

Antioxidant Enzymes Level Detection in Cisplatin Treated Iraqi Lung Cancer Patients and *In vitro* Estimating the Cytotoxic and Reactive Oxygen Species Generation in A549 Cell Line

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ABSTRACT

Cisplatin is one of the most important and effective antitumor drug used as treatment against different types of tumors. Cisplatin directly affects DNA integrity as well as the generation of reactive oxygen species (ROS) and cell death induction. Changes in the activities of antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were investigated in healthy controls and lung tumor patients before and after cisplatin treatment. Untreated patients showed a significant reduction in CAT, GPx, and SOD levels, while cisplatin-treated patients retained the normal levels of antioxidant enzymes compared with healthy controls. Cisplatin exhibited cytotoxic activity against A549 lung tumor cells in a dose-dependent manner with an IC_{50} of $10.33 \mu\text{g mL}^{-1}$. Cisplatin was found to induce the generation of ROS in cisplatin-treated A549 cells compared with a negative control. Such an increase in ROS production was significantly correlated with increasing cisplatin concentration.

Keywords: ROS generation, Cisplatin, A547, antioxidant

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INTRODUCTION

Normal eukaryotic cells have developed variety of defense mechanisms that control the balance between the generation of reactive oxygen species (ROS) and their elimination to normal levels that required for different cellular functions.¹ Antioxidant enzymes subsist in cells to conserve versus the poisonous influence of “oxygen-derived species” created through ordinary cellular metabolism or pending oxidative stress.² These antioxidant enzymes or defenses implicate superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).³ Malignant cells have been the revelation to have the abnormal standard of antioxidant enzymes and efficacy when contrasted with normal cells.⁴ However, antioxidant enzyme performance also differs between different tumors.⁵ Antioxidant enzymes used to be found in different isoforms, intracellularly and extracellularly, and their action inspires an integrated antioxidant defense system. Cells are also conserved versus “oxidative stress” by a system of non-enzymatic “free radical scavengers,” like ascorbic acid.⁶ Oxygen is an element

major for life; however, mature to its strong chemical efficacy, some by-product of oxygen metabolism are pestilent to the living organisms. ROS are reactive molecules, which generated as free radicles or non-radicles molecules from molecular oxygen like superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (OH), singlet Oxygen (1O_2), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).^{7,8} ROS generated in mitochondria of mammalian cells as results of oxygen reduction, electron transport, oxidation-reduction activities and other processes during the life cycle of a cell. In many types of cancer cells, ROS detected at very high levels due to the higher metabolic activities, increased cellular enzymatic activities, oncogene activation, and mitochondrial dysfunction.⁹

Lung cancer is one of the leading diseases that cause death worldwide. In Iraq, the incidence of lung cancer is 8.1% among all types of cancers and ranked the second type of cancers after breast cancer with the highest mortality rate over all other types.¹⁰ Cisplatin is well known chemotherapy that used to treat many types of tumors including non-small cell

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lung cancer-causing DNA damage and eventually inducing apoptosis in cancer cells.¹¹ Part of cisplatin toxicity is the increased ROS generation, which results in alteration of mitochondrial membrane potential (MMP) and disturbs the electron transport chain related to mitochondrial respiration and finally triggering cell apoptosis.⁴ Moreover, patients using cisplatin exhibited significant decreases in concentrations for the antioxidant enzymes. Higher levels of ROS may during cisplatin overwhelmed the antioxidant defense systems, affecting the cytotoxic activity of cisplatin and slowing the rate of tumor cells proliferation.^{12,13}

This study aimed to identify the enzymatic level of SOD, CA and GPx for patients with lung cancer before and after treatment with cisplatin. Detecting the cytotoxic effect of cisplatin *in vitro* (A549 cell line), in addition investigating the role of cisplatin in ROS generation by the distribution of mitochondrial function.

