Neferine increase in vitro anticancer effect of dehydroepiandrosterone on MCF-7 human breast cancer cells

Dingyi Yang1 · Xiaochuan Zou2 · Ruokun Yi2,3,4,5 · Weiwei Liu6 · Deguang Peng7 · Xin Zhao2,3,4,5

Abstract This study was conducted to investigate the in vitro anticancer reinforcing effects of neferine (Nef) on dehydroepiandrosterone (DHEA) and the mechanism was also determined during the investigation. By the growth effects of Nef and DHEA on MCF-7 human breast cancer cells, 8 mg/mL Nef was a non-virulent concentration in MCF-7 cells, and this concentration was used for further experiment. In 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay, 30 mg/mL DHEA showed 49.4 % growth inhibitory effect in MCF-7 cells, whereas Nef (8 mg/mL) + DHEA (30 mg/mL) treatment had the higher effect at 67.8 %. The flow cytometry analysis results showed that 15 and 30 mg/mL DHEA-treated MCF-7 cells had 12.2 and 21.6 % apoptotic cells, respectively, Nef + DHEA could raise the apoptotic cells to 36.7 %. Reverse transcription-polymerase chain reaction assay shows remarkable results according to which DHEA could significantly increase caspase-3, caspase-8, caspase-9, Bax, p53, p21, E2F1, Fas, FasL mRNA expressions and decrease Bcl-2, Bcl-xL, HIAP-1, HIAP-2, survivin expressions as compared to the untreated control cancer cells. Moreover, these effects depend on the concentration of DHEA, and Nef which could further strengthen these effects. From these results, low concentration of Nef could not influence the growth of MCF-7 cells, but using its sensitization effect, Nef raised the in vitro effects of DHEA. Nef could be got easily. With these results we can accomplish that Nef + DHEA might be used as the new anticancer materials combination.

Keywords Anticancer · Dehydroepiandrosterone · Expression · MCF-7 cells · Neferine

Introduction

Dehydroepiandrosterone (DHEA) is a kind of endogenous steroid hormone, which is mainly secreted by adrenal cortex (Samaras et al. 2013). It is confirmed in clinical
reports and animal experiments that the decrease of DHEA can have adverse effect on the body and lead to the increasing morbidity of diseases such as cardiovascular system diseases, nervous system diseases, and osteoporosis (Samaras et al. 2013; Mannic et al. 2015). Studies also showed that long-term supplement of DHEA have inhibition effect on a variety of animal tumors induced by chemical carcinogen, such as colon cancer and cutaneous papilloma, as it has a good anti-original mutagenic activity (Yang et al. 2001; Webb et al. 2006). As its effect is gradually confirmed, DHEA has been used in a variety of health food products, while the extraction of DHEA from natural foods and resources is also established on a vast scale. The value of DHEA in the future use will be further improved.

Neferine (Nef) is a kind of alkaline which is extracted from green germ in mature seeds of nymphaeaceae plant lotus, it has some functional effects (Zheng et al. 2014). Research done by many well-known scientists showed that it has many pharmacological effects, such as expanding blood vessels, reducing blood pressure, anti-arrhythmia, anti-aggregation of platelets, anti-thrombosis, resisting oxidation, resisting poisoning of organophosphate pesticide, and so on (Xia et al. 1986; Hu et al. 1990; Jia et al. 1994). Nef, in particular, has very valuable chemotherapeutic sensitization effect and studies have found that Nef with no significant cytotoxicity can enhance cell cytotoxic effect of adriamicyn (ADM), fluorouracil (5-FU), and cis-platin on human breast cancer cells and enhance the inhibition of ADM on bone sarcoma (Tang and Cao, 2001). Nef with sensitization concentration does not significantly induce tumor cell apoptosis, but can significantly enhance apoptosis induced by adriamycin. Enhancing apoptosis induced by anticancer drugs may be one of Nef sensitization mechanisms (Bao et al. 2003).

