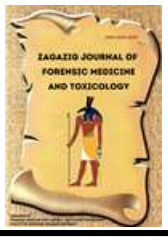

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Original article

Role of Autophagy (Beclin -1) and Apoptotic (Bax) Associated Proteins in Discrimination between Antemortem and Postmortem Burn injuries in Rats Skin Biopsies

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Abstract

Background: Establishing the occurrence of burn injuries during life is crucial in forensic medicine. **Objectives:** This research examined the expression of the autophagy (Beclin-1) and apoptotic (Bax) associated proteins in rats' skin tissues after burning infliction. In addition, the study evaluated the potential application of these changes in distinguishing between antemortem (AM) and postmortem (PM) injury. **Materials and methods:** Twenty-five rats were divided into five groups: the control group, the 0-hour AM injury group, the 24-hour AM injury group, the 0-hour PM injury group, and the 24-hour PM injury group. Burn models were induced using a digital thermostat water bath kettle (100°C) for 10 seconds. Alterations in the levels of Beclin-1 and Bax proteins were assessed using Western blotting and the ELISA assay method, respectively, along with histopathological evaluation. **Results:** There was a reduction in expression levels of Beclin-1 in AM injured groups at time intervals 0 and 24 hours, with a statistically significant decrease observed in the 24-hour AM burned group compared to other studied groups. The determination of Beclin-1 levels in the 0-hour and 24-hour PM injured groups indicated a significant increase in the 24-hour PM injured group. Estimation of the changes in Bax levels after burn infliction among the studied groups illustrated an elevation in both 0-hour and 24-hour AM burned groups, which was significant in 24-hour AM injured rats compared to other groups. Our results demonstrated a significant difference in Bax level found between both AM burn-inflicted groups, and PM burned groups at 24-hour. **Conclusion:** Determining Beclin-1 and Bax-associated protein levels may serve as novel biomarkers for distinguishing between AM and PM burn injuries within forensic investigations.

I. Background

Burn represents a highly common form of traumatic injury globally. This condition is linked to significant morbidity and mortality (Brusselaers et al., 2010; Li et al., 2017). Exposure to hot liquids, fire, and electricity are among the most common causes of burn injury from a forensic perspective (El-Sayed, 2016).

Burn injuries primarily affect children, the elderly, and victims of intimate partner abuse. The medicolegal assessment of burns remains a significant issue in forensic practice, involving various conditions implicated in burn injuries, such as self-inflicted and assault-induced burns (Nisavic et al., 2017).

Medicolegally, one of the most crucial aspects in forensic practice is determining burns' nature, whether

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ante- or postmortem. Comprehensive investigations are essential, as an individual may have died due to causes unrelated to the fatal burn, such as an assault or illness, prior to the burn's occurrence. The differentiation of these conditions depends on the evaluation of vital responses based on the histological assessment (Chawla et al., 2014; Pennisi et al., 2023).

Apoptosis, defined as cell-programmed death, has garnered significant attention in contemporary research (Tan et al., 2013). Cell death may occur due to autophagy, apoptosis, and necrosis, all contributing to burn injury progression. There is a paucity of data concerning the function of autophagy in determining the age and progression of burns, as well as in the mechanism of transporting macromolecular waste to lysosomes, where it can be converted into active molecules, such as amino acids essential for homeostasis maintenance (Xiao et al., 2014). This process is a conservative catabolic mechanism that facilitates the recycling of cellular waste, thereby promoting cell renewal and homeostasis, which is essential for the survival of stressed or starving cells. Various molecules facilitate the activation of autophagy. The Beclin1 protein is a component of the phosphatidylinositol 3-kinase complex (PI3KC3) class III. Beclin1 levels have been proposed as a reliable marker of the activity of autophagy (Tan et al., 2013; Yassa et al., 2019).

Apoptosis is a normal physiological process characterized as a highly regulated form of cell death, facilitating the removal of dead cells from healthy, viable tissue (Elvas et al., 2019). Apoptosis can also be a pathological process in some conditions, such as burn injuries, where pathological apoptosis occurs. It has been found that the apoptosis process is largely affected by the expression of two genes: the B-cell lymphoma 2 (Bcl-2) gene, which acts as an anti-apoptotic, and the Bcl-2-associated X protein (Bax), which is a pro-apoptotic gene (Jiang et al., 2014; Wang et al., 2019).