MATERIALS AND METHODS

Patients

The protocol involved seventy-five blood samples from patients, which were admitted in Medical City–Baghdad Teaching Hospital and was diagnosed for non-small lung cancer at different tumor stages, 25 were newly diagnosed and 50 were already treated with cisplatin at dose range 75–100 mg m⁻¹ (combined with other chemotherapies). The demographic characteristics of patients are listed in Table 1. Blood samples from healthy volunteers were collected as control. All blood samples 5 mL were drawn in a heparinized tube and diluted (1:10) in 0.9% NaCl solution. Triton X-100 (Sigma Aldrich, Germany) was added at a concentration of 0.1% for cell lysis. The blood was kept in ice bath with vigorous vortexing followed by centrifugation at 3000 rpm for 15 min at 4°C.¹⁴ The supernatant (hemolysate) was aspirated and used for enzyme activity detection.

Enzymatic activity of CAT, GPx, and SOD

Antioxidant enzymatic activity of human catalase (CAT), glutathione peroxidase (GPx) superoxide dismutase (SOD) were evaluated and were examined in patient samples as well as healthy controls.

Human Catalase (CAT)

CAT activity was measured via a commercial ELISA kit purchased from MyBiosource, Canada (Cat No: MBS770782). The kit based on the principle of biotin double antibody sandwich technology. Standard and samples were pipetted into the supplied coated plate and the presented CAT bounded by the immobilized Ab. By adding a biotin-conjugated antibody specific for CAT, avidin conjugated horseradish peroxidase and stop solution, the intensity of color development was measured by using plate reader PowerWave STM (BioTek®, USA) at 450 nm and the enzyme was calculated in ng mL⁻¹.

Glutathione Peroxidase

The activity of GPx in samples was calculated in samples using the quantitative colorimetric EnzyChrom™ kit detection of GPx purchased from BioAssay Systems, USA (Cat No:

EGPX-100). This kit employs the reduction of glutathione in samples through the direct measuring of NADPH consumption in GPx coupled reactions. Optical density at 340 nm was determined using plate reader PowerWave STM (BioTek®, USA), and decreased measurement in U L⁻¹ is proportional to GPx activity.

Superoxide Dismutase

SOD activity was measured using an available ELISA kit (Abcam, USA, Cat No: ab119520), for the accurate determination of human Cu/Zn SOD levels using a Cu/Zn SOD specific Abs pre-coated plate following the addition of Cu/Zn SOD HRP-conjugated Ab. The unbound proteins were removed by washing; then, TAB was added, which catalyzed by HRP to produce a blue color after that an acidic stop solution was added to each well. Absorbance was measured at 450 nm using plate reader PowerWave STM (BioTek®, USA) and the higher in color intensity (yellow) is proportional to SOD concentration (ng mL⁻¹) in samples.

Cell Line and Cell Maintenance

The cytotoxicity of cisplatin was tested against human alveolar carcinoma A549 cells. The cells were kindly provided from Biotechnology Research Center / Al-Nahrain University and were maintained in RPMI-1640 medium with HEPES and L-glutamine supplemented with 15% fetal bovine serum (Sigma-Aldrich – Germany), Penicillin G (10³ IU 100⁻¹) and streptomycin (0.001 g 100⁻¹) were added to the culture medium and were supplied by Ajanta Pharm (India). Cells were incubated in a humidified atmosphere at 37°C, 5% CO₂.

MTT Assay

A colorimetric MTT assay (Intron Biotech, Korea) was performed in order to investigate the cytotoxic effect of cisplatin against A549 cells. In brief, 1×10⁴ to 1×10⁶ of A549 cells were suspended in 100 µL of complete RPMI-1640 medium and seeded onto 96-well plate containing another 100 µL of medium with the presence of cisplatin (concentration range from 5 to 80 µg mL⁻¹). All concentrations of cisplatin, in addition to control (untreated cells), were tested in triplicates. After 48 h of incubations, 20 µL of MTT solution was added to each well, and the plate was further incubated for 4 hours. The medium was carefully removed, and 100 µL of solubilization solution was added. The absorbance of the purple formazan crystals resulting from mitotically viable cells was measured at 570 nm using PowerWave STM (BioTek®, USA) plate reader.

