POST MORTEM DNA ANALYSIS FOR DETERMINATING TIME OF DEATH AND SEX IDENTIFICATION

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ABSTRACT

Time of death is usually estimated by evaluating events mostly occur after death. In this paper the potential application of nuclear DNA image analysis in liver tissue used to evaluate post mortem interval. After death, internal nucleases within the cells cause degradation of nuclear DNA of hepatocyte more rapidly than other tissue, where liver cell rich in lysosomes that contain hydrolases enzymes. Nuclear DNA degradation is measurable and quantifiable in relation to time. Three liver samples were taken at zero, 24, 48, 72 hours, 5 days, one and two weeks post mortem interval. DNA ploidy, DNA index (1&2), mean DNA content, nuclear area and DNA histogram were found to correlate with an increased post mortem interval from zero to 72 h. Hepatocyte nucleus and sequensed DNA histogram couldn't be detected at or more than 5 days after death. Three skeletal muscle samples (at zero, 24, 72, 1w, 2w, 19 days and 21 days) were used for sex identification using polymerase chain reaction (PCR) technique. Sex could be identified till two weeks post mortem interval. From these results nuclear DNA in hepatocyte reveals time dependent alteration. So can be used as a predictor for post mortem interval. DNA in certain tissue (Skeletal muscle) is more stable and allowing to perform DNA typing (sex identification) by polymerase chain reaction.

INTRODUCTION

One of the most important longstanding problems in the field of forensic medicine is the determination of the time of death **(Dokgoz, et al 2001 and Johnson and Ferris, 2002)**. Time of death estimated by evaluating events which happen in a deceased in conjunction with the known behavior of such events. The use of direct DNA testing in forensic analysis has become increasingly common **(Housman, 1995)**. Many recent techniques were used to monitors DNA damage induced during cell death (apoptosis, necrosis and autolysis). Comet assay (single cell gel electrophoresis) and tunel assay are examples of these methods **(Chandana, 2004, Kim, et**

al 2002 and Grasl- Kraupp, et al 1995). Moreover the probe (GACA) 4 could be used to analyse partially degraded DNA **(Poche, et al 1991)**.

DNA founds inside and outside (mitochondria) the nucleus **(Grivell, 1983 and Rush and Misra, 1985)**. Post mortem stability of DNA becomes an important point and has already been studied **(Bar, et al, 1988; Gill, et al 1985, Hagelberg, et al, 1991 and Ogata, et al, 1990)**. DNA analysis have been carried out with human hard tissue and successful examinations of bones **(Hagelberg , et al 1991; Jeffreys, et al, 1992 and Gill, et al 1994)** and teeth **(Potsch, et al 1992 and Smith, 1993 and Pfeiffer, et al, 1999)** have been described. However the success of investigation depend on the degree of DNA degradation **(Graw, et al 2000)**. DNA from brain tissue was detected even after 85 days. In heart or in muscle cells DNA were stable up to one month post mortem period allowing to perform DNA finger printing. In other tissues (kidney, liver and lymph nodes) DNA were degraded after short period and becoming unsuitable for blotting **(Bar et al 1988 and ludes, et al 1993)**. On the other hand under favourable conditions, successful typing is still possible after thousands of years **(Hoss and Paabo, 1993)**. Quality and quantity of DNA depend mainly on the type of tissue used **(Bar et al, 1988 and Ludes, et al, 1993)** and the site in which the body was deposited **(Graw, et al 1998)**.

DNA typing is a useful tool in forensic for determining the remains of body who have been dead from various periods of time, polymerase chain reaction (PCR) method gives good results **(ludes, et al, 1993)**. DNA printing can be applied on dried blood or semen stains up to 4 years old **(Gill, et al 1985)**.

Aim of work: the aim of this article is to use certain remained tissue (liver) for detection of post mortem interval using new quantitative measure of nuclear DNA damage. In addition to using another tissue (muscle) for DNA typing (sex identification) by polymerase chain reaction.

MATERIAL AND METHODS

Materials:

A- Samples: Three specimen of liver and muscle 1000 gm of each were collected from three male bull (just after slaughtered) and brought to the laboratory in a chilled condition using liquid nitrogen. The samples are aged by keeping in the natural environment (summer) in May and June. Liver sections were taken at zero time, 24 h, 48 h, 72 h, 5 days, 7 days and two weeks to be used for determination the time of death. Sections from each muscle sample were taken at zero, 24 h, 72 h, one week, two week, 19 days and 21 days. Specimen were kept separtely in liquid nitrogen for 1 h. Frozen tissues stored at - 20 till examination.

