ORIGINAL ARTICLE

Oxidative Stress-related Biomarkers as Forensic Biomarkers for Burned Skin Vitality in Rats


a Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
b Department of Animal Wealth Development, Faculty of Veterinary Medicine, Mansoura University, Egypt
c Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Benha University, Benha, Toukh, Egypt
d Department of Pathology, Faculty of Veterinary Medicine, Mansoura University, Egypt
e Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt
f Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University, Egypt
f Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Delta University for Science and Technology, Gamasa, Egypt
g Department of Plastic and Reconstructive Surgery, Faculty of Medicine, Mansoura University, Egypt

Abstract

OBJECTIVE: In forensic practice, the diagnosis of whether a skin injury is inflicted in life or not is a challenging research topic. Therefore, new reliable biomarkers of vitality are urgently required. In this study, biochemical, molecular, and histopathological techniques were used to explore the role of oxidative stress biomarkers in differentiating antemortem from postmortem burn injury.

DESIGN, ANIMALS AND PROCEDURES: Eighteen male rats were allocated to three groups: normal unburnt group, antemortem burn group, and postmortem burn group.

RESULTS: The analysis of skin specimens revealed a notable increase in malondialdehyde (MDA) levels together with a decrease in GSH and total antioxidant capacity (TAC) in both burnt groups compared with the controls. The molecular results revealed marked upregulation of nuclear factor-erythroid-2-related factor (Nrf-2) and downregulation of Kelch-like ECH-Associated protein-1 (Keap-1) gene expression in the skin under thermal injury. These oxidative biomarkers were significantly higher in vital burns than in the postmortem burns.

CONCLUSION AND CLINICAL RELEVANCE: Our findings suggest that cellular oxidative injury markers are helpful tools for the forensic diagnosis of vital burn reaction.

Keywords: Forensic diagnosis, Nuclear factor-erythroid-2-related factor/Kelch-like ECH-Associated protein-1 pathway, Oxidative stress, Skin burn, Vital reaction

1. Introduction

Burn injuries are a critical public health problem that result in multiple organ damage and high mortality [1,2]. In forensics, the examination of burn wounds is a crucial step, particularly during the assessment of burned corpses. Forensic pathologists often have to determine whether the burn infliction occurred during life (antemortem) or after death (postmortem) because this contributes to the reconstruction of crime scenarios [3]. The differentiation between premortem from postmortem burns depends on external vital reactions, such as erythema and blisters, as well as internal reactions as elevated carbon monoxide-hemoglobin in the circulation and smoke deposits in the respiratory tract [4]. However, these methods may be unremarkable in some complex conditions such as putrefaction, which can also cause the development of erythema.
and blisters in addition to the absence of carbon monoxide-hemoglobin in the case of open areas [5].

Many cellular mechanisms are involved in burn pathophysiology including increased production of proapoptotic proteins, inflammatory molecules, and thrombus formation as a result of vascular damage [6]. Burn-induced ischemia-reperfusion injury is one of the main causes of cellular oxidative injury through the formation of highly reactive radicals. Therefore, the generation of reactive oxygen species (ROS) is responsible for the progression of oxidative stress during thermal injury [7]. In addition, complement activation and intravascular stimulation of neutrophils increases the production of cytotoxic ROS. The enhanced catalytic activity of xanthine oxidase, causes the production of toxic byproducts, such as hydrogen peroxide and hydroxyl radicals, which directly damage the skin structure [8]. Beiraghi-Toosi et al. [9] found significant declines in total glutathione and reduced glutathione levels with marked increases in oxidized glutathione in the serum samples of patients with more than 15 % burns.

Under the cellular oxidative stress of burn, the production of heme oxygenase-1 (HO-1) enzyme increases owing to the nuclear translocation of nuclear factor-erythroid-2-related factor (Nrf-2) to protect the cellular structure and function [2]. Nrf 2, a crucial transcription factor, is normally located in the cytoplasm and attached to Kelch-like ECH-Associated protein-1 (Keap-1). Nevertheless, it separates from Keap-1 and moves to the nucleus under stress to stimulate the production of phase 2 detoxification proteins such as HO-1 [10]. HO-1, a microsomal enzyme, protects cells via the activation of antioxidant, anti-inflammatory, and antiapoptotic machineries [11].

As the differentiation between pre and postmortem burn injuries is a challenge in forensic pathology, our study explored the forensic application value of oxidative-related markers in identifying vital skin reactions in a rat burn model.

