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SHORT REPORT Open Access

An introduction to the single cell gel electrophoresis assay: a technique resolving issues in forensic science

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Abstract

Time since death (TSD) estimation is a crucial issue in death investigation. In majority of homicide/suicide cases deceased body recovered within the first 48 h, therefore, it is critically important to determine the time of death quickly and precisely. In the present scenario, TSD estimation still remains difficult even for experienced pathologists undertaken with extreme caution. After death numerous factors may be of cadaveric or environmental origin can influence the 'normal' rate of postmortem changes. Therefore, it could be more difficult to estimate longer TSD. However, these environmental influencing factors can also effect on DNA in the form of degradation, which start after 1–2 h of death. This DNA degradation could be work as molecular clock which help to estimate early TSD. Single-cell gel electrophoresis (SCGE), also known as the Comet assay, is the technique by which DNA degradation can be visualized and measured qualitatively as well as quantitatively. Using this assay not only early TSD estimated but many other challenges such as time since deposition of biological fluids, repair of genetic material from degraded biological sample could also be resolved. With the help of this paper an attempt was made to introduce a well-known cytogenetic technique that is SCGE assay, which could be a versatile technique for forensic science.

Keywords: Single cell gel electrophoresis, Comet assay, DNA degradation, Postmortem interval, Forensic science, Time since death

Introduction

In death investigation, accurate time since death (TSD) estimation is a crucial task (Haglund 2002). Physical and biochemical changes which occur in the body after death can be helpful together to estimate the time of death (Donaldson et al. 2013). In the present scenario, various techniques have been used for estimating TSD. But precise estimation of TSD becomes difficult in the presence of numerous influencing factors (cadaveric or environmental origin) which can influence the 'normal' rate of postmortem changes, simultaneously. Generally, TSD can be estimated by two different methods (Pounder 1995). First one is Concurrence-based methodology. This method is based on the occurrence of a known event, which took place at a known time, with the occurrence of death, which took place at an unknown time. For example determining the age of manufacturing of clothing found on a body, tree ring development, dates on personal effects, etc. Concurrence based methods rely on both evidence associated with the body and anamnestic evidence such as the deceased's normal pattern of movements (Pounder 1995).

The second technique is rate of change methodology. This method is to measure some aspect of evidence, directly associated with the body, which changes at a known or predictable rate and is started or stopped at the time of death. This method includes body temperature, tissue decomposition, insect succession and bone weathering (Pounder 1995).

By using the rate-of-change methodology, early TSD can be estimated on the basis of observational changes occur in human body after the death. The cooling of the body after death (*algor mortis*), the gradual stiffening of the body (*rigor mortis*) and the fixed pooling of the blood resulting in discoloration of the lower portions of the body (*livor mortis*) can be easily assessed with minimal instrumentation (Clark et al. 1997).

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These Postmortem changes can roughly estimate the TSD but are unable to provide accurate estimation. Furthermore, these postmortem changes are very complex and influenced by the different internal and external factors (Swift 2006).

All of the above mentioned methods are effective but are insufficient to achieve the precise conclusion regarding to the TSD estimation. Instead of independently, these observational changes can collectively predict well, but all these changes are influenced by cadaveric or environmental origin, so it may vary from case to case and person to person. Therefore, accurate TSD estimation is not possible with these methods. Thus more accurate and reliable method for TSD estimation is urgently needed.

In spite of the tremendous advancement in forensics and life sciences over the past few decades, especially through DNA analysis, TSD estimation is still lacking behind. DNA is a genetic material and present inside a nucleus of the cell, therefore external and internal factors would not be able to affect them easily. Hence with the help of DNA degradation analysis we could reach to the more accurate estimation of the TSD.

