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Capillary electrophoresis analysis of hydrogen peroxide induced apoptosis in PC12 cells

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Abstract

Apoptosis is a highly regulated and controlled process of cell death that plays a fundamental role in many biological processes. Abnormal apoptosis of cells is closely related to some diseases such as cancer. Development of a simple and effective method to detect apoptosis is of great importance. In the present paper, capillary electrophoresis (CE) method was applied to distinguish cell apoptosis and necrosis of rat pheochromocytoma (PC12) cells treated by hydrogen peroxide by characterizing the DNA fragmentation. Firstly, effects of separation conditions (voltage, polymer concentration, temperature and injection time) on DNA separation were studied using 100 bp DNA ladders as the analyte. Under optimal separation condition (polyacrylamide coated capillaries: 57.5 cm × 75 μm i.d., effective length: 50 cm; running buffer: 1× TBE containing 2% PVP and 1.2% HEC; separation voltage: 5 kV; temperature: 25 °C; electrokinetic injection: 10 kV × 10 s), CE was used to monitor the progress of hydrogen peroxide induced apoptosis of PC12 cells by analyze DNA fragmentation. It was found that normal, apoptotic and neurotic cells had distinct DNA fragmentation patterns analyzed by CE. The results by CE were tested by other current methods (DAPI nuclei staining, flow cytometry analysis and TUNEL) to detect apoptosis and correlated well with those methods. Results show that CE can distinguish cell apoptosis and necrosis, quantify the degree of apoptosis in cells and have advantages of high efficiency, fast sample analysis speed, minute sample consumption and reliable results, which provides an accessorial method in the research of multiple diseases with abnormal apoptosis such as cancer and neurodegenerative diseases.

Keywords: CE; Apoptosis; DNA fragmentation; PC12 cells

1. Introduction

Apoptosis is the process of programmed cell death or cell suicide, which is essential for the balance between proliferation, growth arrest and cell death [1]. Apoptosis is distinct from the other form of cell death called necrosis in that it develops in a controlled and regulated fashion. In response to specific signals instructing the cells to undergo apoptosis, a number of distinctive biochemical and morphological changes occur in the cell. Cell apoptosis is morphologically characterized as nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing. The most dramatic biochemical feature of apoptosis is DNA fragmentation as a result of cleavage of the cell's DNA at intervals of 160–200 bp by endogenous endonuclease. The low mass fragments can be observed as a DNA ladder on a gel lane [2]. Other biochemical characters include activation of caspases and the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Since apoptosis is a normal part of biological process and abnormal apoptosis is closely related with diseases such as cancer, the study of it has been attractive [3–5]. Development of reliable and convenient methods to determine apoptosis is of great importance.

According to the biochemical and morphological characters of apoptosis, there are several methods developed for

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the determination of apoptosis, such as microscope [6], flow cytometry [7,8], agarose gel electrophoresis [2,9] and terminal dexovynucleotidyl transferase-(TdT)-mediated dUTP-biotin nick end labeling (TUNEL) [10,11]. Among these techniques, agarose gel electrophoresis has been commonly used to analyze DNA ladder due to its simplicity and low cost. However, traditional agarose gel electrophoresis possesses disadvantages such as low speed, low resolution and limited information about DNA fragmentation.

CE is emerging as a powerful bioanalytical method with the advantages of high resolution, fast analysis time, minimal sample requirement, negligible waste of toxic chemical, good sensibility and advanced automation. CE has been widely applied to DNA separation [12–16] and characterize cell apoptosis based on determination of DNA fragmentation or difference of cell mobility [17–22]. In the present study, non-gel-sieving CE method was established to distinguish cell apoptosis and necrosis by characterizing the DNA fragmentation of rat pheochromocytoma (PC12) cells treated by hydrogen peroxide. Other current methods (DAPI nuclei staining, flow cytometry analysis and TUNEL) were used to detect cell apoptosis as parallel experiments in order to prove the efficiency of the developed CE method. The results show that CE is a reliable and convenient method for identification of cell death.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade and all solutions were prepared with distilled water. Hydroethylcellulose (HEC, $M_v = 90000$) was obtained from Aldrich (Milwaukee, WI, USA); polyvinylpyrrolidone (PVP-40) was obtained from Sigma (St. Louis, MO, USA). TBE buffer (89 mmol/l Tris, 89 mmol/l borate, 2 mmol/l EDTA, pH 8.3) and TE buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 8.0) were prepared using regents from Shanghai General Chemical Reagent Plant (Shanghai, China). The 100 base pairs (bp) DNA ladders (Jingmei Biotech, Shenzhen, China) were diluted with distilled water to concentration of 0.01–0.05 mg/ml and stored at −20 °C until use. All buffer solutions were filtered through a 0.2 μm nylon membrane syringe filter (Fisher Scientific, Pittsburgh, PA, USA) prior to use.

