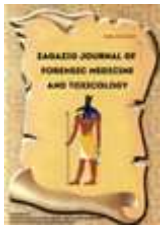

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Original article

Novel approaches for postmortem interval estimation: A biochemical, histopathological, immunohistochemical, and comet assay study of heart tissue

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Abstract:

Background: The postmortem interval (PMI) is a frequently investigated topic. The significance of obtaining a precise PMI in medicolegal death investigations is crucial, as it aids in decision-making concerning suspects, determining the timing of the assault relative to the time of death, and formulating conclusions regarding the suspects involved. **Aim:** The study used a time-dependent experiment utilizing an adult male albino rat model to estimate the postmortem interval by analyzing specific biochemical parameters, histopathological changes, immunohistochemical variations, and comet assay results. **Materials and methods**: This in-vivo experimental study involved 30 rats divided into five groups at the 0, 6, 12, 24, and 48-hour postmortem points. Hearts were extracted from each group, and oxidative stress biomarkers, including catalase (CAT), reduced glutathione (GSH), Nitric oxide (NO), and Malondialdehyde (MDA) were assessed. Additionally, histopathological analysis was conducted using hematoxylin and eosin (H&E) stain, immunohistochemical labeling of Bcl-2, and comet assay to determine the rate of DNA degradation . **Results:** In the cardiac tissue of all rats with increasing postmortem interval, there was a significant increase in MDA and NO levels. Simultaneously, there was a significant decrease in CAT and GSH levels. The histopathological findings revealed normal histological structures in the 0 and 6 hrs postmortem groups. After 12 hours, slight endomysium edema was observed. After 24 hours, there was an increase in interstitial edema as well as a mild presence of mononuclear cell infiltrates and vacuolated sarcoplasm in some myofibers. Additionally, the 48-hour postmortem group exhibited increased interstitial edema, fragmented myofibers, and coagulation necrosis of myofibers. Immunohistochemical staining of Bcl-2 demonstrated a decrease in expression correlated with an increased postmortem interval with minimal expression observed in the 48-hour postmortem group. The comet assay demonstrated a significant increase in Tail length, tail DNA %, and tail moment mean values with increasing PMI. **Conclusion:** The assessment of biochemical parameters of heart tissue, along with histopathological and immunohistochemical alterations, as well as comet assay findings, can serve as supplementary tools for precise estimation of the postmortem interval.

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I. Background

Determining the postmortem interval, commonly referred to as the time elapsed since death, is the most significant medicolegal issue in postmortem assessments (Sutton et al., 2024). Precise PMI estimation is crucial in civil and criminal proceedings since it aids in the determination of the guilt or innocence of suspects, identifying the precise time of a crime, and addressing certain inheritance-related legal matters (Zhang et al., 2024). Numerous factors, such as soil temperature, salinity, humidity, and ambient temperature, can substantially influence postmortem chemical and physical changes. These factors must be considered in determining the time since death (Onyejike et al., 2022).

The assessment of physiological and physical postmortem variations, including rigor mortis, death stains, alterations in body temperature, levels of decomposition, and the distribution and developmental stages of bugs on the corpse, is integral in determining the time since death (Zaki et al., 2017). However, due to the bias introduced by internal and external variables, these adjustments exhibit limitations in the determination of PMI (Noshy, 2021).

Thanatochemistry refers to the examination of the changes in a corpse's chemical composition that occur immediately following death. Paltian et al. (2019), illustrate that these chemical alterations can provide quantitative measures for identifying postmortem intervals in biological tissues. Postmortem, chemical alterations occur due to reduced blood oxygen levels, cellular decomposition accompanied by altered enzymatic reactions, and the halt of anabolic processes within cells (Khater et al., 2020).

In physiological environments, antioxidants benefit from the equilibrium between oxidants and antioxidants. Variations in oxidant and antioxidant levels indicate biochemical disturbances, as it is not feasible to assume that the body maintains oxidant/antioxidant equilibrium postmortem (Shaaban et al., 2017). The heart muscle exhibits a significant susceptibility to oxidative injury from free radicals due to the composition of the myocardium cell membrane, primarily composed of phospholipids. These phospholipids are particularly vulnerable to oxidative stress and possess limited antioxidant capacity (Ping et al., 2020).

Histological and immunohistochemical analyses of detrimental changes in body structures have been developed and integrated with additional methods for precise PMI estimations (Welson et al., 2021). Postmortem tissue anoxia, and apoptosis, known as programmed cell death, occur due to the lack of blood flow (Elmore, 2007). Apoptosis aids in the removal of dying cells from healthy tissues. According to Elvas et al. (2019), this kind of cell death is an essential physiological mechanism supporting normal growth and healthy tissue progression.

The B-cell lymphoma 2 (Bcl-2) protein families have been the subject of extensive research in the past decade due to their significance in the regulation of apoptosis. Its members demonstrate both pro- and anti-apoptotic behaviors. The sensitivity of a cell to apoptosis is influenced by the equilibrium between pro-apoptotic and anti-apoptotic protein groups (Panda et al., 2020; Qian et al., 2022). Elias et al. (2004) proposed that Bcl-2 may be a valuable marker for PMI. Approximately four minutes after death, a process known as autolysis initiates decomposition approximately four minutes postmortem. During autolysis, cellular constituents and metabolites are released and damaged as cells progressively degrade. This preliminary phase has led to research investigating postmortem DNA deterioration in relation to the time elapsed since death (Zapico and Adserias-Garriga, 2022).

Research aimed at estimating the postmortem interval via cardiac tissue is significantly limited. Therefore, addressing this gap would significantly improve our understanding and expertise in forensic science.

II. Methodology

Estimation of lipid peroxidation and antioxidant activity in the heart tissue:

NO, MDA, CAT, and GSH were measured by spectrophotometer using commercial kits in heart tissue homogenate according to the instructions of the manufacturer (Montgomery and Dymock, 1961; Ohkawa et al., 1979; Aebi, 1984; Beutler et al., 1963).

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Histological study

Light microscopic studies included the following (Suvarna et al., 2018):

- Hematoxylin and Eosin stains (H&E) to demonstrate the histological structure.
- Immunohistochemical staining for Bcl-2, a marker for apoptosis.

Pretreatment is essential for immunostaining. First, the specimen was boiled in 10 ml of pH 6 citrate buffer for 10 minutes to extract the antigen. The portions were then allowed to cool for twenty minutes at room temperature. The specimens were incubated with primary antibodies for one hour. Immune staining was performed using ultra-vision detection Finally, slices were counterstained using Mayer's hematoxylin (Suvarna et al., 2018).

Histo-morphometry

Mean area percentage of Bcl-2 positive cells in immunostained cardiac sections (×400 magnification). Measurements were conducted using ten distinct fields from diverse areas within each experimental group. The images were analyzed by the Dentistry Research and Equipment Unit at the Faculty of Dentistry, Cairo University, using an image analysis computer system from Leica Microsystems LTD (DFC 295) in Germany.

