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Mitochondrial pathway mediated apoptosis and cell cycle arrest triggered by cobalt(III) complex in Dalton's lymphoma cells

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ABSTRACT

Cancer is a group of diseases which evolves from uncontrolled growth of abnormal cells. It has become the second leading cause of death worldwide. Many platinum-based compounds like cisplatin and carboplatin are widely used as a therapeutic approach against cancer, but patients are resistant to these therapeutic agents with life-threatening side effects. In this study, we used a non-platinum-based compound, i.e. cobalt complex, on the Dalton's lymphoma cells (DL cells). It is a tumor model of murine T-cell lymphoma of thymic genesis, which is very aggressive. The present study was focused on evaluating the anticancer efficacy of cobalt complex on DL cells. The IC_{50} value of cobalt complex was found to be 80 ±3.21 µM in DL cells'. In addition, cobalt complex promoted condensed nuclei, G1 phase arrest, and induced early and late apoptotic cells. Additionally, flow cytometry examination showed a significant loss in mitochondrial membrane potential and induction of reactive oxygen species after treatment. Furthermore, Western blot revealed the increased p53, cyt-c, Bax protein, and decreased Bcl2 protein level in treated DL cells. These results verified the anticancer properties of cobalt(III) complex against DL cells and propose an alternative therapeutic drug in cancer treatment.

INTRODUCTION

Cancer is an incurable group of diseases which arises due to irregular cell cycle or abnormal proliferation. Cancer has become an emerging cause of death worldwide following cardiovascular disease (Yusuf *et al*., 2015). According to the World Health Organization, 19.3 million new cases were reported and 9.9 million deaths took place in the year 2020 worldwide (Siegel *et al*., 2020). There are more than 100 types of cancers reported, and their risk factor may be internal or environmental, such as diet and obesity, smoking, infection, radiation, stress, and the genetic (Anand *et al*., 2008). There is a benign or malignant type of tumor in which the benign tumor is non-cancerous and can be cured through surgery. On the contrary, a malignant tumor is cancerous in nature and spreads to other parts of the body (Hanahan and Weinberg, 2000). Currently, surgery, chemotherapy, and radiotherapy are the major therapeutic regimens for cancer treatment, but chemotherapy is still key therapy in which chemically synthesized compounds are used to combat cancer. Presently, cisplatin, carboplatin, and oxaliplatin are widely used as anticancer drugs worldwide for clinical purposes. About 50% of platinum-based metal complexes have been used in tumor therapies and exhibit remarkable therapeutic agents in a series of solid tumors (Wong, 2011). Different therapeutics or synthesized drugs, like platinum-based regimes, were used from the last five to six decades, but due to their severe side effects, these drugs are switched to non-platinum-based drugs which give promising results, such as copper, gold, ruthenium, and cobalt. Transition metals of the periodic table have more advantages over the platinum-based compound like wide structural diversity, thermodynamic and kinetic of ligand substitution, coordination numbers, geometry, and also they do not imitate the mode of action of cisplatin (Ndagi *et al*., 2017). Among these transition metals, cobalt (atomic number-27) shows biological interest and is present in the active center of vitamin B_{12}, which indirectly helps in the synthesis of DNA (Ortega *et al*., 2009). Cobalt has become an emerging metal ion in cancer treatment because of its oxidation,

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redox reaction, and coordination with the respective ligand (Richardson *et al*., 2009). Cobalt compounds have been useful in the treatment of anemia, along with being used as a substitute to traditional blood doping in sport (Simonsen *et al*., 2011). Several studies showed that metal-based drugs induce apoptosis in the cancer cell to prevent progression and proliferation. Metal complexes showed higher efficacies in killing cancer cells, which might be linked to targeting various factors such as DNA or RNA and protein (Ratanaphan *et al*., 2016). Apoptosis is an involuntary mechanism of cell death where an unwanted cell is removed from the tissue, and it is also an efficient strategy to kill the cancer cells. Cell shrinkage, heterochromatin condensation, apoptotic cell, mitochondrial membrane potential loss, and reactive oxygen species (ROS) generation are the identifications of apoptosis in cells (Arbiser *et al*., 2017). It may be induced through intrinsic and/or extrinsic pathways (Kroemer *et al*., 2009; Saelens *et al*., 2004). Therefore, the synthesis of different therapeutics or synthesized drugs to initiate apoptosis in cancer cells has become more interesting in the area of oncology. Dalton's lymphoma is a non-Hodgkin's lymphoma which is a highly deleterious invasive tumor and originates from the thymus of a murine host (Cheson *et al*., 2014). It is a rarer cancer of poor prognosis whose clinical management is extremely complicated due to its development of insensitivity to well-established anti-cancer drugs. In this study, we evaluate the anticancer efficacy of the cobalt complex ([Co(ftsm)NH3(o-phen)]·H_2O {ftsm=bis(5-furan-1,2,4-triazole)-3-sulfinamide}) (Fig. 1) on Dalton's lymphoma cells.