Methods :

Nuclear DNA image analysis: Five 50 um sections from each sample were put in centrifuge tubes and deparaffinized by adding 10 ml xylene for 20 minutes and repeated twice. Sections were rehydrated using a sequence of 10 ml of 100 %, 95 %, 70 % and 50 % ethanol for 20 minutes and each step was repeated twice. Each section washed in 2 changes of distilled water for 20 minutes and left in 10 ml distilled water for 24 hours **Bashar (1979)**. Then the nuclear suspension was done according to **Ensley, et al (1990)**. The pellet was flattened on clean glass slides. The slides were air dried then post-fixed in 10 % neutral buffer formalin for 30 minutes and washed in distal water for 10 min. and left to dried over night at room temperature.

DNA staining and analysis: Air dried prepared slides were treated for 60 min. in 5 N Hcl to hydrolyze nuclear DNA. The slides stained by feulgen stain (Schulte and Fink, 1995)and analyzed with Hund CML image analyzer and soft ware (Helmut Hund Gmb HD 6330 Wetztar 21 Germany).

Sex identification

DNA extraction from tissues: DNA was isolated from frozen tissue on three steps cell lysis, protein precipitation and DNA precipitation caccording to **(Davis, 1980)**.

DNA primers: A set of bovine Y- chromosome specific primers **(Peura et al. 1991)** were used.

1- (5' primer) 1- 21: 5' - GGA TCC GAG ACA CAG AAC AGG - 3'

2- (3' primer) 1 - 21: 5' - GC TAAT CCA TCC ATC CTA TAG - 3'.

Oligonucleotide primers dissolved in 10mM tris Hcl and 1mM EDTA (PH 8.0) TE buffer.

PCR reactions: The reactions were carried out as described by **Saiki et al. (1988)**. The reactions were conducted by using Master mix (50 ng DNA as a template. The reaction mixture contained 5 ul of 10X buffer, 0.4 ul of taq DNA polymerase, 200 uM of dNTPS) and 20 pmol of each primer. The amplifications were carried out in Gene AMP PCR system 9700.

Analysis of the amplified product:

Products were analysed by agarose gell electrophoresis according to the method described by **(Sambrook, et al 1989)**. The gel was examined under ultraviolet light as ethidium bromide intercalate between the bases of the DNA and will fluoresce. Photographs were taken.

N.b. Marker give different bands each 50 base pair.

The data were calculated as mean ± stander error by the student's (t) test "statase" programe.

RESULTS

Cytometric parameters of nuclear DNA analysis were cleared in table (1). Such table showed that the stemline ploidy I and DNA Index (content) I were 2 ± 0.4 and 1 ± 0.2 at zero time, 1.29 ± 0.3 and 0.64 ± 0.1 at 24 h, 0.87 ± 0.2 and 0.43 ± 0.1 at 48h and 0.69 ± 0.2 and 0.34 ± 0.1 at 72h post mortem interval and not recorded at 5 days, one and two weeks respectively where there is no cells could be seen . Decrease at 48h and 72h is significant in relation to zero time. Stemline ploidy 2 and DNA index 2 were 4.07 ± 0.2 and 2.04 ± 0.I at zero time, 3.61 ± 0.8 and 1.81 ± 0.4 at 24h, 1.88 ± 0.4 and 0.94 ± 0.2 at 48 h, post mortem interval (significant decrease at 48 h was detected). Mean DNA contents were 2.86 ± 0.37, 1.91 ± 0.15, 1.36 ± 0.3 and 1.13 ± 0.15 at zero, 24h, 48h and 72h respectively which indicat significant decrease and not recorded at 5 days, one & two weeks. Mean area of nuclei were 36.93 ± 2.3, 48.78 ± 0.7, 23.02 ± 2.8 and 16.18 ± 3.5 micron at zero, 24h, 48h and 72h post mortem interval. All data showed significant decrease except at 24 h showed signifcant increase However these data not recorded at 5 days, one and two weaks post mortem interval. These data were cleared in fig 1, 2, 3, 4 , 5 and 6 (A and B).

DNA histogram was considered diploid when peak occupied the diploid position 0.9 - 1.1 (normal DNA indx I) or at 1.9 - 2.1 (normal DNA index 2) and less than 15 % of cells were present at an euploid position. The aneuploid postitions means peak occupied position more or less than 0.9 - 1.1 or 1.9 - 2.1. The term aneuploid is used to describe hypodiploid, hyperdiploid and hypertetraploid .