Previous studies have shown that DNA degradation and repair is mutual interchangeable biological processes occurring in the cells of living human bodies (Reddy 2009; Clancy 2008). When any xenobiotic substance gets internalized into the cellular system, it may interact with genetic material such as DNA. When they interact with DNA, conformational changes, single or double strand breaks, formation of alkali labile sites may occur (Clancy 2008). When these changes are occurring in DNA, repair mechanism starts functioning immediately which is mainly regulating cell division process. These cell division processes stops in the cells, after death, then DNA repair pathway are impeded and DNA degradation process becomes un-repairable (Clancy 2008). In this way, DNA degradation increase with prolongation of time of death and thus, this could be a good molecular clock for TSD estimation. In this order, Xiong et al. (2010) have quantified the rate of DNA degradation in tissue cells with the prolongation of postmortem interval (PMI). This study revealed a positive linear correlation between the rate of DNA degradation and postmortem interval (Xiong et al. 2010). In addition, forensic researchers have used a variety of techniques in this concern with different tissues as sources of samples which demonstrated the positive correlation between DNA degradation and postmortem interval (Xiong et al. 2010; Lin et al. 2000; Chen et al. 2005; Luo et al. 2006; Zhen et al. 2006; Hao et al. 2007; Gomaa et al. 2013; El-Harouny et al. 2008). In this order, forensic scientists have made an attempt to use flow cytometry technique for estimating postmortem interval through the assessment of DNA degradation (Williams et al. 2015; Cordeiro et al. 2015). Additionally, they have found good correlations between TSD and DNA degradation measured using flow cytometry. But limitations to the procedure as it was originally proposed have proven difficult to overcome for forensic samples. Flow cytometry requires a suspension for staining and analysis. This could make scientists difficult to analyze solid tissue without extensive manipulation. Additionally, this assay measures total DNA but cannot be able to differentiate between intact human DNA and bacterial or fungal DNA. Several modifications to flow cytometry have been made due to which it become more specific and sensitive (Goddard et al. 2006), but the approaches have not extended the general acceptance. Therefore, this technique is not commonly used for forensic purposes. Another point is, this instrument is not easily available, due to high cost. For the forensic purposes we need a low cost, rapid, reliable, easily available and accessible technique that can be performed easily in the laboratory. At this instant, single cell gel electrophoresis (SCGE) or the Comet assay is the most appropriate method for TSD estimation via DNA damage assessment.

This technique was first introduced by Ostling and Johnson (1984), using a microgel electrophoresis technique to quantify DNA damage in cells. This technique is also known as "single-cell gel electrophoresis" which is famous by the name of "Comet assay" due to comet like appearance of degraded DNA in result (Fig. 1). They have used this technique in the neutral conditions that only allow the detection of double-stranded DNA breaks. Later on, NP Singh et al. (1998) have modified this technique as to assess the DNA damage in alkaline condition. The single cell gel electrophoresis (SCGE)/Comet assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross-linking with the single cell approach typical of cytogenetic assays. This is a simple, inexpensive, and sensitive technique to test for DNA damage.

Furthermore, Miteva et al. (2009) exhibited the forensic application of SCGE technique in sexual assault cases. This study revealed that SCGE help to measure the kinetics of sperm DNA degradation which help to estimate the time since deposition of sperm or time of ejaculation of sperm. With the help of this study, Miteva et al. (2009) elucidated that SCGE could be a valuable technique which may aid to the forensic investigation especially in the sexual assault cases. This study accurately measured the time since deposition/ejaculation of sperm qualitatively and quantitatively both.

Therefore, with the help of this paper an attempt was made to introduce SCGE assay, with its descriptive methodology and its role in the field of Forensic Science and also discuss about its future perspective.

