Journal of Forensic and Legal Medicine 52 (2017) 148–153

Contents lists available at ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/locate/jflm

Role of inducible nitric oxide synthase and interleukin-6 expression in estimation of skin burn age and vitality

Mona M. Abo El-Noor [a], Fatma M. Elgazzar [a], Hanan A. Alshenawy [b, *]

[a] Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Tanta University, Tanta, Egypt
[b] Department of Pathology, Faculty of Medicine, Tanta University, Tanta, Egypt

ARTICLE INFO

Article history:
Received 17 April 2017
Accepted 10 September 2017
Available online 12 September 2017

Keywords:
Skin burn aging
Skin burn vitality
Inducible nitric oxide synthase
Interleukin-6
Immunohistochemistery

ABSTRACT

Estimation of age and vitality of burn injury both in the living and dead is essential in forensic practice. Nitric oxide and interleukin-6 (IL-6) play an important role in skin burn healing. In this study, the expression of inducible nitric oxide synthase (iNOS) and IL-6 proteins during skin burn healing in rats was studied for purposes of burn dating and to differentiate between ante-mortem and post-mortem burn. Ante-mortem skin burns were created on forty five rats. Normal and burnt skin samples were taken at 1, 3, 5, 7, 9, 11, 13, 15 and 21 days following burn induction (5 rats for each stage). Post-mortem burn was inflicted 6 h after scarification in another five rats. There was a statistically significant difference in both iNOS and IL-6 expression between the different time intervals of the ante-mortem burn. Expression of both iNOS and IL-6 decreased remarkably in the post-mortem burn with a statistically significant difference from ante-mortem intervals. A statistically significant positive association between the two markers was found. These results indicate that both iNOS and IL-6 expression in ante-mortem burnt skin was time dependent and significantly differed from post-mortem burn. Further research on humans is recommended.

1. Introduction

Wound examination is an essential issue in forensic practice. Determination of wound age and vitality is a classic but still popular and pivotal issue in forensic pathology to evaluate accurately its causal relationship to death.[1]

Wound healing is a dynamic process consisting of three overlapping phases: the inflammatory phase; including coagulation and inflammation, the proliferative phase; consisting of angiogenesis, the formation of granulation tissue, and re-epithelialization, and lastly the maturation phase; involving matrix formation and tissue remodeling.[2]

There are many studies investigated determination of mechanically induced skin wounds by sharp or blunt objects using either animal experiments or samples from cadavers.[3–5] Despite burn injuries are considered a major cause of disability and death[6]; limited information is available on the determination of skin burn injury.

To study the age and vitality of burn injury, inflammatory mediators or cells and matrix proteins in injured tissue could be analyzed.[7] Nitric oxide (NO) plays a crucial role as a signal molecule in the healing process of skin burn.[8] It is synthesized during the conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS) family of enzymes.[9]

Three NOS isoforms have been identified; two are constitutively expressed in cells including neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). The third isoform is inducible (iNOS) and is activated in response to various stimuli such as cytokines, endotoxins and physiopathological conditions.[10]

Interleukin-6 (IL-6) is a multi-functional cytokine released by a variety of cells including macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. It could regulate the inflammatory response of wound healing process.[11]

Dating of an injury depending on the subjective naked eye evaluation is highly variable. Therefore, it is important to study the injuries microscopically. Immunohistochemical staining supports the histological findings and makes observations and interpretation more objective.[12]

The aim of this study was to investigate the expression of iNOS and IL-6 proteins during skin burn injury healing in rats by

* Corresponding author. Department of Pathology, Faculty of Medicine, Tanta University, 25 Hamdy Gado street, Tanta, Egypt.
E-mail address: Hanan_alshenawy@yahoo.com (H.A. Alshenawy).

http://dx.doi.org/10.1016/j.jflm.2017.09.001
1752-928X/

immunohistochemistry for its forensic application in determination of skin burn age in addition to their possible role in differentiating between ante-mortem and post-mortem burn.

2. Materials and methods

2.1. Ethical considerations

Experimental procedures were performed according to the guidelines for the care and use of laboratory animals approved by the Ethical Committee of Faculty of Medicine, Tanta University, Egypt; fewer numbers of animals estimated to affair valid results were used and animal painless procedures were conducted with appropriate sedation to avoid pain and stress. This was in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.[13]

2.2. Animals

This study was conducted on fifty adult male albino rats, their weight ranged from 150 to 200 g. During the study, the animals were kept in wire mesh cages with ad-libitum access to water. The room temperature was about 22–24 ○C and the animals were exposed to 12:12 h light dark cycles. Animals were allowed a two week pre-experimentation period to be acclimatized prior to thermal injury.