DNA histograms at zero time and 24 h post mortem interval considered diploid, where peaks occupied the position (1.9-2.1) and (0.9-1.1) respectively. At 48 h the histogram shifted to left and the cases is aneuploid (hypodiploid). At 72 h the histogram is completely shifted to right and the cases is aneuploid (hypodiploid)

Figure 7 and 8 showed results obtained for sex identification by polymerase chain reaction after zero, 24, 72h, 1 w, 2 w, 19 days and 3 weeks post mortem interval from muscle of male bull. Discrete 300 dp band was observed in lanes 1, 2, 3 (zero time) 4, 5, 6 (24h) 7, 8, 9 (72h) 10, 11 and no band at lane 12 (1 w) 13, 14 while no band at lane 15 (2 w). While no sex specific bands were seen at lane 16, 17, 18 (19 days) and lane 19, 20, 21, 22 (3 week) post mortem intervale.

DISCUSSION

There are multiple reasons for trying to estimate accurate time of death. As it important to law men. Also may help in assessing whether a proposed cause of death is reasonable. Development of techniques for measuring the quantity of nuclear DNA in apopulation of cells resulted in refinement of prognostic criteria **(look et al, 1985)**. One of such techniques is the DNA image cytometry. Nuclear DNA fragmentation may be a post mortem consequence of DNA fragility **(Grasl- Kraupp, et al 1995 and Schallock, et al. 1997)**. Studies of nuclei extracted from tissue (liver) at different post- mortem time and analysed by image analysis provided knowledge of prognostic value of measuring DNA content. Data of table (1) showed that stem line ploidy 1 and 2 and related DNA content I and 2 in addition to mean DNA content were decreased as time post mortem interval increased. Mean area of nuclei showed the same relation with exception at 24h post mortem which increased. These results agree with **Bar, et al (1988) and Ludes, et al, (1993)**. They recorded that DNA from tissue (liver) were degraded after short peroid of death and becoming unsuitable for blotting. Also agree with **Genest, et al (1992)** who recorded that at least 1% of nuclear basophilia (hematoxylin staining of nucleoprotein of cell) loss in hepatocytes at 24 hours or more after death. and all nuclear basophilia loss at 96 hours or more post mortem interval. Also **Jenner and Hirsch (2000)** detected disappearance of nuclear basophilia in fetal organs during death process.

These results may be explained as a result of postmortem decay which beginning with autolysis and followed by aerobic and bacterial decomposition of organic material. Autolysis (non — bacterial autodigestion) occur by enzymes liberated from lysosomes (hydrolases enzyme) **(Derzeltod, 1955 and Gossner, 1955)**. DNA in dead cells is degraded by nucleases which belong to hydrolases enzyme that includes endonucleases and exonucleases. Liver cells contain large number of lysosmes **(Grasl- Kraupp, et al 1995)**.

Nuclear DNA not detected at or more 5 day postmortem as clear in table (1) and Fig. (5 and 6). These results are some what disagree with **Bar, et al (1988)** who recorded complet degradation of DNA in liver cells varied from 24 - 36 h; while **ludes et al (1993)** reported that yield of DNA becoming very low beyond aperoid of one week after death. Such variations may be explained as degraded DNA correlated to many factors as environmental T... at the site of death and for infections diseases prior to death **(Bar et al, 1988)**. Also damp condition can cause rapid degradation of DNA **(Graw, et al 2000)**.

Area of nuclei correlated directly with the duration of post mortem peroid as in table (1) and Fig. (1, 2, 3 and 4). This result may be explained as when cell dies, the PH lowers, lysozyme (an enzyme within cell) is activated, and the nuclear matter disintegrates and loss of nuclear sub-

stance occure **(Jenner and Hirsch, 2000)**. Also partialy agree with **Trump , et al (1965)** who observed pale nuclear chromation and indistinct out line in liver tissue incubated at 37°C.

Hepatocyte as clear in fig. (1, 2, 3, 4 and 5) showed sequense changes especialy in nuclei and cell outline. These changes were correlated directly with the duration of the post mortem peroid. These results agree with **Tateyama et al. (1998) and Trump, et al (1965)**. They found that tissue removed from body and left unfixed undergo coagulative necrosis. The cell show slightly eosinophilic cytoplasm with little or no alteration of cellular details in early phase. As necrosis progresses the dead cell swollen and the nuclei become indistinct. Cellular fragmentation occur resulting from degenerative changes of autolysis.