The occurrence and development of tumor is a complex process related to many genes, steps, and stages. Apoptosis mainly plays a negative regulatory role in the occurrence and development of cancer, which can inhibit rapid growth of tumor cells (Su et al. 2015). According to the current understanding of apoptosis regulatory mechanism, cell apoptosis-related genes can be roughly divided into two major categories, pro-apoptosis genes and anti-apoptosis genes. When the activity of pro-apoptosis is inhibited or on the contrary anti-apoptotic genes are activated, as a result the cell cannot show apoptosis but will survive for a long time (Dang et al. 2016). Besides, if cancer genes show abnormal high expression and/or the activity of anti-tumor genes is inhibited, this may eventually lead to canceration and the formation of tumors (Bajwa et al. 2012). The occurrence and development of tumors are not only the results of out-of-control cell proliferation and abnormal cell differentiation, but also have a crucial relation with the imbalance of tumor cells apoptosis. So, an important tumor treatment is required to intrusively regulate tumor cell apoptosis based on the imbalance of tumor cell apoptosis (Hassan et al. 2014). Tumor cell apoptosis is one of the mechanisms of many clinically applied cancer treatments, including radiotherapy, chemotherapy, hormone therapy, heat treatment, and other biological therapies. Using functional food to prevent or treat cancer can also be achieved through inducing cancer cell apoptosis (Bao et al. 2003). Some scholars believe that even in normal body there are a very small amount of cancer cells present, so eating healthy products in daily diet to clear these cells will also be beneficial to health (Fan 2010). Studies have shown that DHEA has a certain anticancer effect (Liu et al. 2010; Teng et al. 2014). As a result, a combination of both will be advantageous to develop functional food with better health care effects if the enhanced sensitization effect of Nef can eventually improve the apoptosis of DHEA cancer cells. By observing the enhancing effect of Nef on the anticancer effect of DHEA in vitro under concentration with no significant toxicology effects, this study tests the expression of mRNA in cancer cells dealt with Nef + DHEA treatment to determine the anticancer mechanism of Nef + DHEA through cancer cell apoptosis. The results ultimately provide the theoretical support to develop functional food by Nef + DHEA, which is advantageous to the development of new functional food.

Materials and methods

Cell Preparation

MCF-7 human breast cancer cells and Hs578Bst mammary fibroblasts were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Both the cancer cells and normal cells were cultured in DMEM medium (Thermo Fisher Scientific Inc.; Waltham, MA, USA) contained 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco-BRL; Grand Island, NY, USA) at 37 °C in a humidified atmosphere with 5% CO2 (incubator model 311 S/N29035; Forma, Waltham, MA, USA). The medium was changed for every three days.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay

Growth of MCF-7 cells of Nef or DHEA or Nef + DHEA treatment were determined by the MTT assay. The 100 μL of MCF-7 or Hs578Bst cells were seeded in 96-well plates at 1.0 × 10^5 cells/mL in each well, then the cells were incubated at 37 °C in 5% CO2. After 24 h, the culture
medium in plate well was aspirated and discarded, then the new culture medium with 0–10 mg/mL of Nef or 0–50 mg/mL of DHEA was added to the plate well. The cells were continuously incubated for 48 h and the culture medium was aspirated and discarded in plate well again. After that, the culture medium containing 5 mg/mL MTT solution (Amresco; Solon, OH, USA) was added in plate well and cultured for 4 h. Followed by removal of the MTT solution culture medium, 100 μL of DMSO was added to plate well and mixed for 30 min. Subsequently, the absorbance of plate well was determined by the enzyme-linked immunosorbent assay (ELISA) reader (model 680; Bio-Rad; Hercules, CA, USA) at 540 nm (Zhao et al. 2013). Then the non-virulent concentrations of Nef or DHEA were chosen for further experiments. And the growth of Nef + DHEA treatment in MCF-7 cells was also determined by MTT assay as the above experiment method.

Flow cytometry analysis

After treatment with Nef + DHEA, the cells were subsequently treated by trypsinizing, collecting, washing with cold PBS (phosphate-buffered saline), and resuspended in 2 mL PBS. DNA contents of the MCF-7 cancer cells were determined by a DNA staining kit (CycleTEST™ PLUS kit; Becton–Dickinson, Franklin Lakes, NJ, USA). Nuclear fractions stained with propidium iodide were obtained by following the protocol of the manufacturer. Fluorescence intensity was determined using an FACScan flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Brea, CA, USA) and analyzed with CellQuest software (Becton–Dickinson) (Zhao et al. 2013).

