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Evaluation the Time of Death by Different Markers in Liver and Brain of Rats

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Abstract

This study was done on 81 rats to evaluate the post mortem interval PMI depending on expression of Gapdh mRNA and protein response to immunostaining.

Gapdh in liver demonstrated less stability °radation with a significant decrease in amplicon detection across the entire transcript after 48 hour PMI with no difference in transcript level until 96 hours, and showed a strong significant positive correlation between PMI and Gapdh expression (r = 0.837, p = 0.000).

Consistent and surprising robustness of Gapdh transcript levels in brain with non significant low correlation between gene expression and PMI (r = 0.129, p = 0.522).

Gapdh showed less stability with significant decreases in transcript levels in liver with increasing PMI (up to 48 hour). However, it shows low correlation between Gapdh transcript and PMI in brain.

PCNA showed positive immunostaining in the hepatocytes cytoplasm and in the nuclei in the first few hours after death (0-9hours), later on only few cells showed positive immunostaing in both cytoplasm and nuclei (12hours). At 24-48 hours the hepatocytes only showed positive cytoplasmic PCNA reaction, while in 72-96 hours the PCNA immunostaining showed negative reaction.

In the brain at 0-12 hours positive PCNA was located in the cytoplasm and nuclei of the nerve cells. At 24 hours only positive PCNA immunostaining was located in the cytoplasm and negative reaction in the nuclei. At 48-96 negative PCNA immunostaining was noticed.

Key word. *PMI-Liver-Brain-PCNA- Gapdh-Gene expression*

Introduction

Post mortem interval is a valuable practice in forensic science. The exact time of death was limited to some simple observation as livor mortis, algor mortis, and rigor mortis. Up till now we can't exactly determined the accurate time of death.

The body temperature was used as primary parameter to defined time of death by using algorithms to identify body cooling behavior. Henssge and Madea [1]. Madea

[2] used different modern methods to determined PMI as MRI, Spectroscopy, and immunohistochemistry and flow cytometry.

In criminal investigations, different tools were used in forensic medicine as RNA degradation profiling. Sampaio-Silva , Magalhaes , Carvalho , Dinis-Oliveira and Silvestre [3].

Analysis of DNA and RNA were used in forensic medicine in wide range. Bauer , Gramlich , Polzin and Patzelt [4]. RNA degradation profiling of body fluid was used as advanced way in determine PMI for one day only because RNAs affected by ribonuclease which lead

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to degradation. The stability of RNAs differed according to types of tissues and organs after death. Hanson , Lubenow and Ballantyne [5]. While the protein degraded slower than RNAs after death and the expression of mRNA is not associated with the protein expression in the organs. Tian , Stepaniants , Mao , Weng , Feetham and Doyle , et al [6]. In human and rats, the protein of the brain still stable for 24 hours after death. Goggins , Scott and Weir [7].

The parameters associated with the changes occur after death move in continuous changes. Liu , Shu , Ren , Zhou and Li Y et al [8].

Using of time dependent degradation of nuclei acid was used as method of analysis in forensic medicine clinic's. Bauer , Polzin and Patzelt [9].

The estimation of the PMI by studying RNA degradation or loss of RNA transcripts after death seems to be rapid and time-dependent. Bauer [10].

Proliferating cell nuclear antigen (PCNA) is a DNA clamp that acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication. PCNA is a homotrimer and achieves its processivity by encircling the DNA, where it acts as a scaffold to recruit proteins involved in DNA replication, DNA repair, chromatin remodeling and epigenetics. Moldovan , Pfander and Jentsch [11]. proliferating cell nuclear antigen (PCNA), may be employed to provide visual and quantitative evidence of cell proliferation. Heron and Rakusan [12].