In the present study DNA image cytometry of the smaples at zero and 24h post mortem interval showed non aneuploid (diploid) histograms. At 48 and 72 h post time interval DNA index is aneuploid (hypodiploid) and DNA histograme become shifted to left. These results suggests that internucleosomal DNA cleavage progresses a ccording to time **(Tateyama, et al 1998)**, where intracellular Ca^{2+} concentration elevates after degradation of cell membrane **(Farber, 1982)**. This process could be followed by random cleavage of DNA mediated by released lysosomal enzymes.

Fascinating reports about DNA recovery from Egyptian mummies gave rise to interesting speculations **(Paabo, 1985 and Margaret and Jones, 1986)**. DNA was recoverd from skeletal muscle at different time post mortem interval (zero, 24h, 72h, one week, two weeks, 19 days and three weeks) showed discrete bands in lanes corresponding to reactions performed at zero, 24h, 72h, first and second samples at one week and two weaks post mortem interval. No bands were seen in samples obtained at 19 days and three weeks after death.

This result partialy egree with **Gill (1987), Bar et al (1988) and Iudes, et al (1993)**. They recorded good DNA stability in brain and muscles over aperoid of three weeks post mortem allowing to perform DNA finger printing. Our results were incomplet agree with those authors due to their samples was kept at different T... from 5 to 25°C. So DNA could be detected for up to 3 weeks while our samples kept in open environment (in summer), where T° reach 35°C or more. High environmental T° at the site of death were the main factor for rapid autolysis **(Bar, et al 1989)**. Fragementation of DNA by non specific hydrolysis affected primarly the longer fragment which were better target than the shorter fragment. This hydrolysis produced polynucleotides shorter and never of identical length. Complete loss of high molecular weight DNA never showed banding patterns after hybridisation **(Bar et al, 1988)**. Also **Wiegand, et al, (1992)** mentioned that environmental condition reduce the chances of success after short time of death.

Conclusion: DNA nuclear changes in tissues at different time of death is important point that need to be studied in relation to environmental condition. Some tissue (liver) found to be suitable

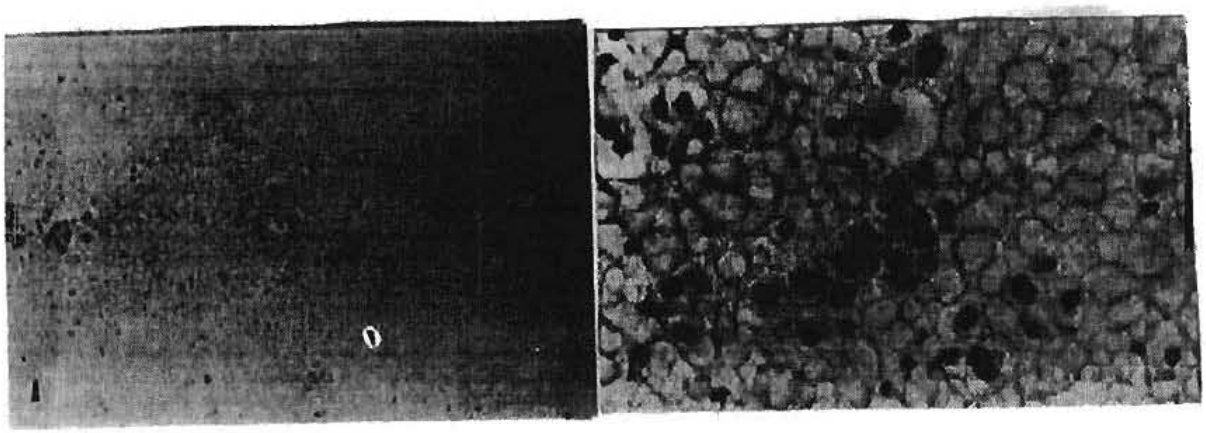
for detection of post mortem interval because rapid degradation of DNA. Other tissues as skeletal muscle found to be good suitable for DNA finger printing. Nuclear DNA image analysis offer significant improvement in monitoing the kinetics of DNA fragmentation induced during cell death coupled with its simplicity and the ability to detect responses of small cell subpopulation this method has a reliable and sensitive analysis of cell death.

Table (1): Cytometric parameters of nucleur DNA image analysis according to post mortem interval. (Mean ± S.E.).

