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Full Length Research Paper

Apoptotic effects of dehydroepiandrosterone sulphate (DHEAS) of the human promyelocytic leukemia cell (HL-60)

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Dehydroepiandrosterone sulphate (DHEAS) has a protective role against cancer; however, the mechanism by which DHEAS has this effect remains poorly understood. The purpose of this study was to investigate the potential effects of DHEAS on HL-60 (human promyelocytic leukemia cell). The percentage of cells undergoing apoptosis was analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate staining. DHEAS internalization into HL-60 cells was visualized by transmission electron microscopy. DHEAS could be used to facilitate uptake to specific cancer cells for cancer therapy and diagnosis. Our results showed that the uptake of DHEAS by HL-60 cells were also much higher than that of the control cells. In this study, the mechanism of DHEAS actions against leukemia involves the inhibition of cell proliferation indicating that DHEAS can be useful in the treatment of leukemia.

Key words: Apoptosis, dehydoepiandrosterone sulphate, human promyelocytic leukemia cell.

INTRODUCTION

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are endogenous steroids largely produced in the adrenal cortex and excreted in the urine. Many studies have demonstrated that administration of DHEAS to animals protects against a variety of chemical carcinogens. DHEAS is the most abundant steroid in the blood of humans (Nasman et al., 1991). DHEAS is adrenal hormone, androgen that have antiglucocorticoid properties on cells of the immune system (Blauer et al., 1991). DHEAS is more commonly measured than DHEA due to its much higher blood levels, long half-life, and most importantly, its absence of diurnal variation, making it possible to take only one blood sample and attain an accurate reading regardless of the time of day (Hornsby, 1995).

DHEAS is a very interesting new diagnostic tool for both scientific research and clinical diagnostics. Moreover, circulating concentrations of DHEAS can be changed by many factors, such as endogenous production, hormone supplementation, many kinds of drugs, and many types of disease states (Leowattana, 2004). Since many beneficial effects have been proven (and even more suggested), DHEAS replacement therapy, as compensation for high age- or disease-related decline of the hormone, has become popular in recent years (Blauer et al., 1991). Epidemiological data indicates an inverse relationship between serum DHEA and DHEAS levels and the frequency of cancer, cardiovascular diseases, Alzheimer disease, immune function, and progression of HIV infection (Kaasik et al., 2001; Leowattana., 2001; Celec and Stárka, 2003; Ceda et al., 2002). DHEA and DHEAS have been reported to have anti-carcinogenic effects in various organs in rodents including mammary gland after chemical cancer induction (Feo et al., 2000). Whether DHEA itself or a metabolite is the compound responsible for this anticarcinogenic effect has not been clarified (Labrie et al., 2000). The human promyelocytic leukaemia cell line (HL-60), derived from a single patient with acute promyelocytic leukemia, provides a unique *in vitro* model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/-

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macrophage lineage. These cells manifest a transformed phenotype, as shown by karyotypic abnormalities, continuous proliferation for over 3 years in culture, tumorigenicity in nude mice, and morphological, histochemical, and karyotypic similarity to the patient's leukemic cells (Collins, 1987; Collins et al., 1980). In the present report, we describe the induction of terminal differentiation of HL-60 cells by DHEAS. These data suggest significant direct effects of different concentrations of DHEAS on apoptosis in different times and indicates that DHEAS may be involved in the differentiation of certain hematopoietic tissues.

MATERIALS AND METHODS

Cell culture

Human promyelocytic leukemia (HL-60) cell lines were used in this experiment. Frozen stock vials of the cells were thawed and used. Cells were routinely cultured at 37°C in a humidified atmosphere with 5% CO_2 in 25 cm^3 flasks containing 5 ml of Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin. The medium was changed every third day. For subculture, the cells were washed twice with phosphate-buffered saline and incubated with trypsin-ethylenediamine tetra-acetic acid solution (0.25% trypsin, 1 mM ethylenediamine tetra-acetic acid) for 2 min at 37°C to detach the cells, and the complete media were then added into the flask at room temperature to inhibit the effect of trypsin. The cells were washed twice by centrifugation and resuspended in the complete media for reseeding and growing in new culture flasks. Cell viability was determined through staining with trypan blue, and cells were counted using a hemocytometer. Subjects were divided into five groups: (I) Group 1 (G1): control, (II) Group 2 (G2): values 15 µg/ml, (III) Group 3 (G3): values 30 µg/ml, (IV) Group 4 (G4): values 45 µg/ml, and (V) Group 5 (G5): values 60 µg/ml. In control cultures, the cells were placed in 5 ml of medium without DHEAS at the same cell density. To study the cellular uptake of DHEAS by flow cytometry and DHEAS was added to the cell culture media at different concentrations. The cells were cultured and then reseeded with the DHEAS culture media. After 24 and 48 h of incubation at 37°C, the cells were washed twice with phosphate-buffered saline, detached using trypsin-ethylenediamine tetra-acetic acid solution, and resuspended in culture media.

