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Original Article

Emblica extract prevents cisplatin-induced apoptosis in dermal papilla fibroblasts

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Abstract

Cisplatin is a widely prescribed anticancer agent that causes hair loss in patients. Since the dermal papilla (DP) fibroblasts are known to be a key mediator in controlling hair growth and loss, understanding the effect and underlying mechanism of cisplatin on these cells may lead to new strategy for hair loss protection in chemotherapy patients. Less is known regarding the effect of cisplatin on DP fibroblasts. We thus treated DP cells with cisplatin (0-250 μmol/L) and found that cisplatin induced DP cell death in a concentration-dependent manner. Our results showed that the mode of cisplatin-induced DP cell death was mainly through apoptosis mechanism. Our results also indicated that the intracellular reactive oxygen species (ROS) induction caused by cisplatin treatment was associated with DP cell apoptosis and cisplatin-induced apoptosis could be attenuated by addition of antioxidant N-acetylcysteine (1 mmol/L) and glutathione (5 mmol/L). Moreover, subtoxic concentrations (0-500 μg/mL) of *Phyllanthus emblica* Linn (emblica) extracts, a known natural antioxidant, showed a strong inhibition effect on cisplatin-induced intracellular ROS induction and subsequently protected DP cells from cisplatin-induced apoptosis.

Keywords: dermal papilla, cisplatin, hair loss, reactive oxygen species (ROS)

1. Introduction

With the incidence rate approximately 65% of all patients who received chemotherapy, alopecia (hair loss), a frequent toxicity of various chemotherapeutic agents, is considered to be one of the most feared side effects for cancer patients (Wang *et al*., 2006). Molecular mechanisms of chemotherapy-induced hair loss have been intensively investigated during the last decade using rodent model as well as cell culture. Although some studies have shown that hair loss induced by chemotherapeutic agents was related to the apoptosis of hair matrix keratinocytes in hair follicle (Paus *et al.*, 1994; Schilli *et al.*, 1998), recent studies have reported that dermal papilla (DP) fibroblast cell, a precursor of a hair follicle, was the most important cell responsible for hair growth as well as hair loss (Botchkarev and Kishimoto, 2003; Rogers and Hynd, 2001). The development of new hair follicle as well as the maintenance of hair growth requires the well interaction between dermal papilla fibroblasts and epithelial keratinocytes (Stenn and Paus, 2001). DP cell is connected to the capillaries to derive nutrients and required growth factors for the cells in hair follicle (Sakita *et al.*, 1995). Moreover, there is evidence that the number of DP cells is correlated with the size of the hair follicle, the proliferation of bulb epithelium, and the volume of the hair fiber (Ibrahim *et al.*, 1982; Elliott *et al*., 1999).

Cisplatin (*cis*-diamminedichloroplatinum II) is a widely prescribed anticancer agent against various cancers including lung, ovarian, and breast cancers (Arriagada *et al.*, 2004; Torri *et al*., 2000; Sledge *et al*., 1988). Mechanisms of

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action of cisplatin-induced cell death are accepted to involve DNA-adduct formation as well as reactive oxygen species (ROS) induction. Moreover, our previous studies have shown that ROS was an important mediator regulating Bcl-2 degradation which induced cell apoptosis in cisplatin-treated lung cancer cells (Chanvorachote *et al*., 2006; Wang *et al.*, 2008). Cisplatin has been reported to possess an ability to induce hair loss (Apisarnthanarax and Duvic, 2003; Botchkarev, 2001); however, very little information regarding the mechanism of cisplatin-induced cell death in DP cells has been reported.

Phyllanthus emblica Linn. or emblica fruits have been shown to contain a number of biologically active anti-oxidants, such as, vitamin C and polyphenols (Khopde *et al.*, 2001). We thus hypothesized that if ROS plays a key role on cisplatin-induced DP cell death, emblica extract could show a protective effect on these cells.

Since the effect of ROS on cisplatin-induced DP cell death is largely unknown and we would like to demonstrate the protective effect of natural antioxidant, emblica extract, our aims of study were to explore the role of ROS on cisplatin-induced DP cell death and to investigate the protective effect of emblica extract on cisplatin-induced DP cell death. The present study found that cisplatin-induced apoptosis in DP cells was strongly related to the induction of intracellular ROS caused by cisplatin treatment. Moreover, emblica extract showed a promising protective effect on this cisplatin-induced cell death. These findings may lead to the development of a new strategy for protection of hair loss induced by cisplatin chemotherapy.

