The relationship between the postmortem interval and the DNA degradation in brain and liver of adult albino rats

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Abstract: Accurate estimation of postmortem interval (PMI) is one of the most important and difficult issues in forensic medicine. After death, the tissues undergo autolysis and biomacromolecules degrade. With the development of molecular biological techniques, DNA quantification methods were applied in estimating PMI. This study aimed to evaluate the time dependant changes of DNA content in rat's brain and liver cells by single cell gel electrophoresis (comet assay) in order to find out an objective and quantitative standard for the estimation of postmortem interval. Thirty six adult male albino rats were used. The rats were sacrificed by cervical dislocation, then divided into 6 groups; first group rats were dissected to obtain organs (brain and liver) immediately after death while the 2nd, 3rd, 4th, 5th and 6th groups were dissected to get liver and brain at 3, 6, 9, 12, 24 hours postmortem respectively. Single cell gel electrophoresis (SCGE) comet assay, was carried out on brain and liver tissue samples to detect the nuclear DNA degradation. The results revealed that, frequency of comet-like cells, the percentage of tail DNA, tail length, tail moment and olive tail moment increased in brain and liver tissues with increasing postmortem interval. In contrast, the head radius, the percentage of head DNA showed a decreasing trend. A high correlation between these parameters and the postmortem interval were statistically detected. These findings suggest that the single cell gel electrophoresis assay is a quick and sensitive method to detect DNA degradation in brain and liver cells, providing an objective and accurate new way to estimate postmortem interval**.**
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1. Introduction

Estimation of the time of death (TOD) is an important task of the forensic pathologist performing the body examination at a death scene. A precise estimation of the TOD enables the verification of witnesses' statements, limits the number of suspects and assess their alibis. Sometimes, estimation of the TOD also has relevance regarding civil law, since it may influence the order of inheritance or possible commitments resulting from the order of deaths (Kaliszan, 2012). Postmortem interval (PMI) is the time elapsed between death of a person and the time of autopsy. Though the exact time of death can rarely be estimated on the basis of autopsy findings alone, an appropriate range of PMI can be deduced by intelligent interpretation of various changes that take place after death (Chandrakanth et al., 2013). Following death, a complex series of biochemical and pathological processes are initiated resulting in a considerable alteration of the structure and composition of the human body. Because many of these changes occur sequentially, it has been proposed that the evaluation of the types and degrees of changes may enable estimation of time since death (Ferreira and Cunha, 2013) The main principle of the postmortem interval determination is a calculation of a measurable date along a time-dependent curve back to the starting point . In studies, the estimation of postmortem intervals is evaluated as a time-based analysis of a parameter, such as hypostasis, rigor mortis, body cooling, electrical excitability of muscle, metabolite concentrations, enzyme activity, immunological reactivity and morphological changes (Kurtulus et al., 2012& Poposka et al., 2011& Smart and Kaliszan 2012) Most of these methods for the estimation of PMI remain relatively inaccurate, and even when applied to the very early post mortem period are of limited practical relevance (Sieger et al., 2000, Prahlow, 2010). Moreover, there are considerable biological variations in individual cases, therefore, the exact time of death cannot be fixed by any method, but only an approximate range of time of death can be given (Abdel Salam et al., 2012). DNA degradation in biological samples starts rapidly after death with fragmentation of DNA caused by endogenous nuclease activity and hydrolytic attack. The persistence of DNA in soft muscle tissue has been shown to correlate with accumulated degree-days, which is the cumulative total of the average daily temperatures (Nazir et al., 2011). During the past decades, advances in forensic molecular biology have led to great success in DNA technology for the

identification of trace evidence in criminalistics. Recently, the application of DNA analyses has been expanded into forensic pathology (Maeda et al., 2013). With advances in molecular biology time-dependent degradation of nucleic acids became a focus of attention because autopsy samples were now used for both DNA and RNA analysis in clinical medicine as well as in forensic science (Ohshima and Sato, 1998). Forensic DNA analysis has advanced considerably over the past 20 years through the development of new techniques, such as the use of miniSTR analysis, which involves amplification of smaller DNA targets (Tate et al., 2012). 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 (Larkin, et al., 2010 ; Lin, et al., 2011). DNA degradation is caused by intracellular enzymes and bacterial proliferation, then its estimation may lead to a diagnosis of the degree of postmortem changes, including the time after death (Swango et al., 2006). Since DNA is known to be stable a long postmortem period, methods for quantification of DNA degradation level, such as flow cytometry or single cell electrophoresis, were described by several authors (Perry et al.,1988 ; Johnson and Ferris, 2002). Single cell gel electrophoresis (SCGE), sometimes referred to as a comet assay, uses migration of DNA from cells encapsulated in agarose to measure the level of DNA fragmentation. A tissue sample is encapsulated in agarose and the DNA within the tissue is denatured. The DNA is electrophoresed through the encapsulating agarose and samples with degraded DNA generate smeared tails (hence the name comet assay). The stronger the signal from the tail, the more DNA damage present. Tail length is directly influenced by the size of the DNA fragments with more degraded DNA generating longer tails. The tail moment is the product of the fraction of DNA in the tail and the tail length and is related to the proportion of non-fragmented to fragmented DNA. (Alaeddini et al., 2010).