The present study aimed to determine changes in autophagy, assessed via Beclin-1, and apoptosis, indicated by variations in Bax levels, following full-thickness 2nd degree burn (scald model) injuries in rat skin. Additionally, it investigated the potential application of these changes for differentiating between AM and PM injuries in forensic practices.

II. Material and Methods

Type of study: an experimental animal study.

Ethical considerations:

This study was conducted in accordance with the guidelines established by the Ethics Committee of the Faculty of Medicine at Banha University, Egypt, following protocol approval (RC. 1.8.2024). The management of animals in this study adhered to the guidelines established by the National Institutes of Health for laboratory animal care and use (Clark et al., 1997).

II.1 Study design:

Experimental equipment and chemicals:

The primary experimental apparatus utilized in this study to examine the expression characteristics of Beclin-1-associated protein included the Biorad gel electrophoresis system, the BioRad ChemiDock Gel documentation imaging system, and image analysis software for determining the band intensities of Beclin-1. We utilized the BMG Labtech FLU Ostar Omega from Germany to estimate Bax associated protein, utilizing a wavelength of 450 nm for Bax measurement.

Animal handling and preparation:

Experiments were performed on 25 healthy adult male albino rats, each weighing between 200 and 250 g, obtained from the animal house, Faculty of Veterinary Medicine, Banha University, Egypt.

Initially, anesthesia was induced by isoflurane inhalation. Upon anesthesia, the hair on the dorsal surface and flank skin of each rat was excised using a sterile shaving razor to expose the skin.

II.2 Methods:

Animal grouping:

The rats were randomly assigned to five distinct groups: a control group consisting of healthy adult rats without burn injuries were used as the control group, the 0-hour AM group, the 24-hour AM injury group, the 0-hour PM injury group, and the 24-hour PM injury group with five rats in each group.

At 0- and 24-hours post-burn in the AM groups, a total of five rats from each group were euthanized at each interval. Both PM groups of rats experienced PM burn, which was applied immediately following scarification, with samples collected at 0- and 24-hours post-scarification.

Tissue sampling:

Tissue sampling involved the collection of all skin samples, which were subsequently divided into two portions. One portion was immediately placed into an Eppendorf tube and stored at −80 °C for Western blotting

analysis and enzyme-linked immunosorbent assay. The remaining portion was preserved in formaldehyde for for histopathological evaluation.

Establishment of burn (scald) model:

The burn model was established through the induction of nonlethal 2nd degree full-thickness skin burn injuries on the dorsal surface of rat skin. The process involved immersion in a digital thermostat water bath kettle at 100°C for 10 seconds, as outlined by Li et al. (2018) for both AM (0 & 24-hours) and PM (0 & 24-hours) injured groups. The microscopic histopathological examination confirmed the burn depth.

Rats in the control group did not receive any treatment and were subsequently sacrificed, allowing for the collection of normal back skin tissues for investigation.

Rats in the 24-hour AM injury group were washed with running water for one minute. After that, to control pain, they received an intraperitoneal injection of acetaminophen (Acetaminophen, GlaxoSmithKline Company) at a dose of 25 mg/kg in a volume of 10 mg/ml. An intraperitoneal injection of isotonic saline (1.5 ml) was administered as a fluid resuscitation measure (Im et al., 2012).

