Research Article

Immunohistochemical markers study from different organs of rats after death for estimation of postmortem interval: An experimental study.

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Abstract
The present study aimed to estimate postmortem interval through immunohistochemical markers and histological examination of the skeletal muscle of rats at different time intervals. Fifty adult male albino rats were divided into five groups with 10 rats in each group. The rats were killed by cervical dislocation and the skeletal muscle samples were taken at times (zero, 24, 48, 72, and 96 hrs PM). The samples were subjected to histological and immunohistochemical examination. The results revealed that histological changes occurred gradually over the time since death. Immunohistochemical results of p53 and caspase-3 showed a significantly strong negative correlation with PMI. It was concluded that postmortem histological changes and immunohistochemistry of apoptotic markers (p53 and caspase-3) in skeletal muscle could be useful for estimating a postmortem interval.
Keywords: Postmortem interval; Forensic science; Caspase-3; P53; Apoptosis; Immunohistochemistry.

Introduction
Determination of the time of death is a vital question in any forensic casework. Precise estimation of the post-mortem interval (PMI) is crucial at both the civil and criminal levels. The PMI assists in proving and excluding suspects, identifying the exact time of a crime, and solving some legal problems related to inheritance (Elghamry et al., 2017).

Several techniques have been used to predict the time of death, including physical, biochemical, and entomological methods. These methods, however, are of limited value. Many environmental influences, such as temperature and insect activity, as well as individual factors, such as age and sex, have a significant impact on these processes (Ali et al., 2017).

Apoptosis is a highly regulated type of cell death that promotes the removal of damaged and dead cells from healthy tissues. As such, this form of programmed cell death is an important biological mechanism that plays a key role in normal growth as well as in normal tissue homeostasis. (Elvas et al., 2019).

The activation of caspases is a crucial component in the signaling cascades that lead to the execution of apoptosis. Both intrinsic (via mitochondria) and extrinsic (death receptors activation) pathways of apoptosis converge in the activation of effector caspase-3; the most studied member of this family (Pfeffer & Singh, 2018).

The apoptotic pathway is activated by both intracellular and extracellular signals. The intracellular signals include DNA damage, growth factor deprivation, and cytokine deprivation, while the most common extracellular signals are death-inducing signals provided by cytotoxic T cells from the immune system in response to cells that are damaged or infected (Elvas et al., 2019).

P53 is the most important tumor suppressor and triggered in response to stress signals−DNA damage, oncogene activation, ribosomal stress, and hypoxia and leads to growth suppression by inducing cell cycle arrest or cell death. The primary feature of the p53 is the transcriptional regulation of target genes that regulate various cellular processes, including the cell cycle and apoptosis (Fischer, 2017). The aim of this study is to estimate the postmortem interval through histological examination and immunehisto-

chemical markers of the skeletal muscle of rats at different time intervals.

Materials and methods

This study was conducted in the Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Minia University from the period January 2020 to December 2020. All aspects of animal care and treatment were carried out under the local guidelines of the ethical committee of the Faculty of Medicine, Minia University.

Fifty male albino rats of 8 weeks of age (weighing 150-200 gm) were used in this study. Rats were obtained from Minia University laboratory animals growing center-Egypt and housed in plastic cages, fed a standard laboratory diet and water. Rats were classified into 5 groups, 10 rats in each group.

The rats were sacrificed by cervical dislocation under light halothane anesthesia and dissected to obtain the skeletal muscle" Gastrocnemius muscle" at 0, 24, 48, 72, and 96 hours after death. The first group (0 hours) was considered as the control group which included the samples were taken immediately after sacrifice at the 0-time interval while in the 2nd, 3rd, 4th, 5th groups, samples were obtained at 24, 48, 72, 96 h postmortem respectively.

The sacrificed rats were kept at room temperature (22 ± 2ºC during the day/ 9 ± 2ºC during the night) throughout the experiment. The collected samples were washed in normal saline, fixed in 10% formal saline for 48 hours, then washed by tap water and processed for preparing paraffin sections for the histological and immunohistochemical studies.

Histological study:

Skeletal muscle specimens were fixed in buffered formalin, dehydrated by alcohol, cleared by xylene, and embedded in paraffin blocks. The paraffin blocks were cut at 5 µm and stained with hematoxylin and eosin (Wick, 2019).

