

ROLE OF CASPASE-3 IN SQUAMOUS CELL CARCINOMA CELL LINE APOPTOSIS USING GRAPE SEED EXTRACT

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ABSTRACT

Background and Objective: Squamous Cell Carcinoma (SCC) of head and neck is a very common malignant tumor with high risk to develop in young individuals. Chemotherapy is limited treatment due to the side effects of the drugs and due to development of resistance to the drug; recently, it has been found that GSE has anticancer effects. Apoptosis is the cytotoxic effect that can be induced by extracts of medicinal plants, Active caspase-3 which is responsible for the morphological changes and fragmentation of DNA that occur during the apoptotic process.

Methodology: Cytotoxicity of GSE was assessed on hep-2 cells using the MTT assay, the results were interpreted and half maximal inhibitory concentration (IC50) was estimated. Apoptotic cells death was measured using the Cell Death Detection ELISA kit, following treatment of cells with GSE for 24 h.

Results: the cell viability percent showed a gradual decrease in a time- and dose-dependent manner. Drug treated cells revealed in microscopic examination nuclear morphological alterations correlated with apoptosis in its various stages, Detection of caspase 3 enzyme assays showed that in drug treated cells the level of caspase 3 increased with increasing concentrations of GSE.

Conclusion: GSE induced the apoptosis of squamous cell carcinoma cells demonstrating the anticancer efficacy of GSE.

KEY WORDS: HEP-2, GSE, Caspase 3, Apoptosis

INTRODUCTION

Squamous Cell Carcinoma (SCC) of head and neck is a very common malignant tumor with high risk to develop in young individuals especially in the developed countries. There are two main methods

for treatment which are radiotherapy and surgery but with common recurrences and high resistance to treatment⁽¹⁻³⁾.

Recent studies have shown that derivatives of natural extracts such as flavones may be used as

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safe drugs for cancer treatment because they are less toxic and have properties that are non-mutagenic in human body ⁽⁴⁾.

Apoptosis is the cytotoxic effect that can be induced by extracts of medicinal plants ⁽⁵⁾.

Apoptosis, which is the programmed cell death, can occur in both physiological and pathological conditions, in the nucleus the hallmarks of apoptosis are the condensation of chromatin and fragmentation of nucleus ^(6,7).

The main role of apoptosis is eliminating damaged cells without harming normal cells ⁽⁸⁾.

Defect in the mechanism of apoptosis allows cells of cancer to survive and permits the tumor to develop angiogenesis and invasiveness which lead to metastasis ⁽⁹⁾. Tumor cells acquire resistance to treatments by evading the apoptotic mechanisms ^(10, 11). Many therapies targeted to inhibit the inhibitory proteins of apoptosis and to induce apoptosis in treatments of many cancers ⁽¹²⁾.

There are two main groups of Caspases according to their functions, caspases of apoptosis (caspase 3, 6, 7, 8, and 9) and those of inflammation (caspase 1, 4, 5, and 12) ⁽¹³⁾.

The initiator caspases in the apoptotic process are caspase-8 and caspase-9. caspase-3 is cleaved and activated by caspase-8 and caspase-9, the active caspase-3 enzyme is formed by cleavage of Caspase-3 into p12 and a p17 subunits ⁽¹⁴⁾.

Active caspase-3 which is responsible for the morphological changes and fragmentation of DNA that occur during the apoptotic process ⁽¹³⁾.

The grape vine is rich in many substances as polyphenols, anthocyanin, flavonoids, procyanidins, proanthocyanidins and trans-resveratrol ⁽¹⁵⁾.

In herbal medicines the seeds and the leaves of the grape are used while the fruit is used as dietary supplement. Recently grape seed extract (GSE) has many properties which are: antioxidant property and scavenging of free radicals, anti-diabetic effect,

cardioprotective effect, hepatoprotective effect, anti-carcinogenic, anti-microbial property, and antiviral activities ⁽¹⁶⁻¹⁸⁾.

In the present study, the cytotoxic effect of grape seed extract (GSE) was investigated and its cell death induction ability on squamous cell carcinoma (hep-2 cell line) was determined.

MATERIAL AND METHODS

Cell line: Head and neck squamous cell carcinoma (HNSCC) HEP-2 Cell line was obtained from Vacsera- Egypt department of cell culture. HEP-2 cells were obtained from the "American type Culture Collection (ATCC) in the form of frozen vial with the reference number "CCL-23".

Fetal bovine serum (FBS): FBS was obtained from sigma Aldrich- Munich- Germany.

Phosphate buffer saline (PBS): It was obtained from Biovision- California- USA imported through EMPA, GE health care- Cairo- Egypt. The pH adjusted by using HCl or NaOH, PBS was dissolved and sterilized by autoclaving for 20 minutes at 4° C.

