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Analysis of postmortem DNA degradation by single-cell gel electrophoresis

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Abstract

One of the most important longstanding problems in the field of forensic medicine is the determination of the time of death upon the discovery of a possible homicide victim. With a majority of homicide victims discovered within the first 48 h, it is critically important to be able to determine time of death quickly, and with accuracy and precision. Current methods of determining postmortem interval (PMI) vary, but none can provide better than an 8-h window time estimate. In this paper, the potential application of single-cell gel electrophoresis (SCGE), also known as the comet assay, to evaluate postmortem cell death processes, specifically nuclear DNA fragmentation, is assessed. Upon the death of an organism, internal nucleases contained within the cells should cause chromosomal DNA to degrade into increasingly smaller fragments over time, and if these fragments can be isolated and visualized, the fragmentation should prove to be measurable and quantifiable. An original study providing proof of the concept of postmortem DNA fragmentation between early and late time periods was conducted using human leukocytes. With an established trend seen in the leukocyte results, this study was then expanded using a porcine animal model, over a longer time period, with more frequent time-points evaluated. DNA degradation in all samples was revealed by SCGE and quantified by the use of DNA-specific quantitative stains, as measured by digital camera affixed to a microscope. The comet 'tail-moment' gave a measure of the proportion of fragmented to non-fragmented DNA, while the 'tail-length' provided the relative size of degraded DNA fragments. In both models, an increase in DNA fragmentation was found to correlate with an increased PMI from 0 to 56 h postmortem, as evaluated by comet-tail-moment and by comet-tail-length, with tail-length providing the strongest statistical correlation, based upon regression analysis. The postmortem DNA fragmentation observed in this study, reveals a sequential, time-dependent process with the potential for use as a predictor of PMI in homicide cases.

Keywords: Forensic science; Forensic pathology; Single-cell gel electrophoresis; Postmortem interval; DNA degradation

1. Introduction

In the investigation of homicide, one of the persistent problems that has long plagued the forensic pathologists, has been the determination of time since death, or the postmortem interval (PMI). Pinpointing the time of death in the immediate PMI is currently imprecise, such that only a wide (8-h) window of estimation is the best that can be obtained [1]. Determining the PMI can contribute to the reconstruction of a crime scene, differentiate between homicide and suicide, pinpoint a suspect, make or break an alibi, and is important for the mourning process of the bereaved relatives of the victim.

Although much is known about cell death as caused by apoptosis and the mechanisms involved [2,3], little is known about the process of nuclear and cellular degradation following organismal death. It has recently been shown by evaluation through image cytometry [4], that following death, nuclear chromatin undergoes specific morphological changes as the time progresses.

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The application of single-cell gel electrophoresis (SCGE), also known as the comet assay, has been well documented for its use as a tool to evaluate the physical characteristics of DNA damage within cells, brought about by various chemotherapeutic and radiation-induced treatments [5–12]. As the chromatin within the nucleus becomes damaged through apoptosis, single- or double-stranded breaks occur in the DNA strands, causing the formation of increasingly smaller fragments. Suspending these cells in an agarose matrix, and running an electric current through them causes the smaller fragments of DNA to travel further through the gel, away from the larger strands of DNA, which remain within the vicinity of the cell nucleus. When stained with a quantitative DNA-specific dye, the result of this electrophoresis is visible microscopically in the shape of a comet. The head of the comet represents the long-stranded DNA nucleus of a single-cell, while the comet-tail is composed of the smaller pieces of fragmented DNA which have electrophoresed down the gel. Smaller pieces travel further, creating a longer tail, while increasingly numerous fragments result in a comet-tail which is more dense. There are two commonly used quantitative measures of DNA damage used in conjunction with the comet assay; tail-length and the tail-moment. The tail-length is a relative measurement of the fragment size, whereby, the smaller the fragment, the further it travels along the gel, while the tail-moment is a calculation based on the proportion of DNA in the tail of the comet relative to the proportion of DNA in the comet head [5,13]. We have applied this technique to the quantification of DNA degradation in two different models, a human blood (leukocyte) model, and a porcine animal model, in the early (0–72 h) PMI.

