Research Article

Protective Effect of Diosgenin against H_2O_2-Induced Oxidative Stress on H9C2 Cells

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Abstract

Diosgenin is an important compound in pharmaceutical industry. It has various effects such as hypocholesterolemic action or antioxidant activity in HIV infected patients. Biological oxidation pathways are involved in causing or aggravating heart disease. This study investigated the potential protective effect of diosgenin on cell viability and antioxidant defenses of cultured H9C2 cells submitted to oxidative stress induced by H2O2. Viability of cells exposed to H2O2 was detected by MTT assay. The generation of ROS and hydrogen peroxide release after H2O2 were detected using the fluorescent probe H2DCF-DA. The lipid peroxidation product i.e. MDA formation was estimated by assessing the levels of thio-barbituric acid reactive substances (TBARS) using spectrophotometry. SOD activity was assayed with NWLSS (TM) Superoxide Dismutase (SOD) activity assay kit. Pretreatment of cells with 3-25 µM of diosgenin for 24 h before applying H2O2 completely prevented cell damage and significantly enhanced viability of H9C2 cells. Increased ROS induced by H2O2 was dose dependently prevented when cells were pretreated for 24 h with diosgenin. The level of the lipid peroxidation was significantly higher in H9C2 cells exposed to H2O2 as compared to the control and cells pretreated with diosgenin. SOD activity in cells treated with diosgenin significantly decreased compared with cells exposed to H2O2. These results show that treatment of H9C2 cells with diosgenin (3-25 µM) confers a significant protection against oxidative stress.

***Keywords*:** Diosgenin, H9C2 cells, Oxidative stress, MDA, Cell viability

Introduction

Diosgenin is a steroidal sapogenin belonging to the group of triterpenes. It is found in several plants including fenugreek (Trigonella foenum graecum), the roots of the wild yam (Dioscorea villosa) and Costus speciosus (Attele et al., 1999; Liu et al., 2005). Steroidal sapogenins are secondary metabolites and their biosynthetic precursors are sterols, especially cholesterol. They are mainly found as glycosides called steroidal saponins, which constitute a structurally diverse class of natural products and are one of the major components in traditional Chinese medicines (Attele et al., 1999a; Liu et al., 2005). Diosgenin is an important compound in pharmaceutical industry as a natural source of steroidal hormones (Liu et al., 2005; Roman et al., 1995). It has various effects, such as hypocholesterolemic action or antioxidant activity in HIV infected patients (Accatino et al., 1998; Kim et al., 2012; Turchan et al., 2003).
Diosgenin has anticancer effects against a wide variety of tumor cells, including colorectal cancer, breast cancer, osteosarcoma and leukemia (Corbiere et al., 2003; Liu et al., 2005; Srinivasan et al., 2009; Wang et al., 2004). Other researchers have reported that it has estrogenic effects (Aradhana et al., 1992). Diosgenin acts as a megakaryocytic differentiation inducer and could cause changes in lipoxygenase activities in human erythroleukemia cells. Five lipoxygenase activating protein (FLAP), and leukotriene A4 (LTA4) hydrolase gene expression during megakaryocytic differentiation induced by diosgenin (Beneytout et al., 1995; Corbiere et al., 2003; Wei et al., 2001). It induces p53-mediated cell cycle G_1 arrest and apoptosis in osteosarcoma cells (Moalic et al., 2001). It is necessary to study the biochemical and cellular mechanisms of action of this natural product. Hydrogen peroxide is a physiological component of living cells and is uninterruptedly produced via various cellular pathways. The intracellular concentration of H2O2 is strongly controlled by enzymatic and nonenzymatic antioxidant systems.

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Intracellular steady-state concentrations of H2O2 above 1 µM are considered to cause oxidative stress inducing growth arrest and cell death (Antunes and Cadenas, 2001; Stone and Yang, 2006).