Trypsin EDTA solution: obtained from sigma Aldrich- Munich- Germany.

Dulbecco's modified eagle media (DMEM): supplemented with 10% FBS 2 Mm glutamine and sodium bicarbonate (Invitrogen, USA).

Methyl thiazolyl tetrazolium (MTT) (sigma Aldrich- Munich- Germany)

Caspase 3 enzyme assay kit: was purchased from Invitrogen- California- USA.

Drug: GSE was purchased from Bulk Supplements Company (Address: 7511 East Gate Road, Henderson, NU89011 U.S.A), through www.Amazon.com.

Cell culture:

HEp-2 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 2% sodium bicarbonate and 2% streptomycin penicillin in T25 flasks at 37°C in a 5% CO₂ incubator.

MTT assay

GSE cytotoxicity was assessed on hep-2 cells by using the MTT assay according to manufacturer’s protocol. The cells of cancer were seeded in 96-well plates (104 cells/ well) and then incubated for 24 h at 37°C with 5% CO₂. After that The cells were treated with serial dilutions of GSE (100µg/ml, 25µg/ml, 6.25µg/ml, 1.56µg/ml, 0.39µg/ml). they were incubated for 24 hours, 48 hours and 72 hours, then washed by PBS twice, then incubated with medium of 0.5 mg/ml MTT in CO₂ incubator at 37°C for 4 h and maintenance medium containing DMSO.

The results were interpreted and half maximal inhibitory concentration (IC₅₀) was estimated as follows: The means optical density (OD) of each column of the 96 well plate was calculated by dividing the sum of OD of the column wells by the number of wells. The mean OD of each column with specific concentration treatment was divided by the mean of the control untreated cells to get the percent of viability and cytotoxicity of each concentration of each concentration treatment. A graph was plotted between the log concentration on the X axis versus percentage viability on the Y axis. The equation Y= mx + C was used, as (m) and (C) were constants, Y substituted by 50 and hence the x value was calculated. The natural root of the x value was the IC₅₀ value.

Hematoxylin and Eosin Staining

The slides that are fixed were rehydrated in alcohol concentrations of (100%, 90%, 75% then 50%) and then washed in distilled water for about 5 minutes. Then the slides were immersed in hematoxyline stain for about 3 minutes and then washed twice with distilled water. After that the slides were immersed in eosin stain for about 5 seconds and then also washed with distilled water. After that slides that were dried immersed in xylene, mounted with Canada balsam and then cover slips were placed and left to dry.

Assessment of apoptosis

Apoptosis was measured by using the Cell Death Detection ELISA kit, following treatment of cells with GSE for 24 h, Cell lysis for cell culture samples was carried by spinning down cells for 15 minutes at 1200 RPM. Cell pellets were washed once in cold PBS, and then cells were re-suspended in Lysis Buffer to a concentration of 1.5 x 10⁶ cell/ml, incubated for 1 hour at room temperature. Cells were centrifuged at 200 x g for 15 minutes. The supernatant was diluted at least 50-fold in 1 X Assay Buffer (5 µl supernatant + 245 µl 1 X Assay Buffer) for the assay. Hereafter, prepared biotin antibody was added, and then the prepared HRP solution was added after washing. Tetramethylbenzidine (TMB) solution was added and stop solution was also added. Then, readings were done at 450 nm immediately.

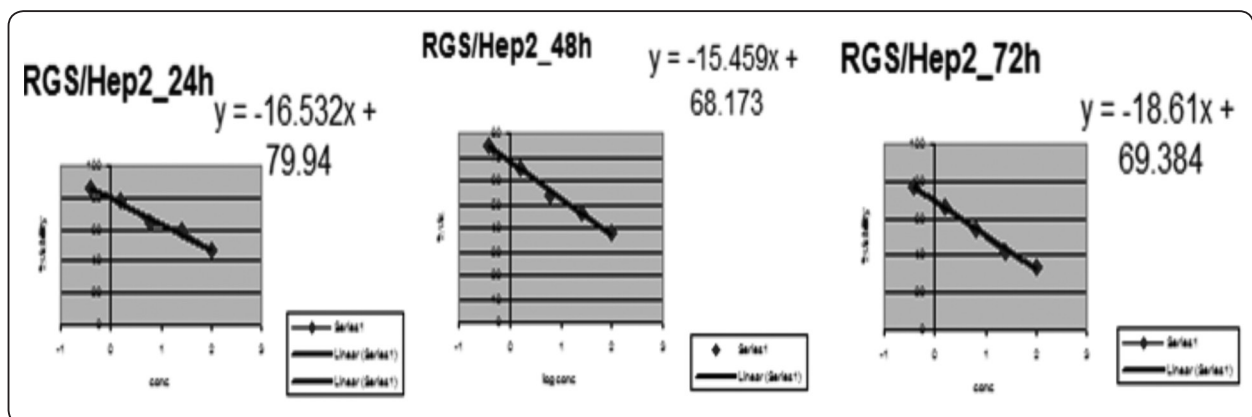


Fig. (1) Graph showing a curve between the log concentrations of GSE versus viability percentage of cancer cells at 24, 48 and 72 h time intervals.