DNA degradation assessment in heart tissue (Single cell gel Electrophoresis (Comet) assay)

The technique was applied at the Animal Reproduction Health Research Institute, Giza, Egypt. Using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) and a CCD camera, we could quantitatively and qualitatively determine the extent of DNA damage in the cells by calculating the length of DNA immigration and the percentage of immigrated DNA. This was achieved by examining (Ethidium Bromide) EtBr-stained DNA with a 40x objective on a fluorescent microscope. The resulting image showed comets with fragmented DNA in the tail and a core spherical shape (the head area), which contained most of the long-stranded DNA still intact in the nucleus (Shukla, 2017).

Data analysis: The 28th version of the SPSS software (IBM Co., Armonk, NY, USA) was utilized to evaluate the biochemical and morphometrical measurements studied statistically. Quantitative parametric data were represented using the standard deviation (SD) and mean. The data were statistically analyzed utilizing one-way analysis of variance (ANOVA) and subsequent post hoc testing using the least significant difference (LSD) method. The level of statistical significance was set at $P < 0.05$.

III. Results

Biochemical results

Regarding the CAT and GSH levels, there was a significant decrease in advanced PMI in the five PM time points by ANOVA. By comparing in-between groups using LSD, there was a time-dependent decrease in heart tissue levels of CAT and GSH (Table 1) and (Figure 1 A & B).

ANOVA indicated a significant increase in NO and MDA levels with advanced PMI in the five PM time points. The in-between group comparison using LSD revealed a time-dependent increase in heart tissue levels of NO and MDA (Table 1) and (Figure 1 C & D).

Table (1): Oxidative stress markers at postmortem intervals 0, 6, 12, 24 and 48 hours:

Period in hours	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	F	P-value
N=6			Mean ±SD				
Catalase (CAT) mu/mg	3.90±0.10	3.20±0.11[a]	3.00±0.11[ab]	2.75±0.05[abc]	2.35±0.05[abcd]	236.964	<0.001**
GSH mu/mg	151.0±2.19	133.00±3.28[a]	129.50±0.54[ab]	122.50±3.8[abc]	98.00±2.19[abcd]	313.411	<0.001**
Nitric oxide (NO) umol/mg	18.20±0.61	29.80±0.98[a]	42.70±1.97[ab]	53.00±2.41[abc]	63.95±4.21[abcd]	342.737	<0.001**
MDA nmol/mg	2.77±0.11	2.98±0.02[a]	3.28±0.08[ab]	3.51±0.02[abc]	3.75±0.05[abcd]	198.137	<0.001**

N.B All values are expressed as mean ±SD. SD: standard deviation, hrs: hours, F One-way ANOVA, **: statistically highly significant ($p<0.001$), N: number of rats=6. LSD following ANOVA expressed as letters: [a]$p<0.001$ significant versus 0 h group, [b]$p<0.001$ significant versus 6 h group, [c]$p<0.001$ significant versus 12 h group, [d]$p<0.001$ significant versus 24 h group.

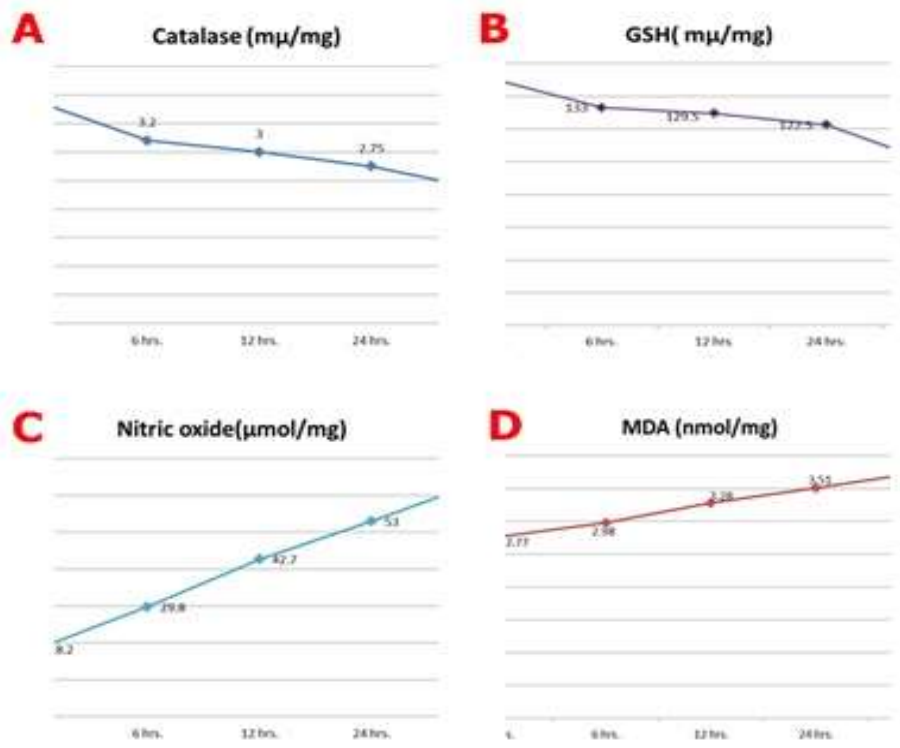

Figure (1): A Line graph displaying the heart's mean CAT levels (mu/mg) (A), GSH levels (mu/mg) (B), NO levels (umol/mg) (C), and MDA concentrations (nmol/mg) (D) at 0, 6, 12, 24, and 48 hours after death.

Histological Results

Light microscopic studies

Heart sections stained with H&E in the 0 and 6-hour postmortem groups exhibited the typical histological structure of striated, branching cardiomyocytes characterized by centrally located solitary oval nuclei and acidophilic sarcoplasm. Additionally, intercalated discs connected the myofibers. Flat, black fibroblast nuclei were observed in the thin interstitial tissue (Figure 2 A & B). Mild endomysium edema was observed in the 12-hour postmortem group (Figure 2 C). The cardiac muscle bundles in the 24-hour postmortem group exhibited elevated interstitial edema and minimal mononuclear cell infiltration. Additionally, degraded myofibers were mainly represented by vacuolated sarcoplasm (Figure 2 D). In the 48-hour postmortem group, the heart muscles displayed coagulative necrosis and myofibers fragmentation characterized by loss of hyper-eosinophilic sarcoplasm and lack of striations with missing or pyknotic nuclei. There was an increase in interstitial edema compared to previous groups. Bacterial colonies were identified, particularly within interstitial tissue (Figure 2 E).