RT-PCR assay

Total RNA of the MCF-7 cancer cells in different groups were isolated using Trizol reagent (Invitrogen; Carlsbad, CA, USA) and isolation was done by the manufacturer’s recommendations. The isolated RNA was digested by RNase-free DNase (Roche; Basel, Switzerland) at 37 °C for 15 min and purified using the RNaseasy kit (Qiagen; Hilden, Germany) by the manufacturer’s protocol. cDNA of MCF-7 cancer cells was synthesized from total RNA (2 μg) by incubating at 37 °C for 1 h with avian myeloblastosis virus reverse transcriptase (GE Healthcare; Little Chalfont, UK) with random hexanucleotides according to the manufacturer’s guidelines. Sequences of primers (Tables 1, 2) were used to specifically amplify the genes of interest. Amplification was performed in a thermal cycler (Eppendorf; Hamburg, Germany). The polymerase chain reaction (PCR) products were separated in 1.0 % agarose gels and visualized with ethidium bromide staining.

Statistical analysis

The in vitro parallel experiments were determined three times and the experiment data were presented as mean ± SD. Differences between the mean values for individual experiment groups were assessed with one-way analysis of variance (ANOVA) with Duncan’s multiple range test. And p < 0.05 was considered to indicate a statistically significant difference. SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used to conduct the statistical analyses.

Results

Inhibitory effects of Nef + DHEA

The growth of Nef and DHEA on Hs578Bst mammary fibroblasts and MCF-7 human breast cancer cells were determined by MTT assay (Figs. 1, 2). After treatment of 0–8 mg/mL Nef, the growth of Hs578Bst mammary fibroblasts and MCF-7 human breast cancer cells was almost 100 %; these concentrations of Nef could not influence the growth of Hs578Bst and MCF-7 cells and in any mean, in the same way, had no toxic effect on these cells (Fig. 1). And by the same experiment, 0–30 mg/mL of DHEA also showed no toxicity on Hs578Bst cells, but DHEA could inhibit the growth of MCF-7 cells depend on the concentration of 0–50 mg/mL (Fig. 2). The 0–8 mg/mL Nef had no effect on both normal and cancer cells. Moreover, 0–30 mg/mL DHEA had also no effect on normal cells, but these concentrations of DHEA reduce the cancer cells growth. Based on the above experiment results, the concentration of 8 mg/mL Nef was chosen to do further experiments as sensitization concentration of Nef, and the 15, 30 mg/mL of DHEA were chosen to do the in vitro anticancer effect experiments. The treatment of both Nef and DHEA in Hs578Bst normal cells and MCF-7 cancer cells was also determined by MTT assay (Fig. 3). Nef (8 mg/mL) and different concentrations of DHEA (0–40 mg/mL) could not significantly (p < 0.05) reduce the growth of Hs578Bst cells, but these treatment could raise the growth inhibition of MCF-7 cancer cells, and in the same concentration of DHEA, Nef adding had the better cancer cells growth inhibitory effect than only DHEA treatment.

By the MTT assay, DHEA-L (15 mg/mL DHEA) and DHEA-H treatment (30 mg/mL DHEA) had 19.7 and 49.4 % growth inhibitory rates on MCF-7 cancer cells (Table 1). Higher the concentration of DHEA, stronger the growth inhibitory effect on cancer cells. Nef (8 mg/mL) could significantly (p < 0.05) raise the inhibitory effect of 30 mg/mL DHEA treatment, causing the inhibitory rate to reach as high as 67.8 %.
Induction of Apoptosis by Nef + DHEA

DNA content of the sub-G1 MCF-7 cancer cells after treated with Nef + DHEA was evaluated by flow cytometric analysis (Fig. 4). The control cells had only 3.8% apoptotic cells (sub-G1 cells), the sub-G1 DNA content of Nef + DHEA-treated MCF-7 cells was highest (36.7%), and the content of DHEA-H treatment (21.6%) that was higher than DHEA-L treatment (12.2%).

mRNA expression of caspase-3, caspase-8, and caspase-9 on Nef + DHEA-treated cancer cells

Two concentration of DHEA raised the caspase-3, caspase-8, and caspase-9 mRNA expressions of MCF-7 cancer cells as compared to the control group cells (Fig. 5). The mixture of Nef and high concentration DHEA-treated cells showed the higher caspase-3 (4.19 folds of control), caspase-8 (5.43 folds of control), and caspase-9 (5.00 folds of control) expressions than only high concentration DHEA-treated cells.