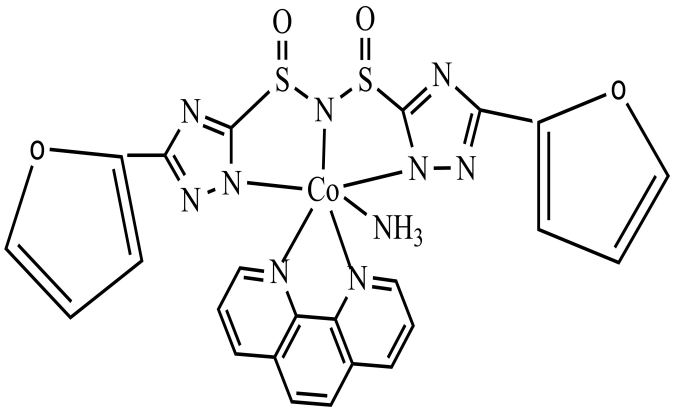

Figure 1. Chemical structure shows centered cobalt metal ion bonded with furan ring bis(5-furan-1,2,4-triazole)-3-sulfinamide.

MATERIALS AND METHODS

Reagents

Chemicals and reagents were obtained as indicated: 3-(4,5-dimethylthiazol- 2-yl)-2-5-diphenyltetrazolium bromide (MTT) for cell viability, 4,6-diamidino-2-phenylindole (DAPI) for morphology, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, USA). Rhodamine-123 and propidium iodide dye (PI) for cell cycle and 2',7'-Dichloro-dihydro-fluorescein diacetate (DCFD-HA) were taken from Sigma Chemical Company (St. Louis, USA), and used for flow cytometry. Roswell Park Memorial Institute-1640 (RPMI-1640) and Fetal Bovine Serum (FSB) were obtained from Thermofisher (South America); Polyvinylidene Fluoride (PVDF) membrane (0.45 um pore size) was purchased from Merck. Apoptosis kit was obtained from Invitrogen (USA). Primary antibodies and secondary antibody Immunoglobulin G (IgG) (H + L), Horseradish Peroxidase (HRP) conjugates, were obtained from Invitrogen^{tm}, ThermoFisher (USA), and other reagents were taken from regional firms, and they were of molecular grade.

Animal and drugs treatment

The Balb/c mouse (25 ± 2 g) was used in the present study. Standard conditions were maintained for Balb/c mice, i.e. 12–12 hours light and dark, 23°C ± 2°C temperature, and provided standard food pellets *ad libitum*. Dalton's lymphoma cells (DL cells) (1×10^6) were transferred intraperitoneally after 12–16 days, DL cells were collected from DL stance mice, and cultured in 5% CO_2 incubator at 37°C in RPMI-1640 added with 10% fetal bovine serum (50 µg/ml penicillin, 50–100 U/ml streptomycin, 1% essential amino acid, and sodium bicarbonate). DL cells (1×10^6 cells/ml) were incubated with different concentrations of cobalt compound in multi-well culture plates. After incubation, cells were washed and various experiments were carried out. Care of mice, animal breeding, and *in vitro* experiments were carried out after the approval of the institutional ethical committee.

Cell viability assay

After treatment with cobalt complex, cell viability or cytotoxicity was evaluated by MTT assay. Briefly, DL cells (10,000 cells/well) were cultured with different concentrations of ligand and cobalt complex in a 96-well culture plate using RPMI-1640 enriched with fetal calf serum (10% *v/v*) for further process. Thereafter, DL cells were cultured with 10 µl (5 mg/ml) of MTT reagent for 3–4 hours at 37°C. Live cells have the ability to convert MTT reagent into visible formazan crystals. These crystals were solubilized with DMSO, and their optical density was taken at 595 nm by a microplate reader. Relative cell viability or cytotoxicity was calculated by comparing with control (Nath *et al*., 2017).

Nuclear morphological assessment

The DAPI staining was executed to assess nuclear morphological changes. Briefly, DL cells (1×10^5–10^6 cells/ml) were cultured in the multi-well plate with cobalt compound at different concentrations. After incubation for 24 hours, DL cells were cleaned three times in phosphate-buffered saline (PBS), and fixed by using 4% paraformaldehyde solution, and permeabilized by 0.1% Triton-×100 reagent and kept at room temperature for 10 minutes each. The fixed DL cells were washed and stained with DAPI solution (1 µg/ml) in the dark condition for 5 minutes at Room Temperature (RTM). The nuclear morphology of the DL cells was studied by fluorescence microscopy (Nikkon E800, Japan), and the images were taken at 20×x magnification (Lee *et al*., 2016).

Acridine orange/ethidium bromide (Ao/EtBr) staining

DL cells (1×10^6 cell/well) were cultured with different concentrations of the cobalt complex for 24 hours at 37°C and 5% CO_2. Subsequently, the DL cells were washed and stained with ethidium bromide (100 µg/ml) and acridine orange (100 µg/ml) in equal ratio. After washing with PBS, cells were examined under a fluorescence microscope (Nikkon E800, Japan), and the images were taken at 20×x magnification (Oliveira *et al*., 2016).