***1*- Beclin-1 expression analysis:**

The expression of Beclin-1 was quantitatively assessed through Western blotting. Proteins were extracted from skin tissue samples using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The colorimetric method was used to determine the protein concentrations using the bicinchoninic acid (BCA) method (Smith et al., 1985). Laemmli SDS sample buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was combined with 25 µg of total protein, followed by boiling for five minutes at 95°C. The same amount (25µg) of protein was added to the 12% mini SDS-PAGE gel wells. The gels were run to stack for five minutes (at 90 V) and thirty minutes (at 120 V) for gel separation. Subsequently, proteins were electrophoretically transferred to the PVDF membrane at 100 V for one hour. The membrane was incubated in 5% skimmed milk in TBST at room temperature for one hour. Incubation of the membrane was performed overnight at 4˚C with primary antibodies against Beclin 1 (1:1000; cat. no. FAS-81447-A; Fagus, Oxford, UK) and β actin (1:1000; cat. no. MA5-11869; Invitrogen, Carlsbad, CA, USA). The blots were washed three times (each for 5 minutes) using TBST. The membrane was incubated for one hour at room temperature using HRP-conjugated secondary antibody (1:3000; cat. no. 61-6520; Invitrogen, Carlsbad, CA, USA), then washed again three times (5 minutes each) with TBST. Bands were visualized using the enhanced chemiluminescent ECL substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions. Signals were captured using the ChemiDoc imager (Bio-Rad Laboratories Inc., Hercules, CA, USA). Band intensities were quantified and normalized to β actin with Image Lab software.

***2*: Bax protein analysis:**

Bax protein in skin tissue samples was detected using an ELISA commercial kit (cat. no. E-EL-R0098; Elabscience, USA). Tissue specimens were homogenized in phosphate-buffered saline (PBS) using a glass homogenizer on ice. Following the preparation of the rat skin tissue homogenate, centrifugation was conducted for fifteen minutes at 12,000 × g and 4°C. The supernatants were collected, and samples and standards were added to the Rat Bax-antibody-precoated ELISA plate. Incubation was done for one hour at 37°C, and washing was performed five times. The avidin-horseradish Peroxidase (HRP) conjugate was successfully added to each well, and the reaction was then incubated and washed. Incubation was conducted at 37°C for 15 minutes following the addition of the substrate solution to each well. Finally, the reaction was stopped by adding the termination solution. Spectrophotometrical optical density (OD) was measured at a wavelength of 450 nm using a plate reader (BMG Labtech, FLU Ostar Omega, Germany). The concentration of Bax protein

in the samples was determined by analyzing the optical density (OD) based on the standard curve.

3*: Histopathological study*:

Skin specimens from various studied groups were fixed in a 10% formaldehyde solution, dehydrated using alcohol and cleared by xylene. Following fixation, specimens were embedded in paraffin blocks, processed to obtain 5 µm-thick sections, and stained with hematoxylin and eosin (Bancroft & Gamble, 2008).

II.3 Statistical analysis and data interpretation:

To analyze the data obtained in the current study, the SPSS software, version 25 (SPSS Inc., PASW statistics for Windows) Chicago: SPSS Inc.) was used. Counts and percentages were used to describe qualitative findings, while mean and standard deviation (SD) were used to display quantitative data after ensuring data normality using the Shapiro-Wilk test. One-way analysis of variation (ANOVA) test was used for the comparison of the study groups, with the post-hoc test used to identify pair-wise significance (Tukey test). The results significance was considered at ≤0.05. Receiver operating characteristics curve (ROC curve) was used to calculate validity (sensitivity & specificity) of continuous variables with calculation of best cut off point. Predictive values and accuracy are assessed using cross tabulation.

III. Results:

Western blot results showed that the expression levels of autophagy (Beclin-1) protein can be detected in uninjured rats' skin tissues of the control group (1.06±0.17). However, after burn infliction, this expression was reduced in both AM injured groups at 0-hour (0.936±0.13) and 24-hour (0.457±0.09). This reduction was found to be non-significant when compared with the 0-hour AM injured group with controls. But, when the 24-hour AM injured group was compared to both the control and 0-hour AM injury groups, Beclin-1 was significantly reduced. On the other side, Beclin-1 protein expression levels in 0-hour (1.30±0.27) and 24-hour (3.35±0.84) PM injured groups were elevated, and this elevation was found to be significant in the 24-hour PM injured group as compared to other studied groups. However, the elevated levels of Beclin-1-associated protein in the 0-hour PM group were found to be statistically nonsignificant as compared to both control and 0-hour AM groups, but significant with the 24-hour AM injured group (Table 1 & Figure 1&2).