Immunohistochemical study (de Alcântara et al., 2017):

Skeletal muscle sections used were mounted on charged slides, deparaffinized in 2 fresh xylene changes, and re-hydrated in graded ethanol (99%, 95%, and 70%) then washed in PBS for 10minutes. Then endogenous peroxidase activity was blocked and antigen retrieval was performed. Then specimens were incubated at room temperature

The sections were incubated with the specific primary antibodies: anti-p53 antibody (Sigma Aldrich Company, Egypt. Cat. # PA5-88098) and Anti-cleaved caspase-3 antibody (Sigma Aldrich Company, Egypt. Cat. #PA1-29157). The sections were incubated in a humidified chamber at room temperature overnight. A labeling antibody was added to each section. Diaminobenzidine was added as a chromogen. Then the sections were counterstained, dehydrated in ascending grades of alcohol, mounted in DPX, and examined with a standard light microscope.

The expression of caspase-3 was scored for percentage and intensity of immunostaining as follows: weak for staining less than 10% of skeletal muscle cells, mild for staining 10- 25 %, moderate for staining 25- 40 %, moderate to high for staining 40- 65%, a high expression for staining in more than 65% of skeletal muscle cells (Noshy, 2020).

The expression of p53 was scored for percentage and intensity of immunostaining as follows: weak for staining less than 10% of skeletal muscle cells, mild for staining 10- 20 %, moderate for staining 20- 45%, moderate to high for staining 45- 65%, a high expression for staining in more than 65% of skeletal muscle cells (Akhter et al., 2019).

Statistical analysis:

Data were coded and and statistically analyzed using SPSS (Statistical Package for the Social Sciences) version 25 software. Data are summarized using the mean and standard deviation and minimum and maximum of range. Distribution of the data was done by Shapiro Wilk test. Analyses were done for parametric quantitative data between different times using Repeated measures ANOVA test followed by Post Hoc LSD analysis between each two times. Correlation was done using **Pearson's correlation coefficient** followed by simple and multiple linear regression analysis for prediction of postmortem interval. P values less than 0.05 were considered statistically significant.

Results

Postmortem (PM) histological findings:

At the time of death, H & E stained sections of the rats' skeletal muscle showed normal histological morphology; striated muscle fibers were parallelly arranged with flat, peripherally located nuclei (Figure 1, A). At 24h postmortem, showing swollen skeletal muscle myofibers with an eccentric nucleus and partial loss of striations (Figure 1, B). At 48 hrs, the skeletal muscle showing loss of cross striations with a wavy nucleus, widely spaced myofibers, and eccentric pyknotic nucleus (Figure 1, C).

At 72 hrs, skeletal muscle tissues showing areas of segmented muscle fibers and loss of cross striations and intracellular nucleus (Figure 1, D). At 96hrs, the skeletal muscle tissue showing extensive areas of tissue loss with replacement by thin collagenous connective tissue, fragmented muscle fibers with an irregular nucleus, and dilated congested blood vessels (Figure 1, E).