Table 1 Sequences of reverse transcription-polymerase chain reaction (RT-PCR) primers used in this study

| Gene name | Sequence |
|-----------|----------|
| Caspase-3 | Forward: 5'-CTG GAA TAT CCC TGG ACA AC-3' |
| Caspase-8 | Reverse: 5'-CTC GCT ACC TCG GTG ACT TGG-3' |
| Caspase-9 | Forward: 5'-GCC CCT TCC TGG ACT CTC AGT-3' |
| Bax       | Forward: 5'-AGG ATG AAA TCC AAA GCT-3' |
| Bcl-2     | Reverse: 5'-AGC CTT TCT CCG CAG TTT CCT-3' |
| Bcl-xL    | Forward: 5'-GCC AGA AAG GAT ACA GCT GG-3' |
| HIAP-1    | Reverse: 5'-GCC CGC TGG ACC CTC AAC AAT-3' |
| HIAP-2    | Forward: 5'-GCC GAT GGG CTT CCT GCA-3' |
| p53       | Reverse: 5'-GCC CAA GCA GAG ACA AAC AT-3' |
| p21       | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| E2F1      | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| Survivin  | Forward: 5'-GCC CCT TCC TGG ACT CTC AGT-3' |
| Fas       | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| FasL      | Forward: 5'-GCC CGC TGG ACC CTC AAC AAT-3' |
| GAPDH     | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| Bcl-xL    | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| HIAP-2    | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| p53       | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| p21       | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| E2F1      | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| Survivin  | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| Fas       | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| FasL      | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| GAPDH     | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| Bcl-xL    | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| HIAP-2    | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| p53       | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| p21       | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| E2F1      | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| Survivin  | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| Fas       | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |
| FasL      | Forward: 5'-GCC ATG AGC TAT CTC GCC TTA-3' |
| GAPDH     | Reverse: 5'-GCC CAA GCA GAC ACA AAC AT-3' |

Table 2 Growth inhibition of MCF-7 human breast cancer cells by DHEA and Nef + DHEA as evaluated by an MTT assay

| Treatment   | OD_{540} | Inhibitory rate (%) |
|-------------|----------|---------------------|
| Control     | 0.466 ± 0.010<sup>a</sup> | 0.0 ± 0.0<sup>e</sup> |
| DHEA-L      | 0.374 ± 0.012<sup>b</sup> | 19.7 ± 2.9<sup>c</sup> |
| DHEA-H      | 0.236 ± 0.009<sup>c</sup> | 49.4 ± 3.1<sup>b</sup> |
| Nef + DHEA  | 0.150 ± 0.010<sup>d</sup> | 67.8 ± 3.5<sup>a</sup> |

<sup>a</sup>-<sup>d</sup> Mean values with different letters in the same column are significantly different (p < 0.05) according to Duncan’s multiple range test. Control: untreated cells; DHEA-L: 15 mg/mL dehydroepiandrosterone-treated cells; DHEA-H: 30 mg/mL dehydroepiandrosterone-treated cells; Nef + DHEA: 8 mg/mL neferine, and 30 mg/mL dehydroepiandrosterone-treated cells.
mRNA expression of Bax, Bcl-2, and Bcl-xL on Nef + DHEA-treated cancer cells

DHEA could raise Bax mRNA expression of MCF-7 cells, however, reduce Bcl-2, Bcl-xL expressions as compared to the untreated control cells (control group) (Fig. 6). The 30 mg/mL DHEA-treated cells showed the higher Bax (4.19 folds of control) expression and lower Bcl-2 (0.58 folds of control), Bcl-xL (0.37 folds of control) expressions than 15 mg/mL DHEA-treated cells (3.31, 0.66, and 0.54 folds of control), whereas Nef + DHEA-treated cancer cells showed the highest Bax (6.03 folds of control) expression and lowest Bcl-2 (0.30 folds of control), Bcl-xL (0.26 folds of control) expressions.