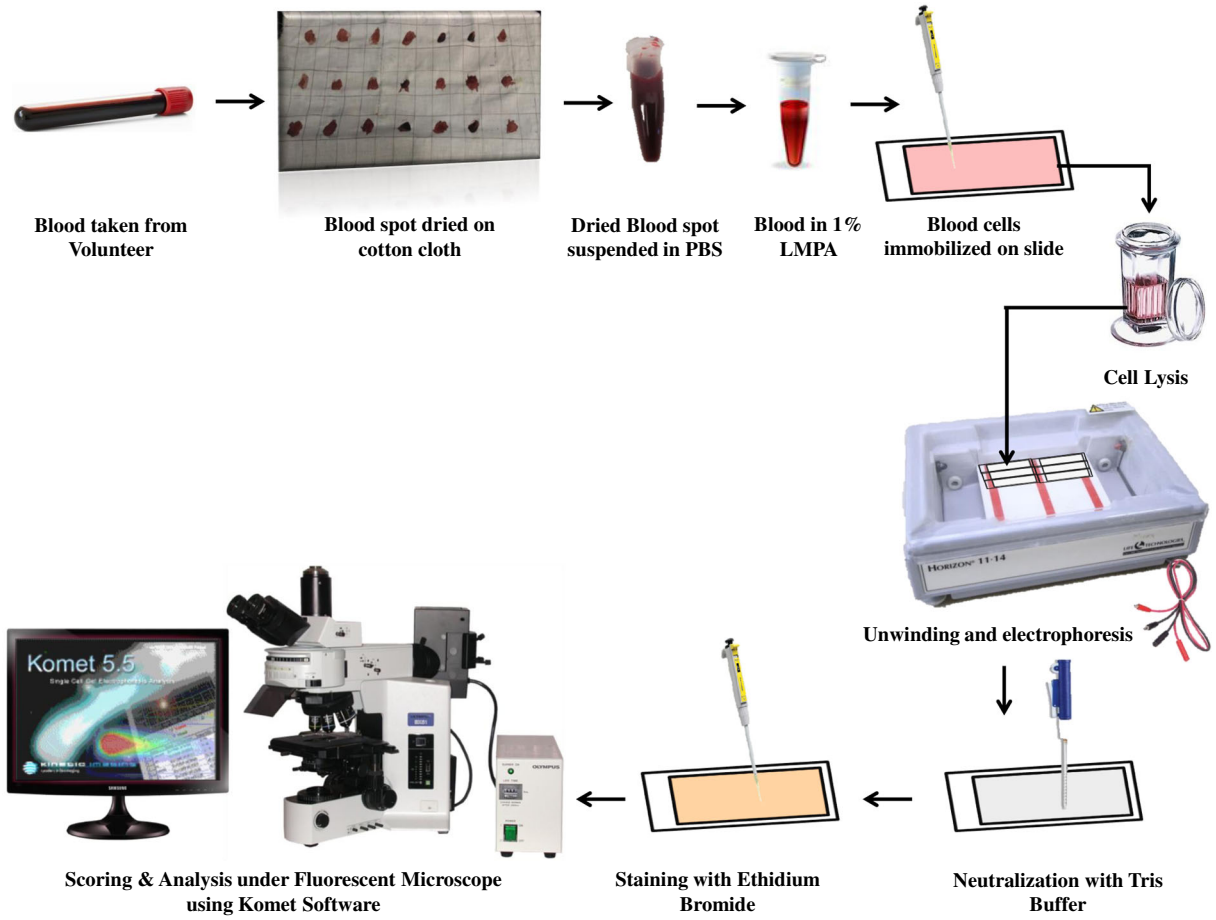

Fig. 1 Schematic representation of Comet Assay Procedure

Methodology of single cell gel electrophoresis (SCGE) assay

Preparation of Agarose gel

Generally, two different type of agarose are used in Single cell gel electrophoresis assay for blood and tissue/organ samples.

1) Low Melting Agarose (LMA) - 1% LMA is mixed into the Phosphate buffered saline (PBS) and heat until the agarose dissolve in PBS. LMA stored at 4 °C until required. When needed, briefly melt agarose in microwave and kept on dry/water bath maintained at 37 °C (Shukla et al. 2011).
2) Normal melting agarose (NMA) - 1% NMA is mixed into the distilled water and heat until the agarose dissolves in distilled water. It always freshly prepared and kept on dry bath at 60 °C during the preparation of base slides (Shukla et al. 2011).

