

## Effect of Grape Procyanidins on Vascular Endothelial Cell Apoptosis by Flow Cytometry Analysis

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**Abstract:** This thesis studied the impact of GPC with different concentrations over the vascular endothelial cells apoptosis by flow cytometry and analyzed the influence of GPC on oxygen free radicals in initiating cell apoptosis. Make use of flow cytometry instrument to detect the apoptosis rate of vascular endothelial cell by adding different dose of GPC separately. Through experiments, the apoptosis rates of normal control group, positive control group, GPC (fifty) group, GPC (one hundred) group, GPC (one hundred and fifty) group, GPC (two hundred) group, separately were 11.89, 12.63, 10.17, 5.45, 4.79 and 4.53%, respectively. The experiment results indicated that GPC possessed distinct inhibitory effect on vascular endothelial cells apoptosis evoked by hydroxyl radical.

**Keywords:** Cell apoptosis, flow cytometry instrument, Grape Procyanidins (GPC), oxygen free radicals

### INTRODUCTION

Cells in human body were destined to die, that some of them were physiological death, while some were pathological death. In recent years, the study of cell death process has been a hotspot in biology and medical research. People found so far that there were at least two cell death ways, they were necrocytosis and apoptosis (Semianov and Tarasov, 2006). Necrocytosis was a kind of cell death way that had been realized very early, while apoptosis was one that been realized gradually in recent years.

Formation of apoptosis concept Australian scientist found that the lysosome of some dead cells scattered in liver parenchyma tissue, observed by electron microscope after ligaturing the portal vein of mouse, was not destroyed in 1965, which was distinctively different from necrocytosis. These cells had volume shrinkage, as well as exfoliated from their surrounding tissues being phagocytized by chromatin agglutination, so as to lose self-function gradually (Soohee *et al.*, 2012). In 1972, three scientists of Kerr, etc., first proposed the concept of apoptosis. Apoptosis is an initiative and regular self-extinction process controlled by gene. The research in recent years indicated that the introduction factor of apoptosis was related to free radical damage (Daraynkiewicz *et al.*, 1992).

Grape Procyanidins is a kind of polyphenol extracted from the skin and seed of grape within strong free radical scavenging and antioxidant activity. Facino

experiment found that GPC can alleviate myocardial damage of rat after ischemia, decrease the sensitivity of heart for reperfusion injury of ischemia, which was related to its ability of improving the oxidation resistance of blood plasma (Le, 2006). The study had a research on the protective effect of GPC to vascular endothelial cell by detecting the influence of different GPC contents on vascular endothelial cells apoptosis through flow cytometry instrument. The research was supported by Qingdao University. The project is funded by Project 2011SJGZ16 of Shandong Provincial Department of Education.

For the past few years, cell separation monitoring technology has been matured gradually. This study analyzed the damage degree of vascular endothelial cell by adopting relative common flow cytometry. Flow cytometry instrument is a kind of far advanced instrumentation that closely integrates fluid jet technology, laser technology, air technology,  $\gamma$  ray spectroscopy, electronic computer, etc., with microscopy fluorophotometer (Iglesias, 2012). It can make a rapid analysis of the physical or chemical property of the particle by detecting the scattered light of cells, as well as other bio-particles and signing fluorescence intensity; have separate collection to cells; rapidly analyze thousands of cells; measure characteristic parameters of numerous cells from one cell; have qualitative analysis and quantitative analysis. It was with characteristics of fast speed, high precision and good veracity.

## MATERIALS AND METHODS

### Methods:

**Grape Procyanidins (GPC):** Dry the fresh grape seeds and crush them. After degreasing by petroleum ether, use ethyl alcohol as the extraction solvent to distill GPC. Then purify and dry the product. The purity of the product should be over 99.9%. The standard is provided by Shimada, Japan Co., Ltd.

**Cell lines:** Human umbilical cord vein endothelial cell line ECV304, provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS. DMEM medium (PH 7.2~7.4) containing 10% fetal bovine serum cultured the cell lines for 72 h in 37°C, 5% CO<sub>2</sub> and sub cultured one time.

**Main reagents:** Propidium Iodide (PI), Np-40RNA enzyme. The reagents were all purchased from Sigma (USA).

**Main instruments:** Low-speed centrifuge, flow cytometry (FACS Vantage type produced by U.S. ECTON DICKINSON), Olympus C-35AD-4 microscopic cameras, Olympus automatic exposure control system.

**Testing site:** Chemistry and biology laboratory of Analysis and testing center, Qingdao University.

**Testing date:** Sep. 26<sup>th</sup>, 2012 - Sep. 28<sup>th</sup>, 2012

### Methods:

#### Monitoring method of flow cytometry instrument:

**Composition:** Flow cytometry instrument mainly consists of four parts, they are: flow chamber and liquid fluid system; laser source and optical system; phototube and detecting system; computer and analytic system (Ho *et al.*, 1999). Its specific monitoring principle was shown in Fig. 1.