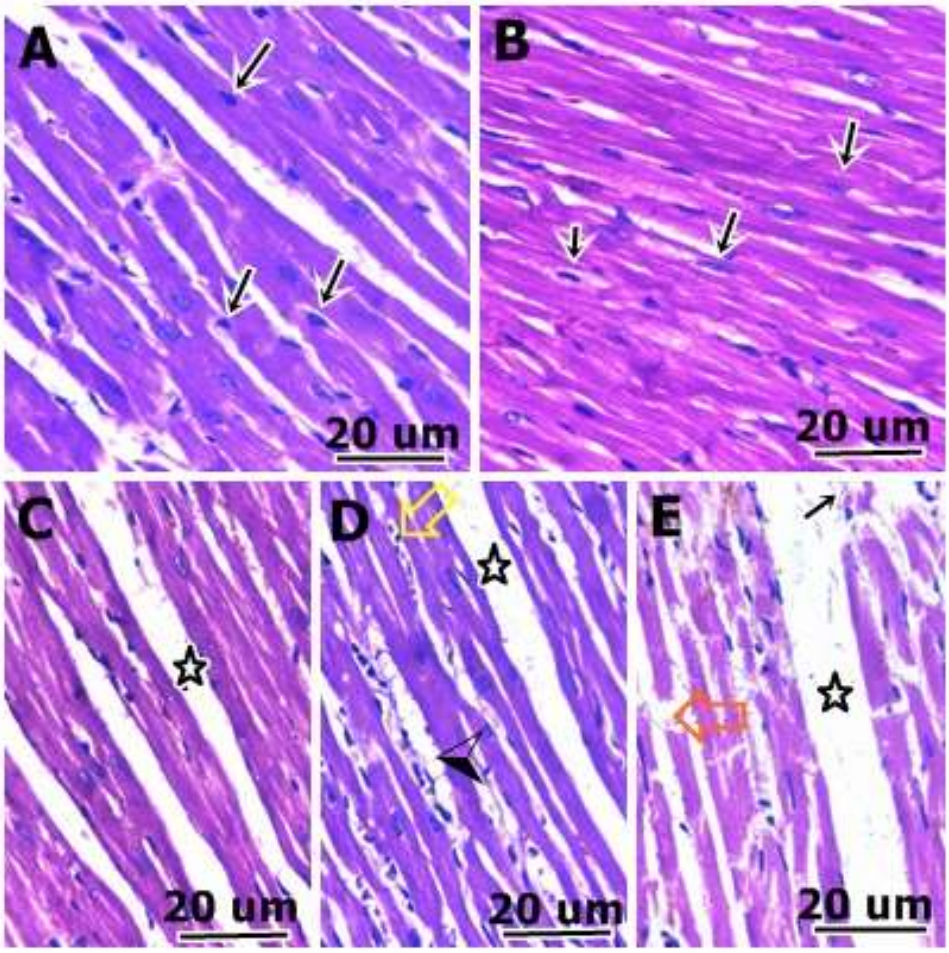

Figure (2): A photomicrograph of H&E-stained heart muscle sections at zero time (A) and six hours postmortem (B) illustrates the characteristic histological features of striated cardiomyocytes, including a single oval nucleus located centrally (arrow). Mild endomysium edema was observed 12 hours postmortem. At 24 hours postmortem, mild infiltration of mononuclear cells (yellow arrow) is observed, accompanied by vacuolated sarcoplasm in certain myofibers (arrowhead) and increased interstitial edema (star). At 48 hours postmortem, increased interstitial edema is observed (star), along with coagulative necrosis of myofibers (arrow) and fragmentation of myofibers (red arrow). (H&E x 400; 20 µm scale bar).

Immunohistochemical results

Immunohistochemical staining of various heart sections from different groups at specific postmortem intervals for Bcl-2 revealed elevated intra-sarcoplasmic expressions in both the 0 and 6-hour postmortem groups (Figure 3 A & B). The number of positively expressed cells was slightly decreased in the 12-hour postmortem group (Figure 3 C). A significant reduction in expression was observed in both the 24-hour postmortem group (Figure 3 D) and the 48-hour postmortem group (Figure 3 E).

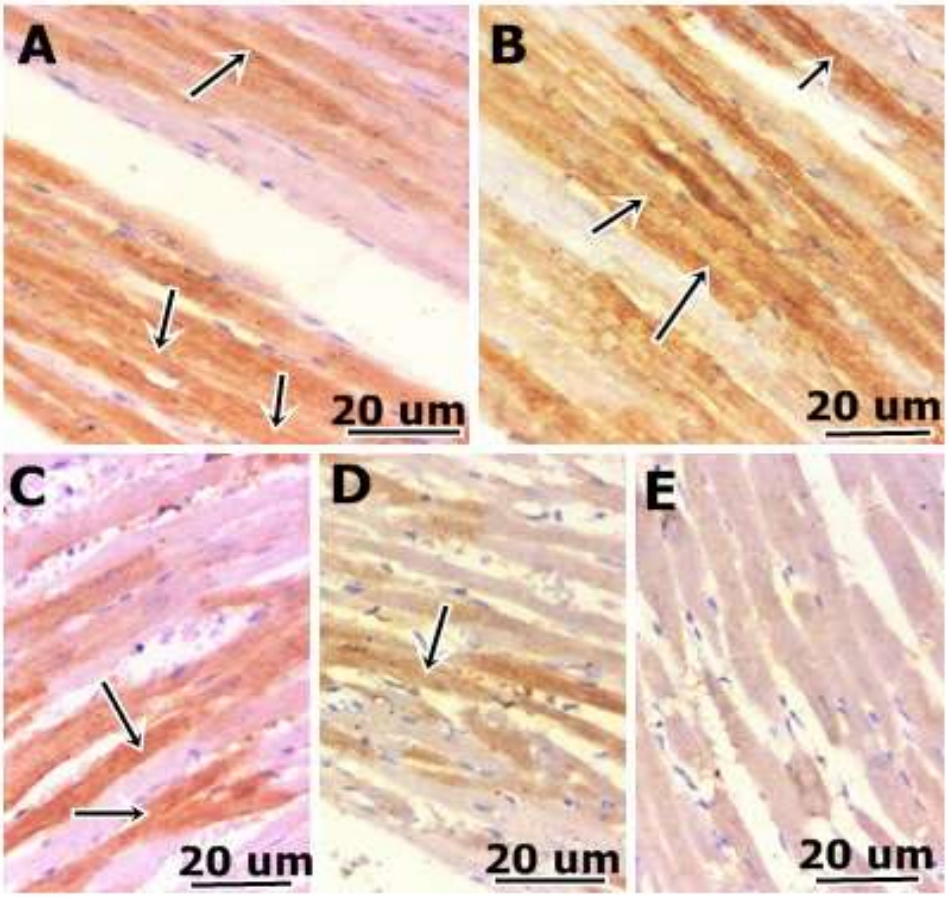

Figure (3): Immunostained sections of heart tissue for the Bcl-2 marker after death show a high level of intra-sarcoplasmic expressions in both zero-time (A) & 6 hrs post-mortem (B). (C): reveals slightly reduced sarcoplasmic expressions at 12 hrs postmortem. (D): demonstrates markedly reduced expression at 24 hours postmortem. (E): with minimally perceptible expressed cells at 48hrs postmortem. (Immunoperoxidase technique x 400; scale bar 20 μm).

