Role of Autophagy Biomarkers In Burn-Age Estimation and Progression

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ABSTRACT

Background: Determination of time passed since burn injury in the living is critical in forensic science. Autophagy biomarkers and vitronectin can play an important role in determination of the age of burn injuries through their levels in the tissue.

Objective: The aim of this study was to investigate the role of autophagy biomarkers in dating burn injury and to correlate them with the histopathological effects of deep second-degree thermal burn.

Method: Fifty-four male rats were used in this study after infliction of second-degree thermal burns to their skin. Samples were taken from them after 30 minutes and one, four, 24, 48, and 72 hours following burn to be examined histologically and also for autophagy biomarkers and vitronectin.

Results: Significant reduction in the autophagy biomarkers ($p < 0.001$) over the first 24 hours then began to increase but still not reach the normal level up to 72 hours after burn. Vitronectin level increased after burn infliction 1.5-fold after first hour, then up to four-fold after four hours and after that began to decline but still did not reach the normal level up to 72 hours.

Conclusion: Autophagy biomarkers can be used as a forensic tool in determination of the time passed since burn infliction in living. *Acad Forensic Pathol.* 2019 9(3-4): 163-171

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As per Journal Policies, ethical approval was not required for this manuscript

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This article does not contain any studies conducted with animals or on living human subjects

STATEMENT OF INFORMED CONSENT

No identifiable personal data were presented in this manuscript

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INFORMATION

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INTRODUCTION

Burn age assessment in forensic medicine is very critical, it is not only important to determine that the burn or wound is vital but also the most accurate age estimation of the wound (1-4).

Burns or burn wounds are different from other types of wounds in many conditions, as in pathway, pathophysiology, and the management (5). Burn healing is a dynamic process with interlaced phases. The initial phase is the inflammatory phase, neutrophils and monocytes infiltrate the site of the injury through fluid extravasation and localized vasodilation. The proliferative phase, which is an important step in wound healing, is characterized by fibroblast and keratinocyte activation by cytokines and growth factors. The vascular network can be closed and restored through keratinocytes migration over the wound (6).

Recent studies have suggested that cell death may occur because of apoptosis, necrosis, and autophagy, all of which lead to burn injury progression (7). Little is known about the autophagy role in determination of burn age and progression.

Autophagy is a pathway that can deliver macromolecule waste to lysosomes. There, they can be converted into active monomers such as amino acids that can be used in maintaining homeostasis (8, 9). Light chain 3 (LC3) is a microtubule-associated protein 1A/1B that can mediate the interactions between microtubules and the components of the cytoskeleton (10). Cleavage of LC3 produces the LC3-I form through lipidation during autophagy; LC3-I is converted to LC3-II to be associated with autophagic vesicles (11). The LC3-II/LC3-I ratio can be used as an indicator of autophagy (12). Beclin-1 level can also be used as an indicator of autophagy (13).

Vitronectin is a glycoprotein synthesized by the liver and found in the extracellular matrix, serum, and bone. Its function is acting as an opsonin and mediates the clearance of thrombin-serpin complexes from both the blood and tissues. In addition, it can support cell adhesion and migration (14). Vitronectin can bind

with growth factor to form a complex that can help in wound healing (15).

The aim of this study was to investigate the role of autophagy biomarkers in dating burn injury and correlate them with the histopathological effects of deep second-degree thermal burn.

MATERIAL AND METHODS

Ethical Considerations

All ethical considerations of the Faculty of Medicine, Assiut University Ethical Committee, Egypt, about animal handling were followed. Appropriate doses of sedation were given to the animals to decrease pain sensation according to the National Institute of Health Guide for the care of Laboratory animals (16).

Animal Handling and Methods

This study was conducted on 54 male Wistar rats, weighing 200 ± 5.5 g. The animals were housed for seven days before the beginning of the study in a well-ventilated room and had free access to water ad libitum. Room temperature was maintained at $24-26^{\circ}\text{C}$, and the animals were exposed to 12 hour light and dark cycles.

Sample Preparation

Rats were grouped randomly into six groups (eight rats per group), with the seventh group served as the control (six rats). Intramuscular injection of ketamine (100 mg/kg) plus xylazine hydrochloride (25 mg/kg) was given after shaving the hair on the rats' dorsal skin in all groups. A metal rod (approximately 5 cm in diameter, 3 cm in height, and 200 g in weight) was heated (100°C) and applied to the skin (for six seconds, with no pressure) to make a deep second-degree thermal burn (17).

The depth of the burn was confirmed by histopathology and microscopic examination (hair follicle destruction, neutrophils infiltration, and microthrombi; **Image 1**). Samples were taken from the first group 30

minutes after burn infliction, from the second group after one hour, from the third group after four hours, from the fourth group after 24 hours, from the fifth group after 48 hours, and from the sixth group after 72 hours.

Skin samples from the wound (center and periphery) from each group were taken according to this period (one-half, one, four, 24, 48, and 72 hours) and the control group. These samples impeded in 10% formaldehyde solution, in a volume equal to ten times the tissue size. Hematoxylin and eosin (H&E) staining was performed to evaluate the histopathologic changes of the burn wounds according to the passed period.