This experiment limited the liquid flow velocity of  $v < 10$  m/s for ensuring the liquid flow was stable liquid. The detected cells were confined on the axis of the liquid flow for the effect of sheath fluid (Ito *et al.*, 2005). The flow chamber installed with piezoelectric crystal can oscillate after receiving oscillation signal. The fluorescence, produced by fluorescence stained cells after proper optical excitation, was measured by transforming into electrical signal through photoelectric converter.

**Groups of experiments and cell processing:** Cell lines were inoculated in 50 mL culture flasks. The density was  $1 \times 10^5$ /mL and each flask was added 5 mL. After

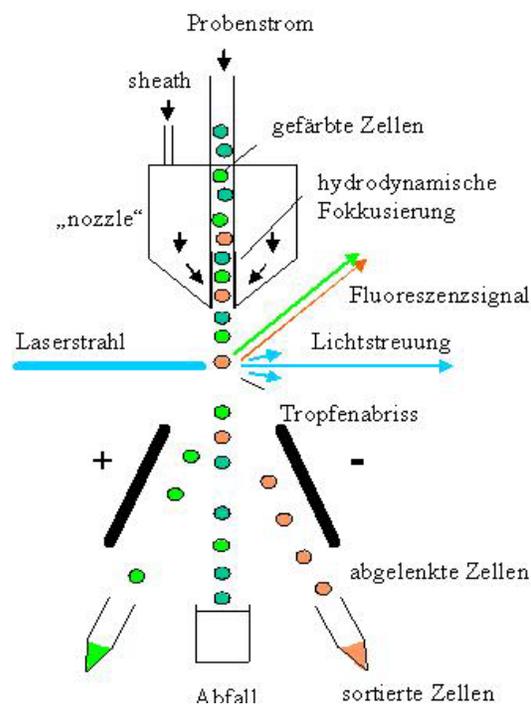


Fig. 1: Schematic diagram of flow cytometry instrument

incubating for 4 h with above conditions, discard original nutrient solution and different reagents were added into different groups (Aina *et al.*, 2012). The experiment was divided into 6 groups, namely:

- Normal control group which was added DMEM medium containing 10% fetal bovine serum
- Positive control group which was added Fenton reagent (the final concentration of FeSO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> was respectively 12 and 3 μmol/L)
- GPC 50 group which was added 50 μg/mL GPC firstly and then was added Fenton reagent
- GPC 100 group which was added 100 μg/mL GPC firstly and then was added Fenton reagent
- GPC 150 group added 150 μg/mL GPC firstly and then Fenton reagent
- GPC 200 group added 200 μg/mL GPC firstly and then Fenton reagent

**Detection of cell apoptosis:** Each group was cultured for 24 h in 37°C. Use 0.25% trypsin to digest the cultured cells taken from above groups. Then after centrifugation 10 min at the speed of 1000 r.p.m, the supernatant was taken out and washed twice with PBS. The cell density of the suspension was adjusted to  $1 \times 10^6$ /mL and then centrifuged at the speed of 1000 r.p.m. for 10 min again. After centrifugation, the supernatant was removed and then PI hypotonic dye concentration of 50 μg/mL (containing NP-40RNA