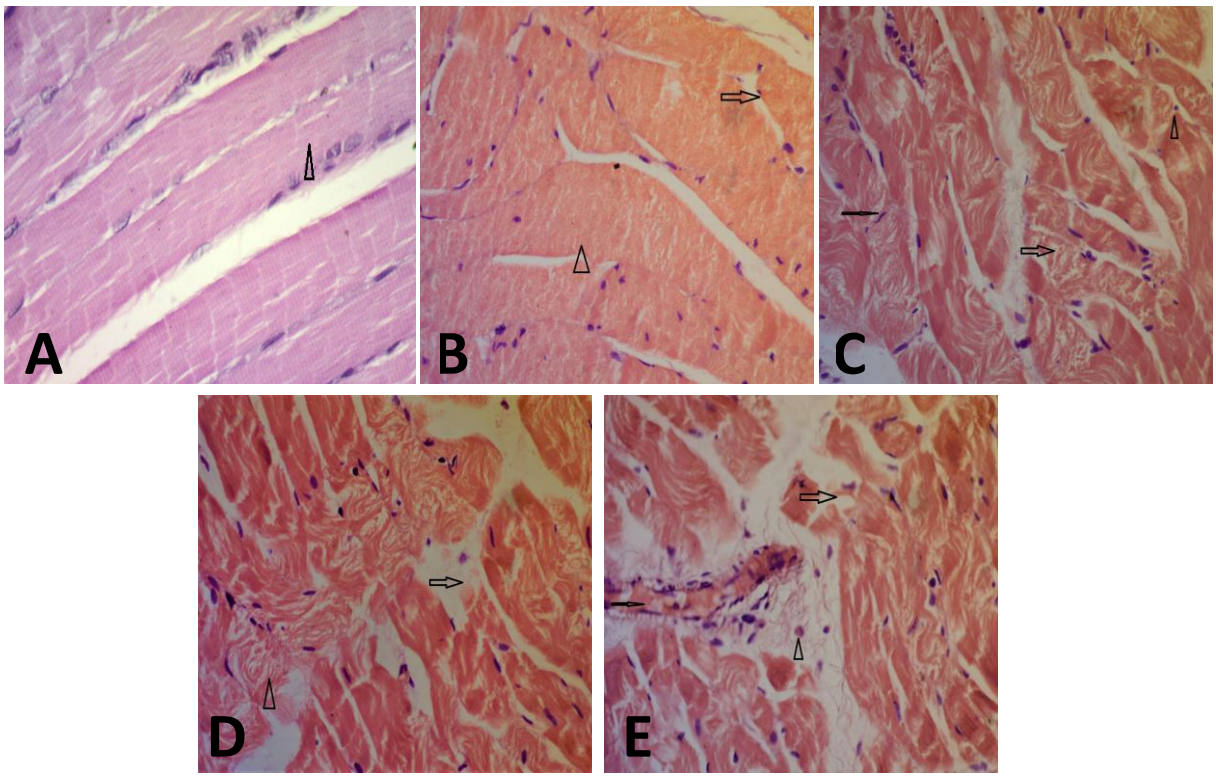

Figure (1): Photomicrographs of H&E stained longitudinal skeletal muscle sections in rats showing: (A) Normal muscle bundles with prominent cross striations and flat, peripherally located nuclei (arrowhead) at time of death. (B) Eccentric nucleus (arrow), partial loss of striations (arrowhead) 24 h PM. (C) loss of cross striations with wavy nucleus (thin arrow), widely spaced myofibers (thick arrow), eccentric pyknotic nucleus (arrowhead) 48 h PM. (D) Segmented muscle fibers (Arrow), loss of cross striations, and intracellular nucleus (arrowhead) 72 h PM. (E) Extensive areas of tissue loss with replacement by thin collagenous connective tissue (arrowhead), fragmented muscle fibers with irregular nucleus (arrow) 96 h PM. (H&E, x 400).

Immunohistochemical findings:

Using cleaved caspase-3:

Caspase-3 immunohistochemical staining showed a gradual decrease of positively-stained areas with increasing PMI. The most significant increase in the caspase 3 expression was at 0 hour postmortem (nuclear and cytoplasmic). Moderate to high cytoplasmic caspase 3 expression was identified at 24 h and then moderate expression at 48 hours postmortem. Beginning from 72 hours after death, there was mild caspase 3 expression then became weak non-specific staining at 96 h after death (Figure 2).