A549 cells growth inhibition by cisplatin was calculated based on the following formula:

- Cytotoxicity (Inhibition Rate) % = 100 – [(Optical density of treated cells – optical density of blank) / (optical density of control – optical density of blank)] x 100.
- The effect of cisplatin against A549 cells was expressed by IC₅₀; the concentration of cisplatin required to inhibit 50% of A549 cell proliferation.

Reactive Oxygen Stress

A549 cells (1×10⁴) were seeded onto a 96-well plate for 24 h. After incubation, the medium was removed, and fresh medium

was added containing cisplatin and DMSO (as negative control) at indicated concentration, cells were further incubated overnight. After incubation, 50 μ L staining solution (RPMI-1640 containing 500 nM Hoechst 33342 and 2.5 μ g mL⁻¹ dihydroethidium) was added and incubated for 30 min at 37°C. Cells were fixed with 3.5% (in PBS) paraformaldehyde for 15 minutes then washed with PBS. The stained cells were analyzed using Cellomics ArrayScan High Content Screening reader (Thermo Scientific, USA).

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD). Significant variances ($p \leq 0.05$) between different groups were analyzed by one way ANOVA (Tukey) and one-tailed unpaired *t*-test using GraphPad Prism version 6 (GraphPad Prism Software Inc., La Jolla, CA, USA). In addition, IC₅₀ was calculated depending on GraphPad Prism software.

RESULTS AND DISCUSSION

Antioxidant activity of CAT, GPx, and SOD

The enzymatic activity of CAT, GPx, and SOD of healthy controls, newly diagnosed non-small lung cancer patients and cisplatin-treated non-small lung patients were summarized in Table 2 and Figure 1. Results showed that the activity of CAT was significantly decreased ($p = 0.0178$) in untreated patients as compared with healthy subjects, cisplatin-treated patients have retained the mean level of CAT and showed no significant difference with healthy subjects. GPx means value in untreated patients was significantly lower than the control

Table 1: Average biochemical parameters of antioxidant enzymes of healthy subjects ($n = 25$), cisplatin-treated subject ($n = 50$) and untreated patients ($n = 25$).

Subjects	CAT ng mL ⁻¹ (Mean \pm SD)	GPx U L ⁻¹ (Mean \pm SD)	SOD ng mL ⁻¹ (Mean \pm SD)
Healthy Control	41.3 \pm 2.70 ^a	39.60 \pm 2.60 ^a	35.40 \pm 3.50 ^a
Cisplatin Treated Patients	39.61 \pm 5.19 ^a	34.52 \pm 0.63 ^b	30.88 \pm 2.24 ^a
Untreated Patients	29.80 \pm 2.00 ^b	17.50 \pm 1.60 ^c	20.90 \pm 4.90 ^b

CAT: Catalase, GPx: Glutathione Peroxidase, SOD: Superoxide Dismutase, SD: Standard Deviation

Letters^{a, b, c} refers to significant result at $p \leq 0.05$

($p < 0.0001$), while in patients treated with cisplatin, the GPx mean activity increased. However, a significant difference was detected between the control group and patients treated with cisplatin ($p = 0.0312$). By comparing the mean value of SOD in patients after cisplatin treatment, we found that no significant differences with the respective SOD level of healthy subjects, while the newly untreated patients were significantly ($p = 0.0070$) differ from that of controls in their SOD mean level.