Sections were deparaffinized and rehydrated, then the slices were washed, hematoxylin was added, then rinsed, 1% HCl alcohol was added (ten seconds), then washed (20 minutes), 0.5% eosin was added (two minutes), and then dehydrated with ethanol (95%). Dimethylbenzene was added (five minutes) twice. Slides were examined with a light microscope.

Western Blot Analysis

To determine the level of LC3 and beclin-1, a small part of each specimen was homogenized for Western blot analysis. The samples were homogenized using a DIAX 900 homogenizer (25 000 rpm, 4°C, for five minutes) in lysis buffer (MgCl₂ 20 mmol/L, Tris-HCl 50 mmol/L pH 7.6, NaCl 150 mmol/L, Triton-X 0.5%, aprotinin 5 U/mL, pepstatin 5 Lg/mL, benzamidine 1

mmol/L, leupeptin 5 Lg/mL, and phenylmethylsulfonyl fluoride 1 mmol/L). A bicinchoninic acid protein assay kit was used to determine the protein levels.

Protein (50 Lg) was extracted by using polyacrylamide 15% gel electrophoresis and transferred to a polyvinylidene difluoride membrane and were kept for two hours in Tris-buffered saline at room temperature with Tween (TBS) buffer, then incubated with anti-LC3 antibodies and rabbit anti-beclin-1 antibodies (1/500 each) and diluted in TBST buffer at 4°C overnight.

Bands were normalized using glyceraldehyde-3-phosphate dehydrogenase after washing and incubating of the membranes at room temperature for one hour with secondary antibody and horseradish peroxidase (1/2000).

Vitronectin Analysis

Another part of each specimen was homogenized for assay of vitronectin by enzyme linked immunosorbent assay kit. According to the kits, reagents, standards, and samples were prepared. At 37°C, a standard or sample was added to each well (one-hour incubation), then aspirated and washed three times, 100 µL biotinylated antibody was added (one-hour incubation), then aspirated and washed three times, 100 µL streptavidin HRP was added (one-hour incubation), then aspirated and washed three times. Ninety microliter TMB substrate solution was added (20-minute incubation). Fifty microliter stop solution was added. Read at 450 nm.

Statistical Analysis

Data were analyzed using the SPSS version 20 program. Data were tested for normality and homogeneity of variance. *p* value <0.05 was considered significant.

Image 1: Burn infliction on rat.

RESULTS

Histopathologic Examination

Examination by H&E stain showed that the control specimen revealed the normal characteristics of dermis and epidermis and thin keratinized stratified squamous of the epidermis and thick dermis, which contains nerves, blood supply, and fibroblasts (**Image 2**). The burnt area showed second-degree burns involving all of the epidermis and most of the dermis. After half an hour, the skin exhibited the early inflammatory stage and infiltration of neutrophils with accumulation of fibrin at the edge of the burn with extensive necrosis (**Image 3**). One hour post-burn, the skin showed greater accumulation of fibrin than that after half an hour post-burn (**Image 4**). Four hours post-burn, the skin showed the appearance of cytoplasmic vacuolization and disruption of the tight junctions between the basal cells of the epithelium. Vasodilatation of the vessels in the hypodermis, vascular stasis, and vascular thrombosis is seen in **Image 5**. After 24 hours of healing, there was a line of demarcation between the dermis and the epidermis (**Image 6**). After 48 hours, scab formation, occurred and the line of the epidermis began to become clear and apparent (**Image 7**). After 72 hours, the skin showed the appearance of new capillaries in the healed tissue and disappearance of the vacuolization (**Image 8**).

Image 2: Skin of the control rats shows the epidermis with its stratified squamous epithelium (granular, basal, spinous, and cornified layers) (A). The dermis contains blood vessels (B) (H&E, x200).

Western Blot Analysis

Western blot analysis showed that the concentrations of LC3 and beclin-1 protein were maintained at a certain level in normal skin (3.7 ± 0.03 and 0.77 ± 0.02 , respectively). After the burn, they declined gradually in the tissue until 24 hours post-burn indicating recent burn infliction. Their concentrations increased gradually but remained significantly ($p < 0.001$) lower than normal skin indicating older burn infliction. Quantitative assay showed that autophagy biomarkers decreased to one-fourth of normal level over 24 hours and then gradually increased but did not return to the normal baseline level (**Image 9**, **Figures 1** and **2**, and **Table 1**).

Vitronectin Levels

The Vitronectin levels increased gradually approximately 1.5-fold over the first hour and four-fold after four hours post-injury indicating recent burn infliction and then decreased slightly, but remained higher than in normal skin ($p < 0.001$; **Figure 3**).

DISCUSSION

Burn injuries are known to have a very high mortality rate. Limited research has been performed to try to identify the patterns, causes, and time passed post-

Image 3: Burnt skin after 30 minutes shows infiltration by neutrophils (A) and accumulation of fibrin at the borders of the wound (B) (H&E, x200).

Image 4: Burnt skin after one hour shows the skin section has a greater accumulation of fibrin at the borders of the burn which is more evident than after 30 minutes. This was indicated by a stronger and extended area (H&E, x200).

