial ROS by DCF fluorescence after treatment of anti γ-GCS cells with 25 ug/ml dox for 24 hours. rTta cells show no change in ROS levels following dox addition (data not shown). * p < 0.05 vs. control at 0 ug/ml. Digitonin alone had no effect on DCF fluorescence levels.

FIG. 5. **Assays of mitochondrial function following GSH depletion in PC12**. (A) Cellular ATP levels (100% value, 15.0 +/- 1.0 nmol/min/mg) and (B) pyruvate-dependent MTT reduction assay in mitochondria isolated from anti γ-GCS cells treated with 0 vs. 25 ug/ml dox for 24 hours. * p < 0.01 vs. control and ** p < 0.001 vs. control at 0 ug/ml dox. (C) State 3 and 4 respiration rates in mitochondria isolated from anti γ-GCS cells following treatment with 0 vs. 25 ug/ml dox for 24 hours; rTta cells showed no change in oxygen consumption rate following dox addition (data not shown). (D) State 3 respiration rates quantified from graph C (100% value, 15.0 +/- 2.0 ng atom 0_2/min/mg mitochondrial protein). *p < 0.01 vs. anti γ-GCS at 0 μg/ml dox.

FIG. 6. **Selective inhibition of complex I (NADH dehydrogenase) activity following GSH depletion in PC12 can be reversed by treatment with the thiol reducing agent, DTT.** Measurement of complex I (100% value, 120.0 +/- 4.0 nmol/min/mg mitochondrial protein)**,**

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Complex II-III (100% value, 130.0 +/- 3.0 nmol/min/mg protein) and Complex IV (100% value, 500 nmol/min/mg protein) activities in anti γ-GCS cells after treatment with 0 vs. 25 ug/ml dox for 24 hours. All mitochondrial enzyme activities were measured at 25 ug/ml dox in the absence and presence of 4 mM DTT. * $p < 0.01$ vs. control at 0 ug/ml. DTT has no effect on complex activities at 0 ug/ml dox (data not shown).

FIG. 7. **Measurement of reduced sulfhydryl groups before and after ND1 immunoprecipitation.** (A) Levels of reduced sulfhydryl residues in mitochondria isolated from anti γ-GCS cells after treatment with 0, 25, and 50 ug/ml dox for 24 hours (100% value, 90 nmol SH/mg protein). (B)Western analysis of NADH dehydrogenase immunoprecipitates isolated from mitochondria of anti γ-GCS cells after growth in 0 vs. 25 µg/ml dox for 24 hrs; band is the 33 kD ND1 subunit. (C) Levels of reduced sulfhydryl residues in immunoprecipitates isolated from mitochondria of anti γ-GCS cells after growth in 0 vs. 25 ug/ml dox for 24 hours.* $p < 0.01$ vs. control at 0 ug/ml.