Mean area percentage of Bcl-2 expression

ANOVA results indicated a significant decrease in the mean area percentage of Bcl-2 expression with advanced PMI across the five PM time points.Comparative analysis of in-between groups using LSD revealed a time-dependent reduction in Bcl-2 expression in heart cells (Table 2) and (Figure 4).

Table (2): Bcl-2 percent at postmortem intervals 0, 6, 12, 24 and 48 hours:

Period in hours	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	F	P-value
N=6			Mean ±SD				
Bcl-2 (%)	68.67±4.92	60±1.78[a]	56±0.89[ab]	17.00±4.47[abc]	4.67±2.25[abcd]	456.638	<0.001**

N.B All values are expressed as mean ±SD. SD: standard deviation, hrs: hours, F: repeated measure ANOVA, **: statistically highly significant (p<0.001), N: number of rats=6. LSD following ANOVA expressed as letters: [a]p<0.001 significant versus 0 h group, [b]p<0.001significant versus 6 h group, [c]p<0.001 significant versus 12 h group, [d]p <0.001significant versus 24 h group.

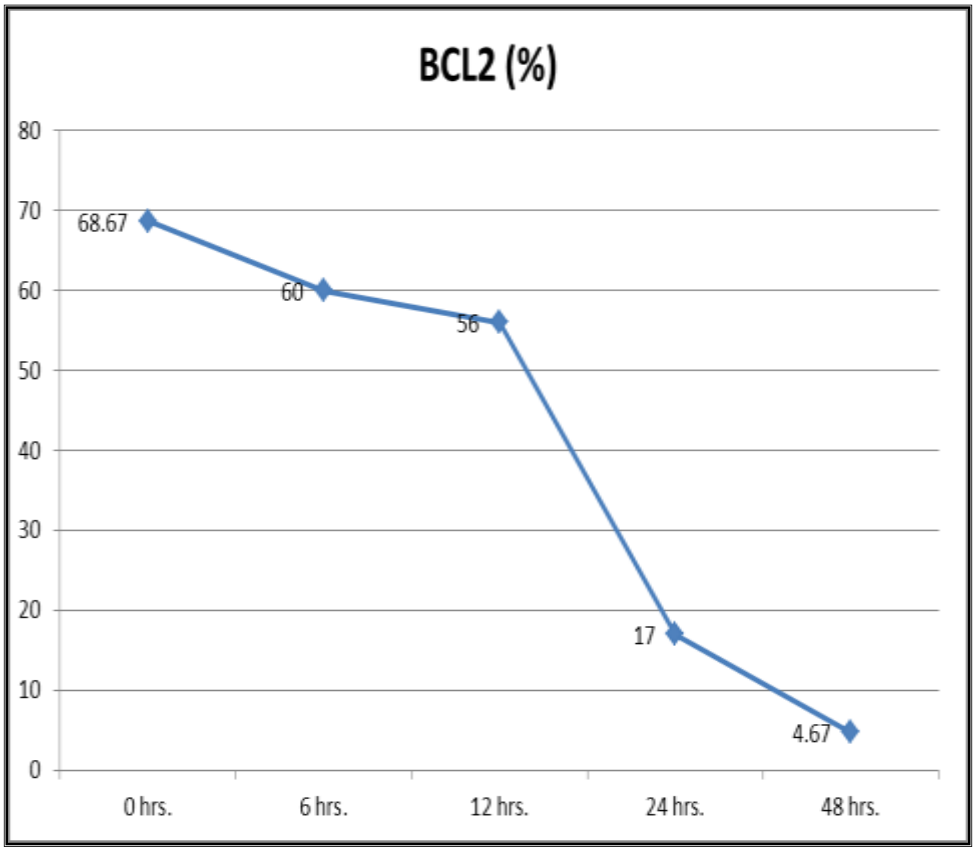

Figure (4): A Line graph displaying the heart's Bcl-2 mean values at 0, 6, 12, 24, and 48 hours after death.

Comet assay (single-cell gel electrophoresis) results

A significant increase in Tail length, Tail DNA %, and Tail moment mean levels was observed with advanced PMI across the five PM time points, as indicated by ANOVA (Table 3 and Figure 5 A, B & C). Comparison of intergroup data using LSD revealed a time-dependent increase in tail length, tail DNA %, and mean values of the tail moment (Table 3).

Table (3): Comet parameters at postmortem intervals 0, 6, 12, 24 and 48 hours:

Period in hours	0 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	F	P-value
N=6			Mean ±SD				
Tail length (µm)	0.73±0.16	1.60±0.03[a]	1.90±0.01[ab]	2.21±0.11[abc]	2.85±0.11[abcd]	339.497	<0.001**
Tail DNA (%)	0.49±0.08	1.57±0.04[a]	1.73±0.09[ab]	2.03±0.2[abc]	2.44±0.11[abcd]	257.381	<0.001**
Tail moment (UNIT)	0.36±0.14	2.51±0.02[a]	3.29±0.21[ab]	4.77±0.97[abc]	6.62±0.59[abcd]	121.743	<0.001**

N.B All values are expressed as mean ±SD. SD: standard deviation, hrs: hours F: repeated measure ANOVA, **: statistically highly significant ($p<0.001$), N: number of rats=6. LSD following ANOVA expressed as letters: [a]$p<0.001$ significant versus 0 h group, [b]$p<0.001$significant versus 6 h group, [c]$p<0.001$ significant versus 12 h group, [d]$p<0.001$significant versus 24 h group.

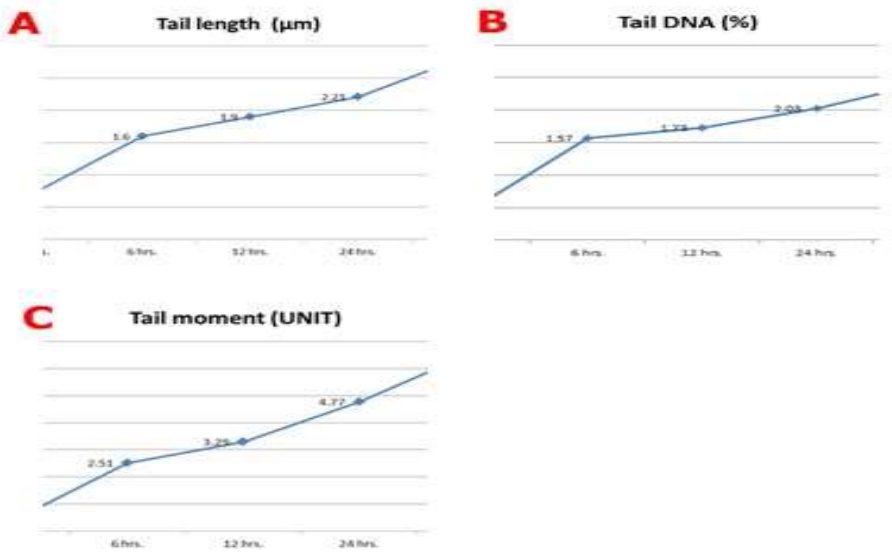

Figure (5): A Line graph showing the comparative magnitude of mean values of tail length (µm) (A), mean Tail DNA percentage levels (B), and mean values of Tail moment (C) in cardiac tissues measured at 0, 6, 12, 24 and 48 hours postmortem.