2. Materials and methods

For sample collection of human leukocytes, whole blood was drawn fresh from a human subject in heparinized tubes. The blood was suspended in freezing solution (15% DMSO, 1.8% w/v NaCl, in Dulbecco's RPMI 1640 (Sigma)). Hypoxia induced by removal of the blood from the body was used to model the effects on white blood cells in the body following death. In vitro at 25 °C, one blood sample was collected after 2 h as an indication of early the PMI effects, while a second sample was collected after 22 h, comprising a late PMI period. All samples were then flash-frozen and stored in liquid nitrogen at −170 °C to prevent any further nuclease activity. Blood samples suspended in freezing solution were thawed in a 37 °C water bath. For electrophoresis, leukocyte density was determined by staining with thionin and counting on a hemacytometer. Approximately 5000–10,000 leukocytes were used per slide electrophoresed.

To further the study, *Sus domesticus*, the domesticated pig, was chosen as an animal model due to its similarity to human body size and basal metabolic rate. Twenty-four specimens of *Sus domesticus*, with an average weight of 85 kg, were obtained freshly sacrificed from an abattoir, and stored in a temperature-controlled environment at 15 °C, where the temperature was continuously monitored using temperature data-loggers. Using standard dissection equipment, 1 cm^3 samples of skeletal muscle, heart, liver and kidney were taken at approximately 8-h intervals, starting at 3 h and continuing until 72 h postmortem (sampling time-points = 3, 16, 24, 32, 40, 48, 56, 64 and 72 h PMI). The samples were placed into 2 ml cryovials containing 1 ml of freezing solution (plus 10% FBS) for 10 min, and then they were flash-frozen and stored in liquid nitrogen. Porcine tissue samples were thawed in a 37 °C water bath and placed into 5 ml test-tubes in 2 ml 1× PBS (Sigma). Using dissecting scissors the sample was sheared for approximately 10 min on ice, forming a homogenous cloudy solution. For muscle tissues, the cells were filtered sequentially through 160 and 100 μm nylon mesh, heart through 160, 100 and 37 μm mesh, and liver and kidney through 160 μm mesh. All samples were treated identically to reduce the effects of any artefactual DNA damage induced by processing.

For electrophoresis and staining, the alkaline comet assay was used to evaluate single-stranded breaks in DNA. Approximately 10,000 cells were suspended in 1% liquid low-temperature agarose in 1× PBS and then pipetted onto a pre-coated microscope slide. The agarose was set at 4 °C for 5 min. The slides were washed in lysis buffer (0.03 M NaOH, 1.2 M NaCl and 0.1 M *N*-lauroylsarcosine) while covered for 1 h and then rinsed in an alkali buffer solution (0.03 M NaOH, 2 mM EDTA) for 1 h, using 3 changes of solution. Using a large (1.5 l) horizontal submarine electrophoresis chamber, the slides were electrophoresed at 0.6 V/cm for 25 min at 40 mA in fresh alkali buffer solution. Slides were then rinsed in distilled water and stained with propidium iodide at 2 μg/ml for 20 min.

The neutral comet assay was used to evaluate double-strand breaks in DNA (modified from Cerda et al. [14]). Approximately 5000 cells were suspended in 200 μl 1% liquid low-temperature agarose in 1× PBS and pipetted onto a microscope slide, covered with a coverslip and chilled at 4 °C for 10 min. The coverslips were removed and the slides were rocked in lysis solution (2.5% SDS in 0.5× TBE) for 30 min. The slides were then rinsed in a conditioning solution of 0.5× TBE for 30 min with 2 changes of solution. Using a large (1.5 l) horizontal submarine electrophoresis chamber, the slides were electrophoresed at 2.0 V/cm for 2 min in fresh 0.5× TBE, rinsed in distilled water for 5 min, and finally silver stained using the BioRad Silver Stain Plus kit as previously described [14].