Oxidative stress that resulting from increased production of free radicals and reactive oxygen species, and/or a decrease in antioxidant defense, leads to damage of biological macromolecules and disruption of normal metabolism and physiology and also pathologies, such as cancer and neurological disorders, as well as in ageing (Bernabucci et al., 2002; Trevisan et al., 2001).

The role of free radicals, reactive oxygen species, and antioxidants in the etiology of chronic diseases, including cardiovascular disease, lung disease, cancer, diabetes, renal ischemia, atherosclerosis, pulmonary pathological states, inflammatory diseases and others, has stimulated research in recent years (Santanam et al., 1998; Trevisan et al., 2001)

It is widely accepted that an excess of ROS is toxic and damages cell components including nucleic acids, proteins and lipids (Pizarro et al., 2009; Thannickal and Fanburg, 2000).

Lipids are important component of the cell membrane. Lipid peroxidation is implicated in the pathogenesis of a number of diseases and clinical conditions (P et al., 2013) which include diabetes, adult respiratory distress syndrome, premature birth disorder, aspects of shock, Parkinson's disease, Alzheimer's disease, pre-eclampsia and eclampsia, various chronic inflammatory conditions, ischaemia, reperfusion mediated injury to organs which include the heart, brain and the intestine, atherosclerosis, organ injury which is associated with shock and inflammation, fibrosis, cancer, inflammatory liver injury, anthracycline induced cardiotoxicity, silicosis and pneumoconiosis (Davi et al., 2005; Riley, 1994; Yagi, 1987).

The lipid peroxidation product, malondialdehyde (MDA), is commonly used as a measure of the oxidative stress in cells. Lipid peroxidation occurs when the hydroxyl radicals, possibly oxygen, react with the unsaturated lipids of the bio-membranes, resulting in the generation of lipid peroxide radicals (ROO•), lipid hydroperoxide (ROOH) and fragmentation products such as MDA (Uchida et al., 1999). This aldehyde is a highly toxic molecule and it should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been referred as a potentially mutagenic and atherogenic agent (Lores Arnaiz et al., 1998; Ueda et al., 1998). Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways. One of the primary antioxidant enzymes in cells that is thought to be necessary for life in all oxygen metabolizing cells is superoxide dismutase (SOD). The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O2) (Peskin and Winterbourn, 2000).

In this study, we investigated the effect of diosgenin on the proliferation rate and diosgenin ability to protect H9C2 cells from cell death when exposed to oxidative stress induced by hydrogen peroxide.

Materials and Methods

Cell Culture and Drug Treatment

H9C2 cells were obtained from Razi Vaccine and Serum Research Institute and were cultured in RPMI (Gibco) supplemented with 20% fetal bovine serum (Gibco) and 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were cultured at 37°C in a humidified chamber with 95% air and 5% CO_2. All experiments were performed in plastic tissue culture flasks. H9C2 cells were seeded in 24 or 96 well plates. After plating, cells were allowed to adhere overnight and were then treated with chemicals. Diosgenin was purchased from Sigma Chemical Co (D1634-5G). Diosgenin (10 mg) was dissolved in 2 ml of ethanol (12000 µM) and mixed with fresh medium to achieve the desired concentration (0, 1, 3, 6, 12, 25, 50, 100 and 200 µM) . The maximum final ethanol concentration in cultures was 0.7%, which did not alter cell growth and cell cycle measurements when compared with untreated control cells.

Determination of Cell Viability (MTT Assay)

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay. The cells were seeded in 96-well plates at a density of 5×10^3 cells/well and after 48 h, they were treated with various concentrations of diosgenin (0–200 μM) for 24h. After the exposure period, media were removed. MTT solution in phosphate-buffered saline (PBS, 5mg/mL) was added to a final concentration of 0.05% for 1 h, thereby allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed and formazan crystals were dissolved in 200 µl of dimethylsulphoxide. Formazan production was measured by the absorbance at 545 nm using a microplate reader (BioRad Laboratories, CA, USA). Viability results were expressed as percentages.

The percentage of cell viability was calculated by dividing the mean absorbance of each treatment to the mean absorbance of its controls multiply by 100.

