Cellular and Molecular Biology ™ **53**, N°1, 56-61
DOI 10.1170/T775

ISSN 1165-158X
2007 Cell. Mol. Biol.™

MANIPULATION OF ENERGY AND REDOX STATES IN THE C6 GLIOMA CELLS BY BUTHIONINE SULFOXAMINE AND N-ACETYLCYSTEINE AND THE EFFECT ON CELL SURVIVAL TO CADMIUM TOXICITY

M.S. YANG*, L.C. YU AND S.W. PAT

Department of Biology, Hong Kong Baptist University, Kowloon Tong, Kowloon, Hong Kong, P.R.C
* Tel: 852-3411-7058; Fax: 852-3411-5995; email: msyang@hkbu.edu.hk.

Received July 3rd, 2006; Accepted October 3rd, 2006; Published April 15th, 2007

Abstract – Changes in cellular energy and redox sates were studied in the C6 glioma cells following exposure to chemicals that affect glutathione metabolism. It was demonstrated that treatment with sublethal concentrations (25, 50 and 100 μM) of buthionine sulfoxamine (BSO) did not affect cellular energy state as measured by total adenosine nucleotides (TAN=ATP+ADP+ AMP), ATP:ADP:AMP and energy charge potential (ECP=[ATP + 0.5 (ADP)]/TAN). However, there was a significantly decrease in cellular GSH/GSSG and total glutathione (TG=[GSH+GSSG]/ TAN). The change was due to a significant decrease in intracellular GSH level without significant change in [GSSG]. Cells exposed to BSO for 24 hr were much more sensitive to subsequent injuries caused by Cd (0.6 mM for 3 hr). The results indicated that while a significant reduction of intracellular redox state did not affect cell viability, it could increase the susceptibility of cells to subsequent chemical stress. N-acetylcysteine (NAC), on the other hand, caused a dose (1, 5 and 10 mM)-dependent increase in GSH/GSSG without significant changes in intracellular energy state. Improvement of intracellular GSH/GSSG offered no protection against subsequent Cd induced cell death unless NAC was present at the time Cd was added. The pattern of cell death also correlated with the increase in intracellular free radial generation as measured by the fluorescence labeling with 2'7'- dichlorofluorescin. Results of the present study demonstrated that intracellular redox states could be manipulated by addition of chemicals that affect glutathione metabolism. While the redox state may not be the sufficient condition to cause cell death, it could modulate the response of cells to subsequent Cd treatment. Furthermore, the action of NAC against Cd cytotoxicity may not be related to intracellular redox status.

Key words: Cadmium, energy state, redox state, glutathione, Buthionein sulfoximine, N-Acetylcysteine, C6 glioma cell

INTRODUCTION

Amongst the different metals, Cd is one of the most toxic in the environment. The action of Cd has been associated with oxidative damage (12, 15, 21, 26). Using the cultured C6 glioma and HepG2 cells as models, acute exposure to Cd resulted in oxidation of glutathione as indicated by a decrease in intracellular GSH/GSSG (26). Glutathione plays an important role in scavenging free radials. In many studies, improving intracellular glutathione content was able to protect cells against environmentally and chemically induced oxidative stress (6, 8, 14, 16, 18, 20). This strategy has been adopted for treatment and protection of a variety of conditions that involved alteration of the redox status (3-5, 11). A decrease in intracellular glutathione, on the other hand, was associated with a number of human diseases including Alzheimer's disease, cancer, Parkinson's disease, liver disease, heart attack, stroke, diabetes as well as infection caused by HIV and AIDS (23).

Intracellular redox states could be changed by chemical treatments. Buthionine sulfoxamine (BSO) caused intracellular oxidation by suppressing the enzyme that synthesizes GSH (1). On the other hand, N-acetylcysteine (NAC), a cysteine analogue, was able to increase intracellular redox state by increasing GSH synthesis (5). Although improving intracellular GSH level was able to protect cells against oxidative stress, some study suggested that the protective action of NAC against Cd induced cell death was not due to an increase in intracellular GSH, but to prevent Cd entering into cells (22). Thus, the exact role of glutathione in protection against cell death is still unclear. To clarify the role of glutathione in protection against Cd induced cytotoxicity the present study aimed to measure the change in cell viability, free radical generation, intracellular energy and redox state in the C6 glioma cell upon exposure to Cd treatment under altered redox states.