The respiratory system is immediately exposed to oxygen with increased generation of endogenous and exogenous oxidative stress. Under such conditions, ROS generates and plays a major role in lung cancer initiation. As a result, various antioxidant activities increased to normalize the ROS generation.¹⁶ In most types of cancer, including lung cancer,

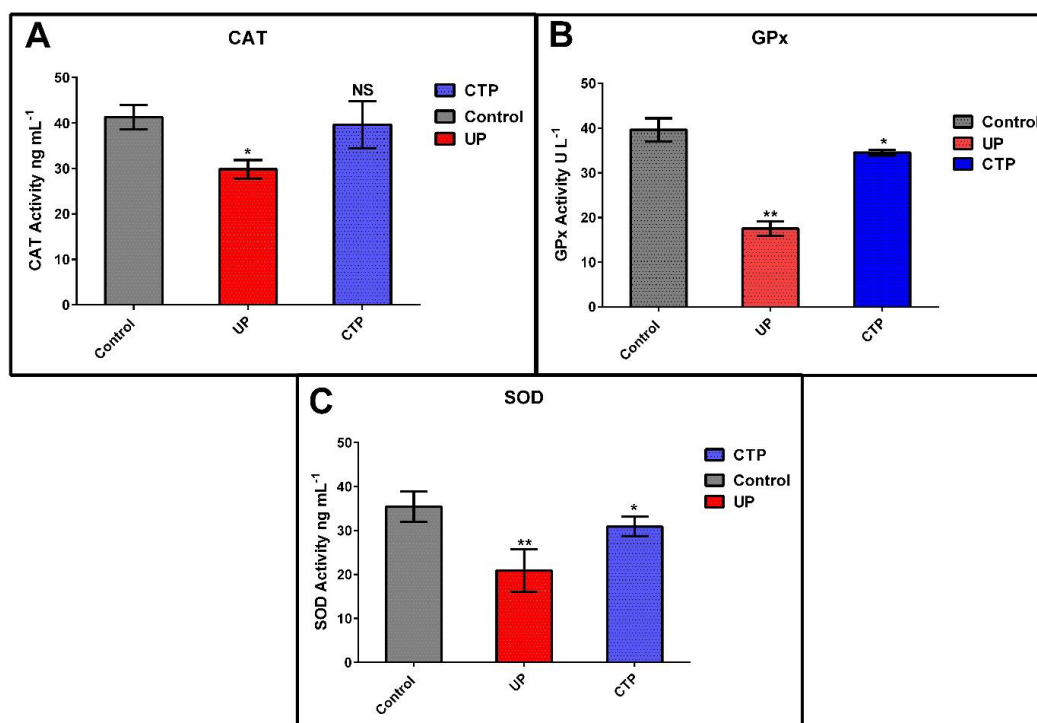


Figure 1: Graphical mean level (\pm SD) of A: Catalase, B: Glutathione Peroxidase and C: Superoxide Dismutase, in healthy control, untreated patients, and cisplatin-treated patients. NS: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$.

it was revealed an increased generation in ROS compared to normal tissues. Treatment with cisplatin as chemotherapy highly associated with ROS production; for this reason, enzymatic antioxidants were dramatically manipulated for detoxifying and decreasing the produced ROS, which in turn affect the toxicity of cisplatin. CAT confirms the scavenging of H_2O_2 by-products resulted from fatty acid oxidation into water and oxygen, which consumed in another metabolic process.¹⁷ CAT activity was significantly reduced in untreated patients; this is in concordance with Ho et al., which were proposed the reduction in CAT activity within the cancerous lung tissue, on the other hand, increased production of intracellular H_2O_2 and lung cancer promoters [18]. The increased activity of CAT in patients treated cisplatin may contribute to the synergistic effect of CAT along with cisplatin; such effect is significantly increased by increasing the production of H_2O_2 [19].

Figure 1: Graphical mean level (\pm SD) of A: Catalase, B: Glutathione Peroxidase and C: Superoxide Dismutase, in healthy control, untreated patients, and cisplatin-treated patients. NS: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$.

