DNA degradation within mouse brain and dental pulp cells 72 hours postmortem

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Abstract

In this study, we sought to elucidate the process of DNA degradation in brain and dental pulp cells of mice, within postmortem 0–72 hours, by using the single cell gel electrophoresis assay and professional comet image analysis and processing techniques. The frequency of comet-like cells, the percentage of tail DNA, tail length, tail moment, Olive moment and tail area increased in tandem with increasing postmortem interval. In contrast, the head radius, the percentage of head DNA and head area showed a decreasing trend. Linear regression analysis revealed a high correlation between these parameters and the postmortem interval. The findings suggest that the single cell gel electrophoresis assay is a quick and sensitive method to detect DNA degradation in brain and dental pulp cells, providing an objective and accurate new way to estimate postmortem interval.

Key Words: DNA degradation; single cell gel electrophoresis; postmortem interval; brain; dental pulp

INTRODUCTION

Along with the rapid progress in molecular biology, automated image analysis and computer technology in recent years, numerous efforts have been made to estimate human postmortem interval by assessing changes in nuclear DNA [1-5]. DNA is one of the most stable components of cells, and its content is similar among different individuals and different cell types within the same species [3-6]. Flow cytometry has been utilized as a means to study changes in nuclear DNA after death, and studies have confirmed that the DNA content in the human body gradually diminishes with increasing postmortem interval. However, many factors restrict the application of flow cytometry, such as the expensive cost of equipment, requirement of complex experimental techniques and the limited number of morphological parameters [7-9]. Single cell gel electrophoresis (SCGE), also known as the comet assay due to the fact that the DNA trail resembles a comet [10-11], is a method for detecting nuclear DNA damage and repair at the single cell level. SCGE determination of DNA migration fluorescence intensity or migration length is the hallmark of quantitative determination of single cell DNA degradation, and thus helps estimate postmortem interval. This study sought to detect changes in DNA degradation in the brain and dental pulp cells of mice within postmortem 0–72 hours, by using SCGE and professional computer image analysis techniques, in a broader attempt to estimate the postmortem interval at the molecular level.

RESULTS

Quantitative analysis of experimental animals

A total of 111 healthy mice were divided into a control group (0 hour) and 36 experimental groups according to the time the tissues were harvested after death (every 2 hours up to 72 hours postmortem). Each group contained three mice.

Nuclear DNA degradation in brain and dental pulp tissue of mice within 72 hours after death

The SCGE assay showed that, at an early stage after death (postmortem 2 hours), the majority of cells in brain and dental pulp tissue displayed a complete head, spherical in shape, and the comet tail was minimal. Within the following 70 hours, the length (μm) and concentration (%) of the tail gradually increased. The brain and dental pulp cells showed more nuclear DNA fragmentation and had a typical comet shape (Figure 1).
The comet images were computer-analyzed to determine the parameters of nuclear DNA degradation in brain and dental pulp tissue. Each parameter was formulated as a regression equation, comprehensively understand changes in DNA and to effectively infer postmortem interval. We found that the degradation rate (frequency of comet-like cells), comet tail length, percentage of tail DNA, tail area, tail moment and Olive moment in brain and dental pulp tissue increased in parallel with increasing postmortem interval, while a reduction in head radius, percentage of head DNA and head area (nuclear DNA parameter in dental pulp tissue is not shown) was also concomitantly observed, all with a strong correlation (Tables 1–3).