On the contrary, ELISA results for estimation change in apoptotic (Bax) associated protein levels by (ng /g) in tissue homogenates among the studied groups illustrated that the mean value of Bax protein levels in normal skin tissue of the control group were (17.64±2.28). However, after burn was introduced, levels of this protein showed an elevation in both 0-hour (19.31±5.09) and 24-hour (182.10±4.53) AM burned groups, and this elevation was statistically significant different in 24-hour AM injured rats as compared to both other groups**.** In PM burned groups, there was a non-significant increase in Bax protein at 0-hour burn injured group (20.63±3.62) when compared to both control and 0-hour AM burned groups; however, there was a significant reduction in 24-hour PM burned group as compared to other groups. Moreover, our results illustrated a significant difference in Bax protein level found between both AM burn-inflicted groups and that PM burned groups at 24-hour (Table 1 & Figure 3).

Microscopic histopathological examination by Hematoxylin and Eosin staining (H&E) of control group specimens demonstrated dermal and epidermal normal features with epidermal thin keratinized stratified squamous epithelium and dermal thickening containing blood vasculature, nerves, and fibroblasts (Figure 4). Group of 0-hour AM burn illustrated full-thickness skin burns that involve the whole epidermis and the majority of the dermis with infiltration of inflammatory cells, especially histocytes (Figure 5A), while after 24-hour AM, the skin exhibited more infiltration of inflammatory cells with fibrin aggregation at the burn edge and vasodilatation occurring at the hypodermis with stasis of the vascular flow (Figure 5B). At the PM stage, the changes at (0-hour) showing skin architecture partially preserved and epidermis can differentiate (Figure 6A), while after 24-hour PM showing epidermis and dermis cannot differentiate with increased necrosis (Figure 6B).

The findings of the current study showed that the sensitivity of both Beclin-1 and Bax proteins for differentiating between AM and PM burn at baseline (0-hour) was 80% with a specificity of 60% and 40% for both proteins respectively (Table 2 & Figure 7). While in 24-hour AM and PM burned groups the sensitivity and specificity were 100% for both Beclin-1 and Bax proteins (Table 3 & Figure 8).

Table (1): Statistical comparison of autophagy (Beclin-1) and apoptotic (Bax) associated protein levels among studied groups by ANOVA, post-hoc tests.

Parameters	**Control group (Mean ± SD)**	**0-hour AM group (Mean ± SD)**	**24-hour AM group (Mean ± SD)**	**0-hour PM group (Mean ± SD)**	**24-hour PM group (Mean ± SD)**	**ANOVA *(F test)***	***P value***
Beclin-1 levels	1.06±0.17	0.936±0.13	0.457±0.09[a]	1.30±0.27[c]	3.35±0.84[abcd]	37.39	<0.001*
Bax levels (ng/ g tissue)	17.64±2.28	19.31±5.09	182.10±4.53[ab]	20.63±3.62[c]	7.62±2.48[abcd]	1946.99	<0.001*

- SD:standard deviation, AM: antemortem, PM:postmortem
-Data expressed as mean ±SD, F: One Way ANOVA test, *statistically significant
- a→ Significant difference between each group and control group, b→ Significant difference between 0-hour AM group and each of studied groups,, c→ Significant difference between 24-hour AM and each of the studied groups, d→ Significant difference between 0 versus 24-hour PM group.

Table (2): Validity of autophagy (Beclin-1) and apoptotic (Bax) protein in differentiation between antemortem and postmortem burn at (0-hour).

	Beclin-1	Bax
AUC (95%CI)	0.920(0.738-1.0)	0.600 (0.219-0.981)
P value	0.028*	0.1602
Cut off point	0.996	17.08
Sensitivity	80.0%	80.0%
Specificity	60.0%	40.0%
PPV%	66.7%	57.1%
NPV%	75.0%	66.7%
Accuracy %	70.0%	60.0%

-AUC:Area under curve , PPV: Positive predictive value , NPV: Negative predictive; -*statistically significant

Table (3): Validity of autophagy (Beclin-1) and apoptotic (Bax) protein in differentiating between antemortem and postmortem burn at (24-hour).

