Tissue Extract Fluid Cytokine Levels as Markers for Wound Vitality: An Experimental Comparative study

Ahmed H. Rizk MD, Mohamed El-Shishtawy MD, Adel F. Al-Kholy MD*

Departments of Forensic & Clinical Toxicology & Medical Biochemistry*, Faculty of Medicine, Benha University

Abstract: Objectives: To experimentally evaluate the ability of estimation of tissue extract levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 for differentiation between antemortem (AM) and postmortem (PM) wounds and for rough determination of time lapsed since death till wound inflection. **Material & Methods**: The study comprised 20 normal healthy growing adult male albino rats. Four skin biopsies were obtained from the back of each animal; one AM and 3 PM. Each PM biopsy was obtained from the edge of new wound inflected at 30-min (PM1), 60-min (PM2) and 6 hours (PM3) after death. The subcutis of skin specimens was removed and skin samples were homogenized and tissue extract fluid (TEF) was used for ELISA estimation of IL-1β, TNF-α and IL-6 levels. **Results**: Mean AM specimens TEF levels of IL-1β and TNF-α were significantly higher compared to their levels estimated in the three PM specimens with significantly higher levels in PM1 and PM3 specimens compared to PM2 specimens. Mean PM2 specimens TEF levels of IL-6 were significantly higher compared to AM, PM1 and PM3 specimens with significantly higher levels in AM specimens compared to PM3 specimens. There was a positive significant correlation between TEF levels of IL-1β and TNF-α with wound vitality. While TEF levels of IL-6 showed a positive significant correlation with time lapsed between death and wound inflection. Using receiver operating characteristic (ROC) curve analysis, elevated TEF level of IL-1β showed significantly high sensitivity for identification of wound inflected since ≤60 minutes PM with area under curve (AUC=0.186, p=0.035) compared versus the null hypothesis, while TNF-α and IL-6 showed non-significant difference. Conclusion: Estimation of TEF levels of IL-1β, TNF-α and IL-6 could differentiate between wounds inflected while animal was alive and PM wound. However, estimation TEF level of IL-1β works better for such differentiation and could determine wound inflected within 60 minutes PM with high sensitivity.

[Ahmed H. Rizk, Mohamed El-Shishtawy, Adel F. Al-Kholy. **Tissue Extract Fluid Cytokine Levels as Markers for Wound Vitality: An Experimental Comparative study**. *J Am Sci* 2013;9(1):188-193]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 30

Keywords: Wound age, Tissue extract fluid, Interleukin-1β, Interleukin-6, tumor necrosis factor-α

1. Introduction

The term wound describes the morphologic-functional disruption of the continuity of a tissue structure. A wound can be inflicted during life—when the cardiovascular and respiratory system is still intact—or after death, i.e. after cardiac and respiratory arrest. Traumatization during life triggers vital reactions that do not occur in postmortem wound **(Grellner & Madea, 2007)**.

Three types of vital reactions in wound healing can be distinguished: reactions of the scavenger type, which are almost exclusively mediated by blood cells; reactions by complex signal transduction pathways, which involves cascade-like release of chemokines, cytokines and adhesion molecules and may influence type 1 and type 3 reactions and reactions of the scarring type, which involve the final repair of the damaged tissue and are carried out primarily by cells residing at the wound edges, i.e. partly concerning mesenchymal cells and partly tissue-specific cells dependent on the involved organ system **(Goldberg *et al.*, 2007)**.

Wound healing is an integrated and complex process involving a large number of regulatory molecules, including proinflammatory cytokines and growth factors, and an orchestrated tissue response. Dysregulation in cytokine or growth factor expression dramatically alters the normal wound healing process, and blocking the inappropriate production of specific proinflammatory cytokines or supplementing the milieu with increased quantities of growth factors has illustrated the central role played by these mediators in healing process **(Kondo & Ishida, 2010)**.

Hu *et al.* (2010) investigated whether interleukin (IL)-1 could affect chemokine production by two different types of cells that are present within wounds and found the level of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 1α, exhibited very large (75- and 463-fold, respectively) differences within wound tissue and genetic variation within Nalp1, an inflammasome component that regulates IL-1 production, correlated with such difference, and consistent with the genetic correlation, IL-1β was shown to stimulate KC production by murine keratinocyte and fibroblast cell lines *in vitro*.

IL-6 is produced in the wound by epidermal keratinocytes, dermal fibroblasts, and macrophages and it affects multiple processes that are related to

wound healing. After wounding, IL-6 is produced and it increases adhesion of neutrophils to dermal fibroblasts and modulates growth factors. IL-6 expression is intimately involved in re-epithelialization, granulation tissue formation, and inflammation. Preliminary data suggest that the mechanism by which IL-6 controls wound healing is indirect; involving the regulation of genes involved in growth factor expression a processes that need its presence for long duration, **(Zubair *et al.*, 2012)**.