2.2. Cell culture and treatment

PC12 cells were purchased from the American Type Cell Collection (ATCC, Rockville, MD, USA). Cultures were plated onto poly-L-lysine coated glass coverslips or flask at 3×10^5 or 1×10^4 cells/cm^2 density. PC12 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (GibcoBRL, USA) and 500 U/ml penicillin/streptomycin in a humidified incubator (Forma Scientific, USA) at 37 °C and 5% CO_2. After flasks or glass coverslips became 70% confluent, cells were treated with 100 μmol/l H_2O_2. All cell culture dishes were then placed to a humidified incubation chamber at 37 °C and flushed by a gas mixture consisting of 5% CO_2/20% O_2.

2.3. DAPI staining

The cells were fixed after exposed to H_2O_2 for different time and stained in 10 μg/ml DAPI (Sigma, USA). After 1 h of incubation, cells were washed with PBS and then fixed by 4% (v/v) formaldehyde for 30 min. The stained cells were observed under fluorescence photomicroscope (Olympus, Japan) to study the morphological change of PC12 cells during apoptosis process.

2.4. FACS analyses

A flow cytometric method was used to assess the percentage of fragmented nuclei [8,9]. For apoptosis assay, the cells were harvested after treatment by H_2O_2 for certain time, and then washed with PBS. The pellets were fixed in 70% (v/v) ethanol overnight at 4 °C. The cells were then resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA and 0.5 μg/ml RNase (Sigma, St. Louis, MO, USA) at 37 °C for 15 min and finally stained with 50 μg/ml propidium iodide (PI) for 1 h. Cell cycle analysis was performed using a Becton-Dickinson fluorescence-activated cell analyzer and data analysis was performed with Modifit LT 2.0 (Becton-Dickinson, San Jose, CA, USA). About 1×10^4 cells were counted for each analysis. The percentage of apoptotic cell accumulation in the sub-G1 peak was quantified with CELLQuest software (Becton-Dickinson, Mountain View, CA, USA).

2.5. TUNEL staining

The TUNEL method was used to detect DNA fragmentation and apoptotic bodies. Briefly, after treated by H_2O_2 for different time, the cells were post-fixed in precooled ethanol:acetic acid (2/1, v/v) for 5 min at −20 °C. TUNEL staining was performed with the APOALERT™ DNA fragmentation assay kit (BD, USA). Slides were incubated in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min at 4 °C. After rinsing with PBS, 50 ml of TUNEL solution containing terminal deoxynucleotidyltransferase and fluorescein–dUTP mixture was applied to slides for 30 min at 37 °C, followed by wash with stop buffer. Stained sections were analyzed using standard fluorescence microscopy (Olympus, Japan).

2.6. DNA extraction

Cultured cells were harvested at certain time after exposed to H_2O_2. The cells were washed with PBS and centrifuged at 5000 rpm for 5 min. The pellets were resuspended in 0.3 ml

water and then treated with 0.3 ml lysis buffer (200 mM NaCl, 20 mM Tris–HCl, 50 mM EDTA, 200 μg/ml proteinase K, 1% SDS, pH 8.0). A 10 μl of 10 mg/ml RNase was added to the mixture and the mixture was incubated in 55 °C for 5 h. After extracted by adding an equal volume of phenol/chloroform, DNA was precipitated with 0.1 volume 3 mol/l NaAc and 2.5 volumes EtOH at −20 °C. After centrifuging, the DNA was washed with cold 75% EtOH, dried in room temperature and finally dissolved in 50 μl TE buffer prior to use.

2.7. CE separation

CE was performed on P/ACE 5000 system (Beckman Instrument, CA, USA). Data were collected and analyzed using P/ACE Station GoldSystem Software. Polyacrylamide coated capillaries (57.5 cm × 75 μm i.d., effective length 50 cm) was prepared as described [20] and used for separation. The sample was introduced by electrokinetic injection and on line detector wavelength was set at 254 nm. The sieving buffer was prepared by dissolving appropriate amount of hydroxyethylcellulose (HEC) into 25 ml solution of 1× TBE containing 2% PVP. Prior to each run, the capillary was washed by H_2O and sieving buffer for 4 min, respectively. All experiments were performed in reversed polarity mode.