2.3. Experimental design

The animals were anesthetized with diethyl ether inhalation in a glass cage. Once anesthetized, the hair on the dorsum of each rat was removed by a sterile shaving razor to reveal a bare region with a diameter of 2 × 2 cm. Thereafter and according to Nagata et al.[14] full-thickness skin burns were made on rats with a heated soldering iron applied for 3 s. The induced burn injuries in all rats were washed with running water for 1 min followed by intra-peritoneal injection of 25 mg/kg of acetaminophen in a volume of 10 mg/ml (Acetaminophen, Perfalgan®, BMS Pharmaceutical, Italy) to overcome pain according to Im et al.[15] They were also injected 1–1.5 ml isotonic saline intra-peritoneal for fluid resuscitation. The animals stopped experiencing any pain by about 12 h after the burn infliction which was evident by their return to normal behavior.

At 1, 3, 5, 7, 9, 11, 13, 15 and 21 days following the burn, a total of 5 rats for each interval were sacrificed by cervical decapitation after being anesthetized by diethyl ether inhalation. These ante-mortem intervals covered the three stages of healing process; inflammatory stage (12hours-2 days), proliferation stage (3–14 days) and the remodeling stage (15–28 days). The remaining five rats encountered post-mortem burn which was inflicted 6 h after scarification and the samples were taken immediately from them.

2.4. Sample preparation and immunohistochemistry staining for iNOS and IL-6

Skin samples were taken from both the center and periphery of the burn and from adjacent non-burned shaved skin of the same animal (served as a control). The dissected tissues were immersed in 10% formaldehyde solution, with a volume ten times the volume of the tissues. Then, they were embedded in paraffin and cut into 5-μm sections for staining with hematoxylin and eosin[16] followed by immunohistochemical staining for iNOS and IL-6.

Immunohistochemistry is readily available and widely used technique in diagnostic and research laboratories.[17] In this technique, sections of skin samples were dewaxed in xylene and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by 3% hydrogen peroxides. The specimens were permeabilized in phosphate buffered saline (PBS) for 10 min, blocked in 20% normal goat serum in 0.01 M PBS, and subjected to antigen retrieval in citrate buffered solution at 92 °C for 15 min. After being washed in PBS, the slides were incubated with the antibody. After washing in PBS, the tissues were incubated by use of biotin-conjugated secondary antibody for 1 h. Then the slides were incubated in streptavidin-biotin horseradish peroxidase complex. Immunoreactivity was visualized by exposing the specimens to diaminobenzidine tetra-hydrochloride (DAB). The sections were counter stained with hematoxylin and then rinsed and mounted. Primary antibodies used were iNOS, Rabbit Polyclonal Antibody (RB-9242-R7, Ready-to-Use: Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA and αIL-6 polyclonal rabbit antibody (Cat-No: ab662 Abcam, Cambridge, UK).

2.5. Semi-quantitative evaluation of iNOS and IL-6 staining

To evaluate iNOS staining, only cells with evidence of cytoplasmic staining were considered positive. The number of iNOS-positive cells was determined by evaluation of 4fields for each slide for each rat per each group at a magnification of ×100 and the results were given as the mean ± SD.[18] Regarding IL-6 staining; the reaction was evaluated in relation to its intensity and its area percentage. IL-6 staining intensity was scored as weak (score 1), moderate (score 2) or strong (score 3), while its area percentage was scored positive if more than 50% of the slide was expressing IL-6 staining in the cytoplasm.[19]

2.6. Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) version 20. The data were tested for normality and homogeneity of variance. One way analysis of variance (ANOVA) and Kruskal-Wallis tests were used to analyze iNOS and IL-6 expression in the different studied groups respectively. Mean rank of scores of IL-6 expression was calculated by ranking all sample data from the smallest to the largest and according to its position in the combined data set the rank was assigned, then the mean of theses ranks was calculated. In addition, Spearmen's rank correlation coefficient was used to investigate the association between the studied two markers. The significance was declared at a P value of less than 0.05.

3. Results

3.1. Histopathological examination

Examination of H&E stained sections at the normal skin revealed its characteristic epidermis and dermis (Fig. 1a while at the burned area revealed deep second degree burns involving most of the dermis. During the inflammatory stage, the most prominent changes were infiltration of neutrophils and extensive edema and necrosis (Fig. 2a).

In the proliferation stage; by the day3, macroscopically, crust was formed from the necrotic tissues and microscopically this crust was rejected from the underlying viable tissues along with the zone of neutrophils infiltration. By the day5, neutrophils were replaced largely by macrophages with the early formation of granulation tissue and new blood vessels. By the day 7, the neovascularization reaches its peak with started scab formation (Fig. 3a). By the days 9, 11 and till 14; decrease in the neovascularization with started deposition of few collagen fibers from accompanied fibroblasts was noticed. The edema fluid started to decrease with increased infiltration with histiocytes, lymphocytes and plasma cells.

In the remodeling stage (14–28 days), the number of