Determination of Diosgenin Effect on Viability of H9C2 Cells Exposed to H_2O_2

Cells were planted into 96-well plates. After incubation for 48 hours, the medium was replaced with fresh medium with various concentrations of diosgenin (0–50 µM) for 24h .

Then, the medium was changed and incubated with or without H2O2 at indicated concentration (200 µM) for 1h. Six wells were included in each concentration.

At the end of treatment, 10 µl MTT was added and incubated for 1 h. Then the medium was discarded carefully and 200 µl DMSO was added. Absorbance was recorded at 545 nm with Universal Microplate Reader.

All experiments were performed in triplicateS. The mean percentage of cell death was calculated as follow:

% inhibition = (A545 of control _ A545 of treated cells)/A545 of control cells × 100%.

Measurement of ROS

Level of intracellular ROS was measured using the fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (H2DCF-DA). Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells/well and after 48 h, they were treated with various concentrations of diosgenin (0–50 µM).

After 24 h incubation, DMEM was replaced by PBS and the cells were treated with 1µM CM-H2DCFDA for 30 min at 37°C in darkness (added from a 20 mM stock solution in dimethyl sulphoxide).

H2DCFDA diffuses across cell membranes, where acetates migrate via intracellular esterases. Oxidation of H2DCFDA occurs almost exclusively in the cytosol, thereby generating a fluorescent response proportional to ROS generation.

After loading the dye, cells were washed in Locke's buffer and fluorescence was measured at a 488 nm excitation wavelength and an emission wavelength of 510 nm, using a Perkin-Elmer Victor 3 fluorometer.

Estimation of MDA

MDA was estimated by assessing the levels of Thio- Barbituric Acid Reactive Substances (TBARS). The TBARS assay was performed by using MDA equivalents which were derived from tetra-ethoxy-propane. MDA was identified as a product of lipid peroxidation which reacted with TBA to give a pink coloured species that gave an absorbance at 532 nm.

Cells were seaded in 12-well plates. 48 hours after incubation, the medium was replaced with fresh medium with various concentrations of diosgenin (3–25 µM) for 24h. Then, the medium was changed and incubated with H2O2 at indicated concentration (200 µM) for 1h.

Afterwards, media was transferred to a fresh tube and the scraped cells with 1 ml TCA was added to the tube and the mixture was centrifuged at 13000 rpm for 5 min.

The method involved heating of the separated supernatant of the treated cells with the TBA reagent which contained Tri-chloro Acetic acid (TCA) (1.5 %) and Thio-Barbituric Acid (TBA)(0.7 %).

After cooling the solution, it was centrifuged at 2000 rpm and the precipitate was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained untreated cells.

SOD Activity Measurement

Superoxide Dismutase (SOD) Activity was assessed by NWLSS (TM) kit which is a sensitive kit using WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.

Cells were planted in 12-well plates. 48 hours after incubation, the medium was replaced with fresh medium with various concentrations of diosgenin (3–25 µM) for 24h. Then, the medium was changed and incubated with H2O2 at indicated concentration (200 µM) for 1h.

Afterwards, media was transferred to a fresh tube, the cells were scraped with cold Tris/HCl 0.1M, pH 7.4 containing 0.5 % Triton X-100, 5mM β-ME, and 0.1 mg/ml PMSF. Cell lysate was centrifuged at 14000 rpm for 5 minutes at 4°C.

Then, the supernatant was transferred to a tube which contains total SOD activity from cytosol and mitochondria. 40 µl of supernatant was poured into 18 tubes and 920 µL of assay buffer was added to each tube.

The solutions were mixed and incubated for 5 minutes. Then, 40 µl Hematoxylin Reagent added to start the reaction. The mixture was vortexed quickly and the absorbance at 560 nm was measured. All experiments were performed in triplicates.

Statistical Analysis

Data were expressed as mean ± SEM. For statistical analysis, one-way analysis of variance (ANOVA) with Tukey–Kramer post hoc test for multiple comparisons were used. *P* value ≤ 0.05 was considered statistically significant.