Aim of the work

This study aimed to evaluate the time dependant changes of DNA content in rat's brain and liver cells by single cell gel electrophoresis (comet assay) in order to find out an objective and quantitative standard for the estimation of postmortem interval.

2. Material and Methods

Animals:

Thirty six adult male albino rats were used each about (150-200 grams). They were obtained from animal house of Faculty of Medicine, Zagazig University.

Experimental design:

The rats were anesthetized by diethylether inhalation, followed by cervical dislocation ,then ,rats were classified into 6 groups; first group rats were dissected to obtain organs (brain and liver) immediately after death while the 2nd, 3rd, 4th, 5th and 6th groups were dissected to get liver and brain at 3, 6, 9, 12, 24 hours postmortem respectively. DNA of rats' organs were detected by single cell gel electrophoresis (The comet assay).

Method of Comet assay:

The comet assay was performed in concordance with the premises established by **Singh *et al*., (1988).** The liver and brain were minced, suspended at a concentration of 1 gr ml in chilled homogenizing solution, centrifuged at 700=g for 10 min at 8^0C, the precipitate was re-suspended in chilled homogenizing buffer at 1 g organ weight per ml. Subsequently 600 ll of Low Melting Agarose (0.8% in PBS) was added to the cell suspension (100 ll). Then 100 ll of this mixture was transferred to agarose pre-coated slides. The coated slides were immersed in lyses buffer(0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining was done by ethidium bromide (20 lg/ml at 4 _C, Sigma). The observation was with the samples still humid, the DNA fragment migration patterns of 50 cells for each organ evaluated with a fluorescence microscope (with excitation filter 420–490 nm [issue 510 nm]). The comets tails lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of ethidium bromide-stained DNA using a 40x objective on a fluorescent microscope using public domain software for image analysis based on comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera. DNA damage was measured as tail length (TL=distance of DNA migration from the center of the body of the nuclear core), and tail intensity DNA (TI =% of genomic DNA that migrated during the electrophoresis from the nuclear core to the tail). By presenting all 3 parameters together, more information on the extent of the DNA damage can be obtained.

Statistical analysis*:*

SPSS Software program was used. Mean values (M) ± Standard Deviation (SD) were calculated,. Pearson correlation (r) was used for testing the association between two continuous variables. The significance level is considered at P value < 0.05

3. Results:

The single cell gel electrophoresis (SCGE) assay showed that, at an early stage after death (zero time), the majority of cells in brain and liver tissue displayed a complete head, spherical in shape, and the comet trail was minimal. Within the following 24 hours, the length (µm) and concentration (%) of the tail gradually increased .The brain and liver cells showed increased DNA degradation rate (frequency of comet-like cells), comet tail-length, percentage of tail DNA, tail area, comet tail-moment and increased in parallel with increasing postmortem interval, while a reduction in head radius, percentage of head DNA and head area (nuclear DNA parameter Tables 1-2). Fragmentation from 3 to 24 hours in brain and liver samples after removal from the body, are shown by the change in comet shape seen in figures 3, As demonstrated in Figs.1,2 a quantitative analysis of DNA fragmentation related to Postmortem interval 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 frequency of comet-like cells, tail DNA % , comet-tail-length, comet-tail-moment provide a stronger correlation with PMI in brain and liver samples than head DNA %, head radius (µm),olive tail moment (Table3). The comet-tail-length, which is determined by the smallest size of DNA fragments created, appears to provide the best indicator of PMI. Brain DNA showed slower degradation than liver DNA.

Table 1: Changes in nuclear DNA parameters in rat brain tissue at different postmortem interval.

Postmortem interval (h)	Head DNA %	Tail DNA %	Head radius (µm)	Tail length (µm)	Tail moment	Olive tail moment
0	99.5	0.5	23.98	0	0	0
3	98.3	1.7	21.55	0.75	0.04	0.02
6	88.7	11.3	20.7	2.2	3.2	2.9
9	73.2	26.8	15.8	4.1	6.2	5.6
12	62.4	37.6	13.0	13.5	4.5	3.3
24	40.0	60.0	10.9	28.4	12.2	11.9

Head DNA (%): the percentage of DNA content in the comet head;
Tail DNA (%): the percentage of DNA content in the comet tail.