3. Results and discussion

3.1. Optimization of separation conditions for DNA fragmentation analysis

CE separation conditions were optimized to improve the separation of DNA fragments of apoptosis cells. In this paper, 100 bp DNA Ladder marker was selected as analyte. In order to assess the effect of parameters (voltage, temperature, injection time and polymer concentration) on the separation, two peak twains including 300 bp/400 bp and 800 bp/900 bp were chosen as the representatives. Effects of parameters on the resolution of the two peak twains were stud-

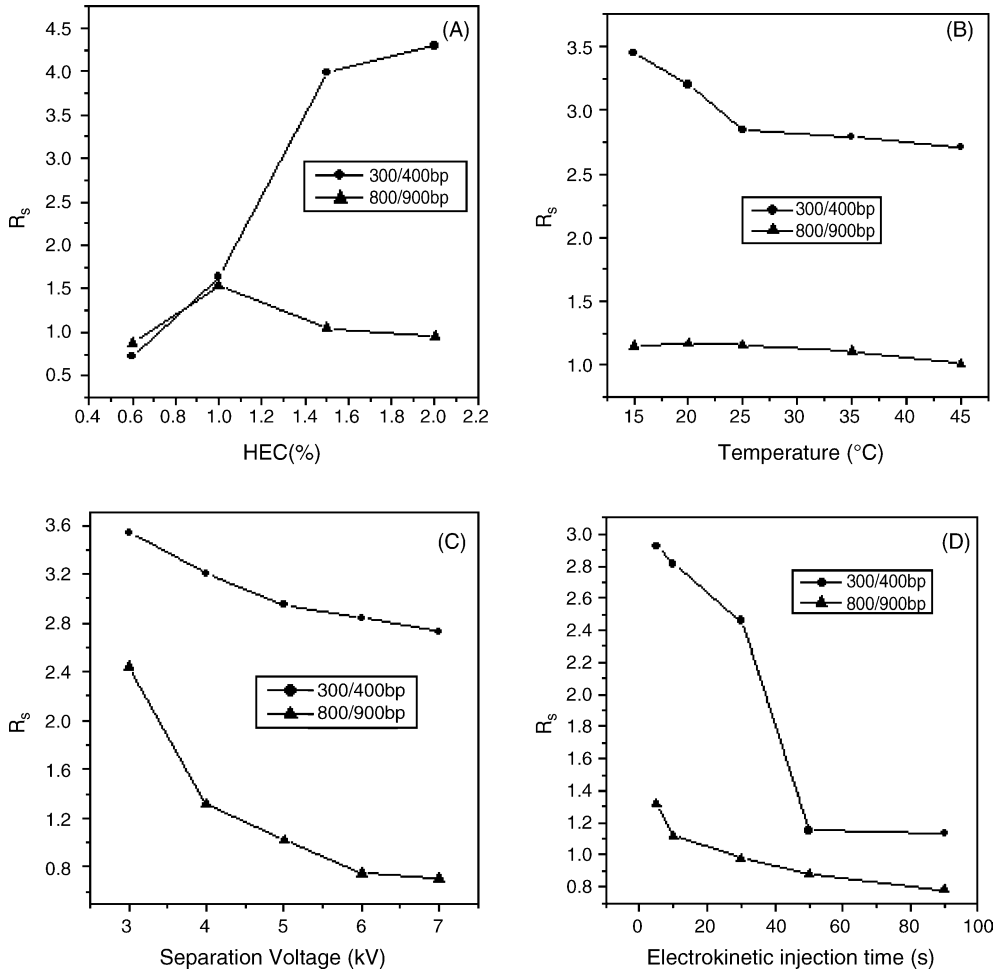

Fig. 1. Effects of separation parameters on separation. (A) Effect of HEC concentration on separation, electrokinetic injection: 10 kV × 10 s, temperature: 25 °C. Separation voltage: 5 kV. (B) Effect of temperature on separation, buffer: 1× TBE containing 1.2% HEC, other conditions are the same as (A). (C) Effect of voltage on separation, buffer: 1× TBE containing 1.2% HEC, other conditions are the same as (A). (D) Effect of injection time on separation, buffer: 1× TBE containing 1.2% HEC, other conditions are the same as (A).

ied. The resolution was calculated as the following equation: $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_2, t_1 represent the migration time and t_{w1}, t_{w2} represent the width of the peaks at the baseline for peak 1 and peak 2, respectively.