Comet figures in cardiac cells

The comet assay results indicated that immediately postmortem (0 h), most cardiac cells exhibited a spherical morphology with a complete head, while the comet tail was minimal. Over the next 48 hours, both the length (µm) and concentration (%) of the tails progressively increased, indicating that greater DNA damage correlates with longer tails. The cardiac cells exhibited an elevated rate of DNA degradation, evidenced by an increase in tail length, tail DNA percentage, and comet tail moment. All these measures increased concurrently with advanced postmortem interval (Figure 6).

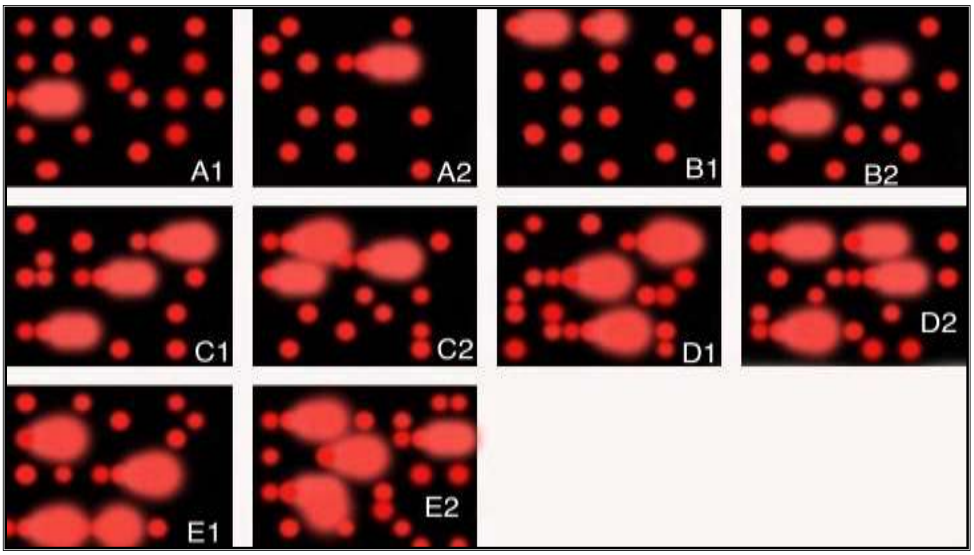

Figure (6): Images of comet assay (single-cell gel electrophoresis) of the cardiac cells of albino rats at different PMI from 0 to 48 hrs (A1&A2 represent 0 hr, B1&B2 represent 6 hrs, C1&C2 represent 12 hrs, D1&D2 represent 24 hrs, E1&E2 represent 48 hrs postmortem groups) demonstrate the nuclear DNA degradation process; as PMI increases, more abnormal tailed nuclei and damaged cells are formed (×400).

IV. Discussion

The biological processes associated with death remain largely unexplored. Typically, death occurs due to irreversible failure in the respiratory and circulatory systems (Emam et al., 2022).

The body undergoes various chemical and physical transformations postmortem. The changes are progressive, irreversible, and inevitable. The events occur in a consistent order as they progress (Brooks, 2016).

Accurate estimation of PMI continues to be a complex process, requiring the integration of various methodologies despite significant efforts to improve it. This study aimed to examine the relationship between PMI and oxidative stress markers, histopathological alterations, Bcl-2 immunohistochemical expression, and DNA degradation in the cardiac tissues of rats within 48 hours.

Oxidative stress is characterized by an imbalance between the production and elimination of free radicals, resulting in an excess generation. The oxidative stress markers measure the degree of oxidative stress (Jomova et al., 2023).

This study demonstrated a notable elevation in MDA and NO levels. A significant decrease in both CAT and GSH levels was observed in the heart tissue of all rats with advanced postmortem intervals in the five PM time points.

Ozturk et al. (2013) examined the rat femoral muscle and found a substantial correlation between oxidant/antioxidant parameters and tissue injury and repair. Their findings are consistent with the current study's results. The levels of oxidants in the tissue were significantly increased, while antioxidant levels in the rat femoral muscle began to decrease two hours PM.

El-Noor et al. (2016) examined the oxidative stress parameters in the deceased rats' heart and kidney tissues, consistent with the current study. The postmortem interval exhibited statistically significant negative associations with CAT and positive correlations with MDA and NO.

Zaki et al. (2017) reported a statistically significant difference in antioxidant and oxidant values in rat brain and skeletal muscle tissues from 0 to 96 hours postmortem. Between 0 and 96 hours postmortem, a substantial decrease in both GSH content and CAT activity was observed. The observed decline aligned with the time elapsed since death, exhibiting maximum inhibition at 96 hours. Shaaban et al. (2017) observed that in the rat brain, GSH levels started to decrease significantly at 5-6 hours postmortem, while CAT levels were noted to decline considerably at 3-4 hours. Regarding oxidant parameters, it was found that MDA showed a significant increase at four to five hours in the rat brain and at three to four hours in the rat liver after death. Conversely, there has been a notable increase in NO levels in the rat brain at 2-3 hours and in the rat liver at 3-4 hours postmortem.

Furthermore, several studies demonstrated a substantial correlation between the PMI and oxidative stress parameters found in rats' hearts, kidneys, and testicles. Nonetheless, they indicated that, at times, the kidney remained unaffected by oxidative injury, while the heart exhibited the most optimal PMI correspondence (El-Noor et al., 2016; Welson et al., 2021). The variations in the amount, composition, and functions of these enzymes in the various tissues may contribute to the variations in the enzyme responses (Shaaban et al., 2017).

Changes in oxidant/antioxidant variables can be assessed through variations in oxidant and antioxidant variables, as the body may fail to regulate endogenous oxidant/antioxidant balance after death (Mostafa et al., 2021). This can account for the highly significant decrease in GSH and CAT activity and the concurrent rise in MDA and NO levels observed in the current study with prolonged postmortem intervals.

The present study confirmed alterations in oxidant/antioxidant status through histopathological changes, including increased interstitial edema, mononuclear cell infiltrates, coagulative necrosis, and fragmentation of cardiac myofibers observed in the 48-hour postmortem group. Additionally, immunohistochemical analysis revealed a reduction in Bcl-2 expression in heart cells correlated with an extended postmortem interval.

Histopathology is typically utilized to determine the cause of death and has been a significant tool in the forensic area. A thorough analysis of all detectable alterations in the decomposition of human tissues and organs would ideally reveal a distinct pattern that can utilized to determine PMI (De-Giorgio et al., 2024). Immunohistochemistry serves as a valuable

supplementary tool in determining the cause of death, establishing causality, and estimating PMI (Huang et al., 2024).