For semen sample, two different types of high resolution agarose gel are prepared which can be used for SCGE assay

1) 0.7% Agarose- 70 mg of 3:1 high resolution agarose mixed with 9 ml of distilled water in a 100 ml glass beaker and microwaved until clear solution formed. Then add 1 ml of PBS in this clear solution and microwave the solution again. Keep this solution on dry bath and maintain the temperature at 55 °C for the experiment (Simon et al. 2013).
2) 1% Agarose- 1% agarose is also prepared with similar manner as preparation of 0.7% Agarose and finally kept on dry bath at 55 °C for the experiment (Simon et al. 2013).

Preparation of base slide

For blood and tissue/organ sample

End frosted microscopic slides are used for the SCGE assay. Slides are first dipped in methanol and burnt over a blue flame to remove machine oil and dust. Thereafter, slides are vertically dipped in 1% NMA gel up to two-third of the frosted area (the frosted end prevents the gel from slipping off the slide). Wipe the underside of slide to remove agarose and lay the slide in a tray or on a flat surface to dry. The slides may be air dried or

warmed at 50 °C for quicker drying. The slides are stored in a dry slide box at room temperature until needed, and are generally prepared the day before use. (NOTE: Slides should be labeled at the frosted end with pencil before storage to avoid confusion of the side with/without gel) (Bajpayee et al. 2005; Shukla et al. 2014).

For semen sample

In case of semen sample, slide is pre-coated with 0.7% agarose instead of NMA. Thereafter, a microgel layer of agarose is prepared prior to put cells on the 0.7% agarose pre-coated slide. In this step, 200 μl of 1% agarose is placed on the pre-coated slide and covered with cover slip. Agarose is allowed to solidify at room temperature for 5 min (or 1 min, if on ice). Before adding cell suspension onto the slide, cover slip is gently removed (Simon et al. 2013).

Sample preparation

Blood- 50 μl of blood obtained from the deceased body/crime scene to perform this assay. This sample is first diluted with 50 μl PBS and then mixed with equal volume of 1% LMA and proceed for the experiment (Bajpayee et al. 2005).

Sperm- Spermatozoa cells are extracted from dried cloths/crime scene by soaking for 5 min at 37 °C and then subsequent washings with 200 μl PBS. Thereafter, spermatozoa cells are collected through centrifuged and re-suspended in 1 ml PBS. This suspension is then mixed with 0.7% agarose and proceeds for the experiment (Simon et al. 2013).

Solid Organs/tissues- A small piece of blood rich organ (eg Spleen, liver) placed in freshly prepared chilled mincing solution (PBS, with 20 mM EDTA and 10% DMSO) and chopped into pieces with a pair of scissors. The pieces are allowed to settle and the supernatant containing the single cells is mixed with 1% LMA and preceded further for the experiment (Shukla et al. 2016).

Slide preparation

For blood and tissue/organ sample

Slide preparation is done according to a modified method of Tice et al. (2000). Cells embedded in Low melting agarose (LMA) are layered on normal melting agarose (NMA) pre-coated base slides. A cover-slip is placed on the slide to ensure that the gel is evenly spread, and the slide is kept on ice to allow the gel to solidify. A third layer of 90 μl LMA (0.5%) is added and again allowed to solidify on ice. Duplicate slides are prepared for each sample (Bajpayee et al. 2005).

For semen sample

Cell embedded in the agarose (50 μl of 0.7% agarose is mixed with 0.5 μl of fresh semen) first layered onto the pre-coated slide and covered with cover slip. The slides are left at room temperature to solidify the gel for 5 min (or 1 min, if on ice), then cover slip is gently removed and 200 μl of 0.7% agarose is added on each slide and covered with cover slip. Again the slide is kept on ice to solidify the gel and the cover slip is finally removed to proceed for lysis (Simon et al. 2013).