Recently, non-gel sieving CE using linear polymer solutions as the sieving matrix has been widely used for the analysis of DNA fragments due to high separation speed and replaceable operation mode [20–22]. In this work, a cellulose derivative solution of hydroethylcellulose (HEC) was chosen as the sieving matrix. The effects of HEC concentration on the separation speed and resolution was investigated in the range from 0.6 to 2.0% while other parameters were kept constant (separation voltage: 5 kV; temperature at 25 °C and electrokinetic injection: 10 kV × 10 s). The polymer concentration influences both the separation speed and resolution. As HEC concentration increased, the migration time was prolonged resulting from the shrinkage of the sieving cores and the increase of the buffer viscosity. As shown in Fig. 1A, the resolution of peak pair 300 bp/400 bp increased with the change of HEC concentration in the range from 0.6 to 2.0%. However, the resolution of peak pair 800 bp/900 bp increased firstly with the increase of HEC in the range from 0.6 to 1.0% then decreased in the range from 1.0 to 2.0%. Taking analysis speed and resolution in consideration, 1.2% HEC was selected for the following experiments. 2% PVP was added in the running buffer in order to avoid the interaction between DNA fragments and capillary wall and improve the reproducibility [20].

Fig. 1B illustrates the effect of the temperature on separation. As depicted in Fig. 1B, in the temperature range of 15–45 °C, the temperature had little influence on the resolution. Separation speed increased with the increase of temperature due to the decreased buffer viscosity at higher temperature. However, at high temperature above 35 °C, the separation system became unstable and bubble peaks appeared. Therefore, 25 °C was set for optimized parameter for further analysis.

The advantage of CE over traditional slab gel electrophoresis is high efficiency and high speed due to the use of high voltage. The applied voltage was studied in the range from 3 to 7 kV. As indicated in Fig. 1C, the lower voltage was helpful to improve the resolution. However the migration time became longer at lower voltage. Separation voltage of was 5 kV selected in later experiments.

In the present study, electrokinetic injection mode was used for sample introduction due to the simplicity and the potential stacking effect when the ionic strength of the sample was lower than and that of the separation buffer. The effect of injection time on the resolution was studied in the range from 5 to 90 s. From Fig. 1D, it can be seen that injection time had little influence on the resolution when the injection time was less than 30 s. Further increase of injection time would reduce the resolution. Taking both the resolution and reproducibility in consideration, 10 s electrokinetic injection was used for optimized separation.

In conclusion, the optimal separation conditions for the analysis of DNA fragments were as follows: polyacrylamide coated capillaries; the running buffer was 1× TBE containing 2% PVP and 1.2% HEC. Separation voltage was 5 kV and temperature was set at 25 °C. The sample was injected by electrokinetic mode at 10 kV × 10 s. As illustrated in Fig. 2A, 100 bp DNA ladder was effectively separated under optimal conditions. The relationship between the migration time and DNA fragments length was showed in Fig. 2B, which was used for confirmation of peak identity and size determination.

The reproducibility of the system was determined by measuring the migration time of 500 bp DNA analyte under optimal separation condition. The daily run-to-run R.S.D.