Material and Method

The experiments were performed on 81 Wistar albino rats weighing 200 – 250 gm (These rats were sacrificed in humanity methods according to the ethical standard of (Faculty of veterinary medicine Benha University Egypt)

Experimental design

The rats were randomly and equally divided into nine groups (9 animals each) according PMI interval time (0- 3- 6- 9- 12- 24- 48- 72- 96 hrs). The rats were euthanized by isoflurane anesthesia followed by cervical dislocation .

1st group: Represented control group

2nd group: collect sample at 3h

3rd group: collect sample at 6h

4th group: collect sample at 9h

5th group : collect sample at 12h

6th group: collect sample at 24h

7th group: collect sample at 48h

8th group: collect sample at 72h

9th group: collect sample at 96h

The samples of group (2-9) were collected after death at the determined time.

Samples collections

Brain and liver were taken put in sterile ependorff tube and incubated at 25°Cin electro thermal incubator (MTI corporation, USA) according to PMI in each group, after each time immediately placed in Cryo tubes and stored in RNA Later solution (by 10 µL per 1 mg of tissue) (Qiagen- GmbH, Germany) at-80°C.

Total RNA Extraction

Total RNA extraction was done by using total RNA Purification Kit from Easy Red TM kit (Intron Biotechnology, Korea), about 100 mg tissue put in a micro centrifuge tube with 750 µL of Trizolsolution was homogenized using rotor Tissue Ruptor (Qiagen, GmbH, Germany). Ten µL RNA (2 µg) from each sample of the same group were taken for Synthesis of cDNA.

Spectrophotometric Quantification of RNA

The concentration and purity of RNA were determined by measuring the absorbance in a Spectrostarnano (BMG Lab Tec,GmbH, Germany). An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to an RNA concentration of 40 µg mL − 1. Pure RNA has an A260/A280 ratio of 1.8-2.0.

Synthesis of cDNA using 2X Reverse Transcriptase Master Mix (Applied Biosystem,USA)

About 2 µg RNA in 10 µL used with 10µL reverse transcription mixfor each sample.

Relative Quantitation of m RNA of the respective genes by real time PCR using SYBR green:

About 20 µL PCR components mix was added to

each well. The real-time cycler was programmed as 95°C for 10 min followed by 40 cycles of 95°C for 15 sec then 60°C for 1 min.

Sequence of Gapdh was 5'-TGC ACC ACC AAC TGC TTA GC -3 ' (5'-3' sequence forward) and 5'- GGC ATG GAC TGT GGT CAT GAG -3' (5'-3' sequence reverse). The PCR primers were synthesized by (Metabion international AG, Germany).

Data Analysis

According to the RQ manager program ABI SDS software (ABI 7500 fast), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale). The threshold Cycle (CT) is defined as the point at which fluorescence rises above the background fluorescence & serves as a tool for calculation of the starting template amount in each sample. Dissociation (melting) curve analysis was performed to verify PCR specificity using the real-time cycler software.

statistically analyzed. The results are presented as means ± SD (ANOVA test) .All analysis was performed using the statistics package St. for social sciences (SPSS) and Microsoft office Excel is used for data processing and data analysis. Differences are considered as statistically significant for p value less than 0.05.

Histopathology

The samples were taken from the liver and brain just few minutes after scarification then exposed to histological techniques according to Bancroft and Gamble [13] .

PCNA immunohistochemical techniques

The monoclonal mouse anti-proliferating cell nuclear antigen (Clone PC10 : Dako) at a dilution of 1:800 with the DakoCytomation EnVision+ System-HRP (DAB) kit according to the manufacturer's instructions. Moldovan , Pfander and Jentsch [11].

Results& Discussion

Post mortem degradation of nucleic acids (RNA decay) has been suggested as an elegant alternative to classical methods for PMI estimation Bauer , Gramlich , Polzin and Patzelt [4] .Nevertheless, the integrity of RNA among different post mortem tissues or organs is known to behave differently . Bahar , Monahan , Moloney , Schmidt and MacHugh , et al [14]. adding a new level of complexity to such analysis.