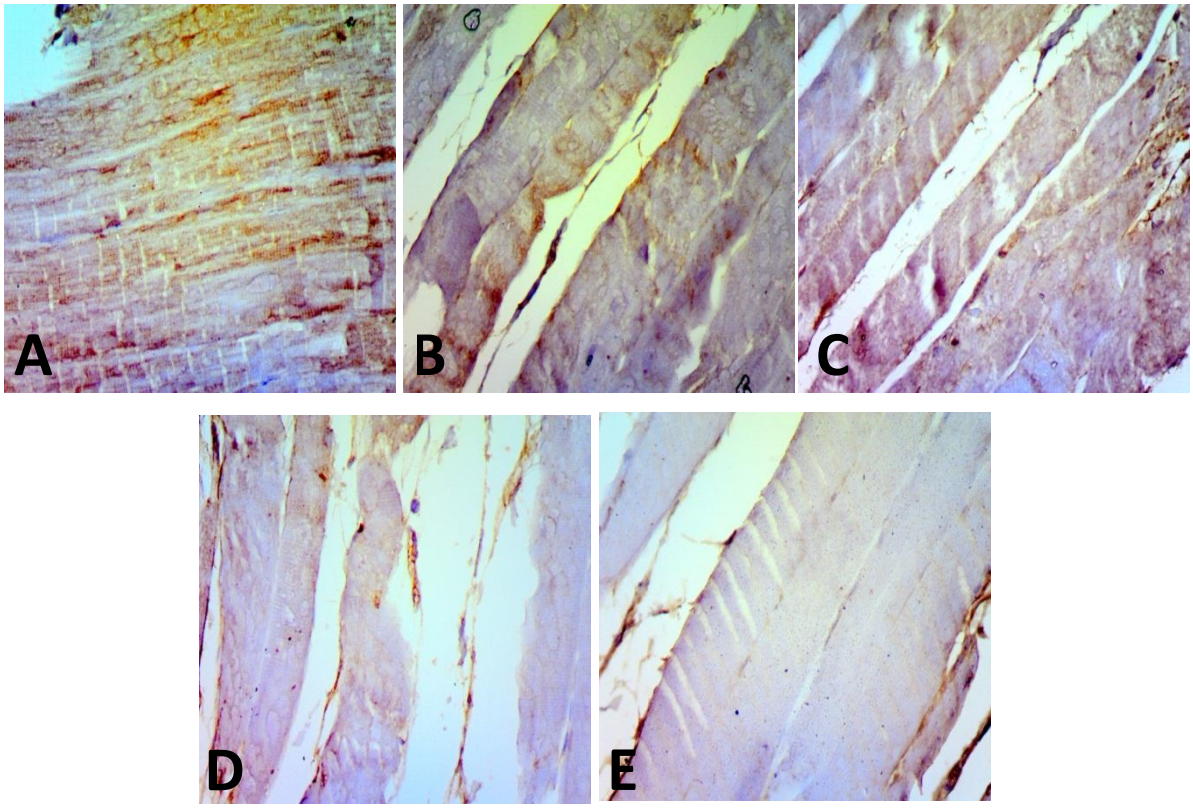

Figure (2): Photomicrograph describing caspase-3 IHC staining in gastrocnemius muscle of rats, Brown color [positive] denotes specific caspase3 immunostaining. Caspase-3 expression among different intervals PM: (A) Strong caspase-3 expression 0 h PM. (B) Moderate to strong caspase-3 expression 24 h PM. (C) Moderate caspase-3 expression 48 h PM. (D) Mild caspase-3 expression 72 h PM. (E) Weak caspase-3 expression 96 h PM. Immunohistochemistry × 400.

Using p53:

The value of p53 expression was gradually decreased with increasing the PMI. The skeletal muscle showed strong p53expression at 0 h PM then moderate to strong expression of p53 at 24h PM was detected. At 48 h PM, moderate p53 expression was demonstrated. Also, at 72 h PM, there was mild p53 expression ending with weak p53 expression at 96 h PM (Figure 3).