2. Materials and Methods

2.1 Cells and reagents

Human follicle dermal papilla fibroblast cells (DP cells) were obtained from PromoCell (PromoCell, Heidelberg, Germany). Cells were cultured in DP cell growth medium containing bovine pituitary extract 0.004 mL/mL, fetal calf serum 0.05 mL/mL, basic fibroblast growth factor 1 ng/mL, recombinant human insulin 5 μg/mL, phenol red 0.62 ng/mL from Promocell (PromoCell, Heidelberg, Germany), and 100 units/mL of penicillin/streptomycin in a 5% CO_2 environment at 37°C. *cis*-diammine-dichloroplatinum II (Cisplatin), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-acetylcysteine (NAC) and glutathione (GSH) were obtained from Sigma Chemical, Inc. (Sigma Chemical, St. Louis, MO, USA). Hoechst 33342, prodidium iodide (PI) and dichlorofluorescein diacetate (H_2DCF-DA) were obtained from Molecular Probes, Inc. (Molecular Probes, Eugene, OR, USA).

2.2 Extraction and sample preparation

Emblica fresh fruits were freed from foreign matter such as dust or other organic matter. The cleaned raw material was commuted to reduce its size. The commuted raw material was agitated with water. The liquid part was separated and then converted to powder form by spray drying. Various concentrations of emblica extract were prepared by dissolving the dry emblica extract powder in deionized water.

2.3 Cytotoxicity assay

Cell viability was determined by MTT colorimetric assay. Briefly, 70-80% confluence of cells were seeded in 96-well plate and incubated with treatments for an appropriate time. After that cells were further incubated with 500 μg/mL of MTT for 4 hours at 37°C. The intensity of the MTT product was measured at 550 nm using an ELISA microplate reader. Cell viability was calculated from optical density (OD) readings and represented as ratio of the non-treated control value in the term of relative cell viability.

2.4 Apoptotic and necrotic evaluations

Apoptotic and necrotic cell deaths were evaluated by cell morphology characteristics. Two different fluorescent dyes which are Hoechst 33342, cell membrane permeable dye and PI, cell membrane impermeable dye, were used for apoptotic and necrotic detection, respectively. Following the incubation period of treatment, cells were incubated with 10 μg/mL of Hoechst and 5 μg/mL of PI for 30 minutes and then visualized under fluorescent microscope (Olympus IX51 with DP70). PI thus stained only DNA of cell membrane-damaged cells which were considered as necrotic cells whereas Hoechst 33342 stained nuclei of all cells. Cells that displayed intensely condensed and/or fragment nuclei stained by Hoechst 33342 were considered as apoptotic cells and represented as percentage of apoptosis.

2.5 ROS determination

Intracellular ROS generation was determined using ROS specific probe H_2DCF-DA. Following the incubation period of treatment, cells were washed with phosphate buffer saline (PBS) and then placed in growth supplement-free media. H_2DCF-DA was added to a final concentration of 10 μg/mL, then incubated for 30 minutes and visualized under fluorescent microscope (Olympus IX51 with DP70). Fluorescence intensity values of green product were measured and represented as fold-induction versus non-treated control value.

3. Results

3.1 Morphology of dermal papilla cells in culture.

An aggregative behavior of DP fibroblast cells has been shown to be associated with DP cell functions (Messenger *et al*., 1986; Jahoda *et al*., 1984). In order to investigate role of ROS and the protective effect of emblica extract on

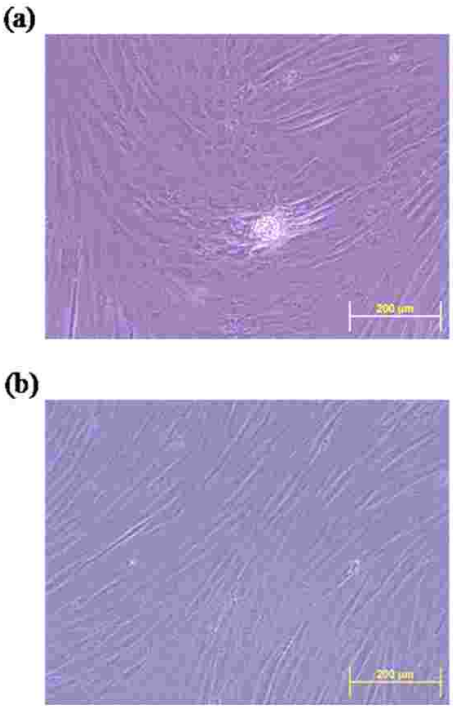

Figure 1. Characterization of cultured dermal papilla cells. DP cells were seeded at high density after subcloning for 2 passages. a), DP cells showed an aggregative property in the maintained culture. b), DP cells remained monolayer in some areas.