	Beclin-1	Bax
AUC (95%CI)	1.0(1.0-1.0)	.0(1.0-1.0)
P value	0.009*	0.009*
Cut off point	1.62	93.94
Sensitivity	100.0%	100.0%
Specificity	100.0%	100.0%
PPV%	100.0%	100.0%
NPV%	100.0%	100.0%
Accuracy %	100.0%	100.0%

- AUC:Area under curve , PPV:Positive predictive value , NPV:Negative predictive value, -*statistically significant

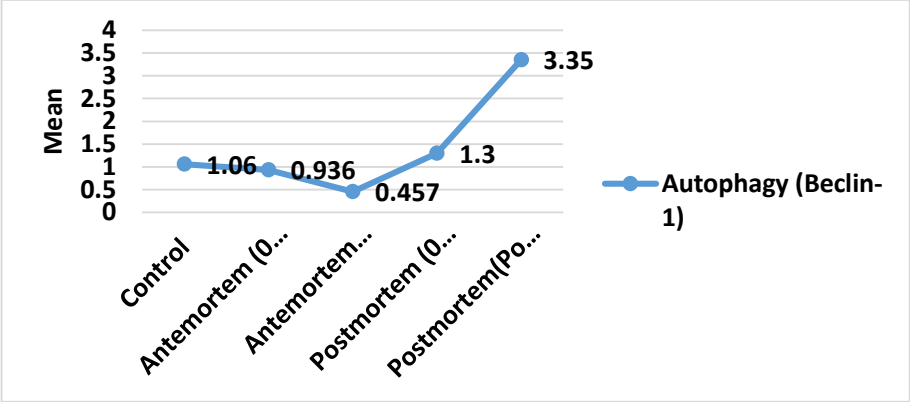

Figure (1): Comparison of autophagy (Beclin-1) expressed protein levels among the studied groups.

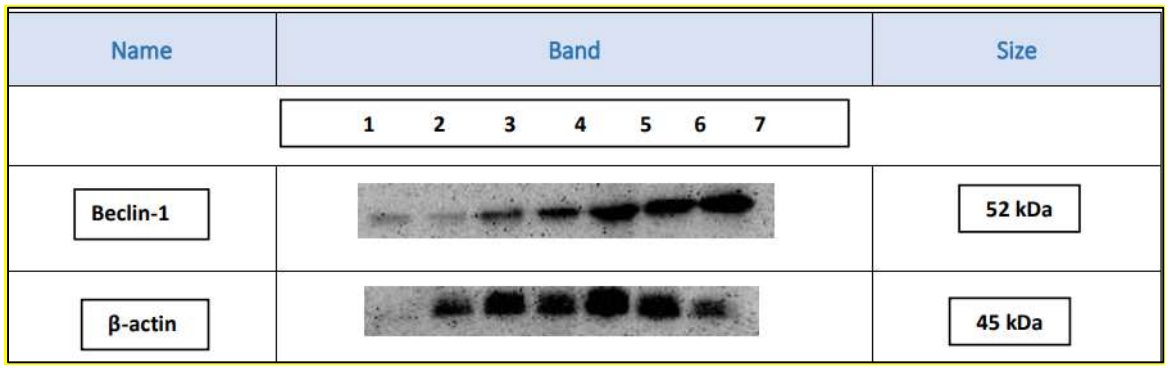

Figure (2): Western blot analysis for Autophagy (Beclin-1) protein shows decreased after 24-hour antemortem while their levels increased after 24-hour postmortem compared to control group.