Omran et al. (2022) found that cardiac samples collected from all study groups immediately postmortem and after four hours exhibited preserved histological arrangements, thereby validating these histological findings. The myocardium exhibited hyaline degeneration, moderate interstitial edema, and round cell infiltrations, which deteriorated with an extended PM interval at 12 and 24 hours after death.

In addition, Welson et al. (2021) reported progressive autolysis and the deterioration of cellular structure and organization resulting from anoxia and oxidative stress postmortem, aligning with our findings. The longitudinal cardiac muscle fibers exhibited expanded interstitial spaces and a wavy morphology within the first 24 hours postmortem. However, the fibers were reduced in size, and nuclear flattening was observed as a consequence of necrosis. The heart tissue exhibited widely spaced myofibers with blood leakage after 48 hours.

El-Noor et al. (2016) demonstrated a progressive decline in the histological analysis of the cardiac tissues of rats with an increasing postmortem interval, which is consistent with the current findings. However, their study focused on a shorter PMI, lasting only seven hours. The current study's findings align with a related investigation by Yahia et al. (2018), which observed that the histological structure of dog heart tissue was normal at the time of death. Characteristic autolytic alterations were observed 24 and 48 hours postmortem, including an increase in cytoplasmic eosinophilia due to protein denaturation, nuclei exhibiting pyknosis with altered positions, and a widening of interfibrillar gaps.

Ozturk et al. (2013) examined the onset of autolytic changes in muscles in relation to significant increases in DNA damage, noting a correlation between the extent of degeneration and alterations in oxidant and antioxidant parameters. Mitochondrial dysfunction leads to cellular death, subsequently resulting in lysosomal membrane rupture and enzymatic activity. These alterations occur sequentially and may be associated with PMI (Rajkumari et al., 2020).

The histological degradation of heart tissue in this investigation agrees with the findings of Mostafa et al.(2021). Their study revealed that postmortem hypoxia causes ATP loss, resulting in the cessation of various energy-dependent cellular processes. This includes the dysfunction of ion pumps, which contribute to cellular swelling and Ca2+ influx, as well as glycogen depletion, which elevates lactic acid levels and decreases cellular pH. These conditions result in atrophic changes, degradation, and a decrease in protein synthesis. Prolonged hypoxia intensifies ATP depletion and facilitates the accumulation of free radicals, leading to cellular membrane damage by cross-linking proteins and fatty acids peroxidation.

The anti-apoptotic protein B cell lymphoma-2 (Bcl-2) is found in the outer mitochondrial wall and regulates mitochondrial permeability to limit the intrinsic apoptotic pathway (Azad and Iyer, 2014). Our findings align with those of Xie et al. (2024), who investigated Bcl-2 as an inhibitor of apoptotic protein in mouse skin. Additionally, Welson et al. (2021) reported that Bcl-2 immunostaining of adult male rats' excised heart sections revealed high expression in the majority of the heart muscle fibers 12 hours after death, which began to diminish 24 hours later and reached negative expression at 96 hours.

The current study's results agree with those of Mohamed et al. (2017), who found that an immunohistochemistry analysis of the apoptotic inhibitor Bcl-2 in rabbit brain tissue showed a time-dependent decrease in expression values from six to twelve hours postmortem. Our study, however, followed all the measured postmortem alterations over a prolonged period. Consistent with the current findings, a study by Elias et al. (2004) on deceased human skin indicated a significant reduction in Bcl-2 levels associated with postmortem interval (PMI) data. Additionally, both p53 and Bcl-2 expressions exhibited variations in response to PMI, implying that Bcl-2 may serve as a valuable marker for PMI estimation.

Following death, tissue anoxia and apoptosis or programmed cell death are exacerbated by the lack of blood flow. The two main routes for apoptotic signaling are extrinsic and intrinsic (mitochondrial). Various intracellular conditions can initiate the intrinsic route, including hypoxia, DNA damage, and free radicals (Noshy,2021).

The Bcl-2 family of proteins mostly synchronizes apoptosis or programmed cell death. The proteins are

categorized into two primary subgroups: anti-apoptotic and pro-apoptotic members. Anti-apoptotic proteins, including Bcl-2, Bcl-XL, Mcl-1, and Bcl-2L10, inhibit pro-apoptotic factors to maintain the integrity of the outer mitochondrial membrane (Opferman and Kothari, 2018).

Two primary variables were identified as the cause of the delayed decrease in Bcl-2 expression: (1) the cell's resistance to undergoing apoptosis and (2) the high concentration of free radicals causing surface cell protein degradation. According to Elias et al. (2004), there was evidence that the cells were attempting to suppress the process of apoptosis by up-regulating the expression levels of Bcl-2.

DNA is a fundamental and robust component of cells, exhibiting uniformity across individuals. DNA alterations are initiated by endogenous nuclease activity and hydrolytic damage, occurring immediately and maintaining consistency irrespective of temperature and the method of death. Therefore, a potential metric for assessing PMI is DNA (Sangwan et al., 2021). Numerous studies have indicated a linear relationship between DNA degradation and the time elapsed since death, yet there remains a limited understanding of the factors affecting the DNA deterioration process (Wenzlow et al., 2023).

Several techniques have been developed in recent decades. Numerous methods have emerged for assessing DNA and its relationship to postmortem interval estimation, as well as for the qualitative and quantitative

Consistent with the current results, Zheng et al. (2012) examined DNA disintegration in mouse brain and dental pulp cells through the comet test for durations extending up to 72 hours postmortem. The study utilized linear regression analysis to establish a significant correlation between comet parameters and PMI.

Zhen et al. (2006) investigated comet characteristics that indicate DNA degradation in rat heart samples at varying PMI for a maximum of three days. Additionally, they found a significant association between the expansion of PMI and the rates of DNA degradation.

Postmortem DNA degradation occurs due to exogenous nucleases produced by microbes, ambient invertebrates, or endogenous nucleases released from host cells. Furthermore, additional modifications to DNA structure will proceed at a markedly reduced rate as a result of spontaneous degradation, hydrolysis, and oxidation. DNA fragmentation is a time-dependent process that can

analysis of nuclear DNA degradation. One of the prominent methods in this area is single-cell gel electrophoresis, commonly known as the "comet assay" (Jellinghaus et al., 2019).

The values of the current work revealed a significant increase in DNA degradation represented by comet parameters (tail length, tail DNA%, and tail moment) in cardiac tissues of rats as postmortem interval increased from zero to 48 hours.

Consistent with our findings, Mostafa et al. (2021) observed a significant increase in the proportion of DNA disintegration in rat muscle tissues from 6 to 144 hours postmortem, as evidenced by the comet assay results. Comparable results were observed by Mohamed et al. (2020), who used the comet technique to examine DNA fragmentation in the salivary gland and palatal mucosa of albino rats 48 hours after death. The findings revealed a significant correlation between PMI and the length and moment of the broken DNA tail in every sample from both tissues.

The results corroborate the previous study by Zaki et al. (2017), which examined the connection between DNA alterations in rat skeletal muscle samples. They found a strong association between these alterations and PMI (0-96 h postmortem). Furthermore, Larkin et al. (2010) found an inverse relationship between the time passed since death and the DNA recovered from muscle samples.