Lysis

This step helps to lyse the cells using lysing solution with detergent. Presence of high salt in lysing solution helps to remove cell membranes, bulk of proteins, cytoplasm and nucleoplasm. It further disrupts nucleosomes and almost all histones being solubilized by the high salt. Furthermore, it form nucleoids consisting of a nuclear matrix composed of ribonucleic acid (RNA), proteins and containing negatively charged super coiled loops of DNA (Collins 2004; Shukla et al. 2011). In this step cover-slips are removed and the slides immersed in a freshly prepared and chilled lysis solution (146.1 g NaCl, 37.2 g EDTA, 1.2 g Tris, pH 10) with 1% Triton X-100 and 10% Dimethyl sulfoxide (DMSO) added just before use. The slides are kept in lysis solution overnight at 4 °C (Bajpayee et al. 2005; Shukla et al. 2014).

NOTE: The purpose of the DMSO in the lysing solution is to scavenge radicals generated by the iron released from hemoglobin when blood or tissues are used.

In case of semen sample, 0.5 mg/ml of proteinase K (PK needs to be incubated at 37 °C to inactivate DNAse impurities which may induce additional DNA damage) and 2 mg/ml reduced glutathione (antioxidant) should be added into the fresh to pre-warmed (37 °C) lysing solution. In case of semen sample despite of Triton X 100, Sodium lauryl sulphate (SLS) can be used as detergent (Simon et al. 2013).

DNA unwinding

With the help of this step, any breaks present in the DNA cause the super coiling to relax locally and loops of DNA are then free. This step proceeds after lysis of the cells embedded on base slide. In this step, the slides are placed first in a horizontal gel electrophoresis tank and then tank pour with fresh and chilled electrophoresis solution (1 mM EDTA and 300 mM NaOH, pH > 13). The slides are left in this solution for DNA unwinding for 25 min.

Electrophoresis

In case of blood and tissue samples, electrophoresis is performed at high pH (> 13) which facilitates denaturation of DNA (because of the disruption of hydrogen bonds between double-stranded DNA) and expression of alkali labile sites as frank breaks. The electrophoresis would pull the damaged DNA towards the anode thus

making the distinct 'tail' of the comet. Electrophoresis is conducted at 4 °C with constant voltage 0.7 V/cm for blood (Bajpayee et al. 2005) and organs/tissue for 30 min. All steps are performed under dimmed light (Shukla et al. 2014).

In case of semen sample electrophoresis is performed at pH 10 and electrophoresis solution contain 0.5 M NaCl, 1 mM EDTA, 0.1 M tris base and 0.2% DMSO (Simon et al. 2013).

Neutralization and staining

After electrophoresis, the excess alkali is neutralized with Tris buffer (0.4 M, pH 7.5). When neutralization step occur, extruded DNA (single and double strand breaks) which are migrate towards anode, formed hair pin loop like structure. And when it stained with DNA binding dye, dye molecules are intercalate in between loop of extruded DNA and become visualized under fluorescent microscope. In the staining, slides are stained with any one of the DNA binding dye (Ethidium Bromide, Propidium Iodide, DAPI or YOYO) and stored in a humidified slide box until scored (Paul and Bhattacharya 2012). Ethidium Bromide is most commonly used DNA binding dye in the Comet assay.

Scoring of slides

Slides are scored using an image analysis system (Andor Technology Belfast, U.K.) attached to a microscope equipped with fluorescence attachment and appropriate filter (N2.1 excitation wavelength 515–560 nm - emission wavelength 590 nm). The microscope is connected to a computer through a charge coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. Generally, 100 randomly selected cells are analyzed per sample. The comet parameters recorded are Olive tail moment (OTM, arbitrary units), tail DNA (%) and tail length (migration of the DNA from the nucleus, μm; TL) as shown in Fig. 2.