mRNA expression of HIAP-1 and HIAP-2 on Nef + DHEA-treated cancer cells

The control cancer cells showed the noticeable HIAP-1 and HIAP-2 mRNA expressions (Fig. 7). But after the treatment of DHEA, these expressions were reduced and the HIAP-1 and HIAP-2 expressions of DHEA-L, DHEA-H, Nef + DHEA-treated cells showed 0.77, 0.46, 0.29 and 0.72, 0.35, 0.13 folds expression of control, respectively.
mRNA expression of p53 and p21 on Nef + DHEA-treated cancer cells

The p53 expression of DHEA-L (2.85 folds of control), DHEA-H (4.43 folds of control), Nef + DHEA (6.76 folds of control) was higher than that of control (Fig. 8). p21 expression in these group cells (1.31, 1.64, and 2.93 folds of control) was also higher than control group.

mRNA expression of E2F1 and survivin on Nef + DHEA-treated cancer cells

After treated with Nef + DHEA, the E2F1 (4.45 folds of control) expression activity increased whereas survivin (0.35 folds of control) expression activity decreased but significantly higher as compared to only treated with DHEA (Fig. 9). On the other hand, DHEA or Nef + DHEA-treated cells had stronger E2F1 expressions and weaker survivin expressions than control cells.
mRNA expression of Fas and FasL on Nef + DHEA-treated cancer cells

Nef + DHEA-treated cancer cells had the highest Fas (8.56 folds of control) and FasL (1.37 folds of control) expressions, and DHEA-H-treated cells had the higher Fas (6.66 folds of control) expression than DHEA-L (5.34 folds of control)-treated cells, but showed the similar FasL (1.15 folds of control) expression to DHEA-L (1.15 folds of control) treated cells (Fig. 10). The Fas/FasL rate of Nef + DHEA (1.29) group also highest, and DHEA-H (1.19) group also higher than DHEA-H (0.96) and control (0.21) groups.

Discussion

Cell apoptosis is closely related to tolerance of anti-tumor cell materials. The inhibition of tumor cell apoptosis may be a more common tolerance mechanism, so besides anti-tumor cell materials, sensitization materials also play an important role in tumor inhibition (Zhu et al. 2015). This study shows that DHEA itself has certain valuable anti-tumor effects, and the effect basically based on the inducing cancer cell apoptosis, while Nef under concentration without toxic effect can significantly enhance the inducing effect of DHEA on cancer cells apoptosis.

Study finds that apoptosis mainly has two independent apoptotic pathways. One is activation path through death receptors. Death receptors are certain proteins on the surface of cell membrane, which are having the ability to combine with specific ligands that carry apoptosis signals and quickly transduct apoptosis signals into cells to induce apoptosis. Another pathway is mitochondria-cytochrome c path. Cytochrome c released by mitochondria can form complex (apoptotic body) with Apaf-1 and caspase-9 and activate caspase-3 with the existence of dATP and ATP to start caspase cascade reaction which ultimately induces apoptosis (Hu et al. 1999). In the activation pathway which is through death receptors, there are specific death receptors, on the surface of apoptotic cells, which can accept extracellular death signals to activate cell apoptosis. Stimulated by certain apoptosis signals, Fas can combine with its specific ligand (FasL) and start polymerization in the membrane surface. Then the compound combines with caspase-8 which specifically form a structural domain by
Fig. 7 Effect of liensinine on the mRNA expression of HIAP-1 and HIAP-2 in MCF-7 human breast cancer cells. Fold ratio: gene expression/GAPDH × control numerical value (control fold ratio: 1). Different letters denote significantly different (p < 0.05) according to Duncan’s multiple range test. Control: untreated cells; DHEA-L: 15 mg/mL dehydroepiandrosterone-treated cells; DHEA-H: 30 mg/mL dehydroepiandrosterone-treated cells; Nef + DHEA: 8 mg/mL neferine and 30 mg/mL dehydroepiandrosterone-treated cells.