### Table 1 Changes in nuclear DNA parameters in mouse brain tissue within 72 hours after death

| Postmortem interval (h) | Head DNA % | Tail DNA % | Head radius (μm) | Tail length (μm) | Tail moment | Olive moment | Head area (μm²) | Tail area (μm²) |
|------------------------|------------|------------|------------------|-----------------|-------------|--------------|----------------|----------------|
| 0                      | 99.71      | 0.29       | 26.50            | 0               | 0           | 0.03         | 1996.00        | 0.50           |
| 2                      | 99.42      | 0.58       | 20.00            | 0.71            | 0.03        | 0.10         | 1962.93        | 30.35          |
| 4                      | 97.84      | 2.16       | 27.05            | 2.40            | 0.09        | 0.51         | 1931.40        | 32.60          |
| 6                      | 95.57      | 4.43       | 24.37            | 1.94            | 0.31        | 1.21         | 1851.37        | 40.57          |
| 8                      | 94.09      | 5.91       | 20.78            | 3.32            | 0.47        | 0.51         | 2390.89        | 38.05          |
| 10                     | 91.66      | 8.34       | 18.86            | 6.13            | 0.84        | 1.30         | 2100.25        | 184.38         |
| 12                     | 89.35      | 10.65      | 20.81            | 14.71           | 1.24        | 1.77         | 2287.43        | 167.38         |
| 14                     | 90.04      | 9.96       | 21.06            | 7.88            | 2.23        | 5.60         | 2391.40        | 177.83         |
| 16                     | 87.96      | 12.04      | 18.74            | 19.34           | 3.83        | 2.08         | 1997.33        | 121.59         |
| 18                     | 66.43      | 33.57      | 15.37            | 9.62            | 4.51        | 11.56        | 910.70         | 355.36         |
| 20                     | 75.47      | 24.53      | 14.48            | 12.18           | 5.08        | 2.72         | 887.65         | 956.75         |
| 22                     | 83.10      | 16.90      | 22.17            | 21.67           | 5.65        | 4.31         | 574.92         | 2017.82        |
| 24                     | 65.37      | 34.63      | 14.25            | 29.28           | 5.17        | 7.64         | 776.40         | 1895.31        |
| 26                     | 84.61      | 15.39      | 13.08            | 28.88           | 6.69        | 2.22         | 591.50         | 2255.10        |
| 28                     | 69.43      | 30.57      | 14.27            | 11.22           | 7.33        | 12.65        | 602.22         | 1725.78        |
| 30                     | 89.14      | 10.86      | 13.90            | 29.69           | 5.42        | 5.74         | 507.10         | 908.03         |
| 32                     | 76.25      | 23.75      | 10.86            | 10.04           | 7.84        | 7.34         | 478.87         | 1210.28        |
| 34                     | 73.67      | 26.33      | 13.76            | 37.25           | 9.37        | 11.18        | 624.94         | 3050.10        |
| 36                     | 78.38      | 21.62      | 17.72            | 34.67           | 9.19        | 10.53        | 363.33         | 3794.67        |
| 38                     | 74.47      | 25.53      | 2.15             | 27.01           | 8.26        | 28.00        | 756.25         | 3540.38        |
| 40                     | 66.06      | 33.94      | 10.98            | 40.15           | 7.42        | 6.16         | 492.33         | 4585.67        |
| 42                     | 46.35      | 53.65      | 8.13             | 32.67           | 20.84       | 14.48        | 446.30         | 1764.32        |
| 44                     | 51.60      | 48.40      | 14.04            | 52.75           | 24.29       | 22.28        | 1235.50        | 1301.25        |
| 46                     | 62.67      | 37.13      | 15.50            | 41.11           | 19.28       | 7.16         | 102.50         | 4860.50        |
| 48                     | 27.34      | 72.66      | 9.39             | 56.21           | 35.18       | 10.67        | 501.18         | 3097.81        |
| 50                     | 43.68      | 56.32      | 10.54            | 53.91           | 27.34       | 20.10        | 587.72         | 2458.70        |
| 52                     | 32.53      | 67.47      | 11.02            | 40.86           | 24.91       | 15.40        | 640.14         | 3046.14        |
| 54                     | 21.36      | 78.64      | 17.03            | 47.85           | 25.55       | 13.72        | 784.00         | 4543.00        |
| 56                     | 30.65      | 69.35      | 17.07            | 42.86           | 26.65       | 17.60        | 735.86         | 2849.29        |
| 58                     | 37.49      | 62.51      | 17.64            | 51.20           | 40.49       | 23.57        | 678.60         | 5356.80        |
| 60                     | 28.47      | 71.53      | 14.00            | 70.75           | 64.57       | 19.35        | 694.02         | 5655.92        |
| 62                     | 35.63      | 64.37      | 11.76            | 81.31           | 68.34       | 33.69        | 637.15         | 5978.13        |
| 64                     | 23.46      | 76.54      | 12.90            | 90.57           | 63.89       | 35.36        | 619.26         | 7659.40        |
| 66                     | 28.27      | 71.73      | 7.18             | 101.33          | 60.75       | 43.78        | 332.01         | 8329.00        |
| 68                     | 31.29      | 68.71      | 9.69             | 77.20           | 53.92       | 35.93        | 621.80         | 4067.80        |
| 70                     | 25.81      | 74.19      | 8.84             | 86.34           | 74.56       | 41.18        | 607.70         | 7412.80        |
| 72                     | 22.55      | 77.45      | 10.25            | 77.37           | 44.56       | 33.72        | 648.12         | 3863.37        |