For comet imaging and analysis, silver stained slides were viewed on a standard light microscope, while propidium iodide stained slides were viewed on a fluorescent microscope, using a 20× objective lens. Comet images were captured using a Pixera digital camera attached to the microscope, and analyzed on computer using NIH Image software with a comet scoring macro. With this software, size and density of the comet head and tail are measured, and

assigned tail-length and tail-moment values accordingly, as previously described [13]. Statistical analysis was conducted by evaluating the mean values of comet-tail-length and comet-tail-moment of 30 randomly chosen comets per sample, experimental replicates were averaged and mean values ± S.D. was calculated for a given time-point.

3. Results

The SCGE of human blood showed an increase in the DNA fragmentation from 2 to 22 h after removal from the body, as shown by the change in comet shape seen in Fig. 1. In samples from the early period (2 h), the comet appears mostly as a single circular shape. This represents the majority of long-stranded DNA remaining in the nucleus of the cell, with very little fragmentation. In samples from the later period (22 h), an increase in the length and density of the comet-tail is seen, representing a decrease in fragment size and an increase in the number of fragmented pieces of DNA. As the DNA becomes increasingly degraded, the fragments travel out of the nucleus and along the gel, carried by the electrical current. At the same time, the nucleus is seen to decrease in size and density, due to the loss of long-stranded DNA by fragmentation. If a quantitative DNA stain such as propidium iodide or silver nitrate is used, the quantity of fragmented DNA that has migrated can be compared to the quantity of long-stranded DNA remaining in the nucleus, as measured by the comet-tail-moment. The double-stranded (neutral) comet assay was also run on these blood samples, which also showed an increase in DNA fragmentation. As expected, when testing for double-strand versus single-strand breakage, the variation between comet sizes produced by the double-strand (neutral) assay for 22 h was much less than that seen in the single-strand (alkaline) assay. This indicates that the single-strand method is a more sensitive method, which should be used when examining low amounts of DNA damage, such as those seen in very early time-points, while for extensive DNA fragmentation (later time-points), the double-strand assay is recommended.

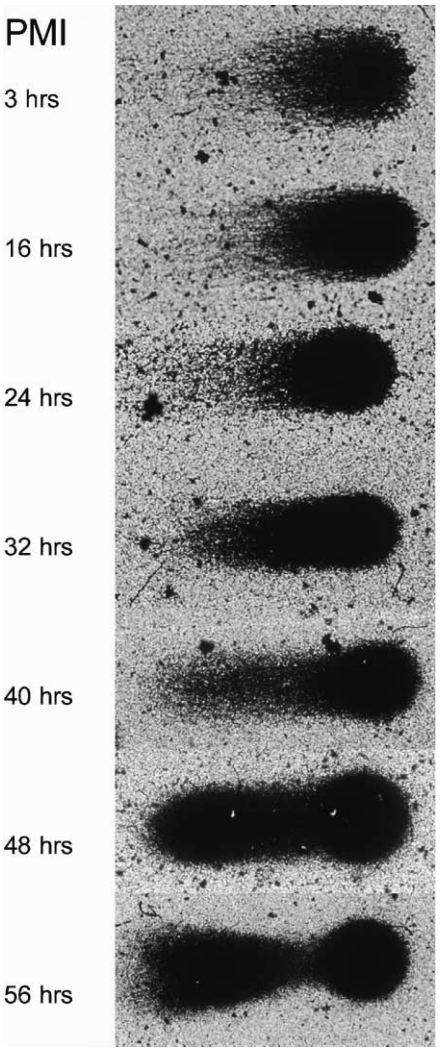

Fig. 2. SCGE of porcine skeletal muscle cells sampled at increasing PMI from 3 to 56 h, showing a pattern of increasing comet-tail formation with time. Slides stained with silver nitrate, original magnification 200×.