Cytometric Parmeters	Time post mortem interval						
	Zero	24h	48h	72h	5 day	One weeks	Two weeks
Stemline ploidy (1)	2 ± 0.4	1.29 ± 0.3	0.87* ± 0.2	0.69* ± 0.2	-	-	-
DNA index (1)	1 ± 0.2	0.64 ± 0.1	0.43* ± 0.1	0.34* ± 0.1			
Stemline ploidy (2)	4.07 ± 0.2	3.61 ± 0.8	1.88** ± 0.4	-	-	-	-
DNA index (2)	2.04 ± 0.1	1.81 ± 0.4	0.94** ± 0.2	-	-	-	-
Mean of DNA content	2.86 ± 0.37	1.91** ± 0.15	1.36 **± 0.3	1.13** ± 0.15	-	-	-
Mean area of nuclei (micron)	36.93 ± 2.3	48.78 ± 0.7	23.02 *± 2.8	16.18** ± 3.5	-	-	-

* Significant at $p \leq 0.05$

** High significant at $p \leq 0.01$



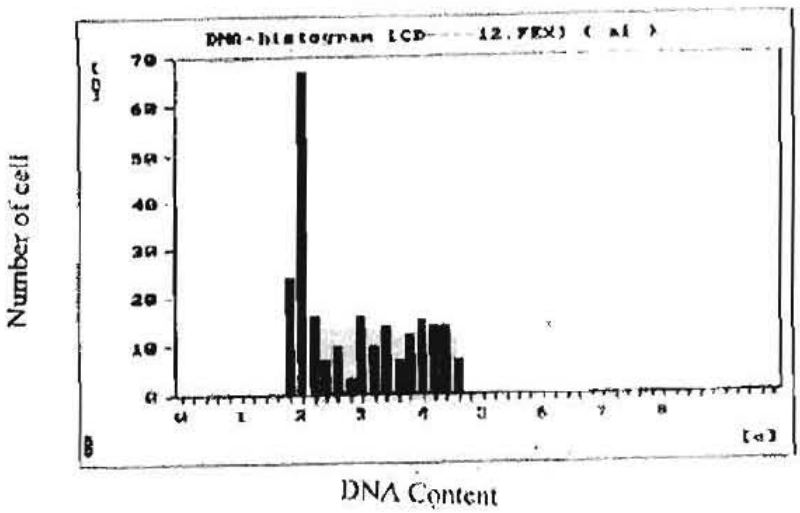
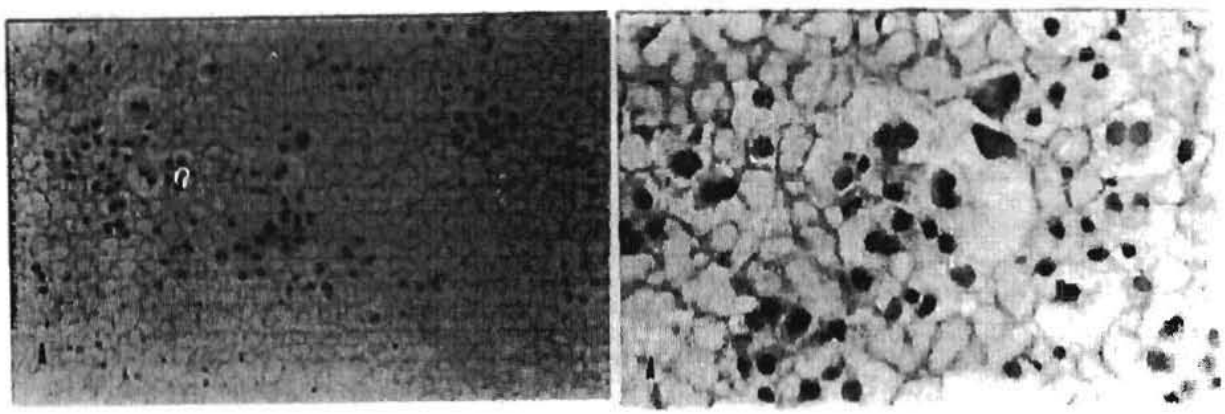


Fig. 1 A) Hepatocyte Showed no alteration of cellular detail nucleus at zero time post mortem interval (Feulgen's stain). X 150 and X 600.

B) DNA histograme at zero time post mortem interval showing normal diploid peak using DNA imaging cytometer.