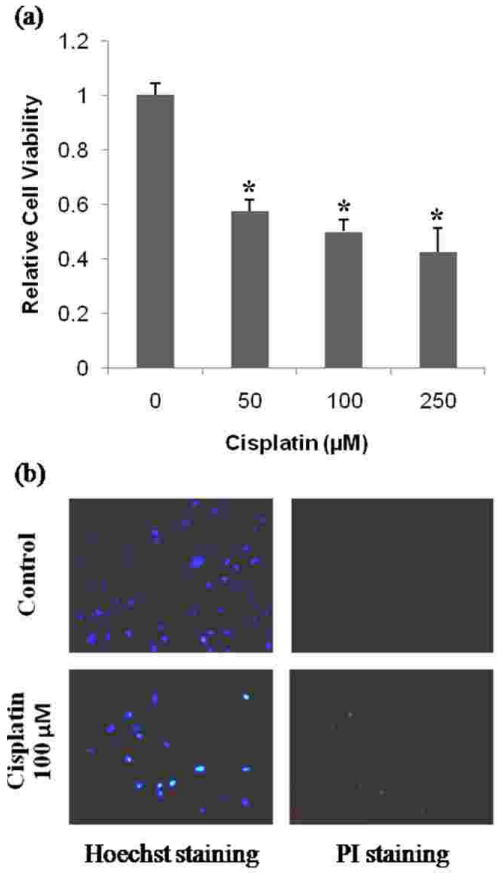

Figure 2. Cisplatin induced DP cell death through apoptosis mechanism. DP cells were treated with various concentrations of cisplatin (0-250 µmol/L) for 24 h. a), effect of cisplatin treatment on cell viability analyzed by MTT assay. b), DP cells were evaluated by Hoechst 33342 apoptotic detection and propidium idodide necrotic detection after treatment with cisplatin (100 µmol/L) for 24 h under fluorescent microscope. Values are means of triplicate samples ± SD. *, $P < 0.05$ versus non-treated control.

cisplatin-induced cell death in DP cells, we first characterized the aggregative property of the maintained cultured DP cells. After subcloning for 2 passages, DP fibroblasts aggregated when seeded at high density (Figure 1a). However, DP fibroblasts remained monolayer in some area of culture plate (Figure 1b).

3.2 Cisplatin decreases dermal papilla cell viability and induces cell death through apoptosis mechanism.

Cisplatin is a widely prescribed anticancer agent for the treatment of many cancers, such as, lung, breast, and ovary cancer (Arriagada *et al.*, 2004; Torri *et al*., 2000; Sledge *et al*., 1988). Previous studies have reported that cisplatin was associated with hair loss (Apisarnthanarax and Duvic, 2003; Botchkarev, 2001). In an attempt to investigate the effect of cisplatin on DP fibroblast cell death, we first determined the cell viability to cisplatin exposure. Briefly, 80% confluence of DP cells were treated with various concentrations of cisplatin (0-250 µmol/L) and further incubated for 24 hours. After incubation period, cell viability was determined by colorimetric tetrazolium MTT assay as described in Materials and Methods. Cisplatin treatment caused a concentration-dependent decreased in cell viability comparing to control group with the LD50 at 100 µmol/L (Figure 2a). Cell death is classified as apoptosis and necrosis, which exhibit some differences in characteristics. In order to investigate the mode of cisplatin-induced cell death, we performed the apoptotic and necrotic evaluations using 2 different fluorescent dyes, which are Hoechst 33342 for apoptotic detection and PI for necrotic detection. After 24-hour cisplatin treatment, cells were stained and visualized under fluorescent microscope. Figure 2b shows that at 100 µmol/L of cisplatin treatment, ~ 40% of cisplatin-treated cells exhibited apoptotic nuclear morphology. However, only slightly PI-stained cells were detected over a wide range of cisplatin treatment. In summary, our results indicated that cisplatin induced DP cell death mainly by apoptosis mechanism.