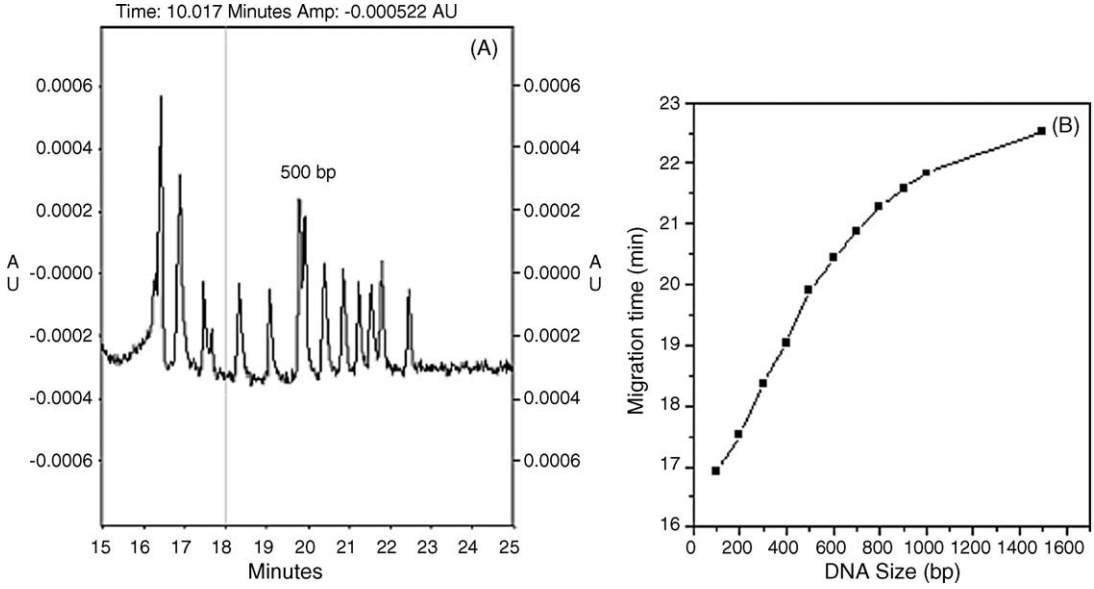

Fig. 2. Electrophoretic separation of 100 bp DNA ladder marker (A) and relationship between DNA size and migration time for 100 bp DNA ladder (B). Conditions: 1× TBE containing 1.2% HEC, electrokinetic injection: 10 kV × 10 s, temperature: 25 °C. Separation voltage: 5 kV.

was around 1.6% ($n = 10$). The day-to-day R.S.D. of the migration time was about 5.6% ($n = 8$), showing the stability of polyacrylamide-coated layer was acceptable in the above operation conditions.

3.2. Determination of hydrogen peroxide induced apoptosis in PC12 cells by CE

The biochemical character of apoptosis is the cleavage of genomic DNA into 180–200 bp fragments. The apoptotic fragments have a size determined by the link between a certain number of nucleosomes that remains together [21]. In this paper, the established CE method was employed to monitor the DNA fragmentation character during the apoptosis process of PC12 cells induced by H_2O_2. The qualitative analysis and identification of the DNA fragments of the apoptosis cells was made by comparison with the migration time of the well-defined fragments of DNA mass markers depicted in Fig. 2B. The degree of cell apoptosis can be quantified by determining the level of DNA fragmentation during apoptosis process.

As shown in Fig. 3A, there is only a broad peak corresponding to the DNA extracted from normal PC12 cells. The peak contains some large pieces of different lengths of genomic DNA that were not resolved by the above CE method. The nuclear morphology of normal cells was shown in Fig. 4A by DAPI staining. Flow cytometry (Fig. 5A) and TUNEL (Fig. 6A1 and B1) results also demonstrate that few cells were apoptotic under normal conditions.

Fig. 3B exhibits the electropherograms of DNA fragments extracted from PC12 cells treated by H_2O_2 for 12 h. As shown in Fig. 3B, many peaks appeared. Comparing the migration time of the peaks with those of the standard DNA ladder marker in Fig. 1B, peak 0 has a size of about 100 bp and can be regarded as cleaved DNA fragments of short length. Peaks 1–5 have approximately sizes of 170, 350, 510, 690 and 860 bp, respectively, corresponding to one to five nucleosomes and their linkers. The peak broadness may