Cell cycle analysis

To analyze cell cycle progression, DL cells were cultured at a density of 1×10^6 cells/well at different time periods with the cobalt complex. After the incubation period, the DL cells were washed, and fixed in chilled 70% ethanol overnight at 4°C. RNase (10 µg/ml), 20 µl of PI (20 µg/ml), and 500 µl of PBS at 37°C for 30 minutes were added after washing with chilled PBS. The red fluorescence (PI) from cells was excited at 488 nm using channel FL2-A (Singh *et al*., 2016). The determination of the distribution of cells in different stages or phases of the cell cycle was achieved by flow cytometry (Becton Dickinson, California, USA) with 10,000 events per sample. The data were analyzed by using Flow Jo software.

Apoptosis assay

To check the effect of cobalt complex on apoptosis in DL cells, annexin V-FITC/PI stain was carried out. In brief, cells were cultured in the multi-well plate with different cobalt concentrations, later washed with chilled PBS three times, and next suspended the cells in the binding buffer. 5 µl of annexin V-FITC and PI dye were added, respectively, and then incubated for 15 minutes. The proportion of apoptotic cells of each sample was determined by flow cytometer (Becton Dickinson, California, USA) and analyzed with Flow Jo software (Wang *et al*., 2018).

ROS generation

To check the possible cause of apoptosis, DCFH-DA fluorescence dye was used in which cells were cultured in the multi-well plate with cobalt complex at different concentrations. After incubation for 24 hours, cells were cleaned three times with chilled PBS and added 10 µl of DCFH-DA dye at 37°C for 30–40 minutes. Then, ROS generation through cobalt complex at different concentrations was examined by a flow cytometer (Becton Dickinson, California, USA) (Rastogi *et al*., 2010).

Mitochondrial membrane potential (ΔΨm)

Loss of ΔΨm due to apoptosis by cobalt complex at different concentrations was carried out by Rhodamine-123 dyes through Fluorescence-activated cell sorting (FACS). Briefly, DL cells were incubated with the cobalt complex for 24 hours. Rhodamine-123 dye was added 30 minutes before the analysis. The DL cells were accumulated and washed with chilled PBS and analyzed through FACScan (Becton Dickinson, California, USA) (Bhushan *et al*., 2013). The data were analyzed using Flow Jo software.

Western blotting

Expression of apoptotic-related proteins like Bcl2, Bax, cyt-c, and p53 were investigated by Western blotting. Briefly, DL cells were cultured with the cobalt complex at different concentrations for 24 hours in a multi-well plate. After incubation, cells were cleaned with chilled PBS, and total protein content was isolated by Radioimmunoprecipitation Assay (RIPA) buffer. The total protein concentration was estimated by Bradford's method; an equal amount of protein was loaded on SDS PAGE; after that, it was transferred onto the PVDF membrane. Blocking was carried out with 5% skimmed milk, and PVDF membrane was incubated with the primary antibody of standard dilution at 4°C overnight. The next day, the membrane was washed with Tris-Buffered Saline Tween (TBST) thrice and added secondary antibody of standard dilution for 2 hours. After that, the band was developed on X-ray film with the help of enhanced chemiluminescent and analyzed by the software ImageJ.

Statistical analysis

Three independent experiments were carried out; mean and standard deviation were used to represent the data. We used one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test and two ways ANOVA, followed by Bonferroni's post-test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered as significant.

RESULTS AND DISCUSSION

Cobalt complex reduced cell proliferation of DL cells

Firstly, *in vitro* cell viability or the cytotoxicity of cobalt complex on DL cell was carried out by MTT colorimetric assay which was used to evaluate the cellular metabolic activities, and also it provided the number of live and dead cells. The results obtained showed that the cobalt complex significantly reduced cell viability of DL cells as compared to ligand Figure 2A and B. Cobalt complex-induced cytotoxicity responses to different concentrations. The IC_{50} values of the ligand and cobalt complex were 150 µM ± 5 and 80 µM ± 3.21, respectively. Therefore, the present investigation shows that the ligand containing metal ion has high cytotoxicity at a lower dose.

Cobalt complex alters the morphology of DL cells

Next, we analyzed the differences in the nuclear structure of DL cells by using the DAPI staining method. In the control, the DL cells were equally stained, rounded, and showed less DAPI stain. After 24 hours treatment with different concentrations of cobalt complex, the DL cells showed morphological changes such as nuclear fragmentation, chromatin condensation, and more DAPI positive cells were observed (Fig. 3).

Cobalt complex-induce apoptosis in DL cells

Apoptosis is crucial for any compound that revealed anticancer activity and leads to the death of cancer cells. Fluorescence microscopy is generally used to examine the cell morphology, and cell death induces by cobalt complex stained with AO/EtBr. This staining was carried out to detect live and apoptotic cells. After treatment with different concentrations, the results show that with the increase in the concentration of complex leads to significant increments in apoptosis of DL cells when compared to the control. Live cells and apoptotic cells were indicated by green and yellow arrows, respectively (Fig. 4).

Cobalt complex-induced G1 phase arrest in DL cells

The event of the cell cycle is very important for the regulation of genomic integrity via the formation of new daughter cells. In order to analyze whether the cobalt complex is involved in anti-proliferative activity by arresting cell cycle, PI staining was carried out. DL cells treated with cobalt complex arrest the cell cycle in G1 phase in a time-dependent manner. The cell cycle analysis of different phases by flow cytometry showed that the cobalt complex significantly induced the percentage of G1 phase cells by 49.7%, 52.5%, and 60.6% at 0, 12 , and 24 hours, respectively (Fig. 5).