MATERIALS AND METHODS

Cell culture models

The C6 glioma cell was used in the present study for its susceptibility to Cd induced glutathione redox change (26). The cell line was purchased from American Type Culture Collection (ATCC) and maintained in the Ham's F-12K medium supplemented with 10% FBS, antibiotics and fungizone, and incubated in a humidified atmosphere of 95% air and 5% CO_2. The cells used were less than 10 passages after received from ATCC. Under microscopic examination, they formed monolayer.

Treatment of cells with BSO and NAC

Cells were cultured in 6 well plates. After confluent, they were washed with PBS and replace with a medium containing different concentration of 1) BSO (20, 50 or 100µM), or 2) NAC (1, 5 or 10 mM). The cells were incubated for 24 hr. The medium was then removed and the cells were washed twice with pre-warmed PBS. Perchloric acid (1 ml of 0.3M) containing Na_2EDTA (1 mM) was added directly to the culture plate. The cells were then scraped off the bottom and transferred to an eppendorf tube. Any precipitated proteins were removed by centrifugation (9000 x g for 5 min). A 950 µl aliquot of the supernatant was transferred to another tube and was neutralized with 170 µl potassium hydroxide ([KOH]=2M). After rigrous shaking, the mixture was centrifuged at 9000 x g for 5 min to remove fine precipitates of perchlorate ($KClO_4$). The neutralized samples were stored at -20^oC. Levels of ATP, ADP, AMP, GSH, and GSSG were analyzed.

Analysis of adenosine nucleotides, GSH and GSSG

Adenosine nucleotides (ATP, ADP and AMP) were quantified by isocratic reversed-phase HPLC on System coupled with a variable wavelength detector. The mobile phase used was 0.1M ammonium dihydrogen phosphate (pH 6.0) with 1% methanol. Using the C-18 analytical column (reversed phase Utraprep^{TM} 10 µm Spherical 80Å pore) and a flow rate 1 ml/min, the peaks of ATP, ADP, and AMP were eluted at retention times, 5.53min, 6.49min, and 13.96 min, respectively. The peak height responses for all three nucleotides were recorded at 206 nm. The concentration of each nucleotide was determined from a 15 µl sample extract and expressed in terms of nmole nucleotide per ml extract calculated from an ATP standard.

The oxidized and reduced glutathione were measured by spectrofluorometry according to the methods described previously (26). Levels of GSH and GSSG were calculated from standard curves constructed with the respective standards.

Acute Cd induced cell death following pretreatment with BSO and NAC

The change in Cd (0.6 mM) induced cell viability was tested in cells pretreated with BSO and NAC. Cells were cultured in 96-well plates. Following a 24-hr pretreatment, cells were washed twice with pre-warmed PBS and a medium containing either 0.6 mM $CdCl_2$, or 0.6 mM $CdCl_2$ together with NAC was added. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT). The reaction product was determined through a S550 filter using a microplate reader.

Analysis of free radical levels in cells

ROS production was studied by measuring the H_2O_2 generated using the fluorescent probe 2'7'-dichlorofluorescin (H_2DCFDA) (17). H_2DCFDA is a non-polar compound that can be converted into a non-fluorescent polar derivative H_2DCF by cellular esterases. H_2DCF is membrane impermeable and readily oxidized to the highly fluorescent DCF in the presence of hydrogen peroxide. H_2DCFDA (10 µl/ml) was added to the cells after various treatment. After a 30-min incubation, they were harvested, and rinsed three times with PBS, and passed through a FACScan flow cytometer to determine the amount of fluorescence in each cell. Fluorescence intensity of DCF stained cells was analyzed using the CellQuest^{TM} software (Becton Dickinson, FACScan). Each study was repeated three times and approximately 100,000 were counted in each test.