GPx is an intracellular antioxidant scavenging enzyme that catalyzes the reduction of free H_2O_2 into water and oxygen; in addition, GPx reduces lipid hydroperoxides into alcohol.²⁰ The role of GPx in maintaining the generation of ROS is well known among different types of cancer.²¹ Our study found that newly diagnosed patients showed significantly lower levels of GPx compared to control, this result is in agreement with Oh et al. .²² The accumulation of DNA damaging ROS agents in lung cancer cells dramatically affect the cell genomic integrity and cause expression down-regulation or inactivation of genes involved in ROS scavenging including GPx.²³ GPx also plays a significant role in developing cisplatin chemo-resistance upon successive treatment with cisplatin,²⁴ this might explain

the overexpression of GPx in cisplatin-treated patients with enhanced GPx enzymatic activity.

SOD considered being the first line of protection against free radicals, which catalyze the dismutation of free radicles (O_2^-) into either oxygen or H_2O_2 [25]. Many studies confirmed our findings regarding the lower levels of SOD in lung cancer patients' pre-treatment with cisplatin.^{14,4} Moreover, cisplatin-treated patients showed no significant differences comparing with control. Little information is known about the correlation between cisplatin and SOD activity. However, Srivastava et al. reported the down-regulation of SOD in lung cancer patients treated with cisplatin.¹⁴

Cisplatin Cytotoxicity

The cytotoxicity of cisplatin was evaluated against A549 tumor cell line using a range of concentrations starting from 5 to 80 $\mu g mL^{-1}$ for 24 h incubation. Results in Figure 2 shows that the cell viability of A549 cells was decreased in a dose-dependent pattern with a maximum inhibition rate (78.51 ± 6.19 %) was at 80 $\mu g mL^{-1}$. The IC_{50} for cisplatin against A549 cells was calculated to be 10.33 $\mu g mL^{-1}$. Cisplatin is a well-known, widely used antitumor chemotherapy for pediatric and adult cancer patients.²⁶ Many studies reported that cisplatin affected many cellular pathways, including: apoptosis, cell proliferation, arresting of the cell cycle, and also DNA damaging via ROS generation.^{27,28} The DNA damage by cisplatin mainly involved mitochondrial DNA (mtDNA). Induction of mtDNA damage by cisplatin affects the mitochondrial function and promotes cell apoptosis via the intrinsic apoptotic pathway, which mainly depends on apoptotic genes (*Bcl-2* and *BAX/BAD*) and activation of caspases.²⁹

Cisplatin-Induced ROS Response

Cell apoptosis induced by cytotoxic cisplatin is highly correlated with increased generation of ROS.³⁰⁺ Results in Figure 3 (A, B) confirmed that increased concentration of cisplatin significantly increased the generation of ROS as compared with DMSO-treated cells after 24 h incubation. It was detected a 12.12% increasing in ROS generation after cisplatin treatment of 25 $\mu g mL^{-1}$ ($p = 0.034$), while treatment with 50 $\mu g mL^{-1}$ exhibited 34.5% increase in ROS. The induction of ROS via cisplatin treatment directly affected mtDNA and thus cause distribution in DNA integrity and mitochondrial redox status.³¹

On the other hand, it was confirmed that mitochondrial damage observed in cisplatin treatment that induced nephrotoxicity and neurotoxicity, resulted in the delivery of antioxidant to mitochondria to reduce ROS generated by cisplatin and thus reduce toxicity.³² This may be related to the increased level of antioxidants regarding the cisplatin-treated group of our study. Increased production of ROS by increasing cisplatin concentration intensely distributed mitochondrial membrane permeability, which at the end induced the release of apoptotic molecules, including cytochrome C.³³

Figure 3: ROS generation in A549 cisplatin-treated cells. A: A549 cells were treated with DMSO (negative control) and cisplatin at 25 and 50 $\mu g mL^{-1}$ for 25 h. Cells were stained

