The Effects of Vitamin E on Apoptosis in HepG2 Human Hepatocellular Carcinoma Cell

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ABSTRACT
The hepatocellular carcinoma cells were cultured in the absence (control) and presence of different dose of VE (Vitamin E) for 24 h. The effect of VE on caspase 3, caspase 9, caspase 1 and cytochrome c in hepatocarcinoma cells were investigated. There were significant increase in caspases 1, 3 and 9 levels in VE treated HepG2 cells. Caspase 1 level significantly increased in HepG2 cells treated with 50 μM VE (p<0,01). Caspase 3 level increased in HepG2 cells treated with 50mM, 50 μM and 10 μM VE (p<0,001). Caspase 9 levels in cells treated with 10 μM VE was found two fold higher than control cells (p<0,05). No differences were found in the cytochrome C levels of the cells and there was no DNA fragmentation pattern in the cells treated with different dose VE. These findings suggest that caspases-dependent cell death occurs in different doses of VE applied HepG2 cells.

Keywords: Vitamin E, Apoptosis, HepG2, Caspase
1. INTRODUCTION

Hepatoma which is the sixth most common cancer worldwide and becomes the leading cause of cancer related mortality in the world due to its incidence increased rapidly (El-Serag and Rudolph, 2007). Clinical studies of the last 30 years on the issue have proven protective effects of individual or combinational antioxidant vitamin and mineral supplements against cancer and other chronic diseases. On the contrary, some results of the previous studies demonstrated that vitamin and mineral supplements did not have any preventive role in development of primary tumors. Since development of malignant tumors may exceed clinical research period and heterogeneity of the subjects may cause unpredictable outcomes, individual or combination of antioxidant compounds should also be studied in model established cell lines especially for in the case of cancer (Yurtcu et. al., 2010).

Vitamin E (VE) exhibits various functions including antioxidant, anti-inflammatory and anti-thrombolytic activities in addition to other therapeutic activities (Azzi et al., 2002; Herrera and Barbas, 2001). In recent years, VE and its isoforms have generated much interest as they have been reported to possess anticancer and tumor suppressing activities (Alqahtani and Kaddoumi, 2015). VE has shown to be highly selective against a variety of cancer types, including breast, prostate, lung, stomach, ovary and colon cancer cells but largely safe to normal cells (Constantinou et al., 2008).

Apoptosis is an important phenomenon in cytotoxicity induced by anticancer drugs. The mechanism of apoptosis includes receptor and mitochondria dependent signaling pathways (Strasser et al., 2000). Caspases have proteolytic activity and are able to cleave proteins. There are ten major caspases with three main subgroups: initiators (-2, -8, -9 and -9), effectors (-3, -6 and -7) and inflammatory (-1, -4 and -5) (Strasser et al., 2000; Weinberg, 2007).

The present study aimed to evaluate the cytotoxicity of VE on hepatocellular cancer cells and investigated the effect of VE on apoptosis in hepatocellular cancer cells.

2. MATERIAL AND METHOD

2.1. Cell culture and α-Tocopherol preparation:

Human HCC cell line HepG2 was purchased from the American Type Culture Collection (ATCC, Cat No. HB-8065). HepG2 cells were maintained in RPMI 1640 containing 10% heat-inactivated FBS and 50 mg/l gentamycin + 300 mg/l L-glutamine. Cells were grown in a 37°C incubator supplied with 95% room air and 5% CO2. After growing to 85% confluence, cells were trypsinized with 0.25% trypsin-EDTA, counted and, were sub-cultured into 25 cm² flasks or 96-well plates according to the selected experiments. Cells were allowed to attach to the surface for 24 h prior to VE exposure. VE (α-tocopherol succinate, 0-4500 μM) was dissolved in medium.