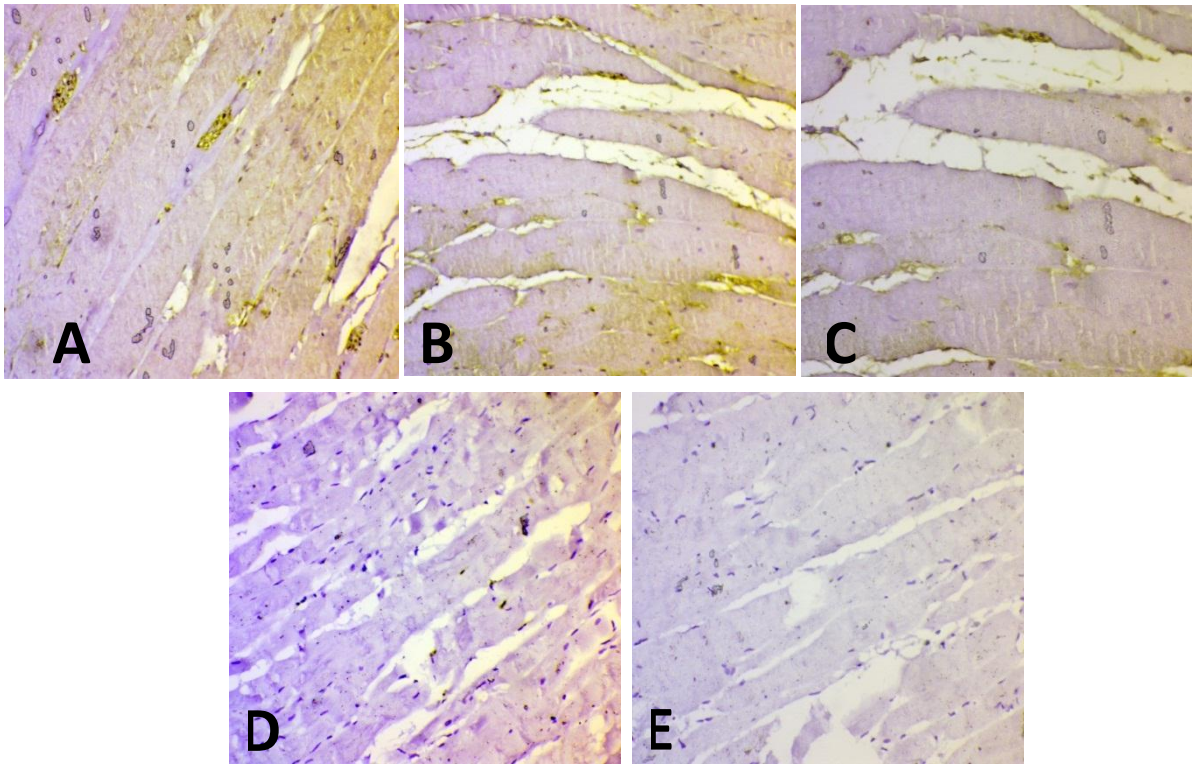

Figure (3): Photomicrograph describing p53 IHC staining in the skeletal muscle of rats, Brown color [positive] denotes specific p53 immunostaining. P53 expression among different intervals PM: (A) Strong expression of p53 0 h PM. (B) Strong to moderate p53 expression 24 h PM. (C) Moderate p53 expression 48h PM. (D) Mild p53 expression 72h PM. (E) Weak p53 expression 96 h PM. Immunohistochemistry× 400.

Regarding the range and the mean of caspase-3 in the skeletal muscle, there is a significant statistical difference between different groups and between each two times, as illustrated in the table (1), chart (1).

Table (1): Showing expression of caspase-3 in the skeletal muscle at different PMI.

Skeletal muscle	**At 0 hr** **N=10**	**At 24 hrs** **N=10**	**At 48 hrs** **N=10**	**At 76 hrs** **N=10**	**At 96 hrs** **N=10**	**P value**
Caspase-3 *Range* *Mean ± SD*	(80-90) 85±3.5	(60-65) 61.7±1.9	(30-40) 32.9±3.1	(20-25) 21.4±2.1	(0-3) 0.6±1.1	***<0.001****
P value between each 2 times						
At 0 hr		***<0.001****	***<0.001****	***<0.001****	***<0.001****	
At 24 hrs			***<0.001****	***<0.001****	***<0.001****	
At 48 hrs				***<0.001****	***<0.001****	
At 76 hrs					***<0.001****	

- ***Repeated measures ANOVA test for quantitative data between different time series followed by LSD Post Hoc analysis between each two times***
- ****: Significant Level at P value < 0.05***
- ***SD: standard deviation, hrs: hours***

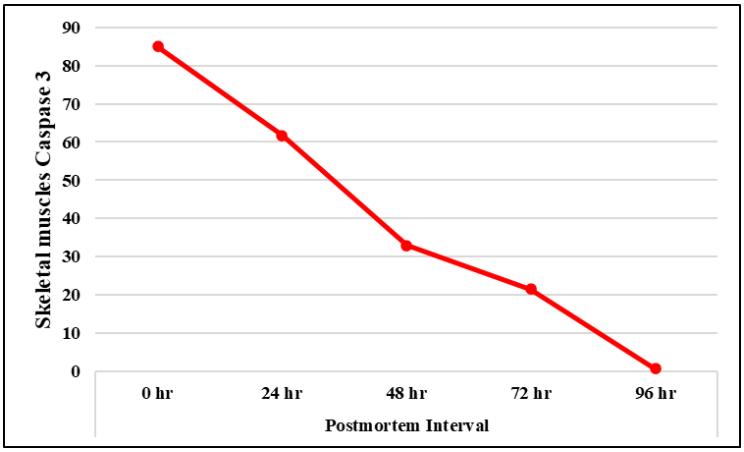

Chart (1): Showing expression of caspase-3 in the skeletal muscle at different PMI.

Regarding the range and the mean of p53 in the skeletal muscle, it had a significant statistical difference between different groups and between each two times, as shown in table (2), chart (2).

Table (2): Showing expression of p53 in the skeletal muscle at different PMI.

Skeletal muscle	**At 0 hr N=10**	**At 24 hrs N=10**	**At 48 hrs N=10**	**At 76 hrs N=10**	**At 96 hrs N=10**	**P value**
P 53 *Range* *Mean ± SD*	(70-80) 75.2±4	(60-64) 61.2±1.5	(30-42) 34.5±4.4	(15-20) 16.5±2.1	(0-9) 2.4±3.3	***<0.001****
P value between each 2 times						
At 0 hr		***<0.001****	***<0.001****	***<0.001****	***<0.001****	
At 24 hrs			***<0.001****	***<0.001****	***<0.001****	
At 48 hrs				***<0.001****	***<0.001****	
At 76 hrs					***<0.001****	