DNA parameters are expressed as mean values.
There is very little forensic data addressing the use of SCGE to correlate nuclear DNA degradation in dental pulp tissue with postmortem interval. Previous studies examined distance indices only, such as comet-like cell frequency, DNA migration length and head radius, and the measurements were mainly performed by eye, directly or following micrography. In this study, we used comet assay analysis software, which provides professional and fast measurement of comet-like cell geometrical parameters, such as head radius, tail length, head area and tail area. Therefore, DNA damage in individual brain and dental pulp cells was evaluated. However, measurements of the comet length and area cannot fully reflect DNA damage. Our results demonstrate that, with increasing postmortem interval, the degree of DNA degradation in brain and dental pulp cells gradually increased, while changes in DNA tail length were not significant. To comprehensively reflect the degree of DNA damage, tail moment and Olive moment were also used as indicators in the comet assay.

In this experiment, images were acquired using the comet assay every 2 hours after the cell suspension was subjected to agarose gel electrophoresis. Based on the cell fluorescence images, cells were divided into comet-like cells and non-comet-like cells. The percentage of comet-like cells was calculated to estimate the degree of DNA degradation in cells. Results of this study showed that the degradation rate (frequency of comet-like cells) increased in the brain and dental pulp tissue along with increasing postmortem interval, indicating a strong correlation with postmortem interval. Nuclear DNA fragmentation rose and the fragments migrated rapidly in the basic environment when they were subjected to electrophoresis, revealing the evident tailing pattern. These experimental findings suggest that there is indeed DNA degradation and fragmentation in dental pulp cells after death, and the process is gradual, progressive and regular. It is well known that comet tail length depends on the amount of small DNA fragments, produced as a result of degradation. The comet tail length, tail area, tail moment and Olive moment gradually increased with increasing postmortem interval, and the degraded nuclear DNA fragments became smaller and their number increased.

In this study, various cells in the same sample showed different measured values of the same parameter at the same time under the same processing conditions, indicating that DNA degradation varies between different cells within the same tissue. Therefore, we calculated the mean values of each parameter to deal with this variability. Individual cell variability may be due to the tissue dissection itself, which may result in physical damage to the nucleus, compromising nuclear and cellular integrity, leading to a massive release of lysosomal enzymes, thereby accelerating the cellular degradation process. Regression analysis of the tested parameters demonstrated that the parameters that are most sensitive to postmortem interval are tail length and tail moment, followed by the head/tail DNA percentage, Olive moment, tail area, head area and head radius. The tail DNA content, tail moment and Olive moment of the dental pulp tissue were more sensitive than other parameters for the estimation of postmortem interval, accurately reflecting the degree of DNA degradation in dental pulp tissue after death. Thus, tail parameters are better indicators than head parameters, and the moment parameters are better than the geometrical parameters. Single cell gel electrophoresis can quickly and accurately analyze a large number of cells; in particular, multi-parameter detection of the same cell group. Thus, we can analyze the correlation of various indicators in a single cell. In this study, we established linear regression equations for various parameters, to comprehensively assess