Flow cytometry

Apoptosis was induced by treating the cells for different times with DHEAS. Annexin V-fluorescein isothiocyanate (FITC) is a sensitive probe for identifying apoptotic cells. It binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine, a membrane phospholipid, than for most other phospholipids (Cao et al., 2006). In apoptotic cells, phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phosphatidylserine to the external environment (Vermes et al., 1995). A FACSAria (BD Corporation, Bedford, MA) flow cytometer was used for analysis of the cells. A recent report suggested that the uptake of Fe3O4 nanoparticles by cells could be quantitatively measured using flow cytometric light scatter (Jing et al., 2010). Annexin V binding was performed using an AnnexinV-FITC kit (BD Corporation) as described by the manufacturer. Cells were washed twice with cold phosphate-buffered saline and were then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml, after which 100 µl of solution (1×10^5 cells) was transferred to a 5 ml culture tube. Annexın V-FITC 5 µl and propidium iodide 5 µl were added, and the cells were then incubated for 15 min at room temperature in the dark, after which 400 µl of 1× binding buffer was added to each tube and analyzed in the FACSAria.

Transmission electron microscopic analysis

Myeloblastic human blood derived HL 60 cell line purchased from ATCC were obtained from American Type Culture Collection (Rockville, MD). Cells (5×10^5/ml) were exposed to the following inducers at concentrations (unless otherwise indicated) inducing maximal differentiation of exponentially growing HL-60 cells. Uptake of DHEAS by HL-60 cancer cells, as well as morphology, was determined using a transmission electron microscopic analysis (TEM). Samples for TEM (FEI Tecnai BioTWIN) measurement were prepared by dispersing the particles in dimethyl sulfoxide. Cells were deposited on Formvar-coated 200 to 300 mesh copper grids and dried, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and left in phosphate-buffered saline overnight at 4°C. After being embedded in agar and after fixation in 2% osmium tetroxide, the cells were dehydrated in graded ethanol, that is, 70, 90, 96 and 100%. The cells were then embedded in EPON 812 epoxy. They were thin-sectioned using a diamond knife to a maximum thickness of 100 nm. The sections were stained with lead citrate and uranyl acetate.

Statistical analysis

Statistical analysis was performed by using SPSS 15.0 software program. Data are reported here as the mean ± SD. The statistical significance of differences between the results in the different groups was evaluated with the Student's two-tailed *t*-test. For all comparisons, significance was accepted at the level of $p < 0.05$.

RESULTS

The cytotoxicity of DHEAS was determined by exposing normal cells and HL-60 cells to various concentrations in Dulbecco's Modified Eagle's Medium for 24 and 48 h. The results and respective percentages of cells in apoptotic regions for HL-60 cells are given in Figure 1. Control group and by increasing the different concentrations of DHEAS (15, 30, 45, 60 µg/ml), the percentages of HL-60 cells treated with DHEAS at 24 h were 2.0, 7.8, 9.9, 10.3 and 9.8%, respectively and by increasing the concentrations of DHEAS (15, 30, 45, 60 µg/ml); the percentages of HL-60 cells treated with DHEAS at 48 h were 3.9, 4.7, 6.3, 6.4 and 3.3%, respectively. When cells were exposed to DHEAS concentration of 15 µg/ml for 24 h, apoptotic index increased to 7.8%. When incubation time was extended to 48 h, apoptotic index increased further to 4.7%. Apoptosis ındex of HL-60 cells were significantly increased after 24 h of being incubated with 45 µg/ml DHEAS treatment (Table 1). HL-60 cells were incubated with DHEAS for 24 h (45 µg/ml), the population of