Furthermore, Gomaa et al. (2013) found a high association between early PMI (0-24 h) and increases in tail DNA, tail length, and tail moment in the brain and liver tissues with advancing PMI.

Shukla's (2017) study aimed to extend the PMI from 0 to 56 hours. The comet assay parameters revealed an increase in DNA fragmentation. The results confirmed the comet assay as a more accurate, consistent, and appropriate methodology that could serve as an alternative to other conventional methods for PMI measurement.

be measured (Zaki et al., 2017). This may explain the significant increase in all comet metrics observed in the current study, which rose with longer postmortem intervals (tail length, tail DNA%, and tail moment).

V. Conclusions

The progression of PMI is directly associated with heart oxidative stress markers, histopathological changes, immunohistochemical expression, and the rate of DNA degradation. Our study revealed a notable increase in MDA and NO levels, accompanied by a significant decrease in CAT and GSH levels. The histopathological analysis indicated normal histological structures at both 0 and 6 hours postmortem. Endomysium edema commenced after 12 hours and progressively intensified along with increased interstitial edema and mild mononuclear cell infiltration. At 48 hours postmortem, coagulative necrosis and fragmentation of myofibers were observed. Immunohistochemical staining of Bcl-2 demonstrated a decrease in expression correlated with an increased postmortem interval, with minimal expression observed at 48 hours postmortem. The comet assay showed a significant increase in tail length, tail DNA percentage, and mean tail moment values with increasing PMI. The identified changes revealed a sequential time-dependent method applicable for precise PMI valuation.

VI. Recommendations

Studying DNA degradation across various causes of death, utilizing multiple tissues and techniques, is recommended to determine the most effective method for identifying the cause of death and its impact on the rate of DNA degradation.

Declarations

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Data availability statement: The data used during the present research are available from the corresponding author upon reasonable request.

Conflict of interest: Not declared.

VII. References

Aebi, H. (1984): Catalase in vitro. Methods in enzymology. Academic Press; 105: 121-12.

Azad, N. and Iyer, A. K. V. (2014): Reactive oxygen species and apoptosis. In: Laher, I. (eds) System Biology of Free Radical and Antioxidants. Berlin; Springer-Verlag Berlin Heidelberg; 113-127. DOI: https://doi.org/10.1007/978-3-642-30018-9_15

Beutler, E.; Duron, O. and Kelly, B.M. (1963): Improved method for the determination of blood glutathione. The Journal of laboratory and clinical medicine; 61: 882-888. PMID: 13967893.

Brooks, J. W. (2016): Postmortem changes in animal carcasses and estimation of the postmortem interval. Veterinary pathology; 53(5): 929-940. DOI: 10.1177/0300985816629720

De-Giorgio, F., Bergamin, E., Baldi, A., Gatta, R., & Pascali, V. L. (2024): Immunohistochemical expression of HMGB1 and related proteins in the skin as a possible tool for determining post-mortem interval: A preclinical study. Forensic Science, Medicine and Pathology, 20(1), 149-165. DOI:10.1007/s12024-023-00634-1

Elias, E. Z. L. A. N.; Osman, K.; Aziz, S. M. A.; et al. (2004): Determination of the time of death based on basic histological stain and immunostain changes. Jurnal Sains Kesihatan Malaysia.; 2(2): 63-70.

Elmore, S. (2007): Apoptosis: a review of programmed cell death. Toxicologic pathology; 35(4): 495-516. DOI: 10.1080/01926230701320337

El-Noor, M. M. A.; Elhosary, N. M.; Khedr, N. F.; et al. (2016): Estimation of early postmortem interval through biochemical and pathological changes in rat heart and kidney. The American journal of forensic medicine and pathology; 37(1): 40-46. DOI: 10.1097/PAF.0000000000000214

Elvas, F.; Berghe, T. V.; Adriaenssens, Y.; et al. (2019): Caspase-3 probes for PET imaging of apoptotic tumor response to anticancer therapy. Organic & biomolecular chemistry; 17(19): 4801-4824. DOI: 10.1039/c9ob00657e

Emam, A.; Mujalid, H.; Altamimi, N.; et al. (2022): Classification of Postmortem Changes and Factors Affecting It. Journal of healthcare sciences; 2 (9):213-218. DOI: https://www.ncbi.nlm.nih.gov/books/NBK554464/

Gomaa, M. S.; Abd El-Khalek, A. M. and Sameer, M. M. (2013): The relationship between the postmortem interval and the DNA degradation in brain and liver of adult albino rats. Journal of American Science.; 9(5): 535-540. DOI: https://api.semanticscholar.org/CorpusID:212545332

Huang, W., Zhao, S., Liu, H., Pan, M., & Dong, H. (2024): The Role of Protein Degradation in Estimation

Postmortem Interval and Confirmation of Cause of Death in Forensic Pathology: A Literature Review. International Journal of Molecular Sciences, 25(3), 1659. DOI: 10.3390/ijms25031659

Jellinghaus, K.; Urban, P. K.; Hachmann, C.; et al. (2019): Collagen degradation as a possibility to determine the postmortem interval (PMI) of human bones in a forensic context–A survey. Legal Medicine; 36: 96-102. DOI: 10.1016/j.legalmed.2018.11.009

Jomova, K.; Raptova, R.; Alomar, S. Y.; et al. (2023): Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. Archives of toxicology; 97(10): 2499-2574. DOI: https://doi.org/10.1007/s00204-023-03562-9

Khater, S. A., ElHakim, E. A., Aboulhoda, B. E., Rashed, L. A., & Shalaby, E. E. D. (2020): Evaluation of role of gapdh, gsk-3, b-catenin, DNA fragmentation and immunohisto chemistry of caspase 3 in estimation of post mortem interval in albino rats. International Journal of Medical Toxicology & Legal Medicine, 23(1and2), 176-190. DOI: 10.3390/diagnostics12092114

Larkin, B.; Iaschi, S.; Dadour, I.; et al. (2010): Using accumulated degree-days to estimate postmortem interval from the DNA yield of porcine skeletal muscle. Forensic science medicine and pathology; 6, 83-92. DOI: 10.1007/s12024-009-9109-5

Mohamed, A.; Elbohi, K.; Sharkawy, N.; et al. (2017): Biochemical and apoptotic biomarkers as indicators of time elapsed since death in experimentally induced traumatic brain injury. SM Journal of Forensic Research and Criminology; 1(2): 1-7. https://doi.org/10.1016/j.bjbas.2018.01.001

Mohamed, M. A.; El Sherbeny, M. and Farag, D. A. (2020): A Comparative Study of Two Gel-Based Techniques to Detect the Relationship Between Postmortem Interval And Nuclear DNA Degradation In Different Tissues In Albino Rats. Egyptian Dental Journal.; 66: 175-186. DOI: 10.21608/EDJ.2020.77533

Montgomery, H. A. C. and Dymock, J. F. (1961): Nitric oxide assay. Analyst; 86: 414.