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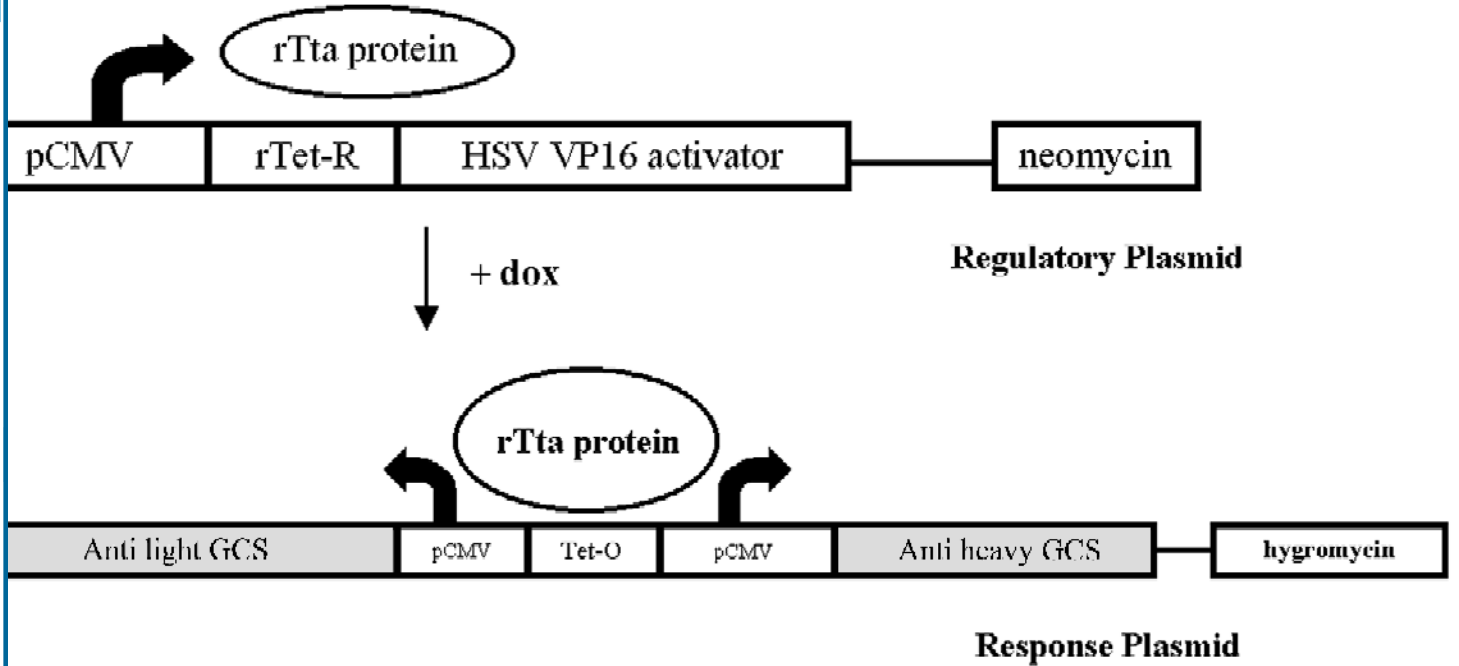
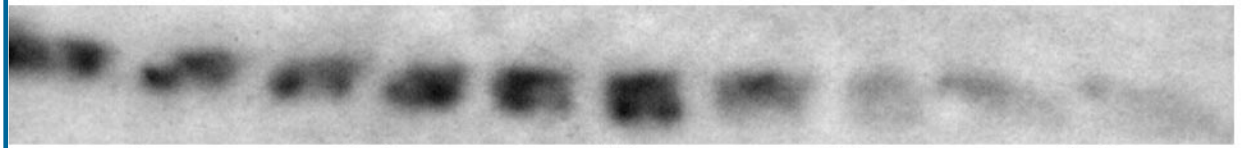

rTta protein
pCMV
rTet-R
HSV VP16 activator
neomycin
Regulatory Plasmid
+ dox
rTta protein
Anti light GCS
pCMV
Tet-O
pCMV
Anti heavy GCS
hygromycin
Response Plasmid



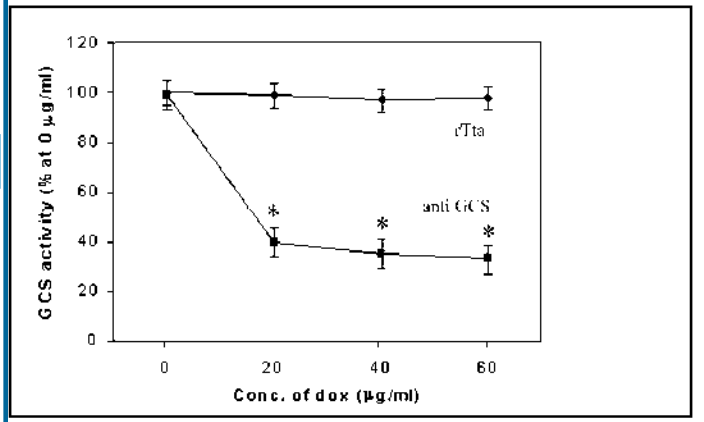
0 20 40 60 80

Dox concentration (μg/ml)