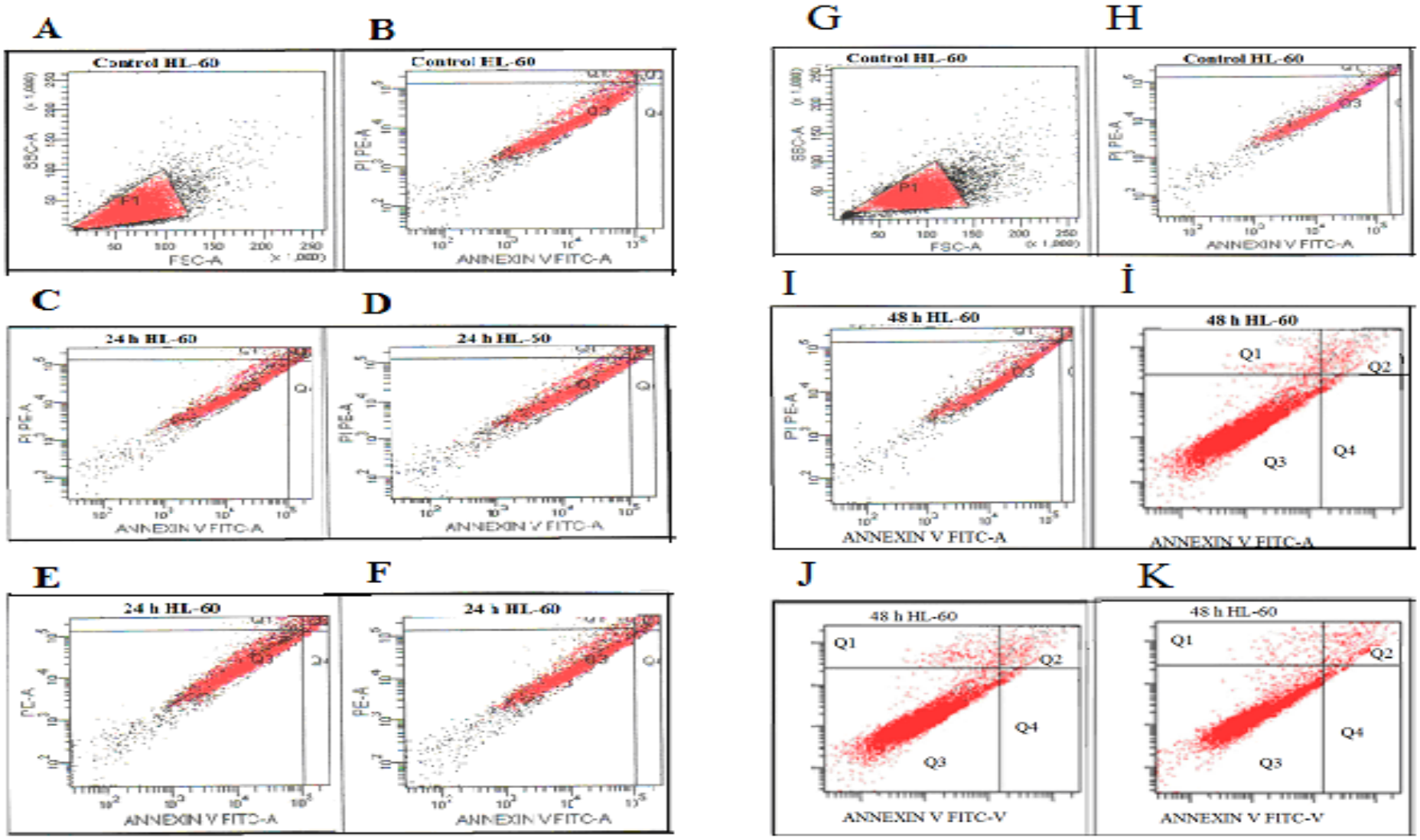

Figure 1. Flow cytometric analysis of HL-60 cells by double labeling with Annexin V fluorescein isothiocyanate and propidium iodide. Unfixed cells from control and treated groups were labeled with Annexin V-fluorescein isothiocyanate and propidium iodide and then fixed and analyzed on a flow cytometer. Dual parameter dot-plot of propidium iodide-phycoerytrin (x-axis), Annexin V-fluorescein isothiocyanate fluorescence (y-axis) showing logarithmic intensity. Quadrants are viable cells Q3 (Annexin V-negative/propidium iodide-negative), early apoptotic cells Q4 (Annexin V-positive/propidium iodide-negative), and late apoptotic and necrotic cells Q2 (Annexin V-positive/propidium iodide-positive). (A) Percentage of apoptotic cells, 24 h; (B) control cells, 24 h; (C) DHEAS, 15 µg/ml, 24 h; (D) DHEAS, 30 µg/ml, 24 h; (E) DHEAS, 45 µg/ml, 24 h; (F) DHEAS, 60 µg/ml, 24 h; (G) Percentage of apoptotic cells, 48 h; (H) control cells, 48 h; (**I**) DHEAS, 15 µg/ml, 48 h; (İ) DHEAS, 30 µg/ml, 48 h; (J) DHEAS, 45 µg/ml, 48 h; K) DHEAS, 60 µg/ml, 48 h.