Image 5: Burnt skin after four hours shows the epidermis with cytoplasmic vacuolization (A) and disruption of the tight junctions between the basal cells of the epithelium (B). In the hypodermis, there was vasodilation, vascular stasis, and vascular thrombosis (C). In both the dermis and hypodermis there was edema (H&E, x200).

Image 6: Burnt skin after 24 hours shows appearance of line of demarcation between dermis and epidermis (A) and thickened edges of epidermis (B) (H&E, x200).

Image 7: Burnt skin after 48 hours shows migration of epithelial cells and scab formation (A) (H&E, x200).

burn (18,19). Vitality and age of injury in the living and dead are of great importance in the field of forensic science. They can help in making a decision as to whether or not the injury date corresponds with the time of crime occurrence (3,20).

The present study was performed to investigate the role of autophagy biomarkers in dating burn injury and correlate them with the histopathological effects of burns. The results in this study indicate that the autophagy level was initially reduced (after one, four, and 24 hours). Autophagy biomarkers reached the lowest

level after 24 hours, then increased (after 48 and 72 hours), but remained below the normal level. The increase in their levels occurred in accordance with the migration of epithelial cells and scab formation (histopathological changes in **Table 1**). The hypothesis about the mechanism is that, at early stages of the burn, the cell number undergoing autophagy is decreased, as necrosis becomes the main event. Later on, when the tissue necrosis ceased, the surrounding tissue was subject to inflammatory and ischemic damage; autophagy may protect against these stress effects as a prosurvival mechanism. This explains the later increase in the

Image 8: Burnt skin after 72 hours showing the appearance of new capillaries in the healed tissue (arrows) and disappearance of the vacuolization in the dermis (H&E, x200).

Image 9: Western blot analysis for light chain 3 and beclin 1 (autophagy markers) shows decline in both compared to normal skin until 24 hours and later when they begin to increase.

Figure 1: Quantitative analysis of light chain 3 (I and II) autophagy markers show that their levels declined after burn infliction until 24 hours. Their levels increased slightly thereafter but remained lower than in normal skin.

Figure 2: Quantitative analysis of Beclin-1 autophagy markers show that their levels declined after burn infliction until 24 hours. Their levels increased slightly thereafter but remained lower than in normal skin.

levels of autophagy. Western blotting supported the prosurvival role of autophagy by significantly increasing autophagy biomarkers. After 24 hours, inflammation decreased and tissue perfusion was restored with higher levels of autophagy biomarkers; this supports the prosurvival role of autophagy in burns.

Debnath et al., Kroemer and Levine, and Tan et al. in their studies support the hypothesis that the autophagy plays a role in cell death (21-23). Autophagy has a role in protecting organisms against neurodegeneration, cancer, infection, and aging. Defects in the autophagy are related to cardiomyopathy, neurodegenerative disease, fatty liver disease, and diabetes. Enhancement of autophagic activity protects against myocardial ischemia, anoxia injury, neuronal ischemia, and slow down aging (24-30).

Also, this study hypothesized that vitronectin has a role in burn wound healing, and this is supported by increase in its level to 1.5-fold after one hour post-burn infliction and up to four-fold after four hours.

Jang et al., in their study about vitronectin deficiency effects, found that loss of vitronectin associated with changes in the fibrinolytic balance led to focal sites of delayed hemorrhage (31). Also, Hashimoto et al. prove that vitronectin contributes in the regulation of fibrinolytic system in wound (32). Upton et al. prove the same by using vitronectin with growth factor as a healing therapy (33).

In conclusion, the current results indicated that the autophagy biomarkers and vitronectin could be used as an indicator for the time passed since burn. Further research on human beings can be done to support the hypothesis of this research.

Table 1: Relation Between Histopathological Changes and the Level of LC3 and Beclin

Time	Histopathological changes	LC3	Beclin
Control		3.7±0.03	0.77 ± 0.02
30 min after burn	Infiltration by neutrophils, and accumulation of fibrin at the borders of the wounds.		
1 hour after burn	Accumulation of fibrin at the borders of the burn, which is more evident than after 30 minutes. This is indicated by a stronger stained and extended area.	3.5±0.02	0.5±0.02
4 hours after burn	The epidermis with cytoplasmic vacuolization, and disruption of the tight junctions between the basal cells of the epithelium. In the hypodermis, there was vasodilatation, vascular stasis, and vascular thrombosis. In both the dermis and hypodermis, there was edema.	1.8±0.03	0.2±0.01
24 hours after burn	Appearance of line of demarcation between dermis and epidermis, and thickened edges of epidermis.	0.9±0.01	0.08±0.03
48 hours after burn	Migration of epithelial cells and scab formation	1.3±0.03	0.11±0.02
72 hours after burn	Appearance of new capillaries in the healed tissue, disappearance of the vacuolization in the dermis.	1.4±0.01	0.15±0.02

Figure 3: The level of vitronectin increased, reaching 1.5-fold one hour after burn infliction and peaked at four-fold after four hours. It then decreased gradually but remained higher than normal.

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