Results

Effect of Diosgenin on The Growth of H9C2 Cells

To determine diosgenin effect on cell viability , H9C2 cells were treated with diosgenin (0–200 µM). Cell viability was evaluated based on the ability of cells to exclude trypan blue.

Diosgenin induced a marked dose-dependent diminution of cell viability as early as 24 h, indicating that the proliferation potential of cells was impaired. After cells were treated with diosgenin, marked morphological changes of cell apoptosis were found (Figure 1).

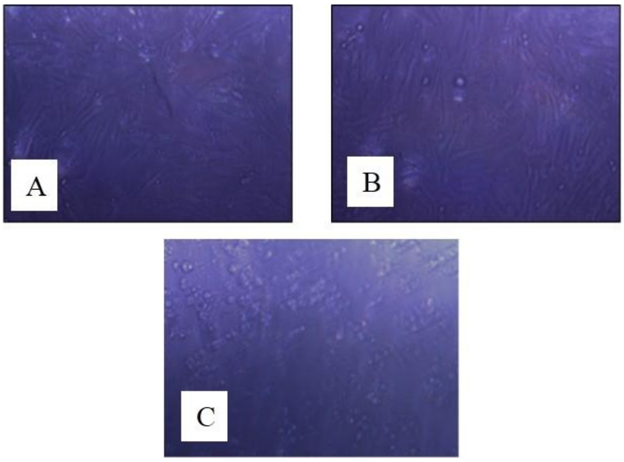

Figure 1. Cytotoxic Effect of diosgenin in H9C2 cells. After cells were treated with diosgenin (0–200 µM) for 24 h, marked morphological changes of cell apoptosis were found. . A: H9C2 cells cultured in RPMI media containing 20% FBS. B: The cells exposed to 25 µM diosgenin. C: H9C2 cells exposed to 100µM diosgenin

The IC_{50} (median growth-inhibitory concentration) determined by the MTT assay, was about 80 µM (Figure 2).

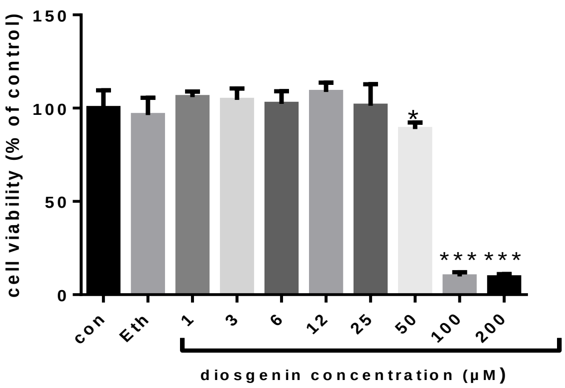

Figure 2. Cytotoxic Effect of diosgenin in H9C2 cells. Cells were treated with different concentrations of diosgenin for 24 h. The ratios of cell viability were measured by MTT assay. Data are presented as mean ± SEM of six replicates from three independent experiments. * $p < 0.05$ and ***$p<0.001$, compared to control

Protective Effect of Diosgenin on H9C2 Cells Against H_2O_2-Induced Cytotoxicity

The viability of H9C2 cells, which was measured by MTT method, decreased significantly ($p<0.05$) to 23.5% of the control values after cells were exposed to 200 µM H2O2 for 1 h (Figure 3).

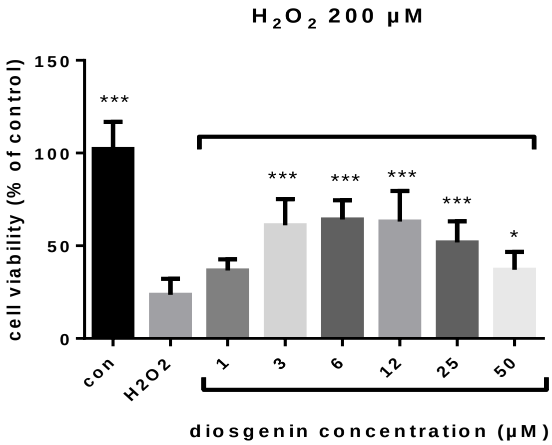

Figure 3. Diosgenin (1-50 µM) attenuated H9C2 cell loss mediated by 200 µM H2O2. Data are expressed as mean ±SEM. *$p<0.05$ and ***$p<0.001$, compared with H_2O_2 group