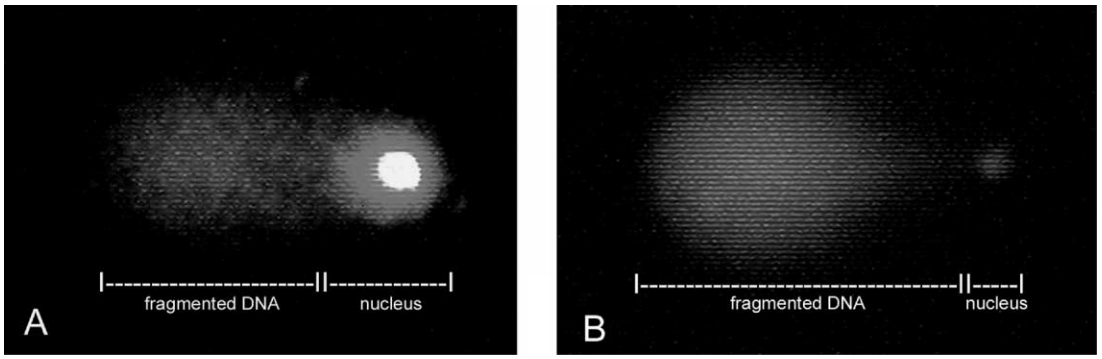

Fig. 1. SCGE of human leukocytes showing an increase in comet-tail size over time after induction of hypoxic conditions. Samples taken at, (A) 2 h after removal from the body; (B) 22 h after removal from the body. Slides stained with propidium iodide, original magnification 200×.

Samples of porcine skeletal muscle tissue taken from 3 to 72 h postmortem were initially evaluated using the alkaline comet assay. The resulting comets formed showed full comet-tails with minimal DNA remaining in the nucleus, even in the earliest samples. This indicated a large amount of single-stranded DNA cleavage occurring early on postmortem. Since a higher level of degradation is required to result in double-stranded DNA breaks, these samples were then evaluated using the neutral double-stranded comet assay in order to assess extensive fragmentation events occurring over a longer PMI. As shown in Fig. 2, the muscle cells show a progressively larger comet-tail-moment, suggesting an increase of DNA fragmentation from 3 to 56 h postmortem. There is a visually evident pattern whereby the comet-tail-length and density are seen to increase with time postmortem. At time-points after 56 h, the comet image does not change significantly, indicating that no further detectable DNA fragmentation occurs. This may represent a maximum level of DNA fragmentation, or it may indicate the sensitivity limit of this particular method. Postmortem porcine liver and kidney tissues were also subjected to both the single- and double-stranded comet assay, however, there was no comet formation visible at any PMI period examined within the first 72 h. This result had been reported earlier [15], and can now be explained as being due to the accelerated nuclear decomposition in these two tissues, caused by inherent enzyme activity. Due to fast-acting internal nucleases, all measurable DNA in the liver and kidney samples had already been thoroughly fragmented by the earliest time-point, to the extent that separate cell nuclei were no longer discernible, therefore the comet assay was found to be not suitable for use on these tissues.

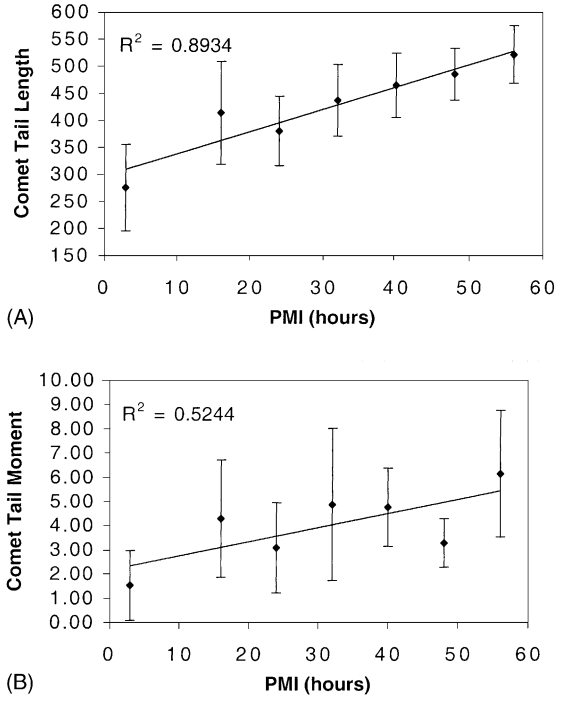

Fig. 3. Regression analysis of DNA fragmentation demonstrated by SCGE of porcine skeletal muscle from 3 to 56 h postmortem. Fragmentation was quantified using, (A) comet-tail-length and (B) comet-tail-moment, relative to PMI. Mean values ± S.D. given.