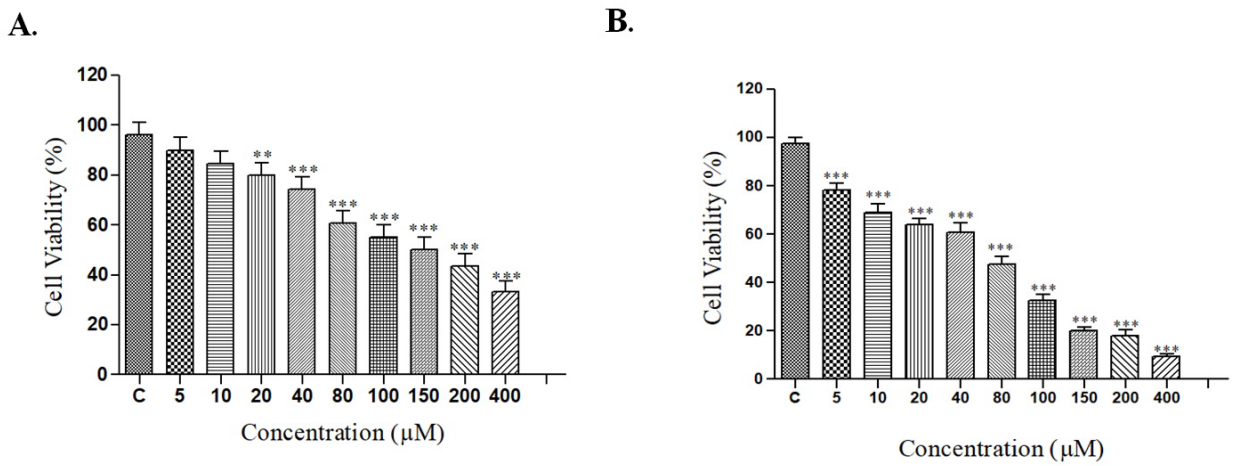

Figure 2. DL cells (1×10^6 cells/ml) were cultured with different concentrations of the ligand and cobalt complex for 24 hours, and cytotoxicity of the compounds was evaluated through standard MTT assay as described in the Material and Methods section. The bar graph represents the cytotoxicity of (A) ligand and (B) cobalt complex against DL cells. Results are indicated as a percentage of mean and SD for the least three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ are studied as statistically significant versus respective control.