Pre-treatment with various concentrations (1 µM to 50 µM) of diosgenin for 24 h, significantly ($p<0.05$) increased cell viability (Figure 3). According to the results, microscopic images showed clearly an increase in the number of H9C2 cells after pre-treatment with diosgenin compared with cells treated with 200 µM H2O2 (Figure 4).

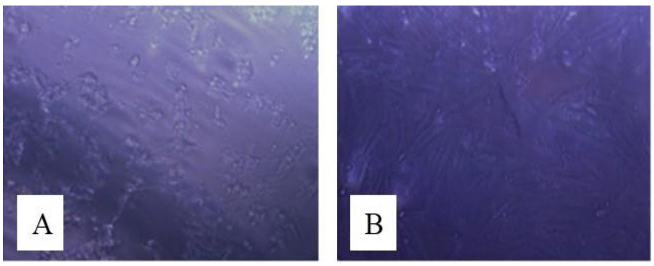

Figure 4. Morphological changes of H9C2 cells exposed to H2O2. Microscopic analysis of H9C2 cells after 1 h of treatment with 200 µM H2O2 in the presence of diosgenin. . A: H9C2 cells were incubated with 200 µM H2O2 for 1 h. B: The cells exposed to H2O2 200µM + Diosgenin 25µM.

Diosgenin Effect on The Reduction of Oxidative Stress

According to the above results, exposure of cells to H2O2 for 1 h (200 µM) caused a significant

increase in intracellular ROS generation. Pre-treatment of cells with different concentrations of diosgenin (3-25 µM) reversed this increase significantly ($p<0.001$) (Figure5).
In order to remove the effect of solvent, a group of cells were treated with ethanol alone. H2O2 increased the number of apoptotic cells and induced changes in the cell cycle phases.
In addition, treatment with diosgenin inhibited the effect of H2O2 on cell cycle phases and apoptosis. Our results show that diosgenin suppresses H2O2-indued cytotoxicity in H9C2 cells.

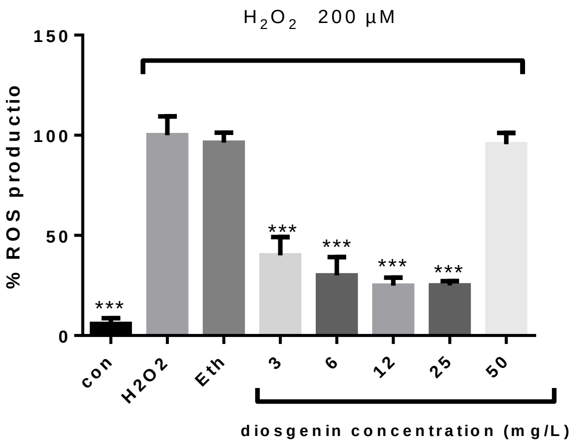

Figure 5. ROS production mediated by 200 µM H_2O_2 was reduced in diosgenin (3-25 µM) treated H9C2 cells. Data are expressed as the means±SEM of three independent experiments; ***$p<0.001$ compared with H_2O_2 group

Effects of Diosgenin on Cell Membrane Peroxidation

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation.
The treatment of H9C2 cells with 200 μM H2O2 during 1 h induced a significant increase of about 100% in the cellular concentration of MDA, indicating oxidative damage to the lipid content of cells.
On the contrary, pretreatment of H9C2 cells with 3-50 µM of diosgenin for 24 h prevented the MDA increase induced by H2O2, indicating a reduced level of lipid peroxidation in response to H2O2 (Figure 6).