These three parameters of SCGE assay are defined as:

Tail Moment: The product of the tail length and % of total DNA present in the tail of migrated DNA is known as Tail moment.

Tail Moment = Tail Length x % DNA Tail

Tail Length: Tail Length is defined as the length of DNA migration from the body of the nuclear core. It is used to assess the degree of DNA damage.

% Tail DNA: % Tail DNA is the fraction of total DNA present in tail of migrated DNA.

Advantages

1. The SCGE assay is a rapid, sensitive and inexpensive method for measuring DNA strand breaks.
2. This method is applicable to both qualitative as well as quantitative analysis.
3. Very less amount of sample is required for processing.
4. Individual cells give their individual result.

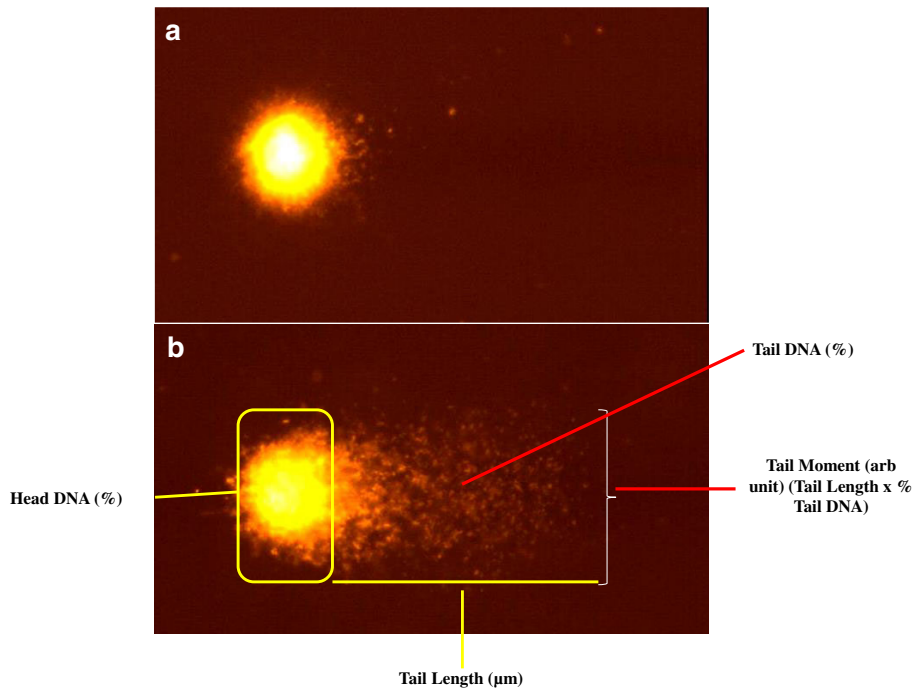

Fig. 2 Photomicrograph of single cell (**a**) Intact DNA; (**b**) Degraded DNA showing Comet parameters for DNA degradation assessment

5. Accuracy of this method is especially significant as there is no much machinery involved in the procedure.
6. Minimal training is required for researcher/expert to conduct this assay and the equipments for the assay are cheap and easy available.

Limitation

Despite great advantages, some limitations are also associated with SCGE assay.

1. The SCGE assay is not able to detect small DNA fragments (smaller than 50 kb) since they are mostly washed during the lysis and electrophoresis.
2. This assay also fails to detect damage associated with mitochondrial DNA as they are also too small.
3. Standardization of this assay is a challenging task. This method exhibits excessive inter-laboratory variability due to a lack of consistent protocols and analysis techniques.
4. This method depends upon the sample condition, processing and temperature throughout the process (should be 4 °C).