Table 1. Effects of DHEAS induced apoptosis ındex in control and HL-60 cells at different times.

Groups	Apoptotic cells (%)		p- Value
	Time points (h)		
	24	48	
Control	2.0 ± 0.40	3.9 ± 0.12	< 0.001
15 µg/ml	7.8 ± 0.22	4.7 ± 1.10	< 0.05
30 µg/ml	9.9 ± 0.80	6.3 ± 0.40	< 0.05
45 µg/ml	10.3 ± 1.24	6.4 ± 0.44	< 0.05
60 µg/ml	9.8 ± 0.92	3.3 ± 0.20	< 0.05

Cells were treated with inducers of apoptosis in the presence or absence of DHEAS. The percentage of apoptotic cells was determined as described in the text. Individual data points represent the mean± S.D.

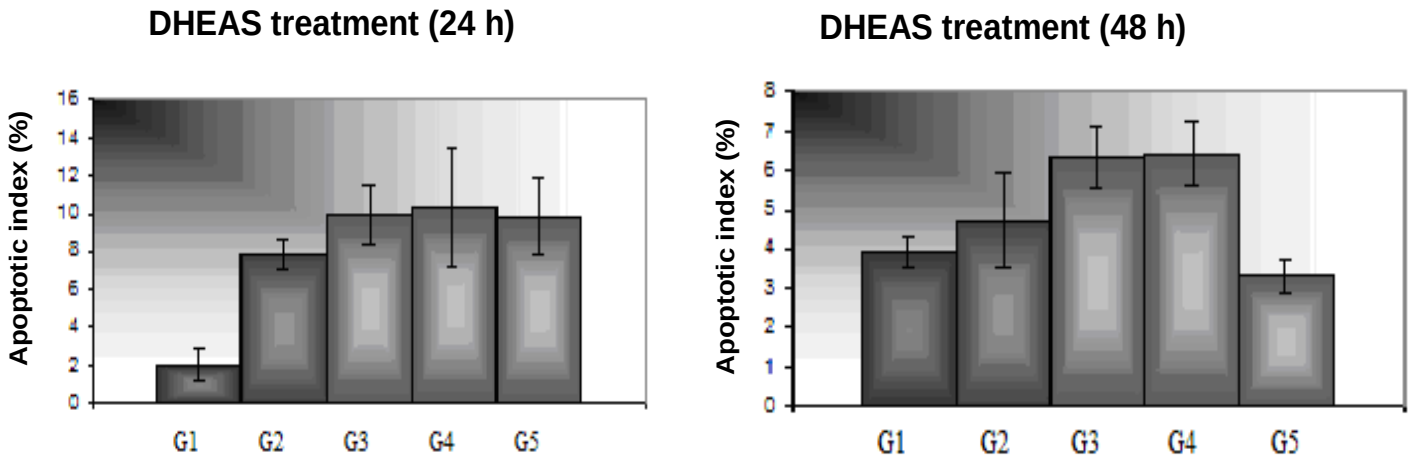

Figure 2. (A) Interrelations of apoptosis ındex of HL-60 cells and control groups after 24 h; (B) Interrelations of apoptosis ındex of HL-60 cells and control groups after 48 h. G1, control group; G2, DHEAS values 15 µg/ml; G3, DHEAS values 30 µg/ml; G4, DHEAS values 45 µg/ml; G5, DHEAS values 60 µg/ml. Individual data points represent the mean ± S.D.