Analysis of Data

The levels of ATP, ADP, AMP, GSH and GSSG were used for calculating the total adenosine nucleotide (TAN=ATP+ADP+AMP), relative ATP:ADP:AMP, Energy charge potential (ECP=[ATP + 0.5 (ADP)] / TAN), GSH/GSSG and total glutathione (TG=[GSH+GSSG]/ TAN) (24, 26). Together with the cell viability and the free radical determination, each experiment was repeated three to six times for calculate of means and standard deviation using the t-test from Sigma Plot. Results from different treatment groups were considered significantly different from the controls at $p<0.05$.

RESULTS

Effect of BSO

In addition to redox state (GSH/GSSG), intracellular energy state was used to monitor the metabolic status of the cell (24). Intracellular energy state represents a set of indicators which monitor the integrity of the cell. High level of total adenosine nucleotides (TAN) with ECP between 0.7-1.0 indicates that the energy status and the viability of the cells are maintained. Figure 1 shows that after treatment with increasing concentration of BSO, cellular energy state was not different from that in control cells. Furthermore, there were no significant changes in cellular ATP/TAN (Figure 1b), ADP/TAN and AMP/TAN (Figure 1c), indicating that the energy production was not compromised. The results demonstrated that following a 24 hr exposure to BSO at concentrations up to 100 µM, cells continue to survive and the energy production system was not affected.

Despite the normal energy state, the redox state of the cells was changed. There was a significant decrease in the intracellular GSH level without significant change in GSSG level. This change resulted in a reduction in GSH/GSSG (Figure 1d). The level of TG was also significantly reduced by half (Figure 1e).

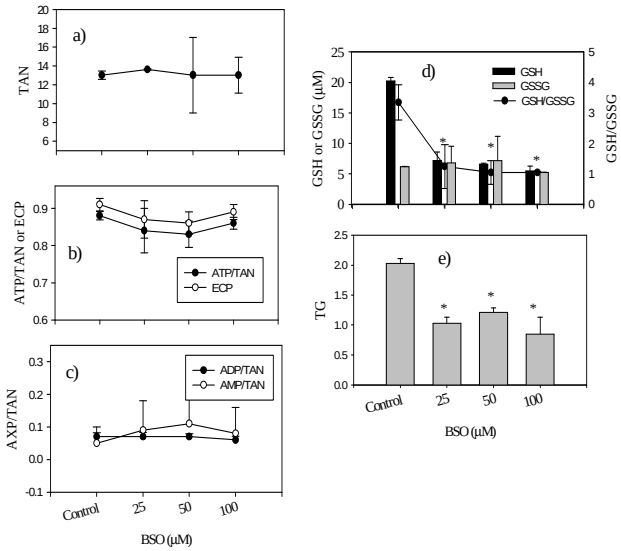

Figure 1. Energy (a, b, c) and redox states (d, e) of cells following exposure to different concentrations of BSO for 24 hr. * indicates that the value is significantly different ($p<0.05$) from that of control. Each value represents the mean and SD of 5 replicates.

Effect of NAC

Opposite to BSO, NAC is a cysteine analogue, which could stimulate glutathione production. Figure 2 shows that after the cells were exposed for 24 hr to up to 10 mM NAC, there was no significant change in intracellular energy state. However, there was a significant increase in cellular GSH and a significant decrease in GSSG level, resulting in a significant increase of GSH/GSSG from 3 to 8. TG was also increased by 50%.