- ***Repeated measures ANOVA test for quantitative data between different time series followed by LSD Post Hoc analysis between each two times***
- ****: Significant Level at P value < 0.05***
- ***SD: standard deviation, hrs: hours***

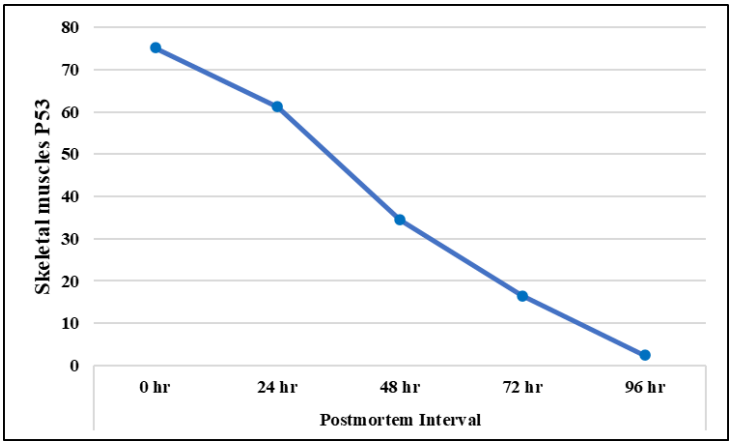

Chart (2): Showing expression of p53 in the skeletal muscle at different PMI.

As regard the correlation of p53 and caspase-3 in the skeletal muscle, it showed a significant strong negative correlation with PMI; (p53, r=-0.988 and P value <0.001*), (caspase-3, r=-0.989 and P value <0.001*), table (3), chart (3, 4).

Simple linear regression analysis for predicting PMI of p53 revealed reliability (R2=0.975, SEE=5.41) and caspase-3 showed (R2=0.977, SEE=5.19), table (4). With the use of multiple linear regression analysis, it revealed a minimal increase in R2 (=0.983) and decrease of SEE (=4.5), as shown in table (5).

Table (3): Showing the Pearson's correlation of p53 and caspase-3 in the skeletal muscle with different PMI.

Skeletal muscles	Postmortem Interval	
	r	P value
P53	***-0.988***	***<0.001****
Caspase-3	***-0.989***	***<0.001****

- ***Pearson's correlation***
- ****: Significant Level at P value < 0.05***
- ***Grade of correlation(r): 0: No correlation, 0.00 to 0.24: Weak, 0.25 to 0.49: Fair, 0. 5 to 0.74: Moderate, 0.75 or more: Strong, 1: Perfect***

Table (4): Showing simple linear regression of p53 and caspase-3 in the skeletal muscle.

Skeletal muscles	Unstandardized coefficients		P value	Adjusted R^2	SEE
	Constant	B			
P53	94.71	-1.23	***<0.001****	0.975	5.41
Caspase-3	93.91	-1.122	***<0.001****	0.977	5.19

- ***Simple linear regression analysis predicting postmortem interval (PMI)***
- ****: Significant Level at P value < 0.05***
- ***Regression equation: PMI = constant + (B * independent variable)***
- ***Where the independent variable is p53 or caspase-3***
- ***SEE: Standard error of estimate***

Table (5): Showing multiple linear regression of p53 and caspase-3 in the skeletal muscle.

Skeletal muscles	Unstandardized coefficients		P value	Adjusted R^2	SEE
	Constant	B			
P53	94.24	-0.576	***<0.001****	0.983	4.5
Caspase-3		-0.605	***<0.001****		

- ***Multiple linear regression analysis predicting postmortem interval (PMI)***
- ****: Significant Level at P value < 0.05***
- ***Regression equation: PMI = constant + (B1 * independent variable1) + (B2 * independent variable2)***
- ***Where the independent variable is p53 and caspase-3***
- ***SEE: Standard error of estimate***