Annexin V-positive live cells was found to be 10.3% and when incubation time was extended to 48 h, apoptotic index increased further to 6.4% (Figure 1). Apoptosis ındex of HL-60 cells were significantly increased after 24 h of being incubated with 45 µg/ml DHEAS treatment ($P < 0.05$). The effect of 45 µg/ml DHEAS on both cell lines on apoptosis was higher than that of the other doses of DHEAS (Figure 2A). Apoptosis of HL-60 cells was significantly increased by 45 µg/ml DHEAS treatment for 48 h ($P < 0.001$) (Figure 2B), but not higher than being incubated after 24 h. In addition, apoptosis ındex of HL-60 cells after 24 h of being incubated with 15, 30 and 60 µg/ml DHEAS treatments were significantly higher than 48 h of being incubated of same doses of DHEAS treatment ($P < 0.05$). The schemes for the uptake of DHEAS display TEM images for this purpose, firstly, DHEAS was imaged. The average particle size is about 30 nm. Figure 3 shows the TEM images of HL-60 cells were treated with DHEAS.

DISCUSSION

The present study investigated the effect of DHEAS on cell viability and apoptosis and the interaction in HL-60 cells. We demonstrated that DHEAS inhibited cell viability in a dose-dependent manner and DHEAS has been shown to induce apoptosis in cells. DHEAS, the major circulating steroid in human, is known for its clinical effects on hypertension, memory disorders, and cancer prevention (Arlt et al., 1999). It is also believed to play an important role in modulating different forms of cellular control, including processes associated with human immune, neural, and metabolic systems. The primary role of steroids is to modulate genetic transcription through binding to their intracellular receptors, which consequently elicit different physiological responses (Chasalow et al., 1989). Flow cytometry is a process whereby physical or biochemical parameters of single biological cells or particles are measured as the cells