RESULTS

The cytotoxicity of grape seed extract (GSE) on hep-2 cell line were determined by MTT, the cell viability percent showed a gradual decrease in a time- and dose-dependent manner. The values at 24 h, 48h and 72 h were shown in table (1) and plotted in figure (2). Regarding IC₅₀, values showed decrease with time, the highest values were obtained at 24h interval whereas the lowest values were obtained at 72h interval as shown in table (1).

TABLE (1): Log concentrations of GSE versus their mean viability percentage of OSCC cells at different times

Study group time dose	Viability % of OSCC cells treated with GSE at different time intervals		
	24h	48h	72h
100 µg	46.32	38.04	33.83
25 µg	58.86	46.55	41.45
6.25 µg	64.33	54.007	53.60
1.56 µg	77.73	65.78	66.78
0.39 µg	86.67	74.98	77.21
IC ₅₀	62.94 ± 4.16	14.98 ± 1.13	11.01 ± 1.22

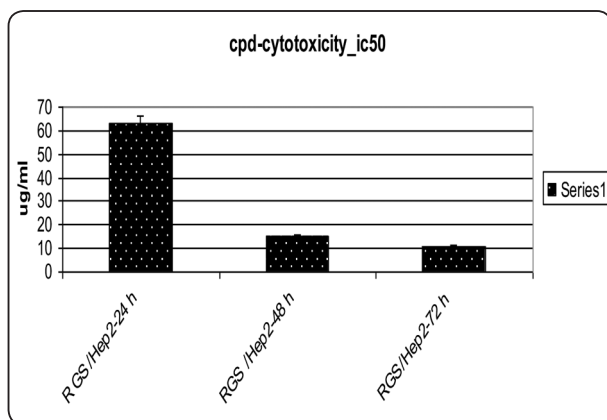


Fig. (2) A column chart showing the different IC₅₀ values for GSE at 24h, 48h and 72h intervals

Microscopic examination of control cells showed regular cellular outline with hyperchromatic nuclei

as shown in fig 2, while drug treated cells revealed nuclear morphological alterations correlated with apoptosis in its various stages. Including peripheral condensation of chromatin against the nuclear membrane, irregularities in the nuclear and cellular membrane, nuclear shrinkage, nuclear fragmentation and apoptotic bodies as shown in fig 3 & 4.

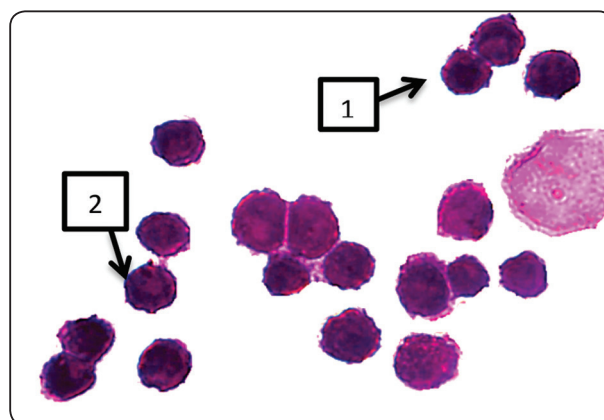


Fig. (3) A photomicrograph of control cells showing hyperchromatism (1) and pleomorphism (2) (H&E x1000 oil).

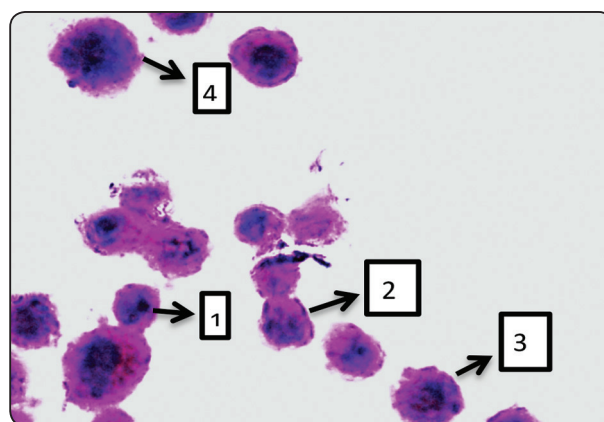


Fig. (4): A photomicrograph of Hep-2 cell post treatment with IC₅₀ concentration of GSE showing: apoptotic body (1), nuclear fragmentation (2), irregular nuclear outline (3) and nuclear shrinkage (4) (H&E x 1000 oil)