3.3 ROS is a key regulator of cisplatin-induced dermal papilla cell apoptosis.

Recent studies indicated that cisplatin-induced cell death was associated with its ability to up-regulate intracellu-

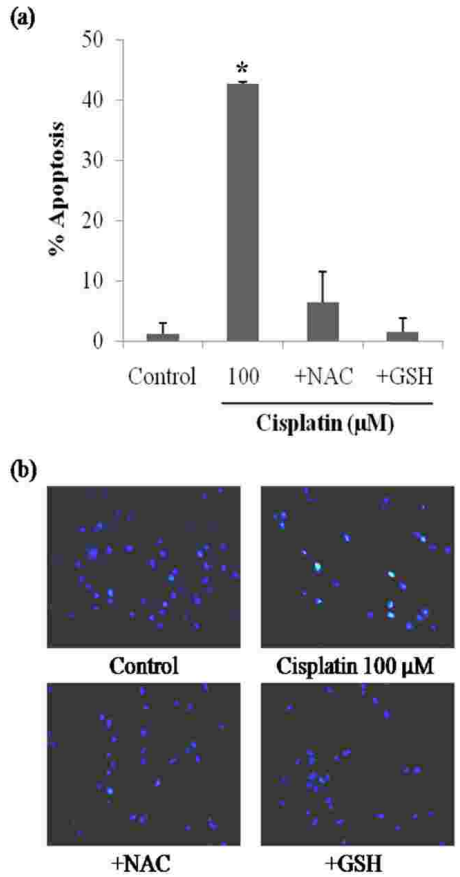

Figure 3. Role of ROS on DP cell apoptosis. DP cells were treated with cisplatin (100 μmol/L) in the presence or absence of NAC (1 mmol/L) or GSH (5 mmol/L) for 24 h. Apoptosis was then determined by Hoechst 33342 assay. a), percentage of cell apoptosis b), morphology of apoptosis nuclei as determined by fluorescent microscope of Hoechst-stained cells. Values are means of triplicate samples + SD. *, $P < 0.05$ versus non-treated control.

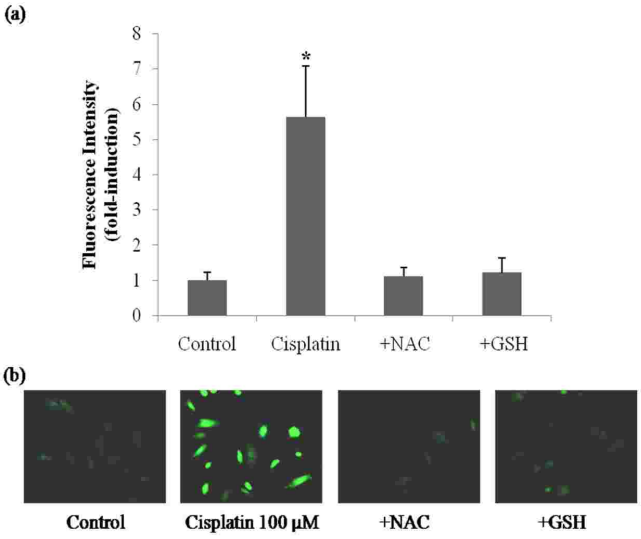

Figure 4. Effect of cisplatin and antioxidants on intracellular ROS level. DP cells were treated with cisplatin (100 μmol/L) in the presence or absence of NAC or GSH for 3 h. Cellular ROS levels were determined by a ROS specific H_2 DCF-DA dye and visualized under fluorescent microscope. Fluorescence intensity was then measured and calculated as fold-induction versus control value. Values represented means + SD. *, $P < 0.05$ versus non-treated control.

lar ROS level (Schweywe *et al*., 2004; Baek *et al*., 2003). These data led to the hypothesis that ROS might play an important role in this cisplatin-induced DP cells apoptosis. We thus investigated the role of ROS using two different known antioxidants, which were N-acetylcysteine (NAC) and glutathione (GSH). Cells were pretreated with NAC (1 mmol/L) or GSH (5 mmol/L) for 30 minutes followed by treatment with 100 μmol/L of cisplatin. The nuclear DNA was stained with Hoechst to assay for apoptosis. In the presence of antioxidants (NAC and GSH), the apoptotic cell death in response to cisplatin treatment significantly decreased comparing to the cisplatin-treated control (Figure 3). These results suggested that ROS plays an important role in cisplatin-induced DP cells apoptosis.