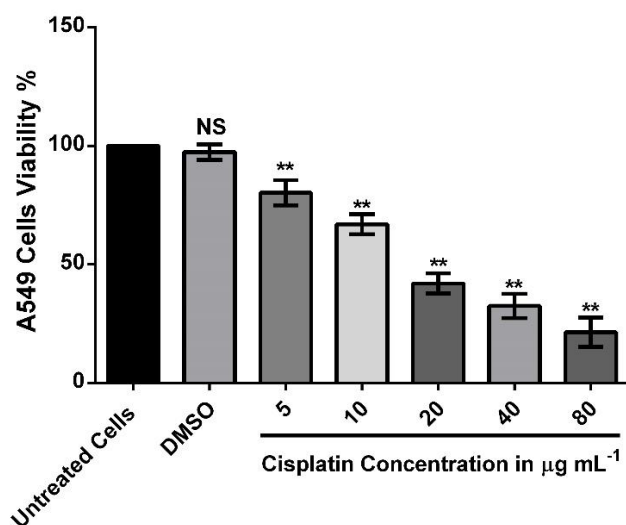


Figure 2: Figure 3 Dose-dependent histogram. The cell survival of A549 cells (%) after treatment with a range of cisplatin concentrations for 24 h. NS: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$.

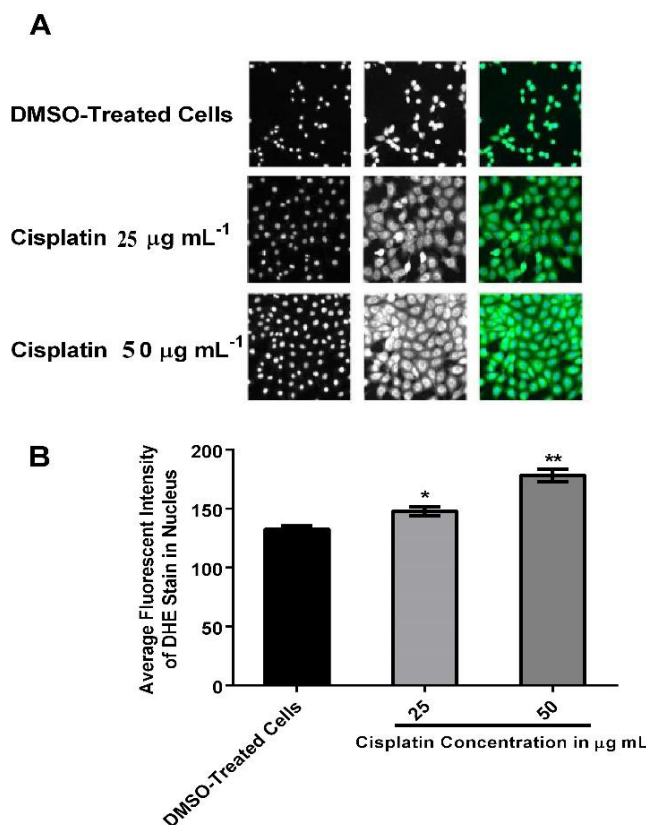


Figure 3: ROS generation in A549 cisplatin-treated cells. A: A549 cells were treated with DMSO (negative control) and cisplatin at 25 and 50 $\mu\text{g mL}^{-1}$ for 25 h. Cells were stained with DHE dye and visualized using HSC array scan. B: Mean intensity of DHE fluorescent dye ($\pm\text{SD}$) in A549 cells treated with DMSO, 25, and 50 $\mu\text{g mL}^{-1}$ of cisplatin. **: $p \leq 0.01$, *: $p \leq 0.05$.

with DHE dye and visualized using HSC array scan. B: Mean intensity of DHE fluorescent dye ($\pm\text{SD}$) in A549 cells treated with DMSO, 25, and 50 $\mu\text{g mL}^{-1}$ of cisplatin. **: $p \leq 0.01$, *: $p \leq 0.05$.

CONCLUSION

In our study, we can conclude that lung cancer patients exhibited a significant reduction in the level of antioxidant enzymes; however, during the process of cisplatin treatment, the enzymatic levels started to maintained to normal range. Cytotoxic activity of cisplatin confirmed the potent inhibition of cell viability via increasing generation of ROS which eventually entering the cell into apoptosis.

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