The endogenous and exogenous ribonucleases, are found to be responsible for the fast in vivo RNA degradation, environmental and storage chemical and thermal conditions can also take place immediately after death and thus influence RNA. Catts , Catts , Fernandez , Taylor and Coulson , et al [15] and Bauer , Polzin and Patzelt [9].

Different attempted to establish the time of death through the quantitative analysis of mRNA degradation by multiplex qPCR in combination with laser-induced fluorescence capillary electrophoresis. Their results showed a significant correlation between RNA degradation and PMI in stored refrigerated human blood and brain samples for up to 5 days Bauer , Gramlich , Polzin and Patzelt [4]

Analyzing the tissue-specificity of RNA integrity loss during different PMI. These organs were chosen due to its high potential in forensic area and their applicability for gene expression studies using post mortem human tissue. Koppelkamm , Vennemann , Lutz-Bonengel , Fracasso and Vennemann [16].

We examined the robustness of mRNA through representative genes across the length of their transcripts to assess the amount of degradation within the molecule across varying periods of PMI. Gapdh is constitutively expressed and considered as a housekeeping or reference gene. Durrenberger , Fernando and Magliozzi , et al [17]. The Ct values and thus the copy number of template molecules varied among the transcripts and PMI.

The stability of Gapdh expression was tested in brain & liver along its transcript across increasing PMI, by establishment of model of different PMI in rat brain & liver.

The transcription of Gapdh gene was showed different variations in the gene expression between all PMI and organs. After normalization, the Pearson correlation (r) and p value were assessed within each organ to find correlation between CT of Gapdh gene and PMI which calculated by SPSS 16.

Our result showed consistent and surprising robustness of Gapdh transcript levels in brain with non significant low correlation between gene expression and PMI ($r = 0.129$, $p = 0.522$; Fig. 1). These results

supported by the findings of Corina, Marissa, Juan, Kathryn, Cristiana, et al. [18]

Also, Partemi , Berne , Batlle , Berruezo and Mont , et al [19] did not find any correlation between PMI and the gene transcription decay in brain and skeletal muscle .

Furthermore, Gapdh in liver demonstrated less stability °radation with a significant decrease in amplicon detection across the entire transcript after 48 hour PMI with no difference in transcript level until 96 hours, and showed a strong significant positive correlation between PMI and Gapdh expression ($r = 0.837$, $p = 0.000$; Fig. 2). As reported by Sampaio-Silva , Magalhaes , Carvalho , Dinis-Oliveira and Silvestre [3]. Who found that RNA recovered from femoral quadriceps, liver and stomach have a faster degradation rate, as observed with the time dependent decrease of the RNA integrity values.

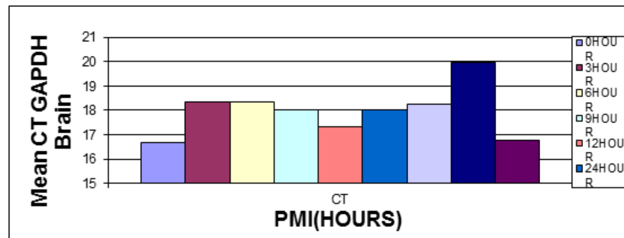


Fig (1): Mean CT of Gapdh in brain.

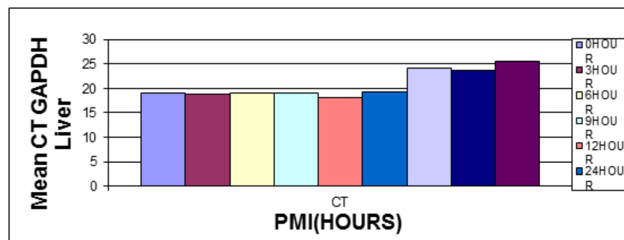


Fig (2): Mean CT of Gapdh in liver.