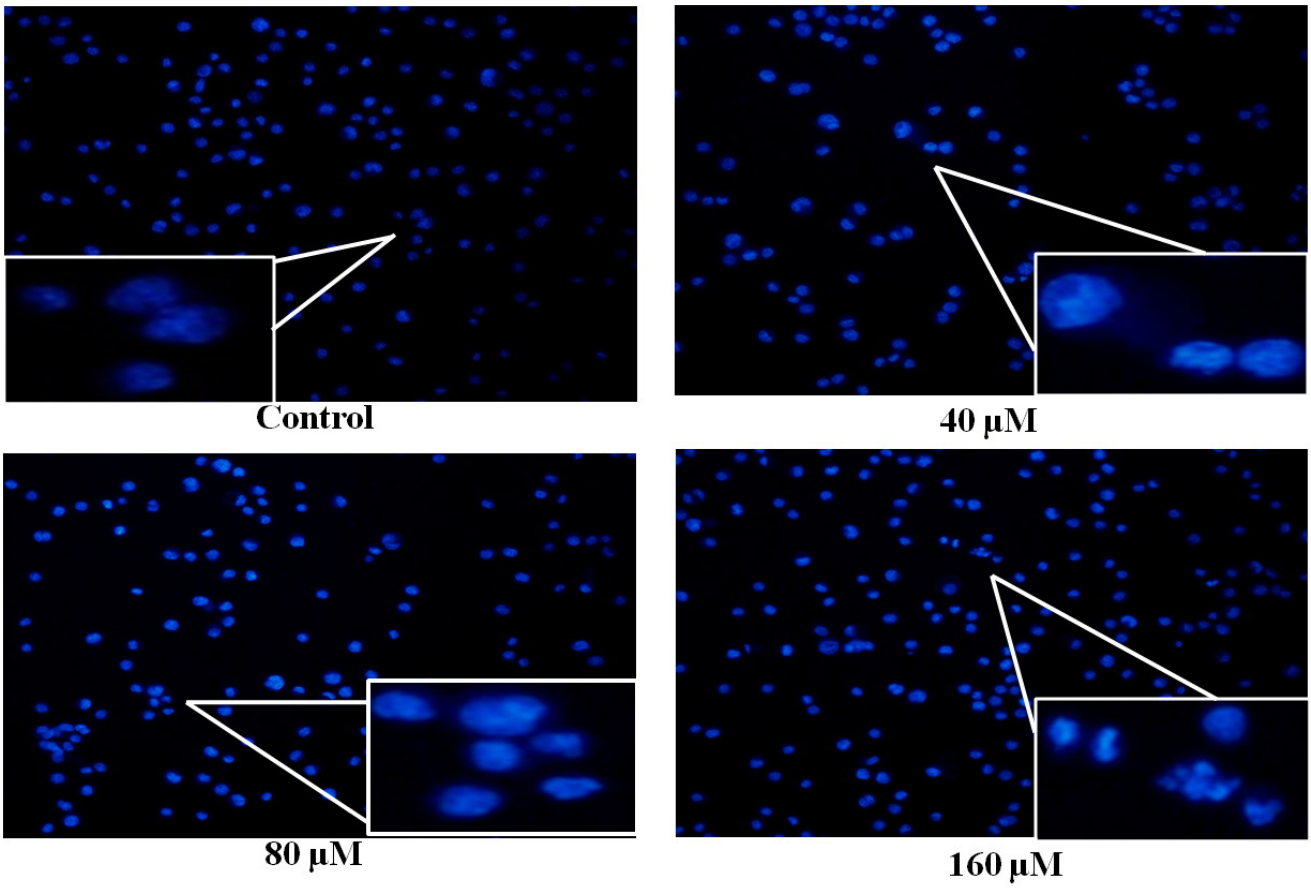

Figure 3. DL cells (1×10^6 cells/well) were incubated with (40, 80, and 160 µM) concentrations of cobalt compound for 24 hours and evaluation of nuclear morphology by DAPI staining using a fluorescence microscope.

Cobalt complex-induced apoptosis in DL cells

A little broader and vicenary evaluation of apoptosis in DL cells was conducted through Annexin V/PI which detects the externalized phosphatidylserine on the plasma membrane of dead cells. Different cell stages were identified by counterstaining with membrane-impermeable dye PI that checks the membrane integrity of cells with Annexin V. As shown in Figure 6, it was found that untreated conditions (control) have 99.4% viable cells, whereas in treated groups, the percentage of early and late apoptotic cells significantly increases as the concentration increased. It was seen that the treatment

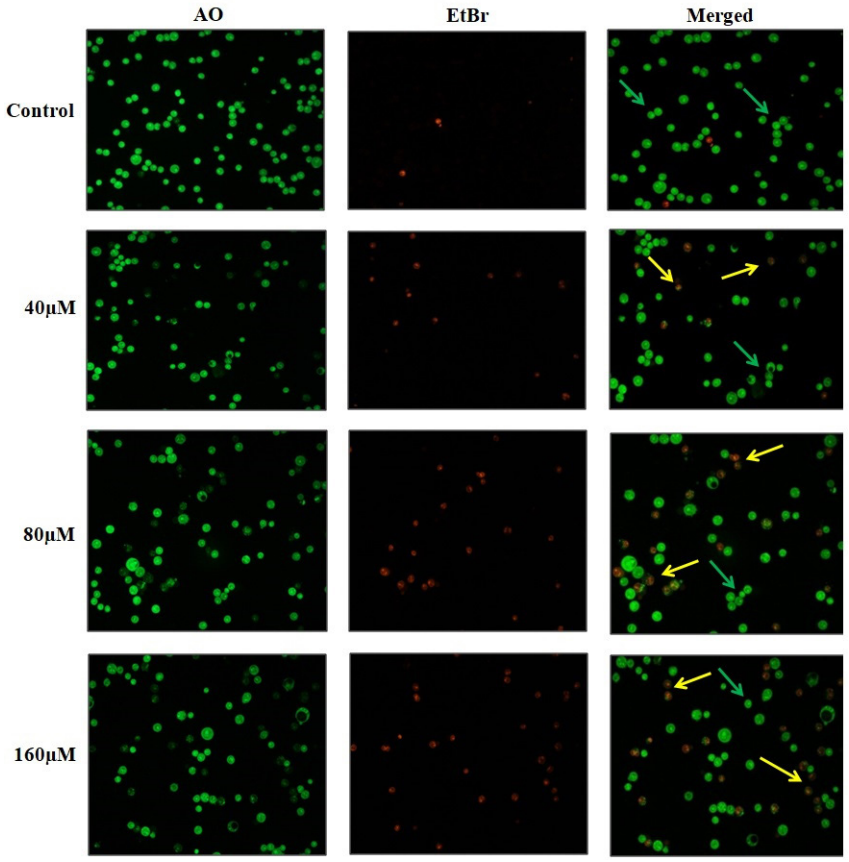

Figure 4. Composite image of A0/EtBr-stained DL cells after treatment with cobalt complex at different concentrations. The green and yellow arrows show live and early apoptotic cells, respectively. At least three similar experiments were executed, and data are presented in the magnification of the 20×x fluorescence microscope.