Role of SCGE in forensic science

Existing literatures have demonstrated that Single Cell Gel Electrophoresis assay play an important role in estimation of time of sperm ejaculation in rape and other sexual assaults cases by analyzing degradation of DNA content in sperm cells (Miteva et al. 2009). In this study, results revealed that DNA degradation rate was increased with time since deposition of stain. In addition, some studies have reported that SCGE is applicable to evaluate postmortem cell death processes, specifically nuclear DNA fragmentation (Xiong et al. 2010; Lin et al. 2000; Chen et al. 2005; Luo et al. 2006; Zhen et al. 2006; Hao et al. 2007; Gomaa et al. 2013; El-Harouny et al. 2008; Johnson and Ferris 2002). Among these studies, Johnson and Ferris (2002) shown that upon the death of an organism, internal nucleases contained within the cells caused degradation of chromosomal DNA into increasingly smaller fragments over time, and when these fragments were isolated and visualized, the fragmentation remarkably proved to be measurable and quantifiable of postmortem interval (Johnson and Ferris 2002). Moreover, some of these studies exhibited the strong statistical correlation between Comet assay parameters and postmortem interval/time since death estimation (Lin et al. 2000; Chen et al. 2005; Luo et al. 2006; Zhen et al. 2006; Hao et al. 2007; Gomaa et al. 2013; El-Harouny et al. 2008). Among these studies, Chen et al. (2005) reported that DNA degradation from heart, liver and kidney could be a hallmark for early time since death estimation. Rate of DNA degradation in first 6 h after death exhibited a linear correlation with postmortem interval. Similar findings also observed by Luo et al. (2006) and Zhen et al. (2006) who stated that tail length of DNA (indication of DNA degradation) increased with prolongation of PMI. In addition, Hao et al. (2007), El- Harouny et al. (2008) and Gomaa et al. (2013) have also demonstrated that the Comet assay is an appropriate method for early PMI estimation as evident by comet assay parameters (OTM, tail length). Apart from applying in PMI estimation and time since deposition estimation, this assay can also be used for environmental degradation assessment of biological sample & its repair (Lehmann 2003; Hall et al. 2004; Hall et al. 2014; Nelson et al. 2009). Some of these studies have successfully developed DNA repair systems (direct reversal by photolyase and single strand break/gap repair) which help to repair the DNA of degraded samples for certain extent (Lehmann 2003; Hall et al. 2004; Hall et al. 2014). In addition, instead of developing new DNA repair system, some of the studies have used commercially available repair enzyme (fpg, Endo III) to resolve the issue of degradation of biological sample (Nelson et al. 2009). In this case, advantage of Comet assay is that DNA repair enzyme can be incorporated in the procedure easily just before the electrophoresis step. In this way, Comet assay not only helps to measure DNA damage but DNA repair can also be measured.

Conclusion and future perspective

Last two decade SCGE assay has been developed as an internationally recognized method to detect DNA damage or degradation in various animals and human cell types. Although it is believed that, the SCGE assay is still growing in use and has high potential for clinical, Toxicological and cancer research, yet in forensic science it is still new and latest.

The above mentioned studies (Miteva et al. 2009; Johnson and Ferris 2002) have successfully demonstrated that SCGE could play an important role in the field of Forensic Medicine but not frequently used. Apart from time since death estimation, SCGE can also be useful to repair slightly degraded biological samples, estimation of time since deposition of blood, semen, saliva, assessment of adverse effect of hazardous chemical on human (toxicovigilance) and biological samples.

In conclusion, from this article an attempt was made to open a new research avenue and awareness to estimate early post mortem interval using SCGE assay. Although SCGE assay has its some advantages and disadvantages, yet some modifications made this assay useful, reliable and consistent for variety of forensic applications.

In the forensic point of view author suggested that SCGE assay is a versatile technique which could be

helpful to resolve issues like DNA degradation, time since death estimation, genotoxicity assessment of hazardous chemicals on biological sample. Introducing SCGE in forensic science will open a new avenue in the field of forensic research & innovation.

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