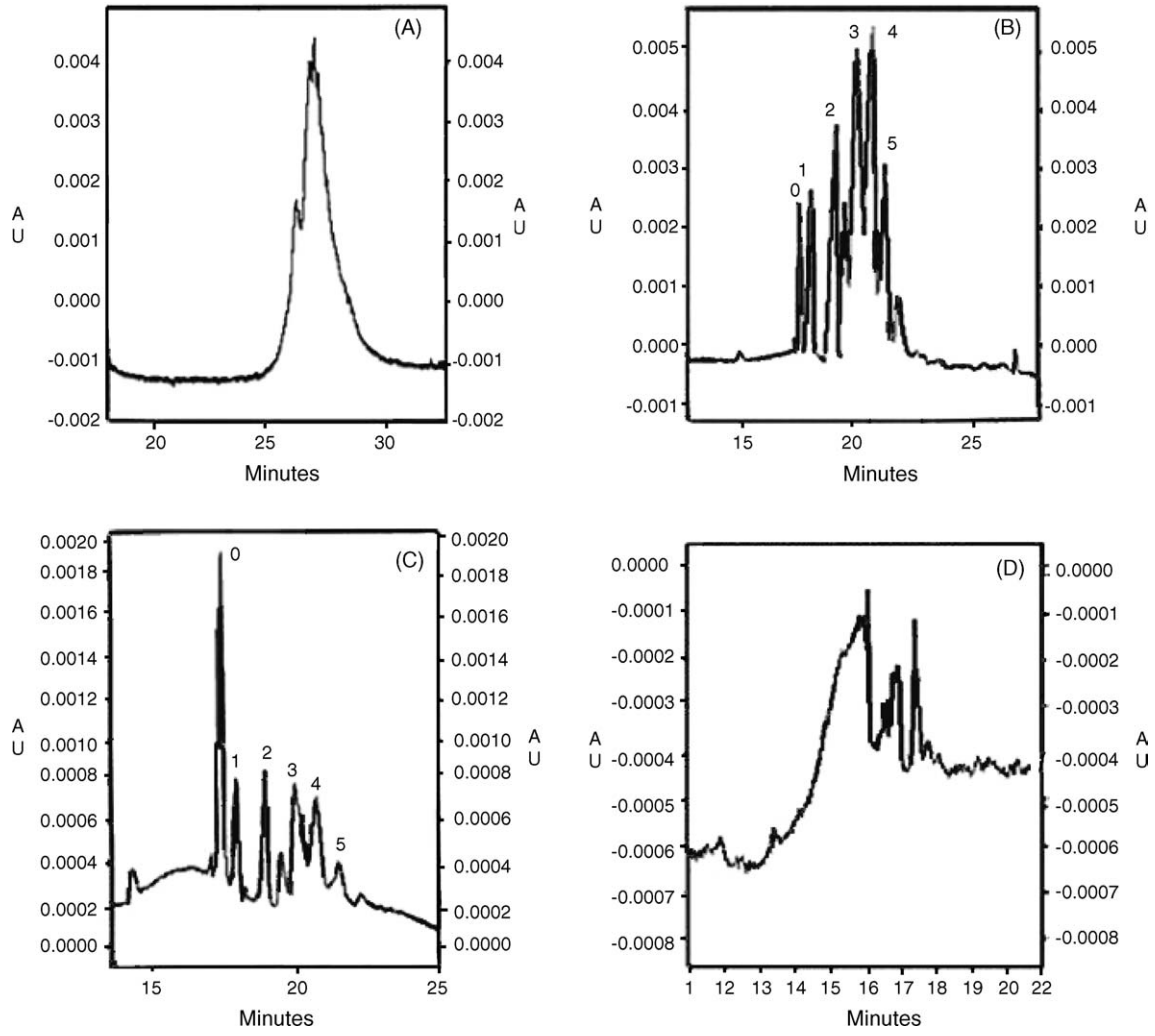

Fig. 3. Capillary electropherograms of DNA samples extracted from PC12 cells: (A) normal cells, (B) cells treated with H_2O_2 for 12 h, (C) cells treated with H_2O_2 for 24 h, (D) cells treated with H_2O_2 for 72 h. Separation conditions are the same as in Fig. 2.

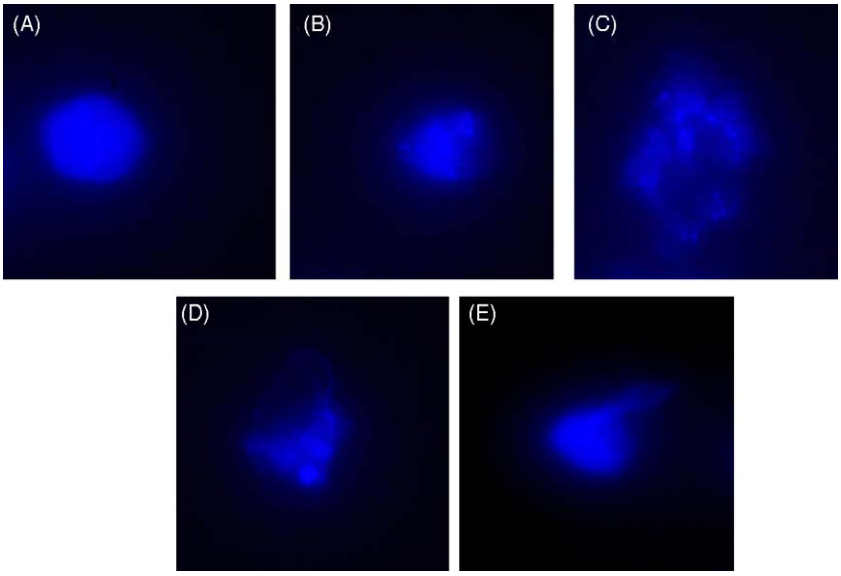

Fig. 4. The nuclear morphology changes of PC12 cells induced by H_2O_2 under fluorescene microscope (original magnification 800×). (A) Control: normal cells; (B) treated for 6 h: nuclear chromatin condensation; (C) treated for 12 h: nuclei fragmentation and migration to the nuclear membrane; (D) treated for 24 h, apoptotic bodies appearance; and (E) treated for 72 h, cells lysis.