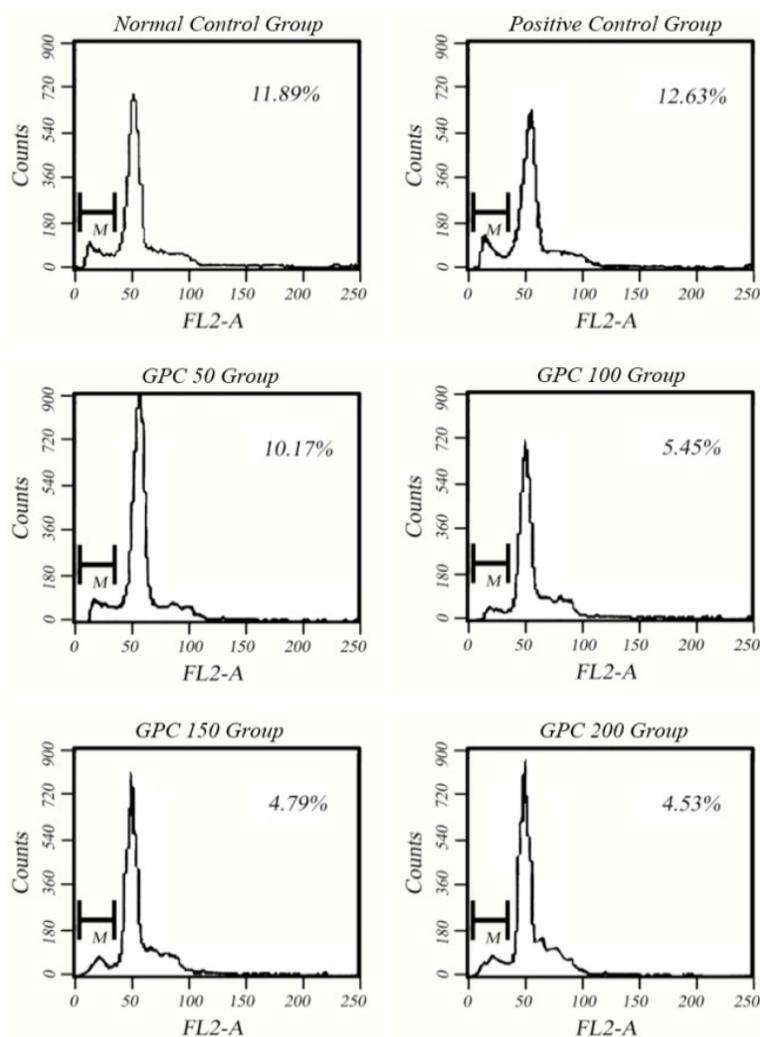


Fig. 2: Flow cytometry test results

every 200 mL) was added. After that, the mixture was stained for 30 min in cryogenic and dark condition. Then apoptotic cells were detected by flow cytometer and the apoptosis rate was analyzed by Ftf software.

## RESULTS

Through 5 times experiments, the results detected by flow cytometer showed that the mean value of the apoptosis rates was 11.89, 12.63, 10.17, 5.45, 4.79 and 4.53%, respectively (Fig. 2). As it could be seen, the apoptosis rate of positive control group was higher than the normal control group. Compared with the normal control group, apoptosis rates of group (3), (4), (5) and (6) which were protected by GPC decreased and the degrees of reduction were related to GPC doses. It indicated that GPC could protect vascular endothelial

cell from apoptosis induced by hydroxyl radical and the protective effect of GPC had a relationship with the dose-response.

Figure 2 shows the single parameter histograms of cell numbers. The Y-axis shows relative number of the testing cells and X-axis shows intensities of the fluorescence signals.

## DISCUSSION

The flow cytometric DNA analysis is to estimate the percentage of apoptosis cells based on the DNA contents in the apoptosis cell lower than the diploid cell. Because DNA degrades and leaks from the cells and the DNA-specific fluorescent staining is low, the apoptosis cells could be detected by flow cytometric DNA analysis. Because of its efficiency and high sensitivity,

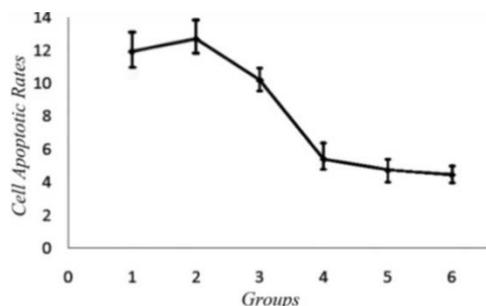


Fig. 3: Cell apoptotic rates comparison in the groups

this method is increasingly used in the study of apoptosis.

Recent studies indicate that many reagents inducing programmed cell death are almost oxidants and cell activation stimulants. Reactive Oxygen Species (ROS) may be the media or delivery device for programmed cell death. In some cases, the study of apoptosis initiated by antioxidants or radicals has great values. The protective effects of GPC on apoptosis induced by  $\bullet\text{OH}$ , which was produced by Fenton system, were discussed in this experiment by using flow cytometer. Furthermore, GPC antioxidant activity and its mechanism were further researched (Fig. 3).

From the experimental results, the cell apoptosis rate of group (3), (4), (5) and (6) which were protected by GPC declined dramatically compared with the positive control group and the degree of declination was related to the content of GPC (Fig. 2). The apoptosis rate of higher dose GPC group was reduced to 5.45% decreased by 7.18% compared with the positive control group. It indicated that GPC efficiently cleared a large amount of hydroxyl radicals generated by the Fenton system and inhibited vascular endothelial cell apoptosis caused by hydroxyl radicals. At the same time, the results also showed that the apoptosis rate of GPC groups was respectively 10.17, 5.45, 4.79 and 4.53%, respectively lower than the normal cells (the apoptosis rate of the normal control group was 11.89%). The reason was that the normal cells in the process of aerobic metabolism were capable of producing ROS and easier to react with biological macromolecules and then damage biological structure directly or through a series of oxidation chain reaction. The experimental results demonstrated that GPC not only prevented the apoptosis induced by alien hydroxyl radicals but also cleared radicals generated by the cells themselves to prevent apoptosis due to autoxidation. The data further proved the inhibitory effect of GPC to heart muscle damage of rat after ischemia and that the reason of reducing the sensitivity of heart to the damage of ischemia reperfusion injury was related to the free radical generated by scavenger-cell in Facino

experiment. The experimental results further confirmed that GPC has a strong anti-free radical damage activity and could protect cells from oxidative damage and apoptosis induced by ROS efficiently.

## CONCLUSION

This study results showed that when GPC concentration was 100  $\mu\text{g}/\text{mL}$  in culture fluid of the vascular endothelial cells, cell apoptosis rate decreased more than 50% to the normal control. This indicates that GPC has strong inhibition effect on vascular endothelial cells apoptosis. Based on this study, further studies on GPC's applications in Health food, medicines and cosmetics could also be carried out.

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