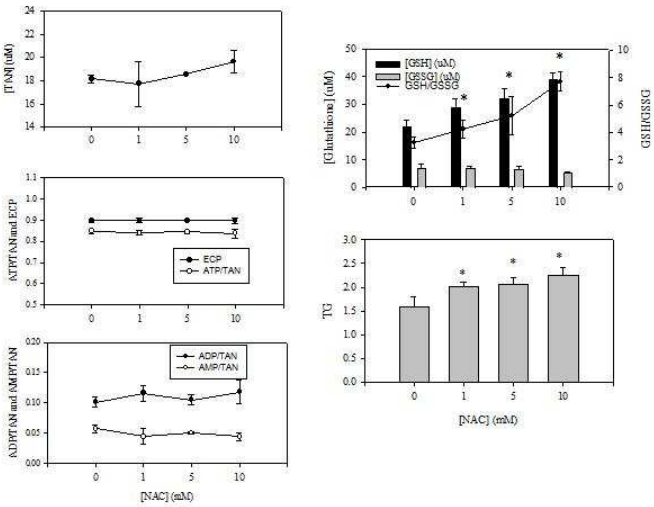

Figure 2. Energy (a, b, c) and redox states (d, e) of cells following exposure to different concentrations of NAC for 24 hr. * indicates that the value is significantly different ($p<0.05$) from that treated with no (0 mM) NAC. Each value represents the mean and SD of 5 replicates.

Change in Cd induced cell death following pretreatment with BSO

Cd is a toxic metal that caused cell death. Figure 3 shows that approximately 50% cell died upon treatment with 0.6 mM of Cd for 3 hr. The data is consistent with the LC_{50} determined for this cell type in previous studies (26). Despite the apparently normal energy state, cells pretreated with BSO were more susceptible to a subsequent high dose of Cd treatment. The viability of cells exposed to 0.6 mM $CdCl_2$ for 3-hr was decreased to <10% in cells pre-exposed to BSO. The results showed that pretreatment of cells with BSO predisposed the cells to Cd cytotoxicity.

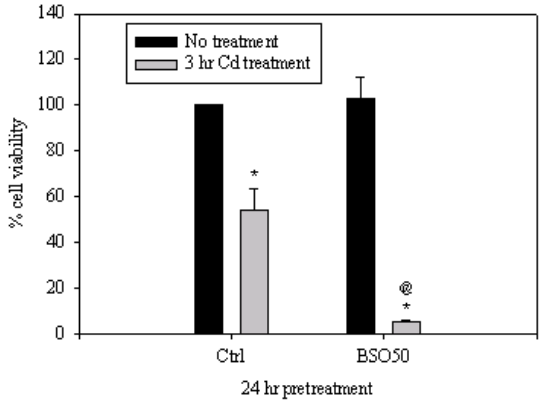

Figure 3. Viability of the C6 glioma cells exposed to BSO (50 μM) for 24 hr followed by a 3 hr treatment with a high concentration (0.6 mM) of $CdCl_2$. * indicates that the value is significantly different ($p<0.05$) from the corresponding control (black bar). @ indicates that the value is significant different ($p<0.05$) from control cells without BSO pretreatment. Each value represents the mean and SD of 8 replicates.

Change in Cd induced cell death following pretreatment with NAC

Figure 4a shows the change in the viability of the C6 glioma cells after pretreatment with 5 mM NAC. Pretreatment with NAC did not improve the tolerance of cells to subsequent Cd treatment. The results indicated that improvement of intracellular GSH/GSSG could not prevent the Cd induced cell death. But when cells were exposed to both Cd and NAC, the toxicity of Cd was completely prevented. Consistent with that previously reported in the LLC-PK_1 cell (22), the major action NAC against Cd cytotoxicity would be to prevent Cd from entering the cells.

In addition to the viability of cells, Figure 4b shows the levels of free radical generation in cells following the different treatments. There was no significant increase in intracellular free

radical generation 5 mM NAC. Cd (0.6 mM) was able to cause a significant increase in intracellular free radical production. Pretreatment with NAC was unable to prevent free radical generation following subsequent exposure to Cd. However, if NAC was present at the time Cd was added, there was a complete suppress the Cd induced free radical generation.