To evaluate the intracellular ROS levels in response to cisplatin and antioxidant treatments, cells were treated with cisplatin for 3 hours in the presence or absence of NAC or GSH. The specific probe for ROS detection, dichlorofluorescein (H_2DCFDA), was used to determine the intracellular ROS level. Figure 4 shows that cisplatin significantly increased DCF fluorescence intensity comparing to the non-treated control. Fluorescence intensity dramatically decreased in the presence of antioxidant NAC or GSH (Figure 4). Taken together with the previous apoptotic assays, our study revealed the association between the apoptotic response and ROS generation induced by cisplatin in DP cells.

3.4 Emblica extract protects dermal papilla cells from cisplatin-induced apoptosis.

Since emblica extract has been reported to have high antioxidant activity (Khopde *et al*., 2001) and our results have shown that ROS was a key regulator of cisplatin-induced DP apoptosis, we further investigated whether emblica extract could protect against cisplatin-induced apoptosis in these cells. We determined the cytoprotective properties of emblica extract on cisplatin-treated cells using colorimetric tetrazolium MTT and Hoechst assays. Cells were co-treated with cisplatin 100 μmol/L and various concentrations of emblica extract (0-500 μg/mL) for 24 hours and determined for cell viability by MTT assay. The results indicated that emblica extract significantly increased cell viability in cisplatin-treated cells in a concentration-dependent manner (Figure 5a). Our results showed that emblica extract at concentrations of 250-500 μg/mL completely inhibited cisplatin-induced cell death. In addition, Hoechst staining apoptotic assay was performed in order to confirm that emblica extract could protect apoptotic cell death following cisplatin treat-

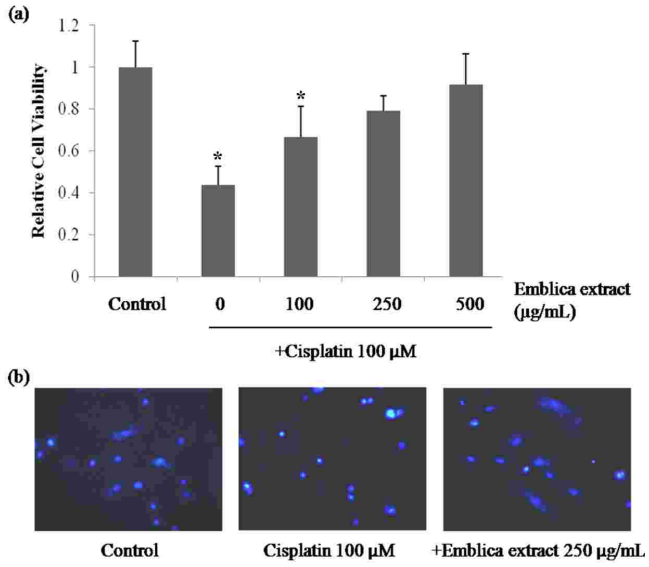

Figure 5. Effect of emblica extract on cisplatin-induced cell death. a), DP cells were co-treated with cisplatin (100 µmol/L) and various concentrations of emblica extract (0-500 µg/mL) for 24 h. Cells viability was then determined using MTT assay. b), morphology of apoptotic nuclei of Hoechst staining cells treated with cisplatin alone (100 µmol/mL) or in combination with emblica extract (250 µg/mL) under fluorescent microscope. Values are means of triplicate samples + SD. *, $P < 0.05$ versus non-treated control.

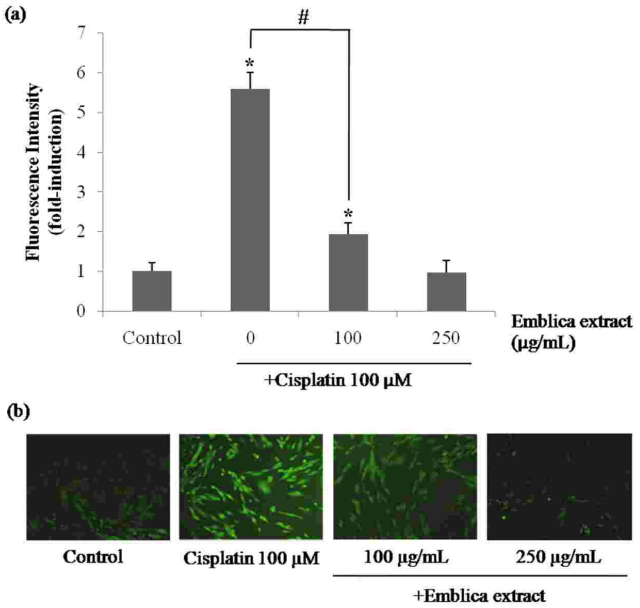

Figure 6. Effect of emblica extract on intracellular ROS induced by cisplatin treatment. DP cells were co-treated with cisplatin (100 µmol/L) and emblica extract (0-250 µg/mL) for 3 h. Cellular ROS levels were determined by a ROS specific H_2DCF-DA dye and visualized under fluorescent microscope. Fluorescence intensity was then measured and calculated as fold-induction versus control value. Values represented means + SD. *, $P < 0.05$ versus non-treated control and #, $P < 0.05$ versus cisplatin treated-cells.

ment (Figure 5b).