| Comet parameters | Regressive analysis | Regressive equation | r  | P     |
|------------------|-------------------|-------------------|----|-------|
| Frequency of comet-like cells | y = 0.032x^2 + 3.607x - 1.727 | 0.937 < 0.001 |
| Head DNA (%) | y = 0.003x^2 - 0.928x + 101.45 | 0.884 < 0.001 |
| Tail DNA (%) | y = 0.003x^2 + 1.928x - 1.454 | 0.884 < 0.001 |
| Head radius | y = 0.005x^2 + 0.551x + 26.207 | 0.642 < 0.001 |
| Tail length | y = 0.011x^2 + 0.434x + 1.590 | 0.897 < 0.001 |
| Tail moment | y = 0.018x^2 - 0.334x + 1.913 | 0.883 < 0.001 |
| Olive moment | y = 0.008x^2 - 0.033x + 1.689 | 0.819 < 0.001 |
| Head area | y = 0.678x^2 - 70.578x + 2366.8 | 0.701 < 0.001 |
| Tail area | y = 0.456x^2 + 62.249x - 354.6 | 0.758 < 0.001 |

y: The time of death (h); x: the measured parameters. Head DNA (%): the percentage of DNA content in the comet head; tail DNA (%): the percentage of DNA content in the comet tail.

| Comet parameters | Regressive analysis | Regressive equation | r  | P     |
|------------------|-------------------|-------------------|----|-------|
| Frequency of comet-like cells | y = 0.016x^2 + 0.410x + 0.282 | 0.912 < 0.001 |
| Head DNA (%) | y = 0.019x^2 - 1.103x + 96.831 | 0.912 < 0.001 |
| Tail DNA (%) | y = 0.002x^2 + 1.103x + 3.169 | 0.972 < 0.001 |
| Head radius | y = 0.002x^2 - 0.024x + 16.054 | 0.918 < 0.001 |
| Tail length | y = 0.013x^2 + 1.917x + 0.956 | 0.899 < 0.001 |
| Tail moment | y = 0.011x^2 + 0.224x + 1.237 | 0.964 < 0.001 |
| Olive moment | y = 0.001x^2 + 0.363x + 0.154 | 0.910 < 0.001 |
| Head area | y = 0.091x^2 - 1.019x + 77.27 | 0.790 < 0.001 |
| Tail area | y = 0.106x^2 + 48.501x + 73.552 | 0.912 < 0.001 |

y: The time of death (h); x: the measured parameters. Head DNA (%): the percentage of DNA content in the comet head; tail DNA (%): the percentage of DNA content in the comet tail.
DNA damage after death, in an effort to efficiently and accurately estimate postmortem interval. In summary, single cell gel electrophoresis, as a rapid, simple, sensitive assay for single cell DNA fragmentation, can be used to examine the correlation between DNA degradation and postmortem interval, with the ability to detect mild DNA damage.

**MATERIALS AND METHODS**

**Design**
A repeated measurement and self-controlled experiment.

**Time and setting**
Experiments were performed from December 2009 to November 2010 in the Laboratory of Forensic Pathology, School of Forensic Medicine, China Medical University, China.