2.2. Cell viability assay

The inhibition of cell proliferation by α-tocopherol was determined by assaying the reduction of MTT to formazan. After incubation with α-tocopherol (for 24 h, the cells (10⁴/well) in 96-well plates were washed with phosphate-buffered saline (PBS), and MTT (10 μl) was added to each well and incubated at 37°C for 2-4 h. 10% SDS (100 μl) was added to dissolve the formazan crystals. The absorbance rate of each well optical density (OD value) was measured at 570 -650 nm by a spectrophotometer. The cell proliferation rate was calculated as (average OD value of wells with administered VE/average OD value of control wells) x 100.
2.3. Quantification of Caspase activities:
Caspase-1, caspase-3 and caspase-9 enzyme activities were examined in VE treated and control cells using Bio-Vision colorimetric assay kits. Crude cell extract was prepared as described in the manufacturer’s instruction. In brief, cells were cultured in a 75-cm² culture flask and exposed to different concentrations of VE (10µM, 50µM and 50mM) for 24 h. At the end of the exposure time, cells were harvested in ice-cold phosphate buffer saline by scraping and were then washed with phosphate buffer saline at 4 °C. The cell pellets were then lysed in cell lysis buffer [1 × 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton, 2.5 mM sodium pyrophosphate]. Centrifugation (15,000g for 10 min at 4 °C) was performed to avoid cell debris and the supernatant (cell extract) was maintained on ice for caspase assays. This assay was based on the principle that activated caspases in apoptotic cells cleave the synthetic substrates to release free chromophore p-nitroanilide (pNA), which is measured at 405 nm. The pNA was generated after specific action of caspase-1, caspase-3 and caspase-9 on tetrapeptide substrates YVAD-pNA, DEVD-pNA and LEHD-pNA, respectively. The reaction mixture consisted of 50 μl of cell extract protein, 50 μl of 2× reaction buffer (containing 10 mM dithiothreitol) and 5 μl of 4 mM YVAD-pNA (for caspase-1), DEVD-pNA (for caspase-3) or LEHD-pNA (for caspase-9) substrate in a total volume of 105 μl. The reaction mixture was incubated at 37 °C for 2 h and the absorbance of the product was measured using a microplate reader at 405 nm according to manufacturer’s protocol. The specific caspase activity, normalized for total proteins of cell lysates, was then expressed as % fold of the baseline caspase activity of control cells.

2.4. Detection of cytochrome c release from the mitochondria to the cytosol:
Cytochrome c determination in cytosolic and mitochondrial fractions was done by western blotting using Bio-Vision kits. The cells were harvested without or with α-tocopherol (10µM, 50µM and 50mM) for 24 h and then washed once with ice-cold PBS. Mitochondrial and cytosolic isolation was prepared as described in the manufacturer’s instruction.

2.5. DNA fragmentation assay:
DNA was isolated using Apoptotic DNA ladder kit (Millipore, APT151) according to the manufacturer’s protocol. The extracted DNAs were electrophoresed through a12% agarose gel and stained with ethidium bromide.

2.6. Protein assay:
The total protein content in the cell extracts was estimated by the Bradford method using bovine serum albumin as the standard.

3. STATISTICAL ANALYSIS
Statistical comparison between the treated and control groups were performed using one-way ANOVA with post hoc Duncan test. P-values <0.05 were considered statistically significant.

4. RESULTS
The results of cell viability assay in HepG2 cells treated with different doses of VE presented in figure 1. The percent viability of cells receiving different doses of VE decreased with increasing concentrations. In other words, VE had antiproliferative effects on HepG2 cells in dose dependent manner. According to results of cell viability assay, 10µM, 50µM, and 50 mM concentrations were chosen as application doses for other analysis.
The result of caspase -1, caspase-3 and caspases-9 activity assays were shown in figure 2,3 and 4 respectively. There were significant increase in caspases 1, 3 and 9 levels in VE treated HepG2 cells. Increment of the caspase 3 levels in HepG2 cells treated with 50mM, 50 μM and 10 μM vit E were found 216 %, 164% and 272% compare to control cells, respectively. Caspase 1 level significantly increased in HepG2 cells treated with 50 μM VE, increment of the caspase 1 levels in the cells treated with 10 μM VE and 50 mM VE were observed insignificantly. Caspase 9 levels in cells treated with 10 μM VE was found two fold higher than control cells. No change was observed in the cells treated 50 mM VE and 50 μM VE. No differences were found in the cytochrome C levels of the cells treated with different dose VE. There was no DNA fragmentation pattern were found in the cells treated with different dose.