Histopathology and immunohistochemistry results

The liver at 0 and 3 hours consisted of hepatic cords and central vein . The hepatocytes were showed positive cytoplasmic and few nuclear PCNA immunostaining (Fig.1 and 1a). While at 6 and 9 hours showed vacuolar degeneration and pyknotic nuclei. The hepatocytes were positive PCNA immunostaing (Fig.2 and 2a). The liver at 12 hours showed hydrobic degeneration and dark stained nuclei. Some hepatocytes showed positive cytoplasmic PCNA immunostaining (Fig.3 and 3a) while at 24 hours showed loss of architecture of the hepatic cords with increased fibrous formation. Few hepatocytes were positive cytoplasmic PCNA immunostaining (Fig.4 and 4a). At 48 hours, it showed degenerated hepatocytes with diffused edematous blood vessels. Few hepatocytes were positive cytoplasmic PCNA immunostaining (Fig.5 and 5a). The liver at 72 and 96 hours showed fibrous structures with no architecture .Negative PCNA immunostaing of the hepatocytes (Fig.6 and 6a).

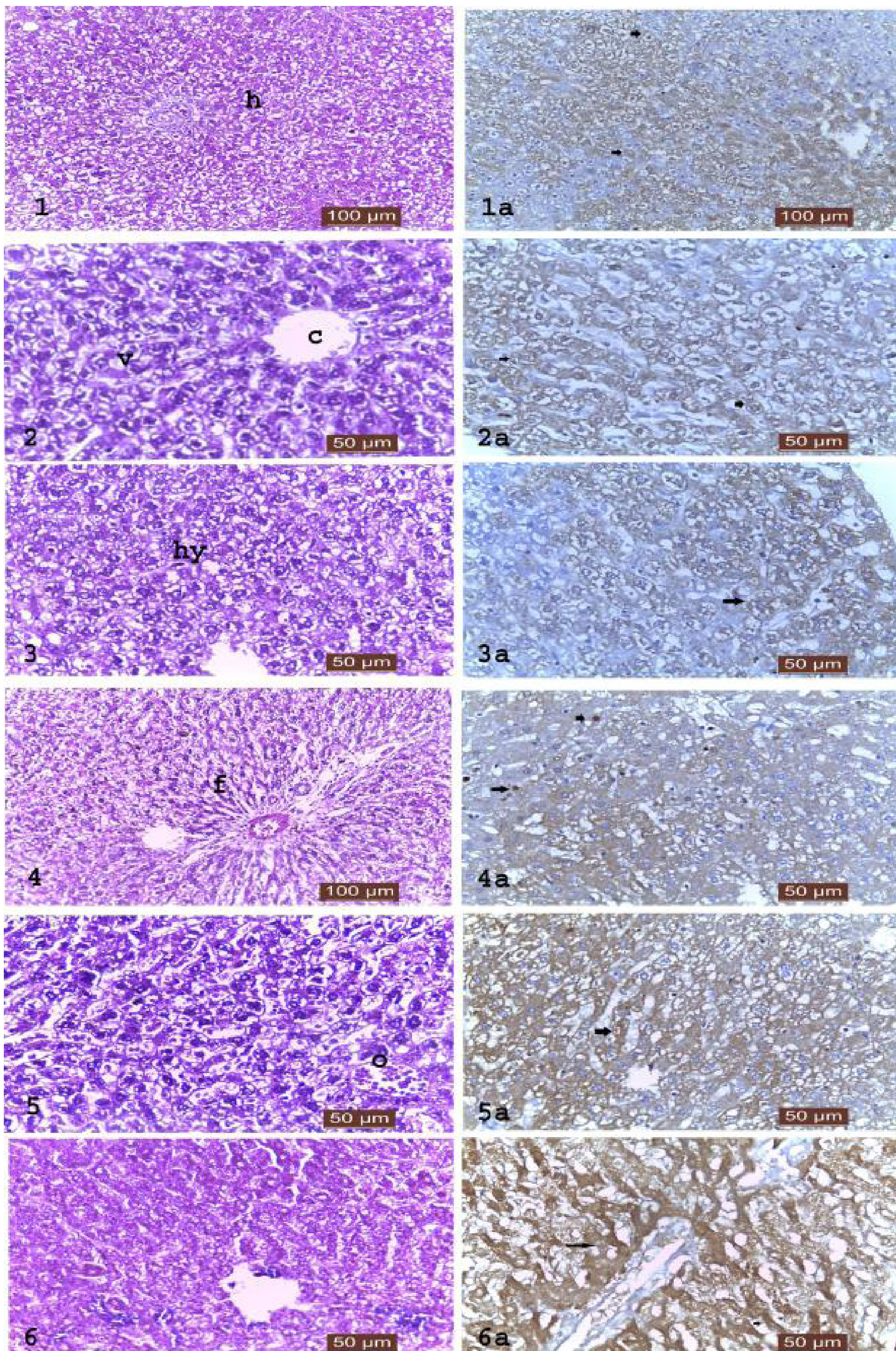
The brain at 0-12 hours showed different sized nerve cells distributed all over the contour of the cerebrum. Positive PCNA immunostined materials in the cytoplasm of the nerve cells (Fig.7 and 7a). At 24 hours the brain showed degenerated nerve cells with hemorrhagic blood vessels. The nerve cells cytoplasm was positive PCNA immunostaing while negative nuclear PCNA immunostaining. (Fig.8 and 8a). The brain at 48-96 hours showed vacuolation , degeneration and liquification in the nerve cells. Negative PCNA immunostaing (Fig.9 and 9a).