Table 2: Changes in nuclear DNA parameters in rat liver tissue at different postmortem interval.

Postmortem interval (h)	Head DNA %	Tail DNA %	Head radius (µm)	Tail length (µm)	Tail moment	Olive tail moment
0	99.22	0.78	26.3	0	0	0
3	94.31	5.69	23.2	0.9	0.09	0.07
6	89.9	10.1	21.1	3.1	1.2	1.13
9	78.4	21.6	17.8	6.3	2.7	3.2
12	61.9	38.1	16.0	18.8	4.2	6.0
24	36.0	64.00	12.9	33.4	13.5	16.9

Table 3: Regression analysis of nuclear DNA parameters in brain and liver samples of rats at different postmortem interval.

	Brain		Liver	
Comet parameter	r	*p*	r	*p*
-Frequency of comet-like cells	**0.926**	< 0.001	**0.932**	< 0.001
-Head DNA %	**0.817**	0.0004	**0.869**	0.0601
-Tail DNA %	**0.905**	< 0.001	**0.925**	< 0.001
-Head radius (µm)	**0.819**	0.032	**0.768**	0.0501
-Tail length (µm)	**0.918**	< 0.001	**0.952**	< 0.001
-Tail moment	**0.927**	< 0.001	**0.951**	< 0.001
-Olive tail moment	**0.800**	0.016	**0.587**	0.021

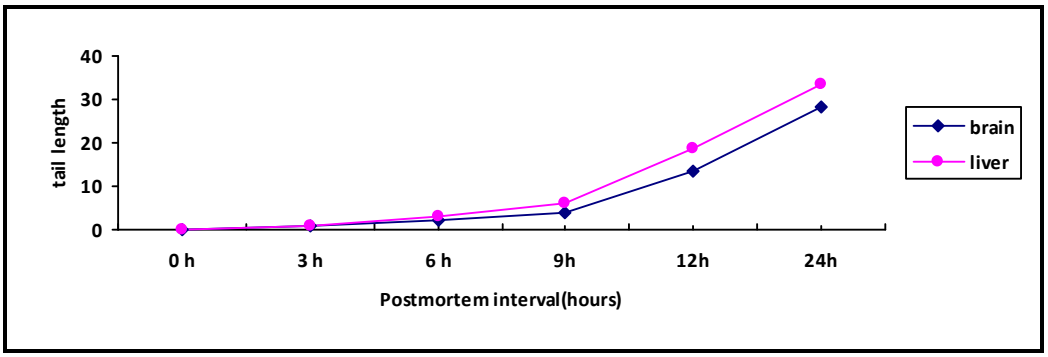

Fig 1: Regression analysis of DNA fragmentation in brain cells of rat from 3 to 24 hours postmortem. Fragmentation was quantified using tail length , relative to postmortem interval.

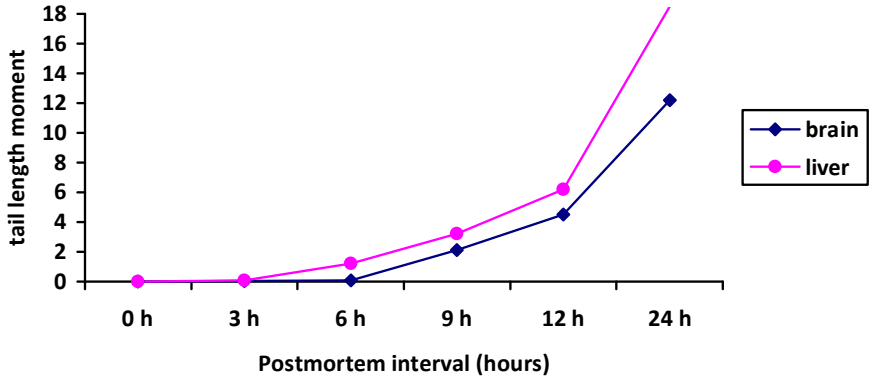

Fig 2: Regression analysis of DNA fragmentation in liver cells of rat from 3 to 24 hours postmortem. Fragmentation was quantified using tail length moment , relative to postmortem interval.