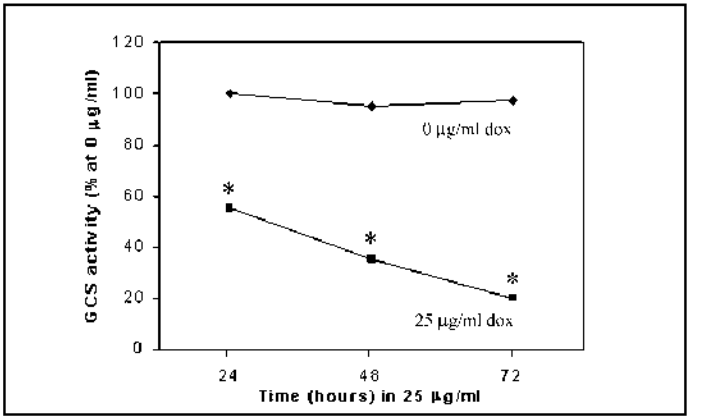
B

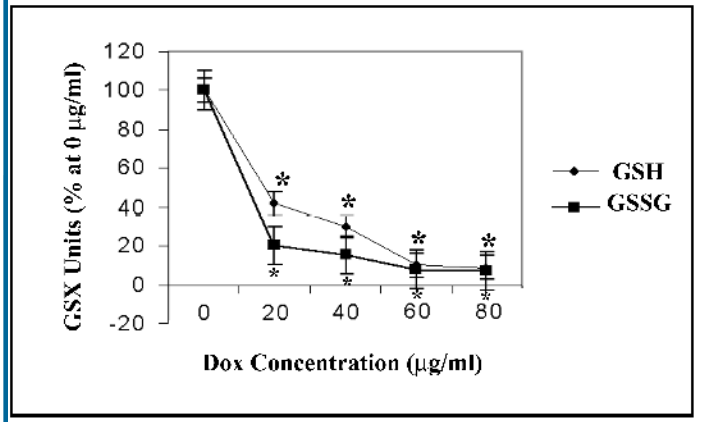
0 20 40 60 80

Dox Concentration (μg/ml)

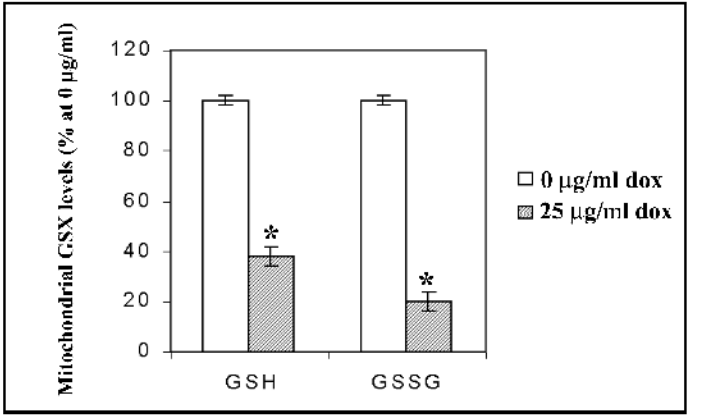

GCS activity (% at 0 µg/ml)
120
100
80
60
40
20
0
rTta
anti GCS
*
*
*
0
20
40
60
Conc. of dox (µg/ml)

B


GCS activity (% at 0 µg/ml)
120
100
80
60
40
20
0
0 µg/ml dox
25 µg/ml dox
*
*
*
24
48
72
Time (hours) in 25 µg/ml


GSX Units (% at 0 µg/ml)
120
100
80
60
40
20
0
-20
*
*
*
*
*
*
0
20
40
60
80
Dox Concentration (µg/ml)
GSH
GSSG