**Materials**
A total of 111 healthy 8-week-old Kunming mice, of either gender, weighing 20-30 g, were used (Experimental Animal Center of China Medical University, China; license No. SCXK (Liao) 2009-0002). After 3 days of adaptive feeding, mice were killed and placed at 22°C, with 70% humidity, under a 12-hour light/dark cycle. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Methods**

**Preparation of brain and dental pulp tissue single cell suspensions**
The whole brain tissue (1.5 g) was harvested, the blood was washed away with cold saline, and the sample was placed in a 5-mL test tube (containing 2 mL phosphate-buffered saline) and triturated in an ice bath for 10 minutes. Samples were digested with trypsin at a final concentration of 0.0125%, in a 37°C water bath for 20 minutes to form a homogeneous turbid liquid, and filtered with sieves with a pore size of 160 and 100 μm, sequentially. The final filtrate was used as the single cell suspension for analysis. Cells were rinsed with cold phosphate-buffered saline and adjusted to 2 × 10⁴ cells/mL.

Dental pulp tissue was exposed from the incisor using an ophthalmic hemostat and tissues were sampled with an ophthalmic tweezer. Dental pulp tissue from four incisors was washed with cold phosphate-buffered saline solution to remove blood and cut into pieces with ophthalmic scissors on an ice bath. Samples were digested with 2 mL trypsin solution, at 37°C for 30 minutes, and the solution was shaken every 6 minutes. The digestion was terminated with D-Hank’s solution, and the solution was filtered with a 300-mesh nylon funnel. Then, 200 μL phosphate-buffered saline solution was added to prepare a suspension and centrifuged at 4°C, 1 000 r/min, for 6 minutes. The supernatant was discarded, and phosphate-buffered saline solution, 200 μL, was added and the sample was centrifuged at 4°C, 1 000 r/min, for 6 minutes. The supernatant was removed and the cell pellet was resuspended and viewed with an optical microscope (Olympus, Tokyo, Japan). Typically more than 10⁴ cells were obtained.

**SCGE assay**
Layer preparation: There were three layers of gel. Layer 1: the matte surface was prepared with 5% normal-melting-point agarose, 100 μL (dissolved in Ca²⁺ and Mg²⁺-free phosphate-buffered saline); layer 2: low-melting-point (37°C) agarose containing brain and dental pulp tissue cells (10⁴). 0.5 mL cell suspension was added to 1.5 mL pre-prepared 1% low-melting-point agarose in a test tube and quickly poured onto the pre-cooled glass slide; layer 3: 0.5% cell-free low-melting-point agarose, 100 μL. The agarose gel was kept at 4°C for 5–10 minutes to completely solidify. Cell lysis and alkaline denaturation process: The completely solidified gel plate was soaked in freshly prepared alkaline hydrolysis solution (1 L; containing 1.2 M NaCl, 0.03 M NaOH and 0.1 M SDS; pH 10–12, 4°C) for 1 hour and then rinsed with bleaching solution (0.03 M NaOH + 2.0 mM EDTA) three times, each 20 minutes.

Gel electrophoresis: The treated gel was placed in a submersible electrophoresis chamber filled with electrophoresis solution (0.03 M NaOH + 2.0 mM EDTA) and electrophoresed at 30 V, 300 mA, in the dark for 25 minutes.

Fluorescence staining: After electrophoresis, the gel plate was rinsed with deionized water for 15 minutes and stained with 0.25% ethidium bromide in the dark for 20 minutes. The stained gel plate was washed with deionized water to remove excess dye and then placed in a dark closed wet box. Image analysis was performed within 24 hours.

Comet image analysis and data processing: After ethidium bromide staining, the gel plate was placed under the fluorescence microscope (BH-2 type; Olympus, Tokyo, Japan) for fluorescence observation. The non-degraded cells showed a circular fluorescent core, that is the comet head, with no tail (Figure 2).

While the degraded cells showed migration of fragments from the head to the anode, thus forming a comet-like tail (Figure 3). Five fields of vision (> 5 cells/field) for each smear slice were randomly selected and observed at 200 x magnification under a fluorescence microscope.
with each group containing three slices. Images were collected with a cold CCD digital camera (Beijing Chuangmei Weiye Technology, Beijing, China), and the degree of DNA degradation was automatically detected with LUCIA COMET ASSAY Version 4.81 analysis software (Beijing Chuangmei Weiye Technology). Analysis parameters included comet tail length, tail DNA%, tail area, tail moment, Olive moment, head radius, head DNA% and head area.