In order to evaluate intracellular ROS levels in response to cisplatin and emblica extract treatments, the H_2DCFDA fluorescent assay was conducted. Cells were treated with cisplatin for 3 hours in the presence or absence of various concentrations of emblica extract (0-250 µg/mL). Results showed that addition of 250 µg/mL of emblica extract dramatically decreased fluorescence intensity comparing to the cisplatin-treated control (Figure 6).

4. Discussion

Chemotherapies were reported to induce hair loss, which is considered as one of the most feared side effects on cancer patient perception. Degree of chemotherapy-induced hair loss depends on the drug used, dosage and route of administration. Most of the studies on chemotherapy-induced hair loss particularly pay attention on keratinocytes, the major population of hair follicles. However, DP cells have recently garnered attention since there is increasing evidences indicating the regulatory roles of these cells on the development of hair follicle as well as the maintenance of hair growth (Matsuzaki and Yoshizato, 1998; Stenn and Paus, 2001). The mechanisms of minoxidil in prevention of hair loss have been shown to be tightly associated with DP cell proliferation and apoptosis (Han *et al*., 2004). Minoxidil also showed evidence of reducing the duration of hair loss following adjuvant chemotherapy in women who had breast cancer surgery (Duvic *et al*., 1996). Collectively, DP cells might be associated with chemotherapy-induced hair loss. Since the widely prescribed cisplatin has been shown to have high potential to cause hair loss and less is known regarding its effect on DP cells, we thus investigated the effect of cisplatin on DP cells *in vitro*. We herein reported that cisplatin induced DP cell apoptosis in a concentration-dependent manner. Our results suggested that ROS played an important role on cisplatin-induced apoptosis in these cells. In addition, we demonstrated that addition of emblica extract significantly protected DP cells from cisplatin-induced apoptosis. Increasing evidence suggested that ROS plays an important role in many cellular responses including cell apoptosis (Simon *et al*., 2000). Accumulation and up-regulation of ROS have been shown to be strongly associated with mitochondrial membrane potential disruption followed by an alteration of the balance between pro- and anti-apoptotic protein members of Bcl-2 family protein. Consequently, this balance alteration leads to the release of cytochrome c to the cytoplasm. Cytosol cytochrome c then activates the caspase cascade leading to cell apoptosis (Woo *et al*., 2003). In case of cisplatin, apoptotic cell death has been long known to be tightly associated with ROS induction and mitochondrial disruption (Siddick, 2003). Moreover, cisplatin induced

intracellular ROS up-regulation has been demonstrated in many cells (Chanvorachote *et al*., 2006; Yoshiko *et al*., 2006; Baek *et al*., 2003). The reports that DP cells have high level of Bcl-2 protein (Muller-Rover *et al*., 1999; Soma and Hibino, 2004) acting as a cellular antioxidant (Haddad, 2004) raised the query that these cells may respond differently to the cisplatin treatment. However, our not unexpected results indicated that cisplatin caused significant up-regulation of ROS level which was dramatically attenuated by addition of antioxidant NAC and GSH. In addition, the apoptotic determination showed a positive correlation with the intracellular ROS level, suggesting an important regulating role of ROS.

Since emblica extract was reported to have high antioxidant activity, we thus demonstrated the protective effect of emblica extract on cisplatin-induced DP cell apoptosis. Having provided evidence that ROS plays a key role on cisplatin-induced DP cell apoptosis, it is not surprising that apoptotic DP cell death caused by cisplatin treatment was attenuated by the addition of emblica extract. To confirm the involvement of ROS on this protective effect, we performed the intracellular ROS detection. The presence of emblica extract significantly inhibited the ROS generation in cisplatin-treated cells.

In summary, our results revealed the role of ROS on cisplatin-induced apoptosis in DP cells. Moreover, we demonstrated the potential protective effect of emblica extract on this cisplatin-induced DP cell death.

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