D


Mitochondrial GSX levels (% at 0 µg/ml)
120
100
80
60
40
20
0
*
*
GSH
GSSG
0 µg/ml dox
25 µg/ml dox

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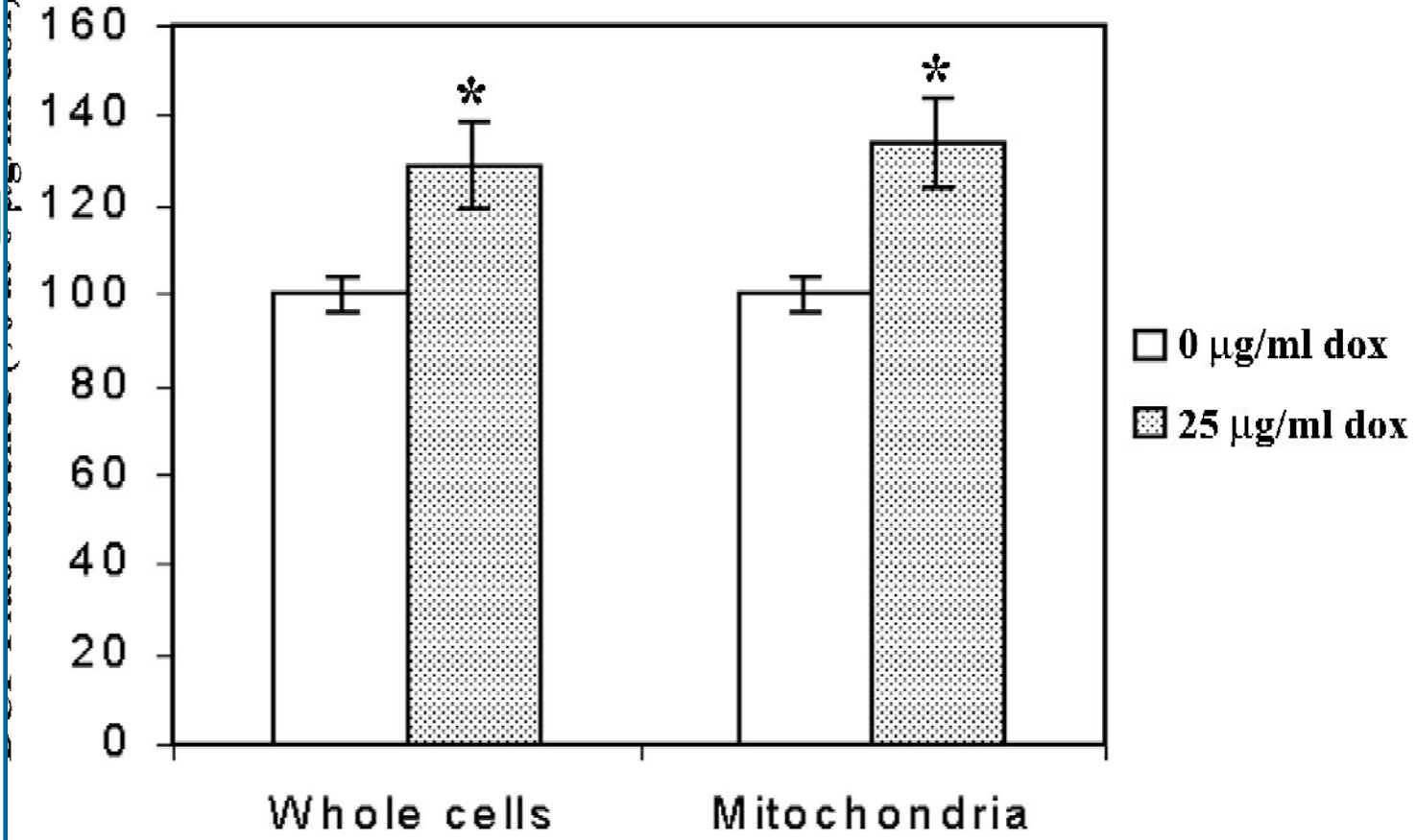
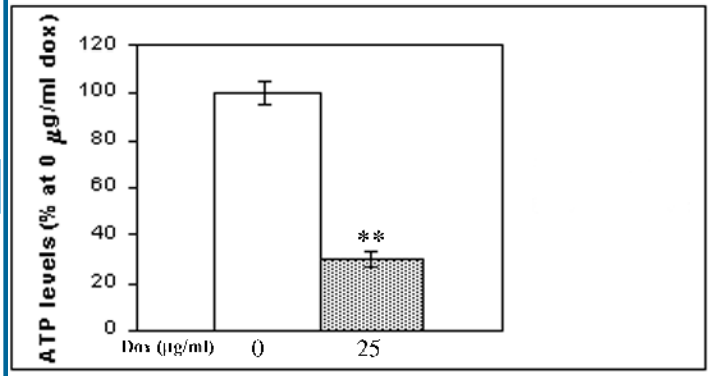
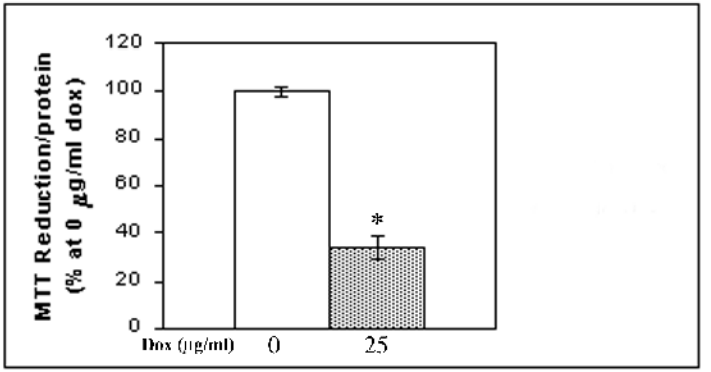
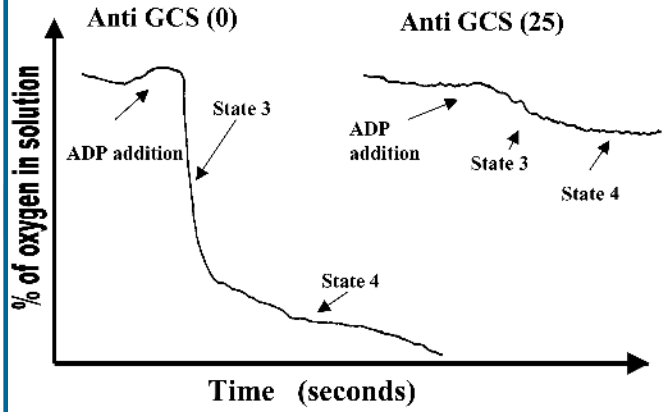