2. Materials and methods

2.1. Experimental animals

Eighteen male Wistar rats, aged 6–8 weeks, weighing 200–210 g were obtained from the animal house of the Faculty of Pharmacy, Mansoura University, Egypt. They were maintained on 12 h light/dark cycle and were provided food and water ad libitum under a controlled environment of 22–26 °C, 50–60 % humidity. Intensive care was provided to reduce animal suffering throughout the experiment.

2.2. Induction of burn model

Rats were assigned to three groups (n = 6/group). The first group (control group) was taken from nonburned rats. Full-thickness burns were induced as previously described [4]. Briefly, the skin of the back was shaved and then exposed to a heated circular metal plate (20 mm in diameter) at 100 °C for 5 s under anesthesia. Rats were directly resuscitated by saline injection at a dose of 0.05 ml/g body weight intraperitoneally.

Five minutes after burn injury, rats (the second group) were anesthetized using an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight). After that, they were sacrificed, and the surrounding tissue within 2 mm from the edge of the burnt skin was sampled. The selection of time depends on the usual interval between thermal injury and death.

Third group (postmortem burn), the dorsal skin of the sacrificed rats was shaved and then exposed to a heated metal plate for 5 s for 15 min under anesthesia. Skin samples were collected. Some skin samples were stored at −80 °C for biochemical analysis. The remaining part was stored in RNA later for molecular analysis. The last portion was fixed in 4 % formaldehyde buffered saline for Masson’s trichrome staining.

2.3. Sample preparation

The skin tissue was weighed and tissue homogenates 10 % (w/v) were prepared in ice-cold isolation buffer. The homogenate was centrifuged at 1000×g for 8 min at 4 °C. The supernatant was collected in fresh tubes and used for analysis.

2.4. Estimation of oxidative stress biomarkers

2.4.1. Measurement of malondialdehyde (MDA)

Lipid peroxidation was estimated by measuring tissue MDA using thio barbituric acid reactive substances (TBARS) assay [12]. The resulting MDA-TBA chromophore was colorimetrically measured at 540 nm using microplate absorbance reader (Bio Rad, Hercules, CABio, USA). The obtained values were expressed as nmol of MDA per mg of protein.

2.4.2. Assessment of reduced glutathione (GSH) levels

GSH in the skin homogenates was determined as described by Ellman [13], depending on the amount of thiol groups in the sample to dissociate the disulfide bond of Ellman’s reagent and then quantified at 415 nm using microplate absorbance reader.
The results are expressed as nmoles of GSH/mg protein.

2.4.3. Estimation of total antioxidant capacity (TAC)
Total antioxidant capacity (TAC) kit (Biodiagnostic, CAT. NO TA2513, Egypt) was used for TAC measurements [14]. The reaction between the antioxidants in the sample and a known amount of exogenous hydrogen peroxide (H$_2$O$_2$) was analyzed. Antioxidants in the sample could eradicate a portion of H$_2$O$_2$ and the residual part was measured colorimetrically at 505 nm. The results were expressed in units/min/mg protein.

2.5. RNA extractions, reverse transcriptions, and qRT-PCR
Total RNA was isolated from burnt skin samples using TRIzol reagent (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions. cDNA was synthesized using a Super ScriptVILO cDNA Synthesis Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Quantification of target mRNA levels was performed by qRT-PCR using SYBR Green MasterMix (Life Technologies, CA, USA) with an Applied Biosystems 7500 Instrument. β-actin was used as the housekeeping gene and the primer sequences are listed in Table 1. Changes in gene expression with respect to the controls were evaluated according to the standard $^{\Delta \Delta C_t}$ method of Pfaffl [15].

2.6. Masson’s trichrome staining
Dermal skin specimens were stained with Masson’s trichrome to examine collagen fibers. The slides were deparaffinized, rehydrated using descending dilutions of alcohol and washed with distilled water. The sections were stained with Weigert's iron hematoxylin working solution for 10 min and rinsed with running warm tap water for another 10 min. Next, the slides were incubated with Biebrich scarlet-acid fuchsin solution for 10–15 min. This step was followed by differentiation in phosphomolybdic-phosphotungstic acid solution for 10–15 min or until the collagen was not red. After that, the sections were left in aniline blue solution for 5–10 min, rinsed with distilled water and differentiated in 1% acetic acid solution for 2–5 min. Finally, the sections were washed with distilled water, dehydrated in xylene and examined under a light microscope (Olympus CX31, Japan).