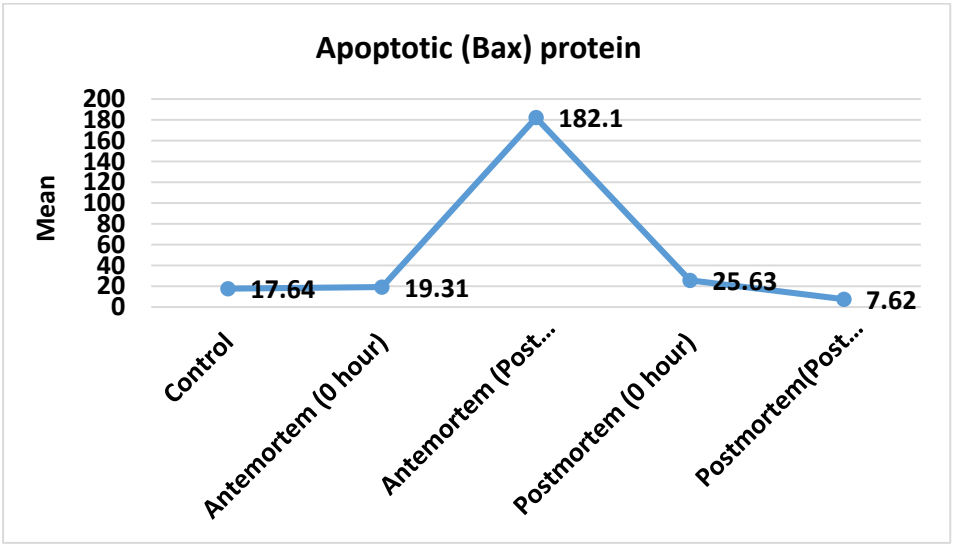

Figure (3): Comparison of apoptotic (Bax) protein levels among the studied groups.

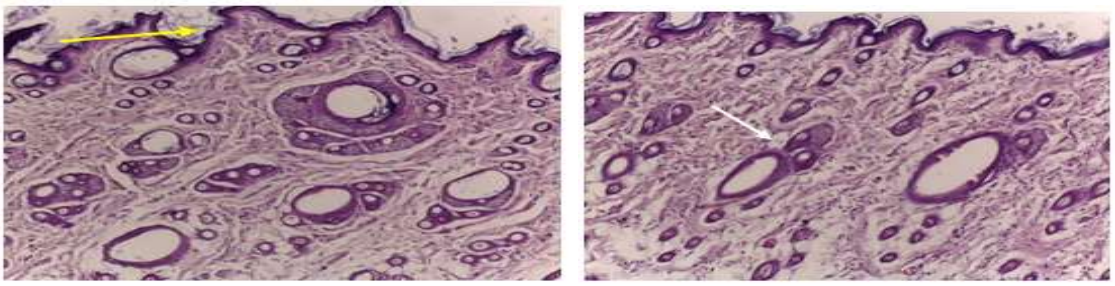

Figure (4): Photomicrograph of normal skin of control group showing intact epidermis [yellow arrow], and dermis showing normal adenxia [white arrow] (H &E; x100).

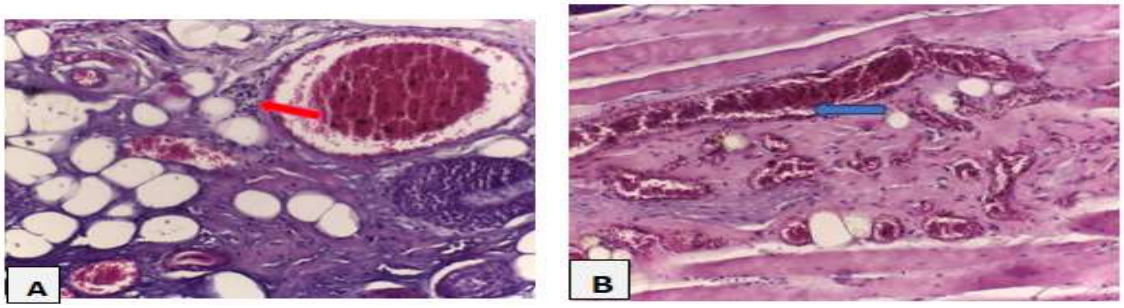

Figure (5): A; Photomicrograph of antemortem burnt skin at (0-hour) showing dermal infiltration by histiocytes [red arrow], B; after 24-hour antemortem showing vasodilatation in the hypodermis with vascular stasis [blue arrow] (H &E; x200).