As demonstrated in Fig. 3, a quantitative analysis of DNA fragmentation related to PMI shows a strong correlation between increased fragmentation and increasing time since death. Both comet-tail-length and comet-tail-moment show an approximate linear relationship, which increases with PMI. Regression analysis shows that comet-tail-length provides a stronger correlation ($R^2 = 0.8934$) than comet-tail-moment ($R^2 = 0.5244$). For the purpose of this experiment, the comet-tail-length, which is determined by the smallest size of DNA fragments created, appears to provide a better indicator of PMI than the comet-tail-moment, which is determined by the ratio of short fragmented DNA to long, non-migrated DNA. At time-points after 56 h, the DNA has generally degraded to a state which cannot be recognized as a 'comet' and therefore does not provide a quantifiable image.

4. Discussion and conclusions

These results indicate that there is indeed a process whereby nuclear DNA is fragmented following death, which is organ and time-dependent. In tissues such as kidney and liver, where enzymes tend to be more active and putrefaction occurs early, neither comet formation nor intact nuclei were observed, suggesting that the DNA within these tissue cells may have already been fully degraded by the first sampling time-point. Proof of concept of PMI DNA fragmentation was demonstrated first in blood and then applied to porcine skeletal muscle. It was shown that fragmentation of nuclear DNA increased with PMI in the 3–56 h postmortem period. In particular, much of this degradation took place early, in the 3–24 h postmortem period. Since the largest change in comet-tail-length/comet-tail-moment takes place by 24 h postmortem, it is possible that this period represents a faster rate of DNA degradation than later time-points. Future experiments with shorter sampling intervals should reveal whether there is a single event, early on, to account for this large change, or whether this is brought about by a generally increased degradation rate which is found over the first 24 h postmortem.

This method should be further pursued to determine whether this DNA fragmentation pattern holds true for all body tissues, at varying temperatures, and also over shorter sampling intervals, particularly in the 0–24 h postmortem period.

It is widely known that, a number of factors can influence the rate of decomposition of a body [1]. Foremost, increases in temperature have been shown to increase decomposition. It would be expected that an increase in temperature would

cause an increase in the rate of reaction which causes the DNA to degrade within the cells postmortem. While 15 °C was chosen for these experiments in order to approximate a room-temperature environment, further experiments in a variety of other environmental conditions (indoor, outdoor, warmer, cooler) must be undertaken to determine the relative stability of this method for practical use.

While the results of these experiments show a potential for use as a future method of estimating time since death, further studies must be conducted to determine the accuracy and precision which can be achieved. Initially, much shorter intervals should be examined, and then a positive-predictive analysis must be done whereby the experimentally determined PMI must be compared to actual PMI.

It is suggested that this genomic DNA fragmentation may be caused by some process other than apoptosis, as the fragmentation continues well after energy in the form of ATP ceases to be made available to the body. The apparently sequential fragmentation seen here also contradicts the currently understood process of necrotic cell degradation, which is believed to be a random fragmentation process [3]. Further steps should be taken to examine whether apoptosis or necrosis is the cause of the postmortem DNA fragmentation. If both of these pathways can be ruled out, then a novel subcellular destruction pathway may be at work.

These results suggest that internal nucleases do act upon a body following death, which contributes to the progressive fragmentation of the nuclear DNA in the early postmortem period. This fragmentation can be quantified and appears to be a time-dependent process, which has the potential for use as a predictor of PMI in the field of forensic pathology.

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