160
140
120
100
80
60
40
20
0
*
*
Whole cells
Mitochondria
0 μg/ml dox
25 μg/ml dox

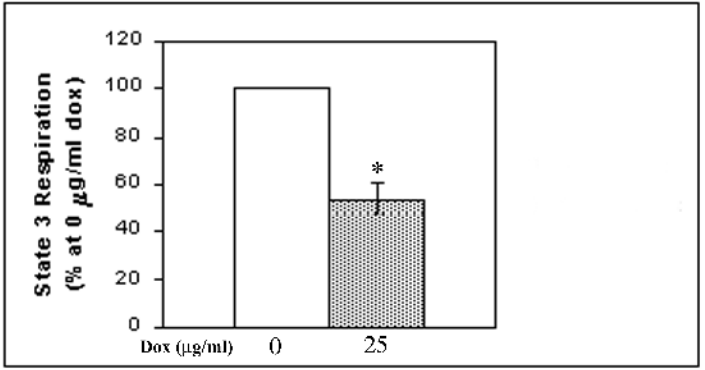

ATP levels (% at 0 µg/ml dox)
120
100
80
60
40
20
0
**
Dox (µg/ml)
0
25

B


MTT Reduction/protein
(% at 0 µg/ml dox)
120
100
80
60
40
20
0
*
Dox (µg/ml)
0
25