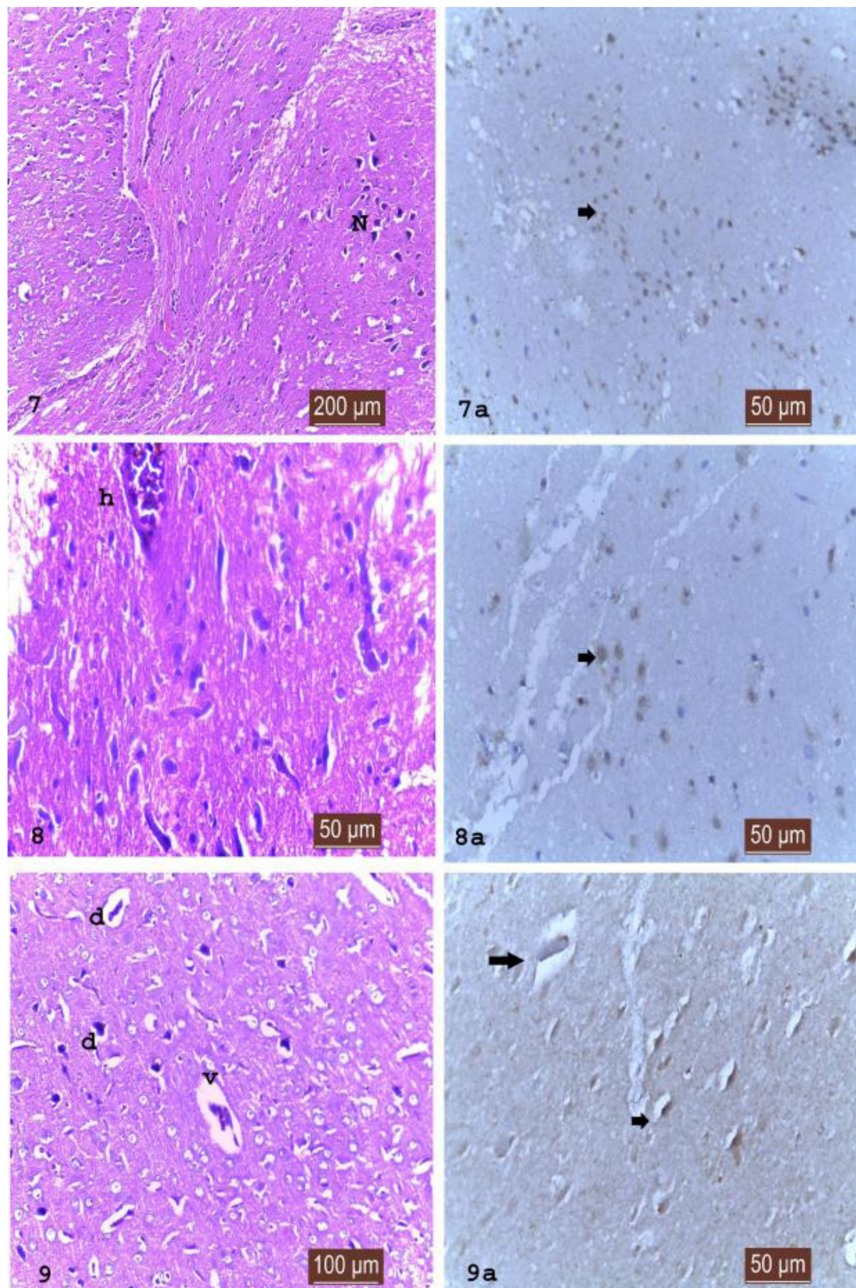
Figure legends

Photomicrograph (1) of the liver at 0 and 3 hours showing; hepatic cords (h). H&E Scale bar 100. While the cells (1a) showed positive immunostaining for PCNA . PCNA immunostaining Scale bar 100 um. The liver at 6 and 9 hours showed vacuolar degeneration (v). Fig (2) H&E Scale bar 50 um. The hepatocytes were showed positive PCNA. (fig.2a) PCNA immunostaining Scale bar 50um. In (fig.3) the liver at 12 hours showed hydrobic degeneration (hy) . H&E Scale bar 50 um. Some hepatocytes showed positive cytoplasmic PCNA (Fig.3a) PCNA immunostaining Scale bar 50um. In (Fig.4) the liver at 24 hours showed loss of architecture with increased fibrous formation (f). H&E Scale bar 100 um. Few hepatocytes showed positive cytoplasmic PCNA (Fig. 4a) PCNA immunostaining Scale bar 50um. In (Fig.5) the liver at 48 hours showed degenerated hepatocytes with diffused odema (o). H&E Scale bar 50 um. Few hepatocytes showed positive cytoplasmic PCNA immunostaining (Fig.5a) PCNA immunostaining Scale bar 50um. In (Fig.6) liver at 72 and 96 hours showed fibrous structures with no architecture. H&E Scale bar 50 um .Negative PCNA immunostaing (Fig.6a). PCNA immunostaining Scale bar 50um.

Photomicrograph (7) of the brain at 0-12 hours showed different sized nerve cells (N). H&E Scale bar 200 um. Positive PCNA immunostined materials in the nerve cells (Fig. 7a). PCNA immunostaining Scale bar 50um. In (Fig.8) brain at 24 hours showed degenerated nerve cells with hemorrhagic (h) blood vessels. H&E

Scale bar 200 um. The nerve cells cytoplasm was positive while negative nuclear PCNA immunostaining. (Fig. 8a). PCNA immunostaining Scale bar 50um. In (Fig.9) brain at 48-96 hours showed vacuolation (v) and liquification in the nerve cells (d). H&E Scale bar 100 um .Negative PCNA immunostaing (Fig.9a). PCNA immunostaining Scale bar 50um





Conclusion

Gapdh showed less stability with significant decreases in transcript levels in liver with increasing PMI (up to 48 hour). However, it shows low correlation between Gapdh transcript and PMI in brain. while PCNA immunohistochemical for cell viability can give stable results about the time of death .

Conflict of Interest. We declare that this paper has no conflict of interest

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