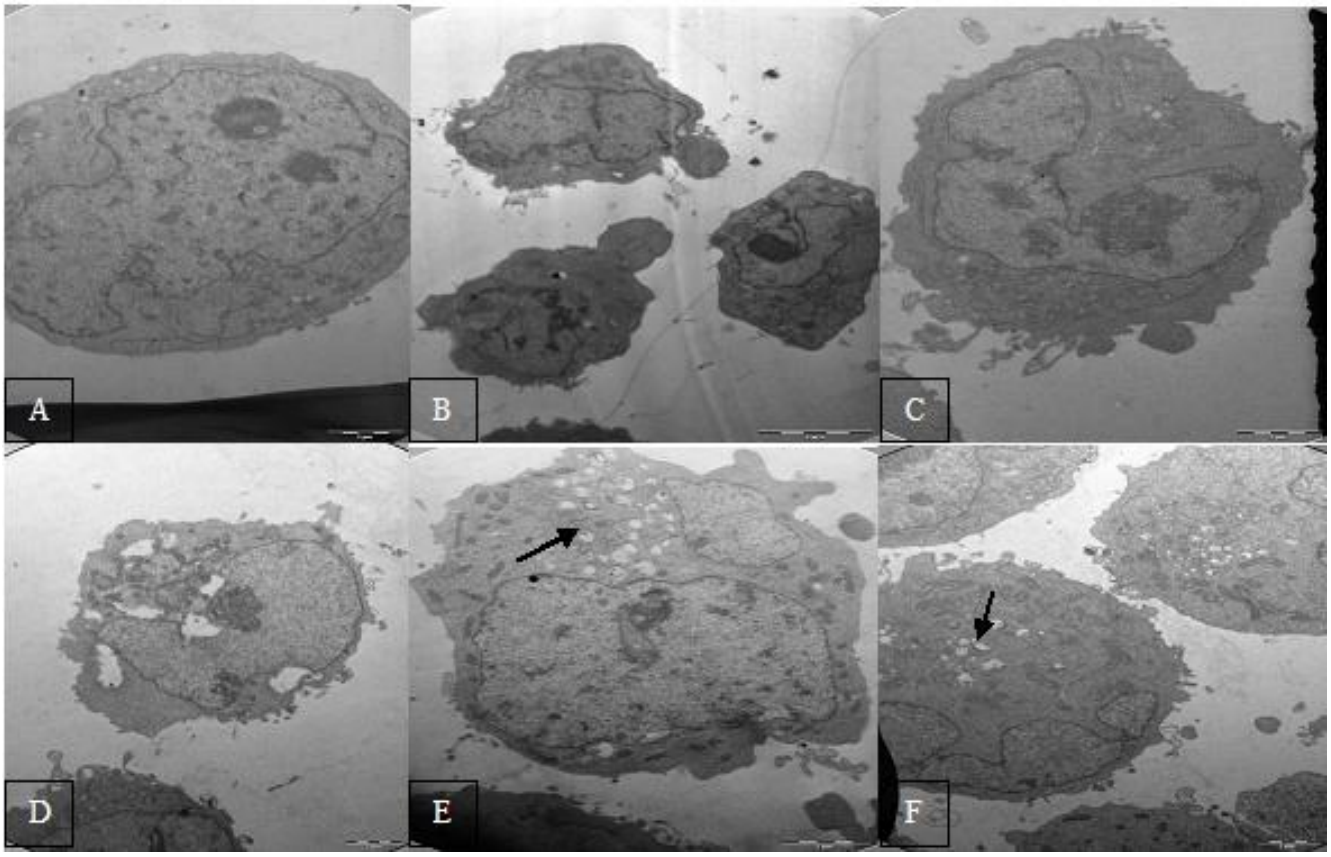

Figure 3. Transmission electron photomicrograph of control (A and B) and untreated control cells. There is normal morphology in the group electron micrographs demonstrating the effects of DHEAS on the HL-60 cells ultrastructure. HL-60 cells were treated with 30, 45 µg/ml for 24 h (C-D) (8200x to 6000x). HL-60 cells treated with 45 µg/ml DHEAS for 48 h revealed extensive vacuolization electron micrographs showing the effect of DHEAS on HL-60 cells (30 to 45 µg/ml) for 48 h. Numerous autophagic vacuoles (arrows) were observed 8200x to 6000x.

move through a fluidic channel (Shapiro, 2003). The Annexin V binding assay provides a very specific, rapid, and reliable technique to detect apoptosis by flow cytometry or by fluorescence microscopy. FITC is a very useful fluorescent moiety that can be used to label essentially any protein. FITC-conjugated Annexin V is used to detect apoptotic cells in a diverse range of cell types and in response to many different proapoptotic stimuli (Brumatti et al., 2008; Martin et al., 1995). Annexin V-FITC allows detection of cell surface changes that occur early during apoptosis (Vermes et al., 1995). Annexin V, an anticoagulant protein, binds to phosphatidylserine with high affinity. Thus, by staining cells with Annexin V-FITC and additionally with DNA-specific fluorochrome, e.g., propidium iodide, it is possible to identify live cells, early apoptotic cells, as well as late apoptotic and necrotic cells, by flow cytometry (Vermes et al., 1995; Zhang et al., 2008). Subsequently, Annexin V-FITC binds to cells expressing phosphatidylserine, an early marker of apoptosis on the cell surface. Target cells are gated upon as propidium iodide-negative and quantified with respect to their Annexin V positivity. The shift from Annexin Vneg to Annexin V high is a discrete event, such that all target cells fall within discernible populations with respect to Annexin V. Dead or dying cells are then stained with Annexin V-FITC. Annexin V has been used to detect apoptotic cells in a wide variety of cell types. Externalization of phosphatidylserine has been demonstrated in plasma membrane permeability changes, as measured by propidium iodide uptake and Annexin V binding, and precedes the morphological features of apoptosis as assessed by flow cytometric analysis of cell shrinkage (Goldberg et al., 1999). The sensitivity and early kinetics of Annexin V binding make it an ideal marker for cell death in a flow cytometric assay. Apoptosis was quantitatively confirmed by analyzing the percentage of early apoptotic cells using Annexin V-FITC/propidium iodide double staining. A marker of early apoptosis, measured by Annexin-V, is phosphatidylserine, which is released as a result of redistribution of the plasma membrane of the cells (Vermes et al., 1995). Three main populations of cells were distributed in dot-plots for viable cells Q3 (Annexin V-negative/propidium iodidenegative), early apoptotic cells

Q4 (Annexin V-positive/ propidium iodide-negative), and late apoptotic and necrotic cells Q2 (Annexin V-positive/propidium iodide-positive).