Anti GCS (0)
Anti GCS (25)
% of oxygen in solution
ADP addition
State 3
State 4
ADP addition
State 3
State 4
Time (seconds)

D


State 3 Respiration
(% at 0 µg/ml dox)
120
100
80
60
40
20
0
*
Dox (µg/ml)
0
25

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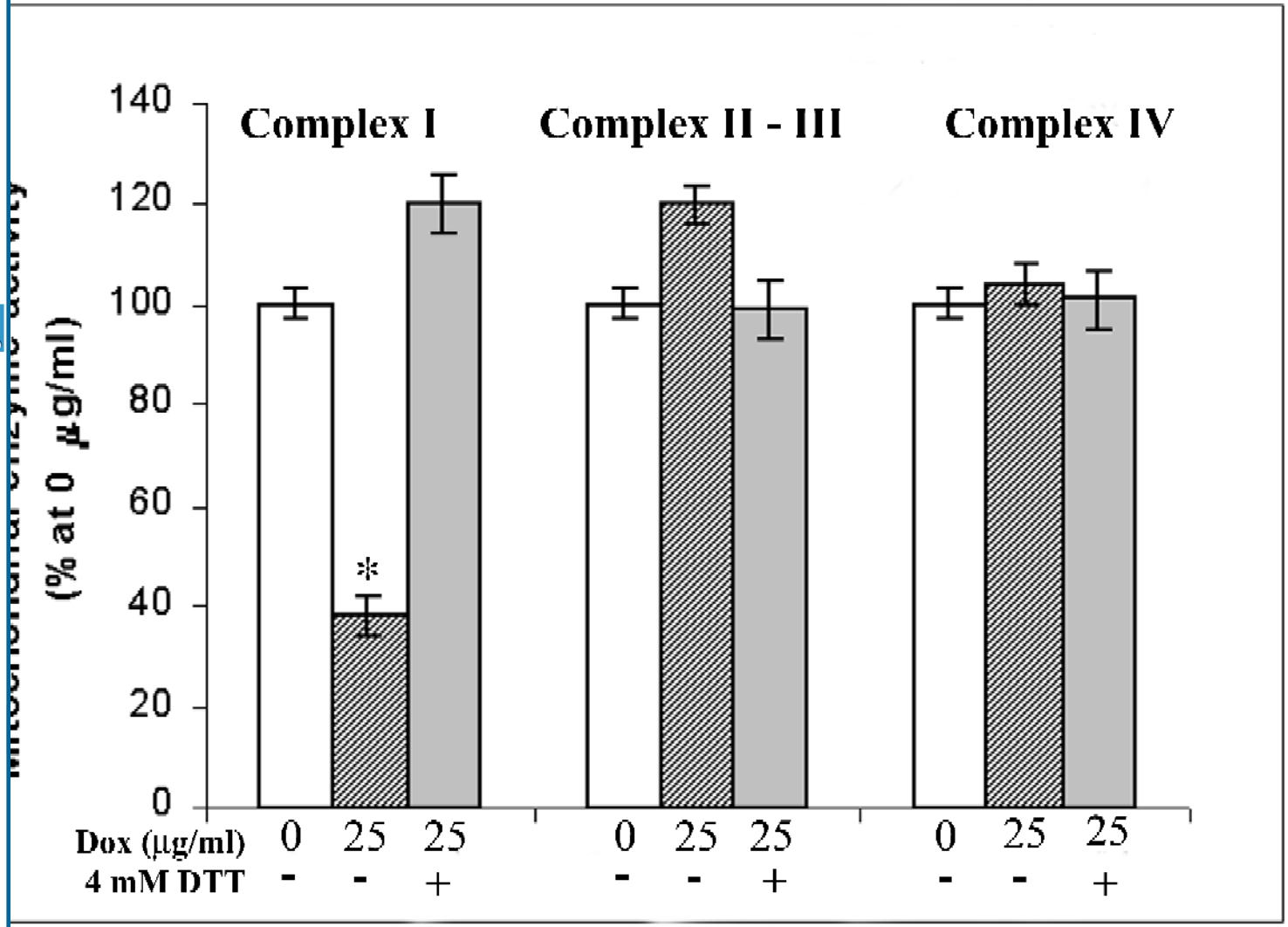