2.7. Image J analysis
Optical photomicrographs of the tested sections were analyzed using the ImageJ software. The tool bar image was opened and clicked for the RGB image, which was then analyzed by adjusting the image tools in the software and thresholding to select the area of Masson’s trichrome positivity. The area of positive staining was analyzed using tool bar measurement. The average percentage of three sections was calculated and all data are presented as the means ± standard error of mean (SEM).

2.8. Statistical analysis
One-way analysis of variance (ANOVA) was used for statistical analysis, followed by Tukey's multiple comparisons test. The data are displayed as mean ± SEM. The statistical significance criterion was set at the $P<0.05$ level. GraphPad Prism (version 8, GraphPad Software, Inc., San Diego, CA, USA) was used for graphical representation.

3. Results
3.1. Biochemical findings
The oxidant/antioxidant status of burnt skin tissues in different groups is shown in Fig. 1. Lipid peroxidation in the burnt skin expressed as MDA was markedly elevated ($P<0.05$) compared with that in the control unburned group. In addition, a remarkable increase ($P<0.05$) was detected in the MDA levels in rats exposed to antemortem burn compared with the postmortem burn group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Oligonucleotide sequence</th>
<th>Annealing temperature (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf 2</td>
<td>NM_031144.3</td>
<td>f5,-GTCCACCCGCGAGTACAACCT-3, r5,-GGAGCCGTTGTCGACGACGA-3</td>
<td>60</td>
<td>119</td>
</tr>
<tr>
<td>Keap 1</td>
<td>NM_057152.2</td>
<td>f5,-GGACGCGCTGAGCAGACGA-3, r5,-GGACGAGATGGCCCTAGATG-3</td>
<td>58</td>
<td>224</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_031144.3</td>
<td>f5,-GGCATGTCAGACGCAGCCGCTT-3, r5,-TAGGAGTCCTTCTGACCATA-3</td>
<td>58</td>
<td>116</td>
</tr>
</tbody>
</table>
In addition, a significant decrease in \((P < 0.05)\) GSH levels was observed in tissues exposed to thermal injury during life compared with the control. Adversely, at the postmortem stage, the tissue GSH levels showed notable increases \((P < 0.05)\) in comparison with the skin samples from the vital burn group.

Furthermore, noteworthy declines \((P < 0.05)\) were observed in TAC levels in the vital burnt tissue with respect to the control unburnt group. However, TAC in the postmortem group was higher \((P < 0.05)\) than that in the antemortem group.

### 3.2. Molecular findings

The fold changes in Nrf-2 showed marked upregulation \((P < 0.05)\) in burned regions in relation to the control unburnt skin. It was also observed that the induced burn before death had a higher mRNA expression levels \((P < 0.05)\) of Nrf-2 than that of the induced injury after death (Fig. 2).

In contrast, the mRNA expression of Keap-1 displayed a marked increase \((P < 0.05)\) in the control group compared with the groups with burn injury. Additionally, the expression level of this antioxidant sensor was significantly higher in the antemortem group \((P < 0.05)\) than its expression level in the postmortem group (Fig. 2).

### 3.3. Masson trichrome findings

Representative Masson’s trichrome staining of the skin from different treatment groups (Fig. 3). The control group exhibited mild collagen deposition in the dermal layer. The antemortem burn group displayed thinner collagen-like threads in dermal layers. However, the postmortem burn group showed marked dense bluish collagen deposition.
The quantitative estimation of the collagen deposition thickness in the different treatment groups is shown in Fig. 4. As shown, significantly higher (\(P < 0.05\)) collagen deposition was seen in the postmortem burn in comparison with the other groups. No significant difference was observed between the control group and antemortem group.

4. Discussion

In the field of forensic investigation, the determination of burn vitality in living and dead bodies has significant medico-legal importance. During the examination of a burnt corpse at a fire scene, the forensic pathologist must determine whether the individual was alive or dead during the infliction of the burn injury. Nevertheless, conventional methods for distinguishing vital burns are generally nonspecific. Consequently, the searching for novel biomarkers of burn vitality is crucially needed [16]. To our knowledge, tissue levels of oxidative stress-related biomarkers for differentiation between antemortem and post-mortem burn have not been previously investigated.