Flow cytometry by this double staining method enables clear detection of three populations of cells (viable, apoptotic and necrotic). Thus, the early apoptotic cells bind only to Annexin-V FITC, and late apoptotic cells to both Annexin V-FITC and propidium iodide, and viable cells do not take up any of the dye. The Annexin V-FITC/propidium iodide population was considered to reflect normal healthy cells, whereas Annexin V-FITC-positive/propidium iodide-negative cells were taken to show early apoptosis. Annexin V-FITC -positive/-propidium iodide-negative cells were in late apoptosis or necrosis (Molecular Station, 2010).

Our flow cytometric analysis has shown that 24 h of treatment with DHEAS caused apoptosis in a HL-60 cell line in a concentration-dependent manner. Apoptosis increased steadily following the increase at a concentration of 45 µg/ml. Flow cytometric determination of cell cycle phase distribution has further confirmed that DHEAS cause cell cycle deformation in a cancer cell line. The results of this study confirm that DHEAS has a protective effect on apoptosis in a cancer cell line. Some animal studies have shown a significant inhibitory effect of DHEAS in vivo tumorigenesis.

Oral administration of the compound inhibited the development of induced mammary cancers in female mice and reduced the incidence and multiplicity of 7,12 dimethylbenz[a]anthracene-induced lung tumors in the same species (Scott et al., 1997). Some studies showed that DHEAS has been shown to stimulate growth in breast cancer cells and however, DHEAS inhibited the cell growth. These results demonstrate that DHEAS has an inhibitory effect on cells. When DHEAS was later removed from the cell growth media, and cells were further cultured it, was shown that while the number of cells was greatly reduced by DHEAS treatment, the remaining cells were capable of further proliferation (Harrell et al., 2006). In cultures that received continuous DHEAS treatment, inhibition of cell proliferation continued. Significant apoptosis was observed in cell lines. These data support of DHEAS suggests that prolonged treatment may be warranted to attain maximal antiproliferative benefit (Hardin et al., 2007).

The viability of normal cells after each incubation time with the DHEAS was close to that of control cells, and was in the 95 to 96% range in flow cytometric analysis. After 48 h of culture in media containing DHEAS, the morphology and viability of normal cells containing the DHEAS was close to that of the control cells, suggesting biocompatibility of the DHEAS. The TEM and flow cytometry results showed that the uptake of DHEAS was internalized into both normal cells and cancer cells. The immobilization of DHEAS was demonstrated by increasing the amount of DHEAS uptake into cancer cells in comparison with normal cells. In our flow cytometry results, the population of Annexin V-positive live cells in the cancer cell line increased in a time-dependent manner, and most of the cells became Annexin V-positive at 24 h at a concentration of 45 µg/ml.

Apoptosis is a normal physiologic process that occurs during embryonic development as well as helps in the maintenance of tissue homeostasis or in the prevention of carcinogenesis. Some authors have reported the protective effect of DHEA on apoptosis in mice or rats; however, the effects of DHEA on chemical-induced apoptosis in human cells have not yet been reported. Many hormones, including DHEA and DHEAS, can affect apoptosis (Takahashi et al., 2004). This study provides evidence of a direct effect of DHEAS on the HL-60 cell line. Together with our recent work where we showed that this androgen induces apoptosis and morphological terminal differentiation of the HL-60 cell line. In our study, maximal apoptosis occurs at a DHEAS concentration of 45 µg/ml after 24 and 48 h. Although, other concentrations of DHEAS increase apoptosis on HL-60 cell. These data suggest significant direct effects of different concentrations of DHEAS on apoptosis in different times.

In conclusion, this study demonstrated that exogenous DHEAS produced anti-proliferative effects on HL-60 cell lines via the apoptosis pathway. Our data suggest significant direct effects of different concentrations of DHEAS on apoptosis in different times. This study has important implications in cancer cell imaging, tumor ablation, and drug delivery and this is the first study showing that DHEAS induces apoptosis on HL-60 cell line; however, DHEAS may be an effective treatment for cancer.

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