Fig. 8 Effect of liensinine on the mRNA expression of p53 and p21 in MCF-7 human breast cancer cells. Fold ratio: gene expression/GAPDH × control numerical value (control fold ratio: 1). Different letters denote significantly different (p < 0.05) according to Duncan’s multiple range test. Control: untreated cells; DHEA-L: 15 mg/mL dehydroepiandrosterone-treated cells; DHEA-H: 30 mg/mL dehydroepiandrosterone-treated cells; Nef + DHEA: 8 mg/mL neferine and 30 mg/mL dehydroepiandrosterone-treated cells.
FADD and make caspase-8 form dimers to activate itself. Caspase-8 is released into cytoplasm to activate effectively caspase-3, 6, 7 which leads to apoptosis (Sun et al. 2013). Besides, caspase-8 can cut bcl-2 family Bid into tBid, which can promote the release of cytochrome c from mitochondria (Wang and Tjandra 2013). Cytochrome c, which is released into cytoplasm, activates caspase-9 that has the ability to activate effective caspases and also lead to apoptosis. In mitochondria-cytochrome c path, if mitochondria are stimulated by substances such as oxidant, ceramide, calcium ion, and certain caspases, it will release cytochrome c, thus activating Apaf-1. Through interaction with CARD–CARD, activated Apaf-1 can activate caspase-9, then caspase-9 enzyme cuts caspase-3, leading to apoptosis (McStay and Green 2014).

According to function and structure, Bcl-2 family is divided into two types: One is anti-apoptotic, such as Bcl-2 and Bcl-xL, while another kind is pro-apoptotic, such as Bax. Bcl-2 which are present in high level can inhibit the death of cells and extend their lives, which is now well-recognized anti-apoptotic gene. The existence of Bcl-2 is beneficial to maintain proliferation of normal cells, while abnormal expression of Bcl-2 in severe hyperplasia area and canceration area increases significantly, which prolongs and accumulates cells that have already become cancerous while normal cells tend to become cancerous, forming cancerous tumors (Um, 2015). The most important two members in Bcl-2 family are Bcl-2 and Bax, which are closely related to tumors. Different from general oncogenes and anticancer genes, Bcl-2 and Bax regulate apoptosis to adjust growth state of tumor cells rather than by regulating cell proliferation. Compared to the original mechanisms, the expression of Bcl-2 in most tumors increase, while the expression of Bax declines in most of them (Adams and Cory, 2007). As another important member in Bcl-2 family, Bcl-xL not only can inhibit apoptosis, but can inhibit cell necrosis. In animal experiments, it has been confirmed that Bcl-xL in T cells can inhibit apoptosis induced by Fas, whereas B cell apoptosis is induced by Fas. In intrinsic apoptotic pathway, Bcl-xL shows its anti-apoptotic effect by blocking the damage of Bax to mitochondrial outer membrane while in extrinsic apoptotic pathway induced by death ligands, Bcl-xL interferes with the assembly of DISC (death-inducing signaling complex) to inhibit the activity of caspase-8 (Scaffidi et al. 1998). In mammals, Bcl-xL and Bcl-2 prevent apoptosis by interfering in the activity of caspase-3 and play their anti-apoptotic role by maintaining the potential of mitochondrial membrane while controlling the toxicity of ROS (reactive oxygen species). As a result, Bcl-xL plays a significant anti-apoptotic function through interaction with a variety of proteins (Jeong et al. 2015).
HIAP-1 and HIAP-2 are important family members of IAPs. HIAP-1 and HIAP-2 have similar structure and function and both are members of TNFR2 complex, interfering in death signals activated by TNF to inhibit apoptosis. The expression of HIAP-2 can also inhibit apoptosis induced by chemotherapy agents and reduce the radiation effect. The activation of HIAP-2 can result in malignant transformation of tumors, including the changes of aggressivity, metastasis, and resistance (Asselin et al. 2001).

In cell cycle, p53 protein repairs damaged DNA or chromosome by preventing cells in G1 phase into S phase. When DNA or chromosome suffers severe damage, p53 can trigger apoptosis mechanism to remove damaged cells so as to regulate and promote the function of radiotherapy and chemotherapy agents. Another important biological effect of p53 protein is to increase the expression of p21 gene (Zhou and Wang, 2015). When cells in body are damaged, p53 protein activates transcription and high expression of p21 gene, making cell cycle stagnate in G1, G2, or S phase. DNA replication and mitosis are restrained, leaving plenty of time for cells to reproduce. If the repair fails, p53 protein mediates p21 gene into apoptosis (Nam et al. 2010). Otherwise, when p53 loses activation, the expression of p21 gene reduces or even stops. The damaged cells cannot be blocked in G1 phase, causing mismatches between DNA of cells and eventually leading to cell alienation or malignancy (Han et al. 2016).