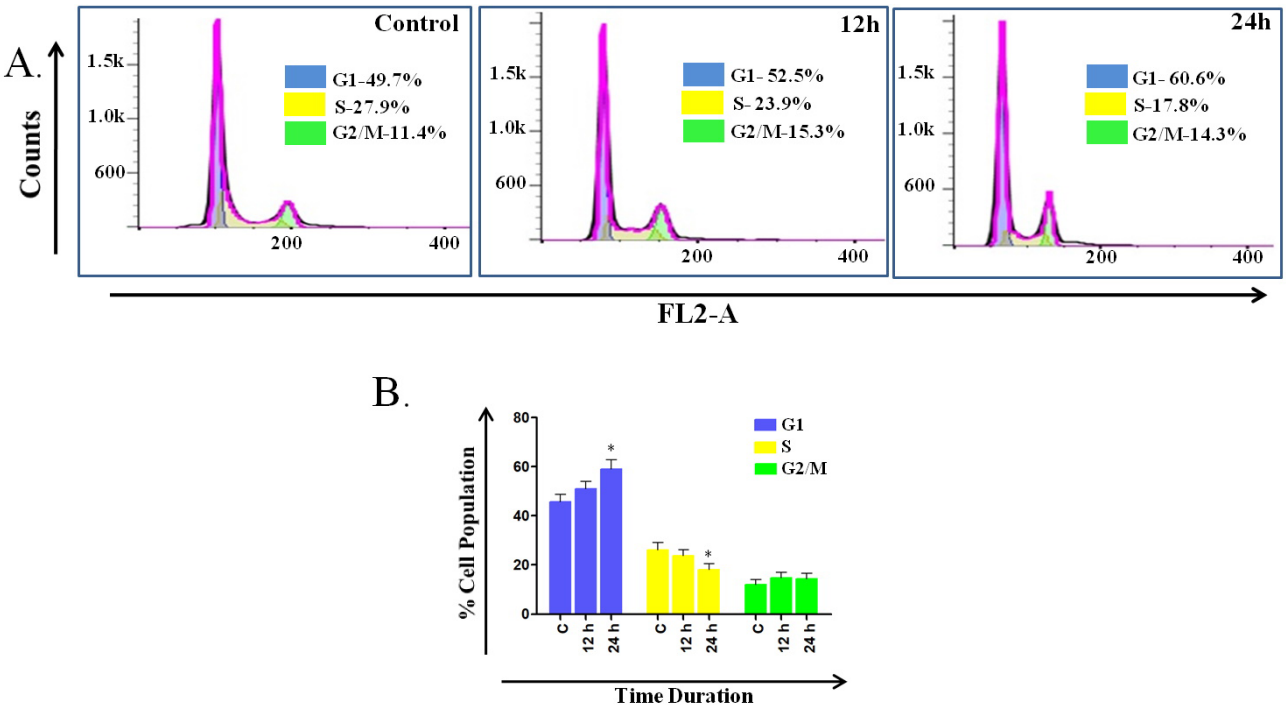

Figure 5. (A) Cell cycle analysis of DL cells labeled with PI dye after treatment with cobalt complex for 12 and 24 hours. (B) Cell cycle distribution is represented as percentages at each time point. *$p < 0.05$ is examined as statistically significant versus control.

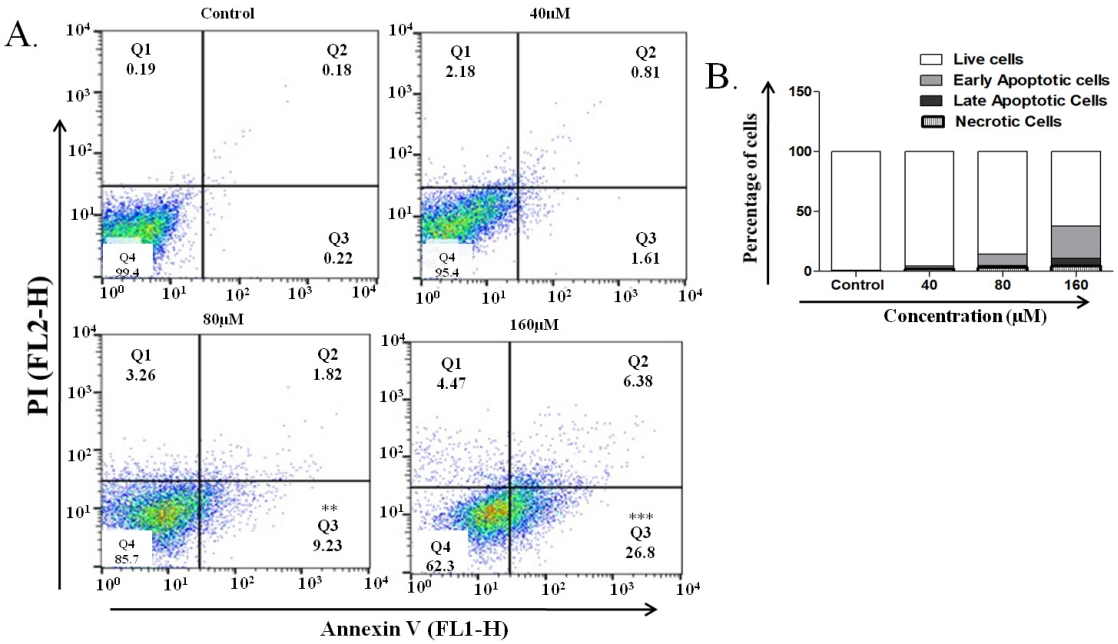

Figure 6. Cobalt complex induces apoptosis in DL cells. (A) Flow cytometry analysis of DL cells after treatment with different concentrations of cobalt complex. Annexin V/PI fluorescence staining differentiated as live, early apoptotic, late apoptotic, and necrotic cells. (B) The histogram represents the percent of different cell stages against a different concentration of cobalt complex. $^{**}p < 0.01$ and $^{***}p < 0.001$ are considered as statically significant versus respective control.