indicate a distribution of fragment lengths since the breakage of DNA could not uniformly be the same site between nucleosides. Different cell type and/or apoptotic induction type results in the difference of nucleosomal repeat lengths that may range from 140 to 240 bp [21]. Therefore peaks 1–5 can be regarded as typical apoptotic DNA fragments, suggesting the cells undergo apoptosis after treated with H_2O_2 for 12 h. Fig. 4C shows that condensed chromatin migrated to the nuclear membrane and nuclei fragmentation took place after the cells were treated with H_2O_2 for 12 h. Flow cytometry (Fig. 5B) and TUNEL (Fig. 6A2 and B2) indicate some cells were apoptotic at this state (apoptosis percentage of cells was about 29.35%).

Fig. 3C demonstrates the electropherograms of DNA fragments extracted from PC12 cells intervened by H_2O_2 for 24 h. Compared with Fig. 3B, the relative intensity of peaks changed obviously. The relative peak intensity was indicated by the ratio of peak area to DNA sample concentration since the sample concentration corresponding to Fig. 2B and C are different. As shown in Table 1, when the treatment time by H_2O_2 was prolonged from 12 to 24 h, the relative intensity of shorter DNA fragments increased while those of DNA fragments with larger length decreased. The DNA fragments with shorter length showed greater enhancement of relative peak intensity. This indicates that fragments with larger size may be more prone to degrade and level of DNA fragmentation and apoptosis of PC12 cells increased as the treatment time increase. The result was also testified by the TUNEL (Fig. 6A3 and B3) and flow cytometry methods (Fig. 5C), which showed the apoptosis percentage increased from 29.35 to 56.17%. DAPI staining showed apoptotic bodies appeared (Fig. 4D) when cells were intervened for 24 h, suggesting the cells were in the later phase of apoptosis.

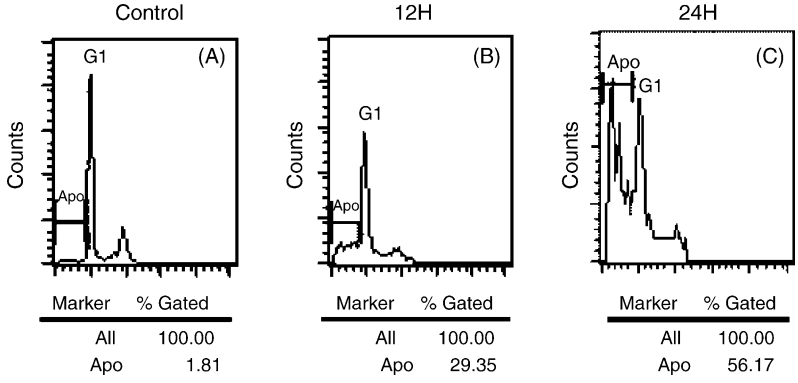

Fig. 5. Flow cytometric DNA analysis of apoptotic PC12 cells treated with H_2O_2. The cell population with a sub-G1 DNA content was calculated. (A) Normal cells; (B) cells treated for 12 h; (C) cells treated for 24 h.

Table 1
Quantification of apoptotic DNA fragmentation in PC12 cells after treatment by H_2O_2

	Relative peak intensity (area/DNA concentration (μg/μL))		
	12 h	24 h	Fold increase
Peak 0	16073	183608	11.42
Peak 1	50514	74505	1.47
Peak 2	85531	91732	1.07
Peak 3	171628	158907	0.93
Peak 4	166044	126165	0.76
Peak 5	51842	31443	0.61

The relative peak intensity was indicated by the ratio of peak area to DNA sample concentration. Peak area was determined by P/ACE Station GoldSystem Software. Sample DNA concentration was calculated by the spectrophotometry value at 260 nm.