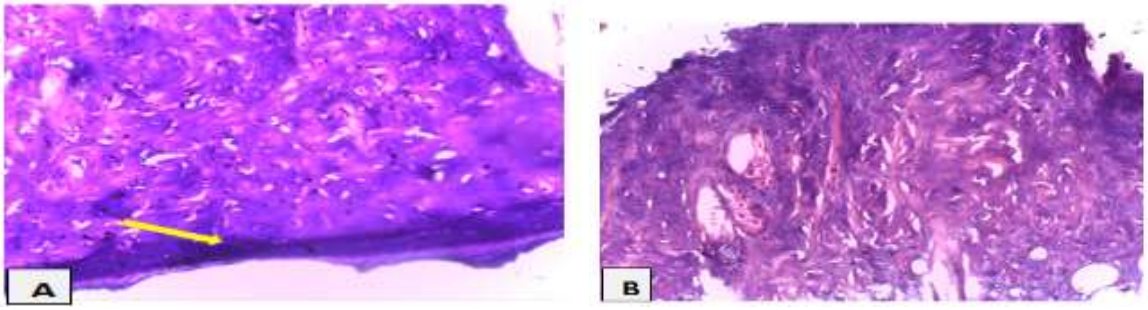

Figure (6): A; Photomicrograph of postmortem burnt skin at (0-hour) showing skin architecture partially preserved & epidermis can differentiate [yellow arrow], B; after 24-hour postmortem showing epidermis and dermis cannot differentiate with increase necrosis (H &E; x200).

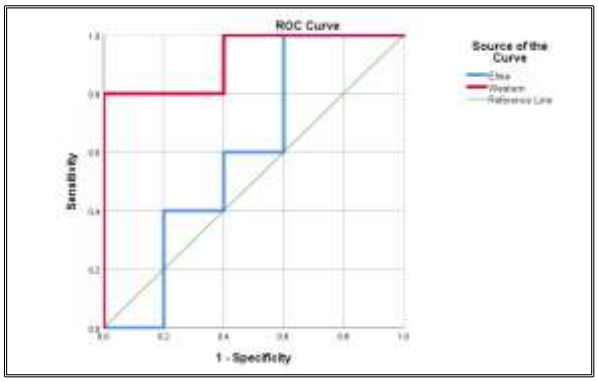

Figure (7): Receiver operating characteristics curve for Beclin-1 by Western blot and Bax by

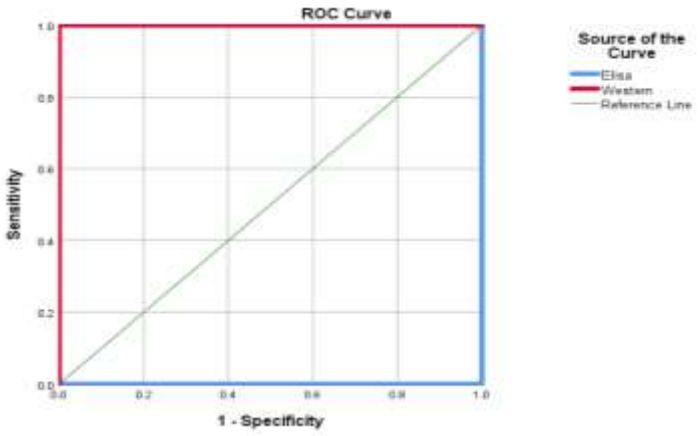

Figure (8): Receiver operating characteristics curve for Bax by ELISA and Beclin-1 by Western blot in differentiating between antemortem and postmortem burns at 24-hour.

IV. Discussion:

Identifying the vitality of burns in dead bodies and living individuals is of considerable medicolegal value in the forensic medicine practice. It is a must for the forensic practitioner to identify whether the victim was dead or alive at the time of fire burning. Using traditional procedures to distinguish burns' vitality is usually not sufficient. Therefore, nonstop seeking for new markers of burns' vitality is crucial (El-Noor et al., 2017).

As regard Beclin-1 protein expression in skin tissue of AM burned rats, the current study stated reduced Beclin-1 expression in 0-hour and 24-hour AM burned groups as compared to control; this reduction was significant at 24-hour. Furthermore, there was a significant reduction of Beclin-1 in the AM burned group at 24-hour as compared to those in the 0-hour group.

These results agreed with Yassa et al. (2019), who found that the markers of autophagy significantly reduced during the first day after burn, then started to elevate again, yet not reaching the normal levels till 3 days following burn. Also, Xiao et al. (2014) indicated the reduction of Beclin-1 protein and LC3 levels in burn injuries to about a quarter of their normal values during the first day, then started to elevate yet not returning to normal values.

These results may be caused by a reduction of the cell number undergoing autophagy at the early stages of the burn, as necrosis becomes the main event. Moreover, inhibiting the autophagy process stimulated the migration of cells, with subsequent acceleration of the wound healing (Joosten et al., 2013).