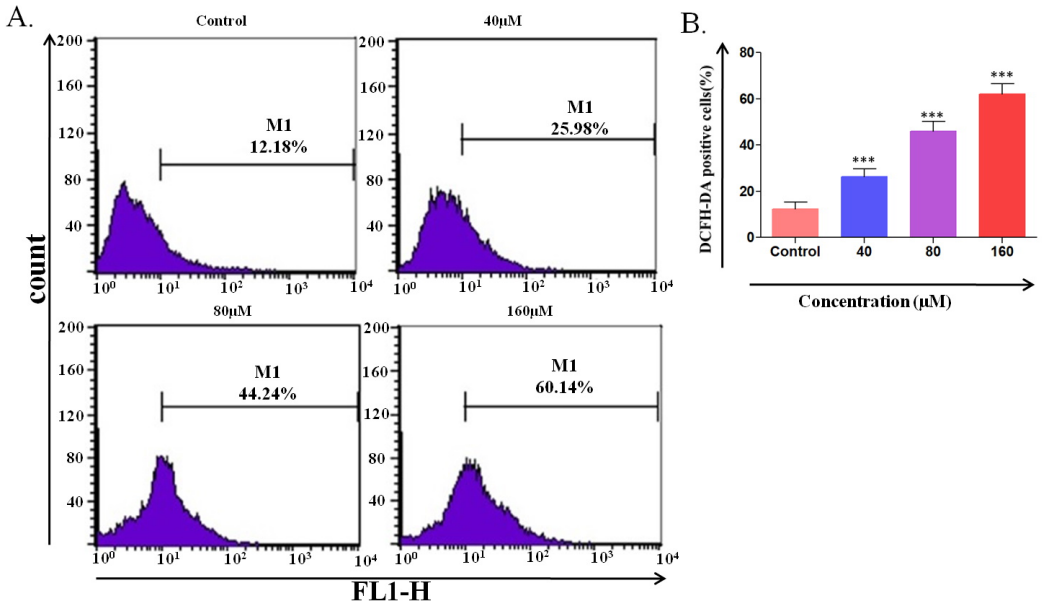

Figure 7. DL cells were cultured with different concentrations of the cobalt complex. Detection of intracellular ROS was carried out through DCFD-HA staining. (A) DCF fluorescent intensity represents the ROS generation in the cell evaluated by flow cytometry. (B) The histogram represents the ROS level in concentration-dependent manners $^{***}p < 0.001$ is considered as statistically significant versus control.

with the cobalt complex increased the percentage of early apoptotic cells by 1.61%, 9.23%, and 26.8%, and late apoptotic cells by 0.81%, 1.82%, and 6.38% at 40, 80, and 160 µM, respectively.

Cobalt complex up-regulate ROS level in DL cells

To evaluate apoptosis of the cell was induced by the cobalt complex is ROS dependent or independent, DCFD-HA staining was performed. ROS generation in cancer cells was generally elevated for their angiogenesis, cell survival, cell cycle progression, proliferation, energy metabolism, and maintenance of tumor stemness, but several studies showed that immeasurable increases in ROS levels can increase apoptosis and arrest cell cycle significantly in cancer cells (Li *et al.*, 2012, 2014). Untreated cells were produced a low level of ROS generation detected by low DCF fluorescence intensity (12.18%). On the contrary, treated cells were generating more intracellular ROS

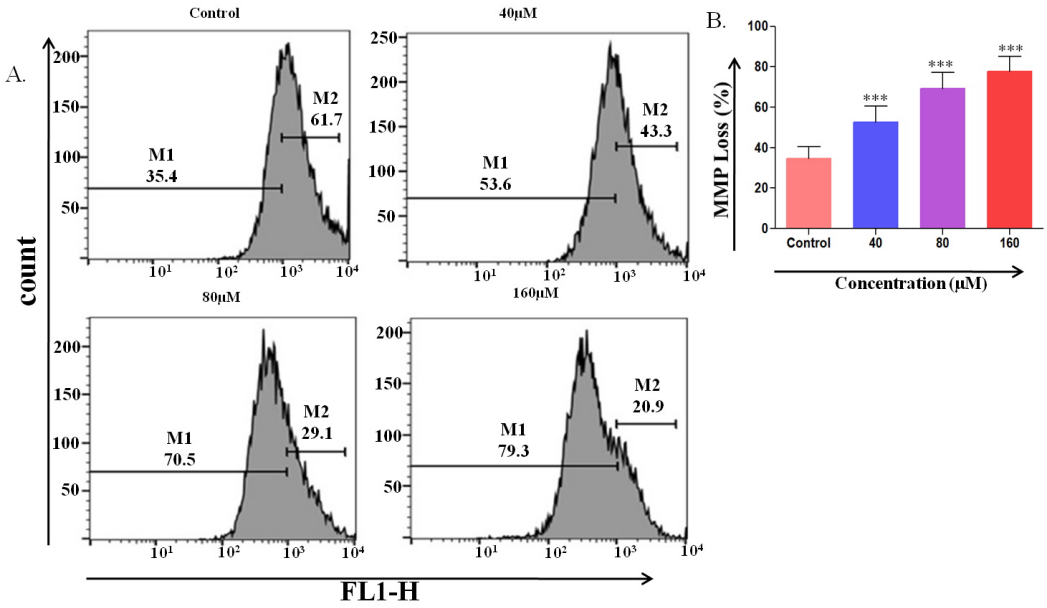

Figure 8. Cobalt complex reduced mitochondrial membrane potential in DL cells. (A) DL cells were stained with Rh-123 dye for 30 minutes and examined by FACS. The histograms showed the left shifting because of the decay of Rh-123 fluorescent in treated conditions. (B) The bar graph represents the MMP loss (%) in concentration-dependent manners. ***$p < 0.001$ is considered as statistically significant versus control.