Detection of caspase 3 enzyme assays showed that the lower level was detected in the control group, while in drug treated cells the level of caspase 3 increased with increasing concentration of GSE as shown in table 2 and fig 5

TABLE (2): Mean values of caspase 3 levels

Code	Cells	Casp3 concentration pg/ml	FLD
RGS/Hep2_ IC50	Hep2	423.2±16.1	8.45
RGS/Hep2_ pre IC50	Hep2	303.3±12.7	6.05
RGS/Hep2_ post IC50	Hep2	442.4±14.3	8.83
Control	Hep2	50.05±1.94	1.0

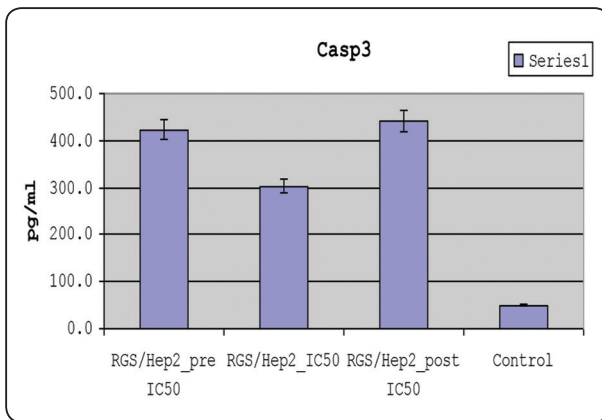


Fig. (5) A column chart showing mean concentrations of caspase 3 in relation to drug concentrations.

DISCUSSION

Chemotherapy is still the main treatment option for invasive malignancies; however, the effects of chemotherapy is limited due to the side effects of the drugs and due to development of resistance to the drug. So it is important to found more effective compounds against cancers with less toxicity. Recently, it has been found that GSE has anticancer effects (19-21).

We studied the effects of GSE on squamous cell carcinoma cell line (hep-2).

By using the MTT assay, we found that the treatment of hep-2 cells with GSE resulted in dose and time dependent reduction in cell viability. These results were in agreement with **Qing Chen et al** (22).

who found that treatment of HeLa and SiHa cervical cancer cells with GSPs resulted in reduction in cell viability in a dose-dependent manner .

Also, **Dinicola et al** (23) found that GSE has anti-proliferative effect and apoptotic effect on CaCO2 and HCT-8 cell lines of colon cancer. And **Sun et al** (24) indicated that GSE has the ability to reduce invasion of HNSCC cells by targeting EGFR and activation of NF-κ B and also by inhibiting the epithelial mesenchymal transition.

The main morphological changes of apoptosis are chromatin condensation and fragmentation of nucleus. As well as fragmentation of DNA is a main feature of apoptosis, Which occur due to activation of endogenous endonucleases and subsequent cleavage of DNA chromatin into internucleosomal fragments of about 180-200 base pairs (bp) (25,26).

The results of the present study revealed that drug treated cells showed by microscopic examination morphological alterations correlated with apoptosis in its various stages. Including peripheral condensation of chromatin against the nuclear membrane, irregularities in the nuclear and cellular membrane, nuclear shrinkage, nuclear fragmentation and apoptotic bodies.

Our results were in accordance with **Qing Chen et al** (22) who found that nuclear condensation of chromatin and formation of apoptotic bodies, by fluorescence microscope in HeLa and SiHa cells treated with GSPs and stained by DAPI.

Results of the present study revealed that levels of caspase 3 enzyme in hep-2 cells treated with GSE was higher than that of control cells. And also, the levels of caspase 3 increased with increasing concentrations of the drug which indicates increase in apoptosis.

The apoptotic process is associated with decrease in the anti-apoptotic proteins as Bcl-2 and increase in apoptosis-promoting proteins as Bak-1(27).

Activation of Bak-1 leads to disturbance in the function of Bcl-2 which leads to loss of mitochondrial membrane and release of Cytochrome c and other apoptosis inducing factors from mitochondria. Cytochrome c which activates pro-caspase-9 leading to activation of caspase-3 which is a key enzyme of apoptosis⁽²⁸⁻³⁰⁾.

These results were also in agreement with **Qing Chen et al**⁽²²⁾ who found that treatment of HeLa and SiHa cells with GSPs resulted in loss of mitochondrial membrane and also increased the activity of caspase-3.

Our results were also in accordance with **Amirala Aghbali et al** who found that apoptosis occurred in the oral cancer cells KB treated with the GSE, and also found that fragmentation of DNA occurred in the late stages of apoptosis⁽³¹⁾.

CONCLUSION

Results of the present study suggested that GSE induced the apoptosis of squamous cell carcinoma cells demonstrating the anticancer potential of GSE.

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