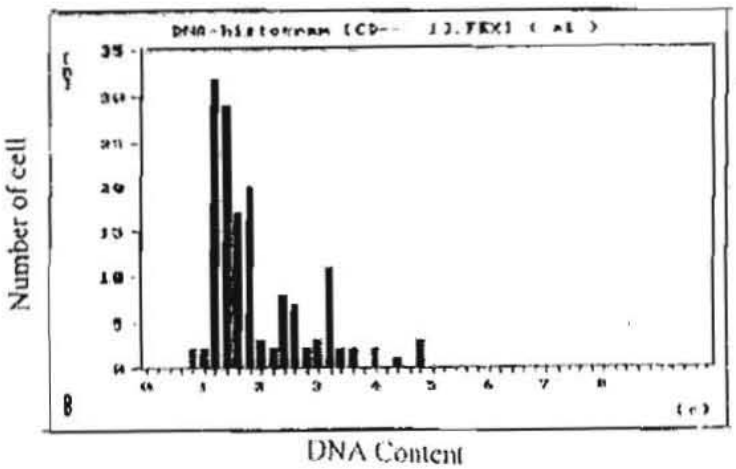
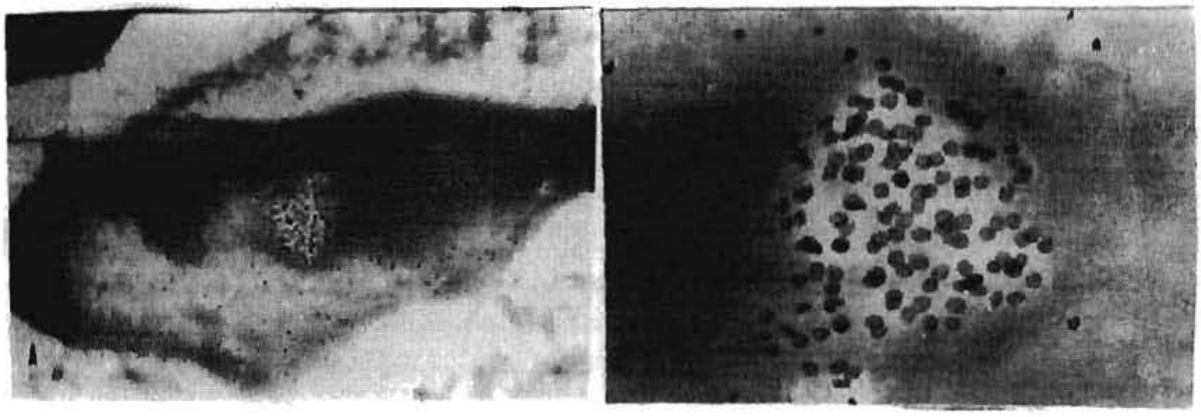


Fig. 2 A) Hepatocyte Showed eosinophilic cytoplasm with little swollen of nucleus at 24h post mortem interval (Feulgen's stain). X 150 and X 600.

B) DNA histograme at 24h post mortem interval showing nearly diploid peak using DNA imaging cytometer.



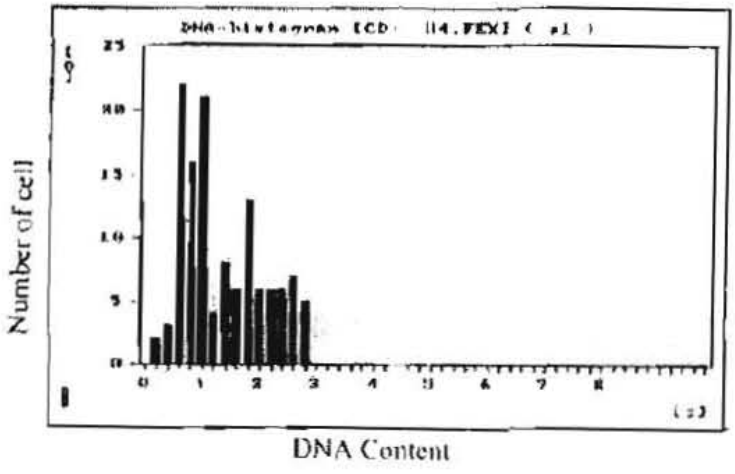
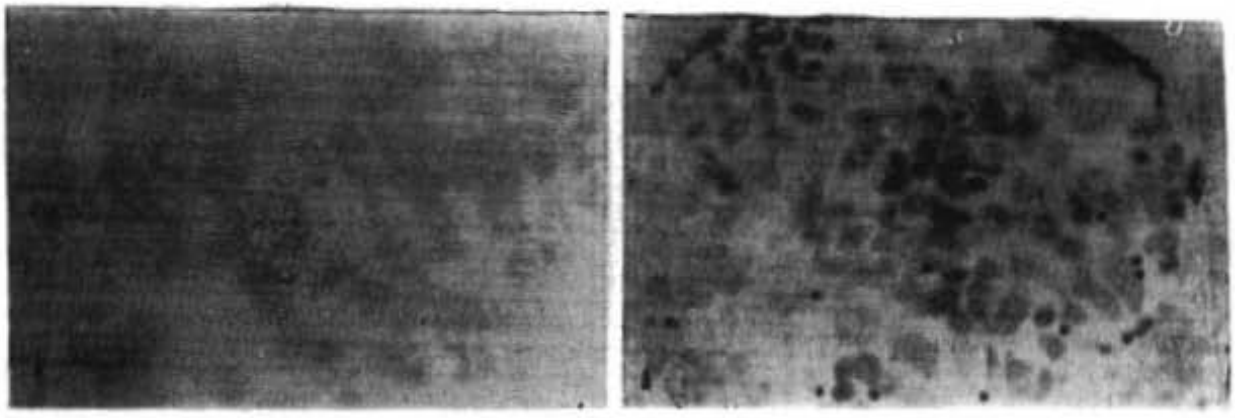