Effects of diosgenin on SOD activity

The results showed that pretreatment of cells with 3-25 µM of diosgenin for 24 h reduced the SOD activity compare to H2O2 treated cells (Figure 7).

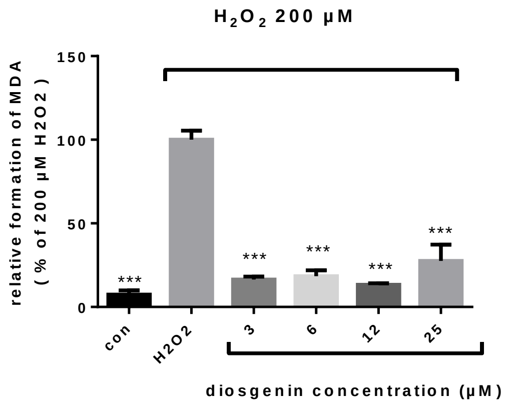

Figure 6. MDA production mediated by 200 µM H_2O_2 was reduced in diosgenin (3-25 µM) treated H9C2 cells. Data are expressed as means±SEM of three independent experiments; ***$p<0.001$ compared with H_2O_2 group

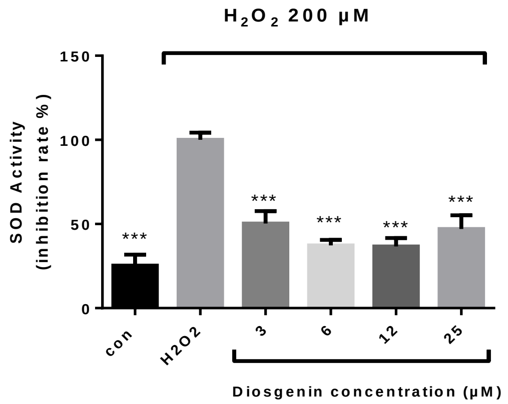

Figure 7. Diosgenin (3-25 µM) reduced SOD activity in H9C2 cells mediated by 200 µM H2O2. Data are expressed as means±SEM of three independent experiments; ***$p<0.001$ compared with H_2O_2 group.

Discussion

The effects of diosgenin on H9C2 cells were investigated in this study. In the first experiment, the toxicity of diosgenin on H9C2 cells was evaluated. Results clearly showed that diosgenin (50 µM) did not have any toxic effect on growth and proliferation of H9C2 cells. The IC_{50} determined by MTT assay was about 80 µM. Liu et al. (2005) reported that the IC_{50} of diosgenin on K562 cells was about 25 µM(Liu et al., 2005). Thus, Concentrations less than 80 µM were evaluated for protective effects on cell injury. The cells were treated with various concentration of diosgenin (1-50 µM) for 24h and then exposed to 200 µM H2O2 for 1h. The results revealed that pretreatment with various concentrations (1-25 µM) of diosgenin, significantly ($p<0.05$) increased cell viability. Consistent with these results, it was determined that increased cell

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viability was due to reduced H2O2-induced oxidative stress by diosgenin. This is because diosgenin reduced the production of ROS to 25% compared with the cells only exposed to 200 µM H2O2 (control). Membrane lipid which contain unsaturated fatty acids, are particularly sensitive to oxidative stress, and their peroxidation leads to disturbance of the membrane integrity (Kaneko et al., 1990; Zaleska and Wilson, 1989). One important repair mechanism of damaged membranes is reacylation of phospholipids in the membrane (Zaleska and Wilson, 1989). In order to investigate the effect of diosgenin on peroxidation of membrane and rate of MDA production, the cells pretreated with diosgenin for 24 h, were exposed to H2O2. Diosgenin reduced peroxidation of membrane as well as the level of MDA especially at concentration 12 µM, especially. SOD activity in cells treated with diosgenin (3-25 µM) was significantly decreased compared with cells only exposed to H2O2.

Conclusion

The results of this study showed that treatment of cardiac H9C2 cells with diosgenin confers a significant protection against oxidative stress to the cells.

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