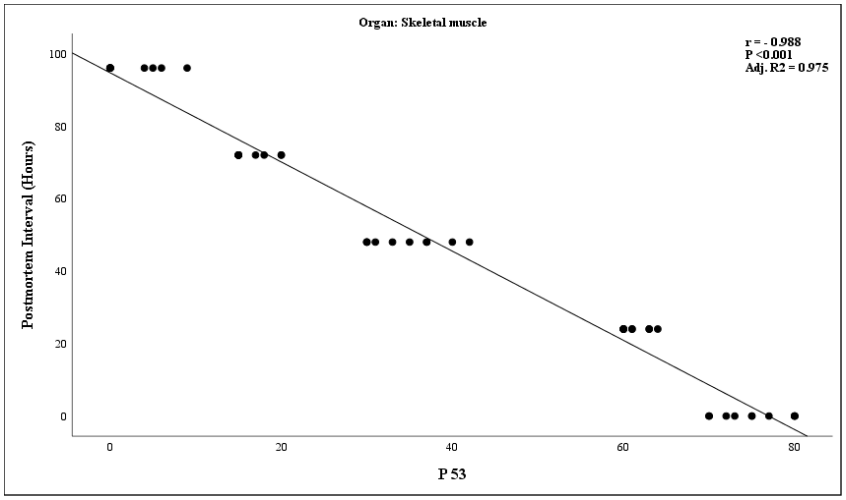

Chart (3): Showing significant strong negative correlation of p53 with PMI in the skeletal muscle.

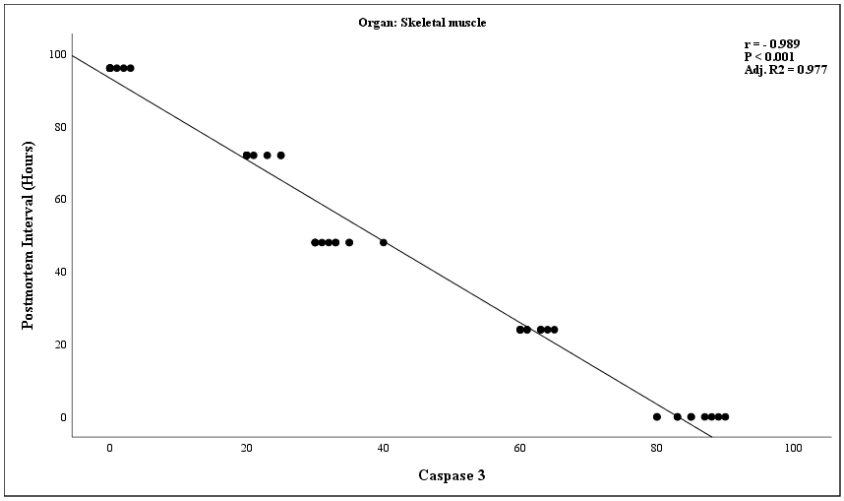

Chart (4): Showing significant strong negative correlation of caspase-3 with PMI in the skeletal muscle.

The following equations were extracted for PMI determination by; simple linear regression analysis **{PMI = constant + (B * independent variable)}**, where the independent variable is p53 or caspase-3 and by multiple linear regression analysis **{PMI = constant + (B1 * independent variable1) + (B2 * independent variable2)}**, where the independent variable is p53 and caspase-3.

Discussion

The most significant medical-legal question of any post-mortem investigation is the assessment of the time passed since death. It's really interesting to know when the crime happened. PMI is the period that has passed following the death of a human. If the time in question is not identified, a variety of medical research methods shall be used to determine it (Lin et al., 2011).

Analysis of electrolytes changes in blood, CSF, intraocular, synovial fluids after death play an important role in evaluation of PMI. While biochemical studies are very important in the determination of PMI, they have been shown to be of little benefit once decomposition occurs. Other studies have shown that histological and histochemical studies of degenerative modifications in different organs and tissues can be a successful tool for estimating PMI (Kawamoto et al., 2013).

The results of this work regarding histological changes by using H&E stained sections of rats' skeletal muscle showed a gradual deterioration with the progression of time after death. The current results coincide with the results of Yahia et al., 2018 who studied histological changes in skeletal muscles of

dogs at different time intervals including 0, 24, 48, and 72 hours postmortem.

The current work agree with Li et al., 2017 who studied histological changes in skeletal muscle of rats but till 144 h after sacrificed by suffocation; these changes are gradually progressive and more obvious with increasing the PMI. Also agreed with Zaki et al., 2017 who studied histological changes in the femoral muscle of rats killed by cervical dislocation at time of (0, 24, 48, and 96 h) after death. Also, with the study of El-Nahass et al., 2017 who reported similar changes in the skeletal muscle at (0, 24, 48, and 72 h).

This study results at 24h agreed with Tavichakorntrakool et al., 2008 who also revealed that changes in the muscle histology are a time-dependent and valuable indicator for assessing the PMI.

These results differed from Kaimbayeva et al., 2019 who reported that; there was an insignificant change in the muscle fibers of cow elk at 24 and 48 hrs, and this may be attributed to different conditions because the present study done at room temperature (22-9°C) but the study of Kaimbayeva et al., 2019 was conducted at 2-4°C (lower environmental temperature) that stop the action of bacteria and delay putrefaction.