Many studies have shown that the over expression of E2F1 can induce wide cancer cell apoptosis (Wang et al. 2015a). E2F1 can induce apoptosis in p53 dependent manner. E2F1 have also the ability to activate ARF and combine with ARF, leading to MDM2 shielded within the nucleus and deactivated (Irwin et al. 2000; Rogoff et al. 2002; Matsumura et al. 2003). Increasing the stability of p53 can cause accumulation and activation of p53, thus transcriptionally activating a series of genes related to apoptosis (Rogoff et al. 2002). Through ATM/ATR-CKH2/CKH2-p53 path, E2F1 can increase the level of p53 protein and induce apoptosis. The expression of p73 can promote cell apoptosis, and the transcription of E2F1 can activate p73 (Irwin et al. 2000). It has been proved that E2F1 is the direct target gene of p73 and E2F1 can directly transcript p73 gene, leading to activation of p73 target gene and promoting cell apoptosis. At the same time, E2F1/APAF1/caspase is one of the important ways of apoptosis.
induced by E2F1. Caspase inhibitor or abnormal APAF1 gene can significantly decrease apoptosis induced by E2F1 (Matsumura et al. 2003). E2F1 does not depend on the release of cytochrome c in mitochondria, but directly mediates activation of caspase-9 by increasing the level of APAF1. High concentration of APAF1 increases the interaction between caspase-9 molecules. Activated caspase-9 activates caspase-3, caspase-6, and caspase-7 in the downstream of caspase-9 molecules. Activated caspase-9 molecules, promoting the release of cytochrome c in mitochondria and inducing apoptosis (Gogada et al. 2013).

Survivin is also a kind of IAPs member with unique structure, which can inhibit cell apoptosis. It blocks apoptosis by inhibiting caspase-3 and caspase-7. Some scholars believe that survivin may be a cancer gene and participates in the development process of tumors, which is a tumor signal with potential value and closely related to tumor diagnosis and prognosis (Cheung et al. 2013). Survivin is the strongest anti-apoptosis factor as ever known, and its effect in inhibiting apoptosis is much stronger than that of other members in Bcl-2 family. A series of antagonists have been designed based on survivin to inhibit tumor growth and enhance the sensitivity of tumor cells to chemotherapies drugs, so the gene may become the next target for cancer treatment (Cheung et al. 2013).

Apoptosis mediated by Fas/FasL is the main way of cell apoptosis. Apoptosis signals activate caspases to take part in apoptosis process and achieve self-regulation through loop amplifier and heterologous oligomerization (Nakajima-Shimada et al. 2000). Combined with ligand Fas or Fas antibody, Fas can induce Fas to express apoptosis, which is one of the most important ways of apoptosis (Lawen 2003). Apoptosis caused by radiation is most important mediated by Fas, and the interaction between Fas and ligand FasL plays an important role in clearing radiation injury, and genes such as p53, Bcl-2, and Bax are involved in the regulation of apoptosis mediated by Fas (Wang et al. 2015a).

High concentration drug may cause toxicity in human; cancer treatment drug can have an effect at high concentration, but may also harm human body (Lo Russo et al. 2016). Sensitizing effects can raise the effects of cancer treatment drug at a low concentration. This course can help protect the body (Atari-Hajipirloo et al. 2016). Neferine is a functional chemical composition from plant; it has no toxicity, many functional or medical effects (Wang et al. 2015a; Yu et al. 2016), has sensitizing effect, and makes DHEA a good anticancer agent as proved in this in vitro study.

In this study, the in vitro anticancer effects of Nef + DHEA were determined: Nef + DHEA showed higher cancer cells growth inhibition effect, i.e., more apoptotic cells, than those treated only with DHEA. And by further RT-PCR assay, Nef + DHEA-treated cells had higher caspase-3, caspase-8, caspase-9, Bax, p53, p21, E2F1, Fas, FasL mRNA expressions and lower Bcl-2, Bcl-xL, HIAP-1, HIAP-2, survivin expressions than only DHEA-treated cells. By enhancing sensitization effect, Nef can significantly improve the inducing effect of DHEA on cancer cell apoptosis and achieve good anticancer effect in vitro. Lotus seeds and sweet potatoes contain a great deal of Nef and DHEA, which have wide resources and cost little. This combination through natural extraction may develop a new kind of functional food having a meaningful cancer-preventing effect.

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