Fig. 3 A) Hepatocyte at 48h. are swollen and som nuclei become indistinct with little preservation of cell out line at 48h post mortem interval (Feulgen's stain).

B) DNA histograme of hepatocyte nuclus at 48h post mortem interval showing aneuploid peak using DNA imaging cytometer.



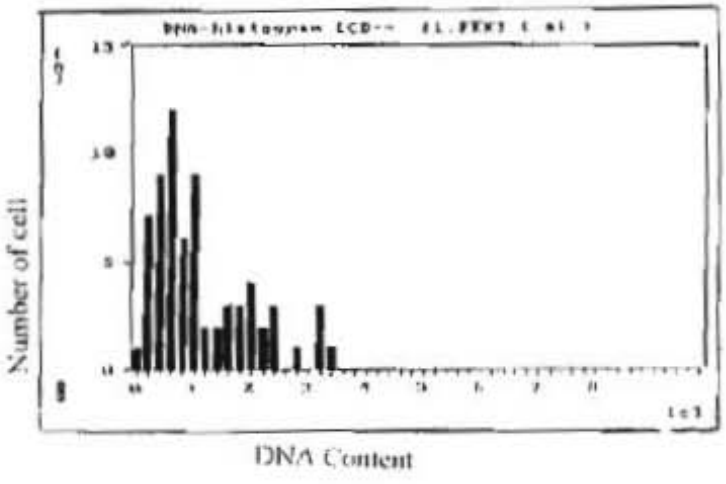


Fig. 4 A) Hepatocyte at 72h. are swollen and become indistinct with out preservation of cell out line (Feulgen's stain). X 150 and X 600.

B) DNA histograme of hepatocyte nuclus at 72h post mortem interval showing aneuploid peak using DNA imaging cytometer.

Fig. 5 : Hepatocyte showed cellular fragmentation at 5 days post mortem interval (Feulgen's stain). X 150.

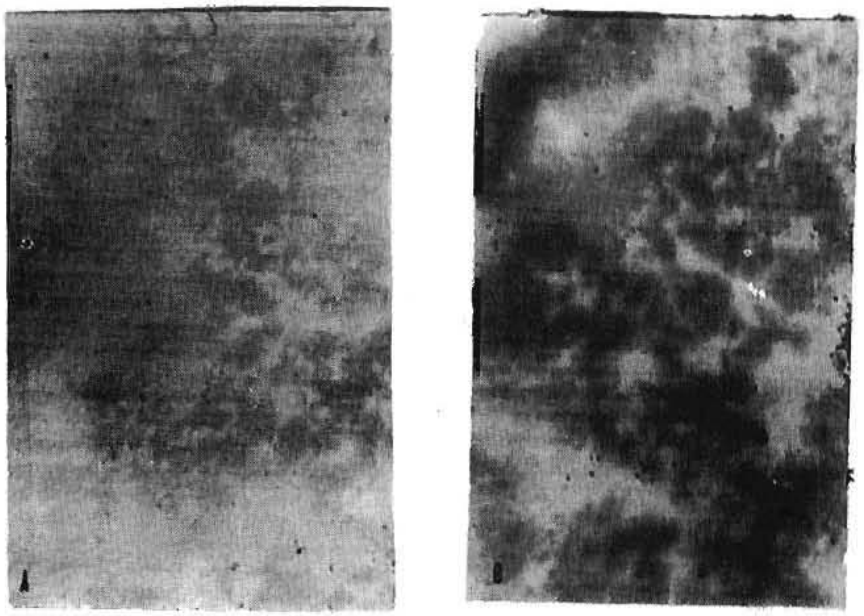


Fig. 6 : Liver tissue at 7 days (A) and 2 weeks (B) post mortem interval showed non identified cell or nucleus (Feulgen s stain). no DNA histogram could be obtained. X 150.