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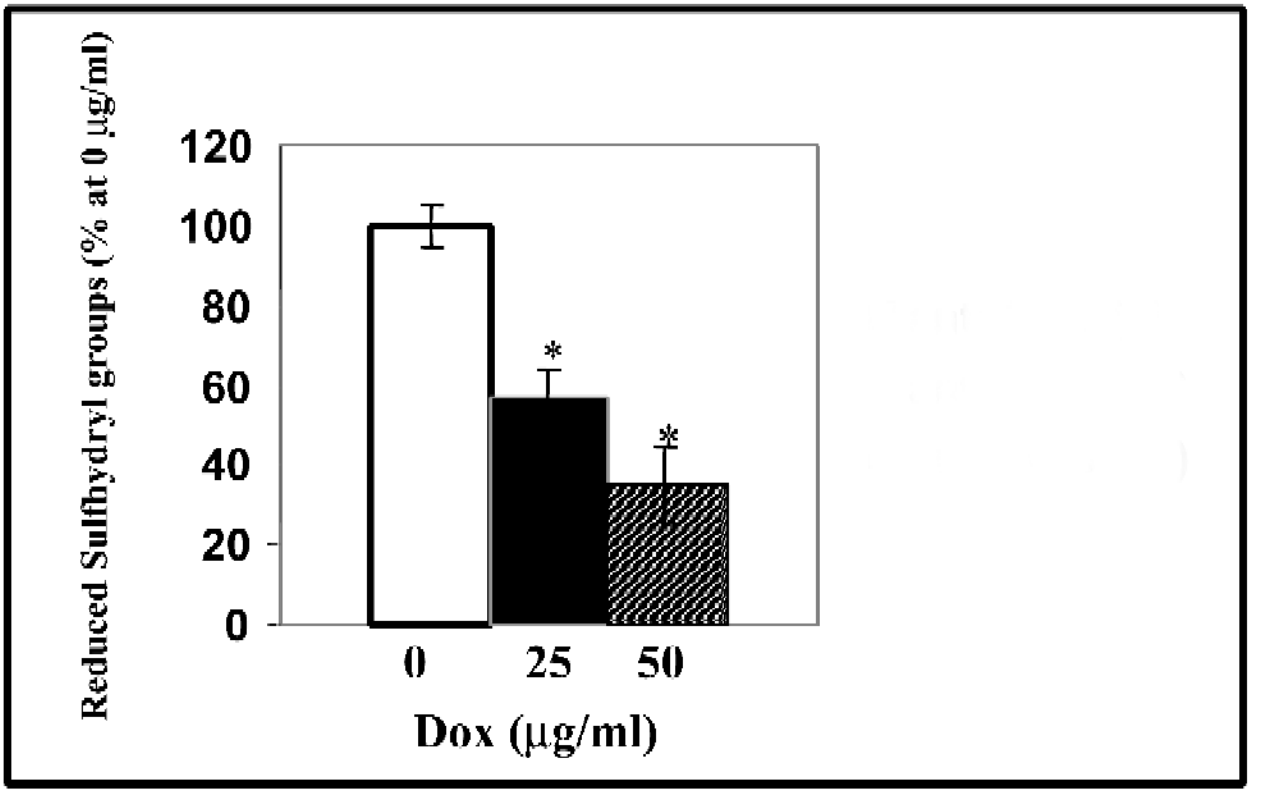
A



B

C