According to the present study, Beclin-1 protein expression in skin tissue of PM burned rats showing an elevation at 0-hour and 24-hour PM as compared to control. This elevation was significant in the 24-hour burned group when compared to both the 0-hour burned rats and the control. Also, there were significant differences between the levels of Beclin-1 protein expression in the burned skin of rats at 0-hour and 24-hour PM when contrasted to the 0-hour and 24-hour AM burned groups, respectively.

Kimura et al. (2015) reported autophagy suppression in terms of p62) protein levels elevation and light chain 3 (LC3) levels reduction in living wounding, compared to the non-wounded areas. Such noting was not certainly demonstrated in wounds occurring PM, confirming their value in identifying the vitality of wounds.

Shi et al. (2020) indicated the role of determining BECN1 expression and the LC3-2/LC3-1 ratio in the injured skeletal muscle tissue for the discrimination of injury whether occurred AM or PM.

Javan et al. (2015) demonstrated the time-dependent increase in proteins associated with autophagy such as Beclin-1, LC3 II, Atg7, and p62 in cardiac tissue of cadavers after death.

Regarding Bax protein level in tissue homogenate of burned skin tissue of AM burned rats, the current study showed increased Bax level at 0-hour and 24-hour in the AM burned groups as compared to the control, which is significant at 24-hour.

These results agreed with Xiao et al. (2014), who found the elevation of apoptosis, as detected by quantitative assay using terminal-deoxynucleoitidyl transferase-mediated nick end labeling (TUNEL) analysis, by about 3.7-fold over the first two days of burn wounds that was followed by a slight decrease remaining higher than normal levels in skin.

Previous studies assessed the apoptosis process in the skeletal muscles' tissues using histological investigations, ELISA, and genetic studies found the number of impaired mitochondria may increase and exceed the capacity of mitophagy, leading to disrupted mitochondrial hemostasis and resulting in cell death (Chao et al., 2019).

Duan et al. (2009) observed a considerable upregulation in the pro-apoptotic proteins/genes' expression and increased caspase activity, indicating that, following burn injury, signaling pathways, which are death receptor-mediated and other apoptosis-related pathways, were involved in skeletal muscle apoptosis.

According to the present study, Bax protein level in tissue homogenate of PM burned groups, there was a non - significant increase in Bax at 0-hour burned group as compared to the control and a significant reduction at their level in PM burned group at 24-hour as compared to other groups (control, 0-hour & 24-hour AM groups & 0-hour PM burned one).

Li et al. (2024) highlighted the immediate upregulation of Bax, Caspase-3, and Bcl-2 expression following AM muscle injury. This up-regulation was obviously slower in PM muscle injury. However, they noted that the PM peak expression levels were higher than the AM ones. This result could be affected by the variation in mortality causes.

In accordance with Saber & Ali (2016) study demonstrated that the levels of gastrocnemius muscle caspase-3 mRNA significantly and positively correlated with time interval from death, with an increase in the levels of caspase-3 mRNA beginning from two hours after death till six hours, with a subsequent marked reduction beginning from eight hours PM. A plausible explanation of these findings was proposed by He et al. (2018) results that indicated the intracellular adenosine triphosphate (ATP) decrease as a result of PM hypoxia, activating apoptotic pathways. This occurs till ATP depletion, after which the apoptosis-related enzymatic activity is limited.

V. Conclusions:

Based on the present study, we concluded that comparing the serial levels of autophagy (Beclin-1) and apoptotic (Bax) proteins might be used as biomarkers for differentiation between AM and PM burn injuries in forensic contexts.

VI. Recommendations:

- Further studies are still needed to elucidate the underlying cellular and molecular mechanisms acting among autophagy, apoptosis for determination of burn vitality, and differentiation between AM and PM burn injuries.
- Clinical trials on human cadavers should be done to prove the applicability of using them in forensic practice.

VII. Limitations:

The limitations of this study are the relatively small sample size, short-term follow-up, and the limitation to making it not generalizable to other species.

Declarations:

Funding: The authors did not receive support from any organization for the submitted work

Ethical approval: This research was commenced after review and approval by the Research Ethics Committee of Banha Faculty of Medicine (RC. 1.8.2024).

Data availability statement: the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest: All authors of this work declared that there was no conflict of interest.

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