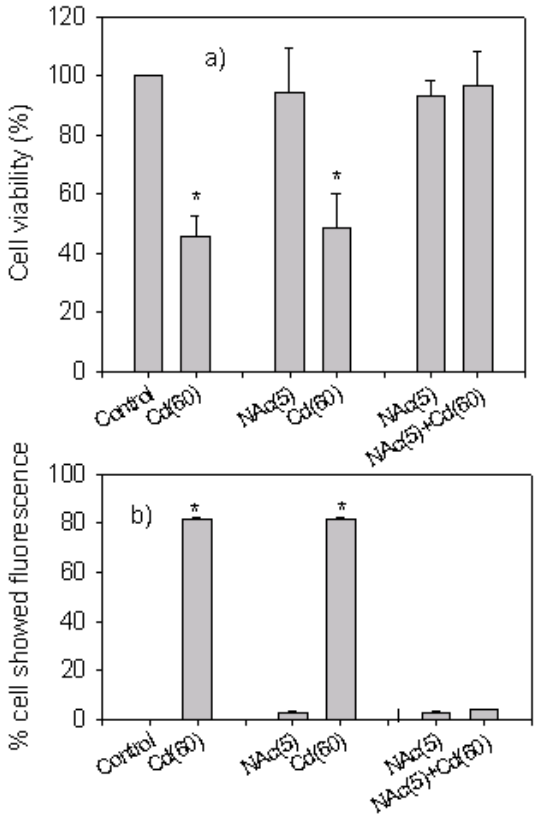

Figure 4. Changes in a) cell viability and b) the degree of free radical generation measured by the DFC fluorescence measured in the C6 glioma cell exposed to 0.6 mM Cd or 5 mM NAC+0.6 mM Cd after pretreatment with 5 mM NAC. * indicates that the value is significantly different from that of the control.

DISCUSSION

Previous studies in our laboratory showed that there are changes in redox and energy states in cells upon exposure to Cd (25,26). In the present study, similar quantitative analysis was conducted in cells exposed to Cd in similar ways following prolonged (24 hr) pre-treatment with BSO and NAC. BSO, an inhibitor of Gamma-glutamylcysteine synthase, could cause significant decrease in intracellular GSH without a significant change in GSSG level. This resulted in a significant decrease in GSH/GSSG ratio. However, the decrease in GSH/GSSG did not cause significant change in intracellular energy state as demonstrated by a steady level of ATP/TAN and ECP, or energy production as demonstrated by an unchanged ATP:ADP. Therefore, a change in GSH/GSSG is not sufficient to cause cell death, but the treatment caused the cells to become more susceptible to subsequent Cd induced injury (Figure 3). Furthermore, the present study also demonstrated that intracellular energy and redox states reflect the activity of two independent intracellular metabolic states. These two systems do not respond similarly, but complement each other on assessing cellular metabolic changes.

NAC, a membrane permeable cysteine analogue, could increase GSH in cell cultures. NAC has been shown to protect against Gamma-ray induced damage in rats (24). It can also prevent intra-acinar oxygen free radical production in pancreatic duct obstruction-induced acute pancreatitis (18); attenuate pyrazole-mediated increases in CYP2A5 mRNA levels (6) and alcohol-induced oxidative stress in the rat (14). Besides, NAC could also protect against inflammation in ischemic rat brain (8), and lung (16). It has also been used clinically for treatment against acetaminophen-induced oxidative damage (4,11). Results of the present study showed that NAC could also protect cells against Cd induced injury. However, the action requires the presence of NAC at the time of Cd administration. This is consistent with that previously reported in the LLC-PK_1 kidney cell (22). Thus, consistent with this previous study, results of the present study support the notion that the efficacy of NAC against Cd induced cell death may not be due to the improvement of intracellular GSH/GSSG. It may be due to the ability of NAC to prevent Cd entry.