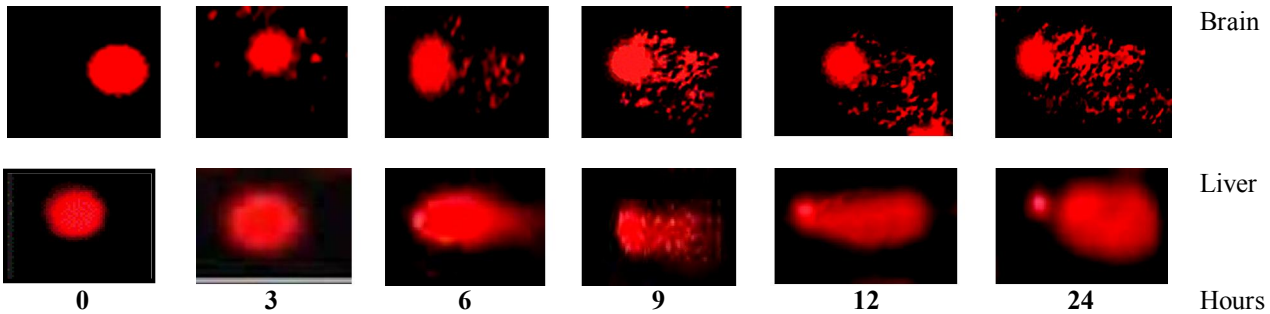

Fig 3: Showing degradation process of nuclear DNA (Comet shape changes) in brain, liver tissues of rat at different postmortem interval (× 400).

4. Discussion

In the field of forensic pathology one of the most important issues is the correct estimation of the PMI (Lendoiro et al., 2012). However, it is often very difficult to accurately determine the PMI in daily practice. An accurate estimation of the PMI requires the evaluation of parameters that change constantly with time after death. This definition seems to fit well in post mortem degradation of nucleic acids (Liu et al., 2007). Indeed, with the advances of molecular biology, the analysis of time-dependent degradation of nucleic acids (both DNA and RNA) became a focus of attention in clinical medicine as well as in forensic science (Bauer et al., 2003). Upon the death of an organism, internal nucleases contained within the cells should cause chromosomal DNA to degrade into increasingly smaller fragments over time . This post mortem degradation of nucleic acids has been suggested as an elegant alternative to classical methods for PMI estimation (Hao et al., 2007) So, this study aimed to profile postmortem degradation of DNA in relation to PMI. DNA was extracted from the brain, liver of rats at different PMI (0, 3, 6,9, 12 and 24 hours postmortem). The DNA was electrophoresed through the encapsulating agarose and samples with degraded DNA generate smeared tails (hence the name comet assay).

The results of the current study revealed that at early stage after death (zero time), the majority of cells in brain and liver tissue displayed a complete head, spherical in shape, and the comet trail was minimal. This represents the majority of long-stranded DNA remaining in the nucleus of the cell, with very little fragmentation (Johnson and Ferris ,2002).

The result of this experiment showed that within the 24 hours post mortem, the brain and liver cells showed increased DNA degradation parameters (frequency of comet-like cells, comet tail-length, percentage of tail DNA, tail area, comet tail-moment). The increase in DNA degradation parameters was in parallel with increasing postmortem interval. Fragmentation from 3 to 24 hours in brain and liver samples after removal from the body, are shown by the change in comet shape suggested that there is indeed DNA fragmentation in brain and liver cells after death, and the process was gradual, progressive and regular. These findings coincide with those of Luo et al. (2006) who reported gradual decrease of bone marrow DNA with prolongation of PMI. And, Zhen et al., 2006 reported that an evident comet tailing was observed in DNA of myocardium cells after electrophoresis, and their changes coincided with the extension of postmortem interval, which indicate that DNA degradation rate has a close relationship with postmortem interval in the periods from 0 to 72 hrs in rats. Also Zheng et al. (2012) reported that comet tail length depends on the amount of small DNA fragments, produced as a result of degradation. These results is also supported by Johnson and Ferris (2002) who suggested 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. However Ferreira & Cunha (2013) stated that PMI estimation is complicated by numerous factors, some endogenous such as cause of death, body build or drug use, and some exogenous, which vary geographically, including temperature, precipitation, pH, oxygen concentration and other soil characteristics.

The result of the current study revealed that brain DNA showed slower degradation than liver DNA , such variability appears to be related to the antemortem ribonuclease activity of the tissue; with relatively “ribonuclease poor” tissues such as brain and retina exhibiting greater nucleic acids stability (Johnson, et al., 1986, Malik et al,. 2003) when compared to ribonuclease rich tissues such as liver, stomach and pancreas (Finger et al., 1987).

The present study used a simple, applicable electrophoresis method (comet assay) that make hopes into a more convenient method in determination of early time of death . So, this method can be used with other conventional methods for a reliable and sensitive analysis of PMI. It is also recommended to study DNA degradation in cases with different causes of death to investigate whether the cause of death has any effect of on DNA degradation rate.

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