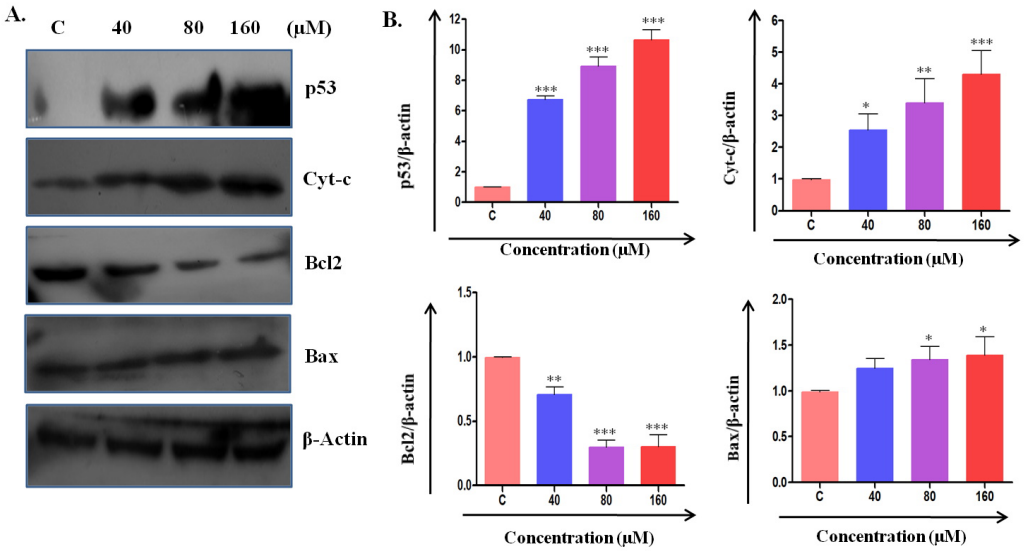

Figure 9. (A) The expression of cyt-c, p53, Bax, and Bcl-2 protein levels after treatment with different concentrations of cobalt complex for 24 hours was conducted by Western blot technique. (B) The expression level of p53, cyt-c, Bcl-2, and Bax. The protein was shown by bar graph against β-actin. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are considered as statistically significant versus control.

in a concentration-dependent manner (25.98%, 44.24%, and 60.14%), as shown by flow cytometry (Fig. 7). These results clearly showed that the cobalt complex produced intracellular ROS, which indicated ROS-mediated apoptosis in DL cells.

Cobalt complex reduce mitochondrial membrane potential (ΔΨm) in DL cells

Mitochondrial membrane potential is tightly regulated in cells; any disturbances in the potential will lead to death. Loss of the mitochondrial membrane potential was examined through Rhodaimne-123 dye which is a cationic lipophilic, and quenched by healthy mitochondria to produce fluorescence that was measured by flow cytometry (Magdalena *et al*., 2010). To understand how the cobalt complexes influence the mitochondrial-dependent apoptotic signaling pathway, the differences in ΔΨm were initially detected. DL cells were incubated with cobalt complex at different concentrations for 24 hours, and it was found that there was a significant reduction in mitochondrial membrane

potential. The cobalt complex treatment at different concentrations (40, 80, and 160 μM) reduces mitochondrial membrane potential by 53.6%, 70.5%, and 79.3%, respectively (Fig. 8).

Cobalt complex-induce apoptosis in DL cells through mitochondrial pathways

To understand the effect of cobalt complex on apoptotic-related proteins, Western blot analysis was executed. The analysis showed that with the increasing concentration of cobalt complex, the expression of Bax, cyt-c, and p53 protein level was upregulated, and Bcl-2 protein level was downregulated. When mitochondrial membrane breaks, cyt-c goes into the cytoplasm, and a series of downward signaling occurs. Our result showed that cyt-c protein level was increased in cells with an increase in concentration which may lead to apoptosis of DL cells. Altogether, we found that cobalt complex induced apoptosis in DL cells via mitochondrial-mediated pathway (Fig. 9).

CONCLUSION

This study concluded that cobalt(III) complex had potent anticancer activity against DL cells which has been confirmed by high cytotoxicity at a low dose, anti-proliferative activity, or cell cycle arrest, generation of reactive oxygen species, loss of mitochondrial membrane potential, and Western blot analysis. Collectively, cobalt complexes could be used to design novel anti-cancer drugs and prevent cancer growth.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors disclose that they have no conflict of interest in this paper.

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ETHICAL APPROVALS

Ethical approval was taken from institutional ethical committee.

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