Fig. 3D shows the DNA fragments character of PC12 cells treated with H_2O_2 for 72 h. Compared with Fig. 3B and C, typical apoptotic DNA fragments disappeared and many randomized cleaved DNA fragments of short length were observed, suggesting the cells were necrotic. Lysis of cells could also be observed (Fig. 4E), proving the related cells were necrotic.

As can be seen in Fig. 3, normal, apoptotic and necrotic cells have distinctly DNA fragments character revealed by CE method. Thus CE would be an effective method to distinguish normal, apoptotic and necrotic cells.

The method precision was also studied. Two identical cell samples received the same H_2O_2 treatment (12 h) and DNA extraction procedure. Then the DNA samples were analyzed separately. The similar degree of DNA cleavage was observed, suggesting the method was producible and reliable.

In conclusion, the developed CE method can distinguish cell apoptosis and necrosis, quantify the degree of cell apoptosis by determining the level of DNA fragmentation during apoptosis process. It offers the advantages of high efficiency, fast sample analysis speed, minute sample consumption and reliable results, which provides a useful accessorial method in the research of multiple diseases with abnormal apoptosis such as cancer and neurodegenerative diseases. Especially

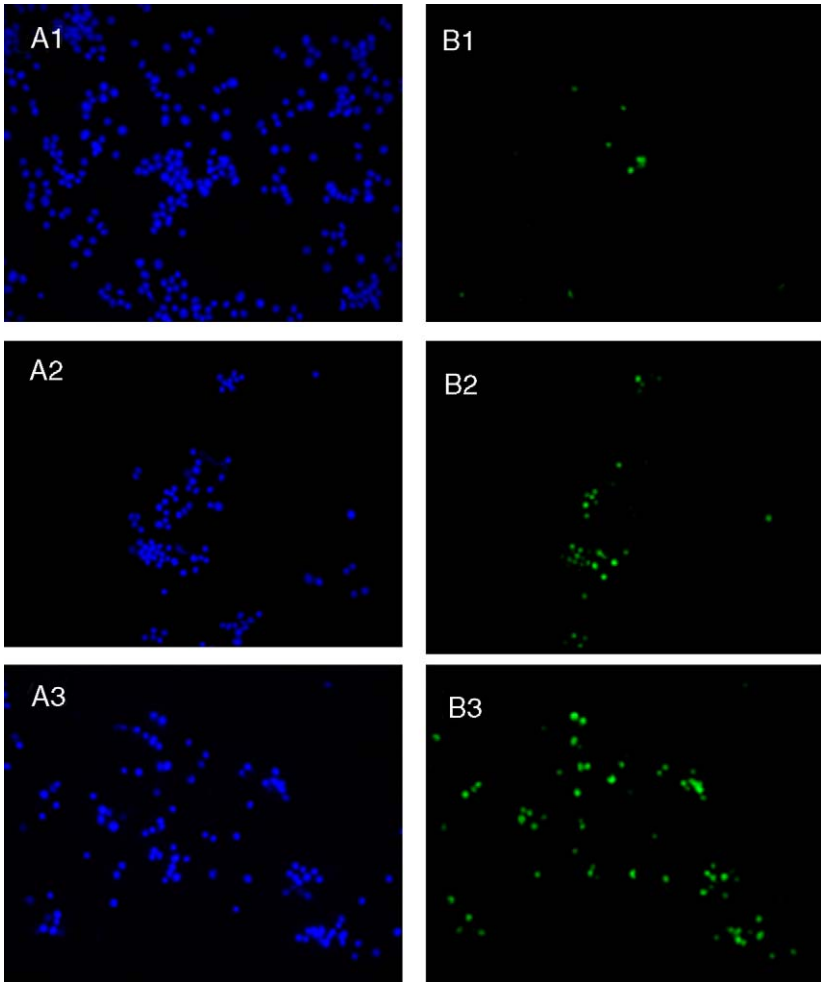

Fig. 6. The analysis of apoptotic PC12 cells induced by H_2O_2 by TUNEL staining under fluorescene microscope (magnification 200×). DAPI (blue, A1–A3) was used to stain the nucleus of all cells while TUNEL positive (green, B1–B3) show the apoptotic cells under the same field. (A1 and B1) Control normal cells; (A2) and (B2) cells treated for 12 h; (A3) and (B3) cells treated for 24 h.

when combined with ultra-sensitive laser-induced fluorescence detector, CE can be used to analysis the early stage of cell apoptosis of limited number of cells, which is important in neuroscience research in our future study.

Acknowledgments

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