Histological modifications found in the current research were due to postortem anoxic conseq uences such as ischemia, glycolysis, and proteolysis. These effects produce changes in the enzymatic activity and ultra-structural cells. The autolytic process involving a change in size, shape, electron density, cell structure localization, and typically causes a progressive loss of highly ordered cell structure (Karadzic et al., 2010).

The results of this study regarding the expression of caspase-3 in the skeletal muscle revealed a significant difference between different groups, with a significantly strong negative correlation with PMI. Immunohistochemical detection of caspase-3 in the skeletal muscle tissue displayed a gradual decrease of positively-stained areas with increasing PMI; ranging from high expression was seen at the time of death to weak non-specific staining at 96 h after death.

The current work results coincide with the results of Lee et al., 2016 revealed that caspase-3 stained area was decreased gradually up to 96h after death by immune-histochemistry and the same pattern was founded by western blot. Also, these results agree with Kemp et al., 2006 who established that the activity of caspase-3 decreases in the pig muscles during the PMI.

A study conducted by Saber & Ali, 2016 revealed a significant positive correlation between caspase-3 mRNA levels in the gastrocnemius muscle with time elapsed after death. They observed a rise in caspase-3 mRNA levels from 2 h postmortem to 6 h, after which a marked decrease occurred at 8 h. Caspase-3 expression in the brain of rats was evaluated by Khater et al., 2020 using immunohistochemistry and proven its valuable role in the determination of PMI at time intervals (0, 24, and 48) h after death. In addition, reported a statistically significant difference between different groups at 0, 24, and 48h PM regarding the expression of caspase-3.

These results can be explained by He et al., 2018 who found that postmortem hypoxia induces a reduction in the intracellular ATP, leading to the activation of apoptosis and caspase-3. The depleted ATP limits the enzyme apoptotic activity.

During autolysis, the action of lysosomal and cytoplasmic enzymes and the destruction of organelles can be required to minimize certain protein antigens causing a decrease in specific immunohistochemical labeling. However, the continued cell metabolism activity shortly after death may contribute to increasing labeling (Scudamore et al., 2011).

Regarding the results of IHC expression of p53 in the skeletal muscle of rats, there was a significant difference between different groups, with a significantly strong negative correlation with PMI. The p53 expression value gradually decreased with increasing the PMI. The Skeletal muscle showed strong p53expression at 0 h PM ending with weak p53 expression at 96 h PM. Little literature was available on the postmortem immune-histochemical analysis of p53 in the skeletal muscle.

A study by Lee et al., 2016 revealed that p53 expression level was constant for at least 96 h postmortem in the rat kidney and psoas muscle by western blot.

While other studies investigated p53 in other different organs other than this study as Mohamed et al., 2017 who studied post-mortem immunohistochemical expression of p53 in the rabbit brain at time intervals (0, 6, and 12h PM). Their study revealed an increase in p53 expression with increasing time elapsed after death. They observed a weak reaction at 0 h, moderate at 6 h, while intense at 12 h. Another study by Elias et al., 2004 investigated p53 expression in human skin and founded downward trend of its expression in decomposing tissue and also concluded; it might be a reliable indicator for PMI.
The increase in p53 at the time of death in the current study results can be explained by the disruption of circulation and respiration that occurs upon death resulting in ischemia and anoxia of tissues which in turn leads to activation of p53 within the first hours of death and apoptosis (Sax & El-Deiry, 2003).

In this study, we conducted both simple and multiple linear regression analyses for predicting PMI for both p53 and caspase-3 in the skeletal muscles, and also equations for PMI determination were performed. Simple linear regression analysis was significant for both p53 and caspase-3 in the skeletal muscles.

The results of using multiple linear regression analysis to predict PMI were better than the simple one; with high adjusted R2 and less SEE. No literature was available for comparing these results.

Conclusion

The current study concluded that changes take place after death in a time-dependent manner according to different postmortem periods. The histological structure of the skeletal muscle tissues showed a gradual deterioration with the progression of time after death.
This study concluded that p53 and caspase-3 showed a significantly strong negative correlation with PMI in the skeletal muscle. So that, p53 and caspase-3 could be used for the prediction of PMI through equations of simple and multiple linear regression analyses with good reliability and low SEE.

Finally, it was concluded that the analysis of histological changes and expression of both p53 and caspase-3 in the skeletal muscles could be used as good predictors of accurate assessment of PMI.

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