Figure 3  Degraded mouse brain/dental pulp tissue cells (fluorescence microscopy, × 400).

Statistical analysis
Data were statistically analyzed using EXCEL software (EXCEL 2007, Microsoft) and SAS 9.1 software package (SAS Institute, Cary, USA) to calculate the binomial regression equation.

Author contributions: Jilong Zheng was responsible for study concept and design, provided and integrated data, performed data analysis and statistical analysis, drafted the manuscript, and was responsible for funding. Xiaona Li, Di Shan and Han Zhang provided technical and information support. Dawei Guan supervised the research.

Conflicts of interest: None declared.

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Ethical approval: Experiments were given approval by the Laboratory Animal Ethics Committee, China Medical University, China.

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REFERENCES

[1] Bortolotti F, Pascali JP, Davis GG, et al. Study of vitreous potassium correlation with time since death in the postmortem range from 2 to 110 hours using capillary ion analysis. Med Sci Law. 2011;51 Suppl 1:S20-23.

[2] Holley S, Fiedler S, Grow M. Macroscopical estimation of the postmortem interval (PMI) and exclusion of the forensically relevant resting period--a comparison of data presented in the literature with recent osteological findings. Arch Kriminol. 2008;221:175-184.

[3] Larkin B, Isachi S, Dadour I, et al. Using accumulated degree-days to estimate postmortem interval from the DNA yield of porcine skeletal muscle. Forensic Sci Med Pathol. 2010;6:83-92.

[4] Kaiser C, Bachmeier B, Conrad C, et al. Molecular study of time dependent changes in DNA stability in soil buried skeletal residues. Forensic Sci Int. 2008;177:32-36.

[5] Zhang JH, Xu M. DNA fragment in apoptosis. Cell Res. 2000;10:105-111.

[6] Lin X, Yin YS, Ji Q. Progress on DNA quantification in estimation of postmortem interval. Fayixue Zazhi. 2011;27:47-49, 53.

[7] Di Nunno N, Costantinides F, Cinas J, et al. What is the best sample for determining the early postmortem period by on-the-spot flow cytometry analysis? Am J Forensic Med Pathol. 2002;23:173-180.

[8] Boy SC, Bernitz H, Van Heerden WF. Flow cytometric evaluation of postmortem pulp DNA degradation. Am J Forensic Med Pathol. 2003;24:123-127.

[9] Di Nunno N, Costantinides F, Melato M. Determination of the time of death in a homicide-suicide case using flow cytometry. Am J Forensic Med Pathol. 1999;20:228-231.

[10] Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the comet assay. Radiat Res. 1990;122:86-94.

[11] Huang P, Olive PL, Durand RE. Use of the comet assay for assessment of drug resistance and its modulation in vivo. Br J Cancer. 1998;77:412-416.

[12] Johnson LA, Ferris JA. Analysis of postmortem DNA degradation by single-cell gel electrophoresis. Forensic Sci Int. 2002;126:43-47.

[13] Deng LB, Yu XY, Yang QE. A study of the relationship between the postmortem interval and nuclear DNA degradation of muscle cells in mice. Zhongguo Fayixue Zazhi. 2003;18:273-275.

[14] Fairbairn DW, Olive PL, O’Neill KL. The comet assay: a comprehensive review. Mutat Res. 1995;339:37-59.

[15] Johnson LA, Ferris JA. Single cell electrophoresis in determining cell death: potential for use in organ transplant research. J Biochem Biophys Methods. 2005;63:53-68.

[16] Zheng JL, Zhang XD, Niu QS. Relationship between the postmortem interval and nuclear DNA changes of heart muscular cells in mice. Fayixue Zazhi. 2006;22:173-176.

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