The action of Cd in mediating cell death has been of particular interest as the metal is an important environmental toxin that can cause serious health hazard to the public. In studying the metabolic changes in both the HepG2 and the C6 glioma cells, it was demonstrated that a 3 hr exposure to Cd caused a concentration-dependent decrease in intracellular GSH/GSSG as well as TAN (ATP+ADP+AMP) (26). But amongst the different nucleotides studied, there was a significant increase in ATP/TAN and decrease in ADP/TAN and AMP/TAN. Thus, upon comparing the ATP and ADP changes, there would be a significant increase in ATP/ADP,

which indicated that there is a significant increase in energy production, probably by activation of the electron transport chain at the mitochondria. This is further supported by the increase in free radicals (H_2O_2) generated (Figure 4b). Taken together, these data suggested that Cd may increase the rate of oxidative phosphorylation. The free radicals generated may be by-products of this activity. The role of GSH is to remove the H_2O_2 that leaked out from the mitochondria. As the activity of oxidative phosphorylation continuous, endogenous glutathione may not be able to handle the continuous excess H_2O_2 generation, thus resulted in a significant decrease in GSH/GSSG, even when the endogenous GSH was improved by pretreatment with NAC.

The present study employed chemicals that modify intracellular redox state to investigate the role of glutathione in protection against Cd induced cell death. The results demonstrated that enhancement of reducing power may not be a sufficient condition to protect against Cd induced cell death. The action of acute Cd exposure may be related to an increase in energy production and the significant reduction in redox state may be a consequence of excess energy production. An enhancement in energy production and generation of H_2O_2 has been well documented in phagocytic cell and is known as oxidative burst. Oxidative burst is a response of the cells to external stimulus (usually bacterial infection) which in turn, activates the enzyme NADPH oxidase, stimulate free radicals as a means for removing the invading bacteria (2). Although oxidative burst was found to be possible in non-phagocytic cells, the activity may not be significant because the NADPH-oxidase activity is much slower (28). Recent studies by (13) found that Cd can induce early oxidative burst in cultured tobacco cells. In mammalian cells, oxidative burst had also been shown to involve in many pathological states (7, 10, 19, 27, 28). Thus, it may not be impossible for the C6 glioma cell to respond to Cd induced injury through oxidative burst.

The present study provides evidence to show that intracellular redox state may not be an important cause of the metal induced cytotoxicity but a depletion of GSH may enhance metal toxicity. Rather, the metal may act by activating the energy production in a way similar to that seen in oxidative burst. Besides Cd, Zn has also been shown to affect energy metabolism in a similar way as Cd (19). Zn has been of particular interest in biomedical research as it is involved in mediating neuronal cell death in a number of degenerative diseases (28). While continuous studies are need to demonstrate the correlation between Cd toxicity and oxidative burst, it may be also of interest to demonstrate the role of oxidative burst in neurons following Zn induced injury.

Acknowldgement: The study was supported by the HKBU Faculty Research Grants.

REFERENCES

1. Armstrong, J. S., Steinauer, K. K., Hornung, B., Irish, J. M., Lecane, P., Birrell, G. W., Peehl, D. M., and Knox, S. J. (2002). Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line. Cell Death.Differ., 9:252-263.

2. Babior, B. M., Lambeth, J. D., and Nauseef, W. (2002). The neutrophil NADPH oxidase. Arch.Biochem.Biophys., 397:342-344.

3. Ben Yoseph, O., Boxer, P. A., and Ross, B. D. (1996). Assessment of the role of the glutathione and pentose phosphate pathways in the protection of primary cerebrocortical cultures from oxidative stress. J.Neurochem., 66:2329-2337.

4. Clark, J. (2001). Acetaminophen poisoning and the use of intravenous N-acetylcysteine. Air Med.J., 20:16-17.

5. De Flora, S., Izzotti, A., D'Agostini, F., and Balansky, R. M. (2001). Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. Carcinogenesis, 22:999-1013.

6. Gilmore, W. J. and Kirby, G. M. (2004). Endoplasmic reticulum stress due to altered cellular redox status positively regulates murine hepatic CYP2A5 expression. J.Pharmacol.Exp.Ther., 308:600-608.

7. Griendling, K. K., Sorescu, D., and Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. Circ.Res., 86:494-501.

8. Khan, M., Sekhon, B., Jatana, M., Giri, S., Gilg, A. G., Sekhon, C., Singh, I., and Singh, A. K. (2004). Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J.Neurosci.Res., 76:519-527.