**Figure 1.** Evaluation of cell viability under the VE treatment. Cells were treated with 0, 10, 25, 50, 250, 500, 1500, 2250, 4500 μM VE for 24 h.

**Figure 2.** Relative caspase-9 activity after 24 h incubation of VE on HepG2 cells. (*indicates the significance of differences as compared with control; p<0.05)


**Figure 3.** Relative caspase-1 activity after 24 h incubation of VE on HepG2 cells. (*indicates the significance of differences as compared with control; p<0.01)

**Figure 4.** Relative caspase-3 activity after 24 h incubation of VE on HepG2 cells. (*indicates the significance of differences as compared with control; p<0.001)

### 5. DISCUSSION

As an antioxidant, Vit E acts in cell membranes where prevents the propagation of free radical reaction, although it has been also shown to have pro-oxidant activity (Herrera and Barbas, 2001). Some studies have shown that treatment with various doses of alpha-tocopherol was also found to inhibit cell growth and induce apoptosis in various cell types (Liu et al., 2001; McIntyre et al., 2000).
An important goal of anticancer strategies is specific induction of apoptosis in cancer cells while shielding normal cells.

The cell growth inhibitory activities of VE were evaluated using the MTT assay. In HepG2 cells, growth inhibition by VE was dose-dependent. We observed significant cytotoxic effect at 24 h incubation and over 250 mM VE dose.

The ability to induce apoptosis by 10 μM, 50 μM and 50 mM VE was examined based on caspase-3 and caspase-9 activation. Caspase-3 was increased 2.7 fold, 1.6 fold and 2.2 fold by 10 μM, 50 μM and 50 mM VE, respectively at 24 h. Caspase 9 levels in cells treated with 10 μM VE was found two fold higher than control cells. No change was observed in the cells treated 50 mM VE and 50 μM VE. Mitochondrial stress-induced caspase activation can be initiated by numerous cellular signals that cause perturbations in mitochondria resulting in the loss of mitochondrial membrane potential and release of proapoptotic molecules such as apoptosis inducing factor (AIF) and cytochrome c from the intermembrane space into the cytoplasm. Cytochrome c then interacts with apoptosis protease activation factor-1 and pro-caspase-9 to form a complex (apoptosome) that induces the activation of initiator caspase-9, and finally leads to the activation of effector caspase-3 and apoptosis (Weinberg, 2007).

DNA fragmentation, mitochondrial and cytosolic cytochrome c were analyzed in HepG2 cells treated with different dose VE for 24 h, a typical ladder pattern of internucleosomal fragmentation and cytosolic cytochrome c was not evident.

VE induced apoptosis occurs independently of mitochondrial stress apoptotic signaling in neoplastic mammary epithelial cells. According to this study that mitochondrial membrane stability and integrity might actually be enhanced for a limited period of time following acute VE exposure, thereby acting to inhibit mitochondrial cytochrome c release into the cytoplasm and prevent mitochondrial stress mediated apoptotic signaling in these cells (Shah and Sylvester, 2004).

Caspase 1 level significantly increased in HepG2 cells treated with 50 μM and 50 mM VE. Caspase-1 has roles in inflammation and mediating inflammatory cell death by pyroptosis, a lytic form of cell death. Activation of caspase-1 occurs in multi-protein complexes termed inflammasomes, which assemble upon sensing of stress signals. Caspase-1 was required for apoptosis and caspase-1-dependent apoptosis is protected the cells. According to researchers, these results suggest a protective role of caspase-1 in keratinocytes during UVB-induced skin cancer development through the induction of apoptosis (Sollberger et al., 2015).

Latest finding suggest that derivatives of VE could be used in combination with anticancer drugs and/or as part of the delivery system for multiple drug resistance (MDR) sensitization (Azzi et al., 2002).

Data presented in this study show different concentrations of VE was demonstrated to be an effective inducer of apoptosis in vitro hepatocellular carcinoma cells, capable of activating caspase-3. VE acts as a pro-apoptotic agent for HepG2 cells.

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CONFLICT OF INTEREST:
The authors declare that there are no conflicts of interest.
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