The response of skin tissue to thermal injury is a complex process. Thermal burn elicits a remarkable change in body metabolism, defined as oxidative stress caused by the generation of ROS with a marked decrease in the TAC of the biological tissue [17]. ROS overgeneration may evoke peroxidation of the lipid components of the cells which is expressed as MDA and is also a marker of oxidative stress [18]. In addition, GSH is a crucial antioxidant molecule that regulates the redox environment, traps ROS, and triggers antioxidative defense inside stressed cells. GSH exerts its ROS scavenging action directly and
through enzymatic reactions. In the latter process, it acts as a reducing agent by donating an electron to produce its oxidized form, GSSG, in the presence of glutathione peroxidase (GPx) [19]. Hence, a decrease in tissue GSH and increase in tissue MDA are indicative of cellular oxidative injury [20,21].

In the ante-mortem burned rats, we observed a significant decline in the burnt tissue TAC compared with the control. Gürünlüoğlu et al. [22] reported marked increases in the serum levels of MDA and total oxidant capacity (TOC) together with decreases in TAC and GSH in partial thermal burn injury in children. In addition, the blood levels of MDA in rats with third-degree burns were significantly increased and the levels of TAC were significantly decreased [23]. Likewise, notable decreases were detected in SOD and CAT enzymatic activities as well as GSH levels, in addition to significant increases in MDA levels in rat's skin exposed to 90 °C bath for 10 s to induce severe skin scald injury [24]. Additionally, Tammam et al. [2] reported that burn injury evoked a noticeable oxidative microenvironment as represented by declines in SOD, CAT, and GSH levels together with an increase in MDA levels in a rat model of skin burn. Upon comparing the burned groups, remarkable increases were observed in the levels of MDA with declines in TAC and GSH in the pre-mortem burned skin tissue with respect to the postmortem one. After death, body tissues undergo to a sequence of biochemical changes due to oxygen deficiency, impairment in enzymatic reactions, lack of metabolites, and collapse of cell components [25]. These results may be useful for understanding the molecular vital reaction to burn injury in the skin. Thus, these markers may aid in the diagnosis of burn vitality.

Evidence suggests that Nrf-2/Keap-1/HO-1 signaling pathway is involved in the pathogenesis of skin wound progression and healing [26,27]. Under stress condition, Nrf-2 is detached from Keap-1 and moves from the cytoplasm to the nucleus. In these cells, Nrf-2 triggers the production of phase 2 detoxification proteins such as NAD(P)H: quinone oxidoreductase-1 (NQO1), and HO-1 [28,29]. In our study, the group exposed to vital burns displayed marked downregulation in the expression of Nrf-2 with upregulation in the expression of Keap-1 at 5 min postburn. These results may be related to cellular response against burn-associated oxidative stress. A study performed by Chen and colleagues demonstrated that mice that lack Nrf-2 were more vulnerable to burn trauma-associated intestinal injury and systemic inflammation and had a markedly high lethality rate [30]. Furthermore, down-regulated mRNA expressions of Nrf-2, NQO-1, and HO-1 together with upregulated mRNA expression of Keap-1 was observed in the wound tissue of diabetic rats [26]. Similar findings were reported in other studies [27,31,32]. Comparing with the ante-mortem burn group, substantial decreases in Nrf-2 expression with increases in Keap-1 expression were observed in the postmortem burned skin tissue. Consequently, both molecular markers can aid the forensic diagnosis of burn vitality.

5. Conclusion

In summary, the current study suggested that comparing the levels of oxidative stress-related markers might be helpful for the determination of vital reactions in burned skin in forensic contexts. Remarkably, marked increases were noticed in the levels of MDA with declines in TAC and GSH in the antemortem burned skin tissue with respect to the postmortem burn. At the molecular level, Keap-1 was markedly upregulated, and Nrf-2 was downregulated in the group exposed to vital burns compared with those exposed to non-vital burns. Further studies are highly recommended to support the using of these markers as objective tools for determining thermal injury dating and vitality.

Author contributions

Conceptualization, validation, methodology: O A. H and A A; supervision, formal analysis and data curation: M M. B, and A M. Z; writing-review and editing: A A, A E and I I. All authors have approved the published version of this manuscript.

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Institutional board statement

The study protocol was approved by the Medical Research Ethics Committee for Animal Research Studies at the Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt (Code No. R/MU-ACUC (VM.R.23.09.125).

Conflicts of interest

None.

References