9. Koh, J. Y. (2001). Zinc and disease of the brain. Mol.Neurobiol., 24:99-106.

10. Li, J. M. and Shah, A. M. (2003). ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy. J.Am.Soc.Nephrol., 14:S221-S226.

11. Marzullo, L. (2005). An update of N-acetylcysteine treatment for acute acetaminophen toxicity in children. Curr.Opin.Pediatr., 17:239-245.

12. Oh, S. H. and Lim, S. C. (2006). A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicol.Appl.Pharmacol., 212:212-223.

13. Olmos, E., Martinez-Solano, J. R., Piqueras, A., and Hellin, E. (2003). Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). J.Exp.Bot., 54:291-301.

14. Ozaras, R., Tahan, V., Aydin, S., Uzun, H., Kaya, S., and Senturk, H. (2003). N-acetylcysteine attenuates alcohol-induced oxidative stress in the rat. World J.Gastroenterol., 9:125-128.
15. Pathak, N. and Khandelwal, S. (2006). Modulation of cadmium induced alterations in murine thymocytes by piperine: Oxidative stress, apoptosis, phenotyping and blastogenesis. Biochem.Pharmacol.
16. Rhoden, C. R., Lawrence, J., Godleski, J. J., and Gonzalez-Flecha, B. (2004). N-acetylcysteine prevents lung inflammation after short-term inhalation exposure to concentrated ambient particles. Toxicol.Sci., 79:296-303.
17. Royall, J. A. and Ischiropoulos, H. (1993). Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch.Biochem.Biophys., 302:348-355.
18. Sevillano, S., de la Mano, A. M., Manso, M. A., Orfao, A., and De, Dios, I. (2003). N-acetylcysteine prevents intra-acinar oxygen free radical production in pancreatic duct obstruction-induced acute pancreatitis. Biochim.Biophys.Acta, 1639:177-184.
19. Shiose, A., Kuroda, J., Tsuruya, K., Hirai, M., Hirakata, H., Naito, S., Hattori, M., Sakaki, Y., and Sumimoto, H. (2001). A novel superoxide-producing NAD(P)H oxidase in kidney. J.Biol.Chem., 276:1417-1423.
20. Sridharan, S. and Shyamaladevi, C. S. (2002). Protective effect of N-acetylcysteine against gamma ray induced damages in rats--biochemical evaluations. Indian J.Exp.Biol., 40:181-186.
21. Watjen, W. and Beyersmann, D. (2004). Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. Biometals, 17:65-78.
22. Wispriyono, B., Matsuoka, M., Igisu, H., and Matsuno, K. (1998). Protection from cadmium cytotoxicity by N-acetylcysteine in LLC-PK1 cells. J.Pharmacol.Exp.Ther., 287:344-351.
23. Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., and Turner, N. D. (2004). Glutathione metabolism and its implications for health. J.Nutr., 134:489-492.
24. Yang, M. S. and Gupta, R. C. (2003a). Determination of energy charge potential in the C6 glioma and the HepG-2 cell culture. Toxicology Mechanisms and Methods, 13:97-101.
25. Yang, M. S. and Gupta, R. C. (2003b). Modulation of energy metabolism in C6 glioma cells as possible mechanism contributing to zinc neurotoxicity. Toxicology Mechanisms and Methods, 13:269-275.
26. Yang, M. S., Yu, L. C., and Gupta, R. C. (2004). Analysis of changes in energy and redox states in HepG2 hepatoma and C6 glioma cells upon exposure to cadmium. Toxicology, 201:105-113.
27. Yang, S., Madyastha, P., Bingel, S., Ries, W., and Key, L. (2001). A new superoxide-generating oxidase in murine osteoclasts. J.Biol.Chem., 276:5452-5458.
28. Zalba, G., San Jose, G., Moreno, M. U., Fortuno, M. A., Fortuno, A., Beaumont, F. J., and Diez, J. (2001). Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension, 38:1395-1399.