Mostafa, H. E. S.; El-Shafei, D. A.; Abouhashem, N. S.; et al. (2021): Could skeletal muscle changes provide a reliable method for estimating the time since death: A histological, biochemical, and DNA study. Australian Journal of Forensic Sciences; 6 (3): 1-13. DOI:10.1080/00450618.2021.1921272

Noshy, P. A. (2021): Postmortem expression of apoptosis-related genes in the liver of mice and their use for estimation of the time of death. International Journal of Legal Medicine; 135: 539-545. DOI: 10.1007/s00414-020-02419-5

Ohkawa, H.; Ohishi, N. and Yagi, K. (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry; 95(2): 351-358.

Omran, B. H. F.; Masoud, E. S. S.; Hussien, W. F.; et al. (2022): Impacts of Chronic Mesterolone Intake on Postmortem Changes in Heart of Adult Male Albion Rats (Biochemical and Histopathological Study): Egyptian Society of Clinical Toxicology Journal.; 10(2): 121-136. DOI: 10.21608/ESCTJ.2022.178085.1021

Onyejike, D.N.; Fischer, V.A.; Esomonu, U.G.; et al. (2022): Factors that influence decomposition timeline estimation in Anambra state, Nigeria. Egyptian Journal of Forensic Sciences; 12: 29. DOI:10.1186/s41935-022-00281-7

Opferman, J. T. and Kothari, A. (2018): Anti-apoptotic BCL-2 family members in development. Cell Death & Differentiation.; 25(1): 37-45. DOI: https://doi.org/10.1038/cdd.2017.170

Ozturk, C.; Talip Sener, M.; Sener, E.; et al. (2013): The investigation of damage in the muscle tissue with the oxidant/antioxidant balance and the extent of postmortem DNA damage in rats. Life Science Journal; 10(3): 1631-1637.

Paltian, J. J.; da Fonseca, C. A.; Pinz, M. P.; et al. (2019): Postmortem interval estimative through determination of catalase and Δ-aminolevulinate dehydratase activities in hepatic, renal, skeletal muscle and cerebral tissues of Swiss mice. Biomarkers; 24(5): 478-483. DOI: 10.1080/1354750X.2019.1619837

Panda, D.; Ray, D.; Behera, D.; et al. (2020): A review on apoptosis: when death precedes life. European Journal of Molecular & Clinical Medicine; 7: 1174-1182. DOI: 10.1080/01926230701320337

Ping, Z.; Peng, Y.; Lang, H.; et al. (2020): Oxidative stress in radiation-induced cardiotoxicity. Oxidative

medicine and cellular longevity; 2020: 3579143. DOI: 10.1155/2020/3579143

Qian, S.; Wei, Z.; Yang, W.; et al. (2022): The role of Bcl-2 family proteins in regulating apoptosis and cancer therapy. Frontiers in oncology; 12: 985363. DOI: 10.3389/fonc.2022.985363

Rajkumari, S.; Mensudar, R.; Naveen, N.; et al. (2020): Estimation of postmortem death interval from autopsied tongue tissue: A cross-sectional study. Journal of Oral and Maxillofacial Pathology; 24(3): 568. DOI: 10.4103/jomfp.jomfp_479_20

Sangwan, A.; Singh, S. P.; Singh, P.; et al. (2021): Role of molecular techniques in PMI estimation: An update. Journal of Forensic and Legal Medicine; 83: 102251. DOI: 10.1016/j.jflm.2021.102251

Shaaban, D. A. A. E. R.; Farrag, D. I. M.; Bayoumy, A.; et al. (2017): Estimation of early postmortem interval by biochemical changes in brain and liver of rats using some oxidant and antioxidant parameters. The Egyptian Journal of Forensic Sciences and Applied Toxicology; 17(1): 147-162. DOI: 10.21608/EJFSAT.2017.46108

Shukla, R. K. (2017): Forensic application of comet assay: an emerging technique. Forensic sciences research; 2(4): 180-184. DOI: 10.1080/20961790.2017.1379893

Sutton, L.; Gallagher, T. and Russo, H. (2024): A medicolegal approach to postmortem interval estimation. In Methodological and Technological Advances in Death Investigations. Academic Press; 155-187. https://doi.org/10.1016/B978-0-12-819394-5.00002-X

Suvarna KS, Layton C, Bancroft JD (2018): Bancroft's theory and practice of histological techniques E-Book. Elsevier health sciences

Welson, N. N.; Gaber, S. S.; Batiha, G. E. S.; et al. (2021): Evaluation of time passed since death by examination of oxidative stress markers, histopathological, and molecular changes of major organs in male albino rats. International Journal of Legal Medicine; 135: 269-280. DOI: 10.1007/s00414-020-02463-1.

Wenzlow, N., Mills, D., Byrd, J., Warren, M., & Long, M. T. (2023): Review of the current and potential use of biological and molecular methods for the estimation of the postmortem interval in animals and humans. Journal of Veterinary Diagnostic Investigation, 35(2), 97-108. DOI: 10.1177/10406387231153930

Xie, D. G.; Wang, X. M.; Li, J. H.; et al. (2024): Short-term postmortem interval estimation by detection of apoptosis-related protein in skin. Forensic Science, Medicine and Pathology; 1-6. DOI: 10.1007/s12024-023-00757-5

Yahia, D.; El-Amir, Y. O. and Sadek, A. A. I. (2018): Early postmortem biochemical and histopathological changes in the kidney, liver, and muscles of dogs. Comparative Clinical Pathology; 27(6): 1447-1455. DOI: https://doi.org/10.1007/s00580-018-2756-8

Zaki, A. R.; Tohamy, A. F. and Yaseen, N. E. H. (2017): Estimation of postmortem intervals by some biochemical changes and DNA degradation in rat brain and skeletal muscle tissues. Mansoura Journal of Forensic Medicine and Clinical Toxicology; 25(1): 59-78. Doi: 10.21608/MJFMCT.2018.47271

Zapico, S. C. and Adserias-Garriga, J. (2022): Postmortem Interval Estimation: New Approaches by the Analysis of Human Tissues and Microbial Communities' Changes. Forensic Sciences; 2(1): 163-174. https://doi.org/10.3390/forensicsci2010013

Zhang, W.; Dong, K.; Sun, J.; et al. (2024): The relationship between postmortem interval and protein changes in mice. Journal of Forensic and Legal Medicine; 101, 102618. DOI: 10.3390/ijms21103540

Zhen, J. L.; Zhang, X. D. and Niu, Q. S. (2006): Relationship between the postmortem interval and nuclear DNA changes of heart muscular cells in mice. Fa yi xue za zhi.; 22(3): 173-176. Chinese. PMID: 16856335.

Zheng, J.; Li, X.; Shan, D.; et al. (2012): DNA degradation within mouse brain and dental pulp cells 72 hours postmortem. Neural Regeneration Research; 7(4): 290-294. DOI: 10.3969/j.issn.1673-5374.2012.04.009

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