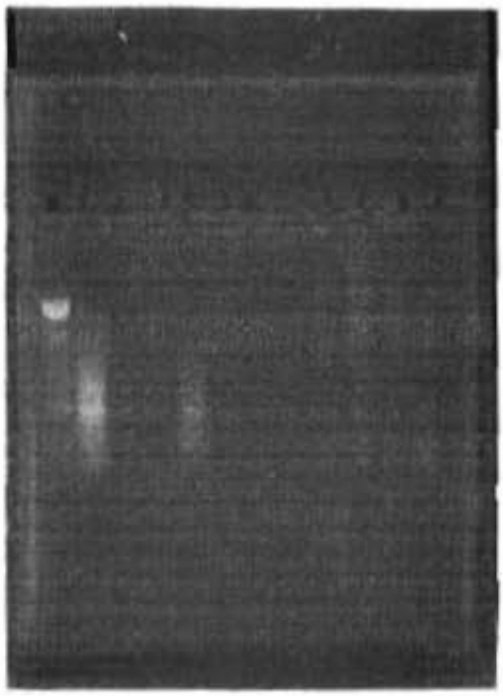


Fig. 7) Banding patteren of Y chromosome - specific sequences in 1.4% agarose gelelectrophoresis of male bull at zero (lane 1, 2, 3), 24h (lane 4, 5, 6), 72 (lane 7, 8, 9) and 1 w. lane 10 and 1 post mortem interval. Lane (M) contains DNA marker.

Fig. 8) Banding patteren of Y chromosome - specific sequences of male bull at one weeks (lane 12), two weeks (lane 13, 14, 15), 19 days (lane 16, 17, 18) and 21 days (lane 19, 20, 21, 22) post mortem interval. Lane (M) contain DNA marker.

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Acknowledge:

To all staff members of clinical pathology dep. and early cancer detection unit, Zagazig university for their help in performing practical parts.

الملخص العربى

تقدير زمن الوفاة والتعرف على الجنس بتحليل الحمض النووى الديزوكس ريبوزى بعد النفوق

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يقدر الوقت الذى مضى على حدوث الوفاة بتقييم حقائق غالباً ماتحدث بعد الوفاة، فى هذا لبحث يدرس إمكانية تطبيق تحليل الحمض النووى الديزوكس ريبوزى الموجود بنواة خلايا الكبد لتقييم الوقت المار على الوفاة يتحلل الحمض النووى داخل أنوية خلايا الكبد أسرع من باقى الأنسجة الأخرى بتأثير إنزيمات النيوكليبز الداخية حيث أن خلايا الكبد غنية بالريزوزوم والتى تحتوى على إنزيمات الهيدروليز لإنزيمات التحليل، تم قياس وتقييم الحمض النووى بنواة خلايا الكبد عند أوقات مختلفة من الوفاة، ت أخذ ثلاثة عينات من أكباد ذكور البقر وعرضت للجو مباشرة.

ثم أخذت عينات بعد الوفاة مباشرة من هذه الأكباد ثم عند ٢٤، ٤٨، ٧٢ ساعة، ٥ أيام، إسبوع وإسبوعين بعد الوفاة لتحليل الحمض النووى داخل هذه الأنوية وتحديد مابها من تغيرات، تم قياس دليل الحمض النووى واحد وإثنين وحجم النواة والرسم البيانى النسيجى للحمض النووى، حيث وجد علاقة بين هذه القياسات والزمن الذى انقضى بعد حدوث النفوق حتى ٧٢ ساعة بعد النفوق، بعد مرور ٥ أيام أو أكثر على النفوق لم يتم تحديد أنوية لنسيج الكبد.

أخذت ثلاث عينات من عضلات نفس الحيوانات السابقة وعرضت للجو مباشرة وتم أخذ ثلاث عينات عند فترات زمنية مختلفة (بعد الوفاة مباشرة، ٢٤، ٧٢، إسبوع، إسبوعين، ١٩ يوم، ٢١ يوم) من النفوق لتحديد الجنس باستخدام تقنية تفاعل البلمرة المتسلسل، تم إثبات جنس الحيوان حتى إسبوعين من النفوق.

ونخلص من تلك الدراسة بإمكانية تقدير الوقت المار على النفوق بتسجيل التغير الحادث للحمض النووى داخل أنوية خلايا الكبد، كما يمكن التعرف على الجنس باستخدام أنسجة يكون فيها الحمض النووى أكثر ثباتاً.