After surgical trauma, burns or injury, a reduction of cellular proliferation and secretion of cytokines by mitogen-stimulated T lymphocytes has been observed, consistent with this, there is defect of stimulated T cells to proliferate and to secrete TNF-α. Interestingly, TNF-α and interferon-γ deficiencies have been shown to result in impaired immune defense against protein antigens and various pathogenic microorganisms **(Sun *et al.*, 2012, Zubair *et al.*, 2012)**.

The current experimental comparative study aimed to evaluate the ability of estimation of tissue extract fluid levels of IL-1β, TNF-α and IL-6 for differentiation between antemortem and postmortem wounds and as a trial for rough determination of time lapsed since death till wound inflection.

2.Materials & Methods

The current study was conducted at Departments of Forensic Medicine and Medical Biochemistry, Faculty of Medicine, Benha University since Jan till June 2012. The study included 20 normal healthy growing adult male albino rats, weighing 200-250 gm. Rats were purchased from the laboratories of Ministry of Agriculture, and kept under standard conditions, temperature 20^{o}C, humidity 60% and 12-hrs day/night cycle, and maintained on standard diet and free water supply till the start of study regimens.

Wounds inflection and specimen collection:

With ether anesthesia, the skin of the back of each rat was lightly shaved and immediately one midline linear incision, 1-cm long, was made through the whole skin thickness with a sharp scalpel. The underlying muscle was not cut. Skin biopsy was obtained from skin tissue surrounding the wound; biopsy dimensions were 1-cm length, 2-mm width and 2-mm depth. The skin edges were then brought together with three interrupted sutures of sterile black silk thread inserted with a sterile curved needle. No dressing was applied to the wound and the animal was returned to its cage. After animals were fully recovered of anesthesia, animals were killed and then new wounds were performed in the healthy part of back skin and similar skin biopsies were obtained from the edge of new wounds inflected at 30-min (PM1), 60-min (PM2) and 6 hours (PM3) after death. Thus one antemortem specimen was obtained at time of wound inflection and 3 postmortem specimens were obtained from 3 PM wounds; PM-1, PM-2 and PM-3, respectively. The subcutis of the obtained specimens was removed and skin samples were stored at −80°C until analysis.

Specimen processing

1. Tissue extraction: the frozen skin specimens were homogenized mechanically and by incubation in an oscillating mill under the permanent addition of liquid nitrogen (1–2 min). The resulting product, which resembled a powder, was transferred to cups, weighed (wet weight) and extracted in a ten-fold volume of phosphate buffered saline (PBS, pH 7.4) and protease inhibitors [2 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM ethylenediaminetetraacetate (EDTA); 1μg/ml of leupeptin, antipain, aprotinin and pepstatin A, respectively]. The extraction took 1 h at 4°C under permanent agitation. Then the samples were centrifuged for 20 min at 15000 g and 4°C. The resulting solution (soluble cytokine fraction) was stored at −80°C until ELISA analysis, **(Nickoloff *et al.*, 1995)**.
2. Determination of sample protein content (mg/ml) by three-fold photometric measurements at 560 nm according to the microtiter plate method of the bicinchoninic acid protein assay (Pierce, USA), **(Wiechelman *et al.*, 1988)**.
3. Measurement of IL-1β **(Dinarello, 1992)**, IL-6 **(Gaines-Das & Poole, 1993)** and TNF-α **(De Kossodo, 1995)** by enzyme-linked immunosorbent assays using commercial kits (Boehringer GmbH, Mannheim, Germany).

Statistical analysis

Obtained data were presented as mean±SD, ranges, numbers and percentages. Results were analyzed using Wilcoxon; ranked test for unrelated data (Z-test) and Chi-square test (X^2 test). Possible relationships were investigated using Pearson linear regression. Sensitivity of estimated parameters as predictors for vitality were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that AUC=0.05. Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. *P* value <0.05 was considered statistically significant.

3. Results

Mean TEF levels of IL-1β and TNF-α estimated in AM specimens were significantly (p<0.05) higher compared to their levels estimated in the three PM specimens. Moreover, mean TEF levels of IL-1β and TNF-α estimated in PM1 and PM3

specimens were significantly (p<0.05) higher compared to their levels in PM2 specimens with non-significant (p>0.05) difference between PM1 and PM3 specimens, (Fig. 1). On contrary, mean TEF levels of IL-6 estimated in PM2 specimens were significantly (p<0.05) higher compared to the levels estimated in AM, PM1 and PM3 specimens. Mean TEF levels of IL-16 estimated in AM specimens were significantly (p<0.05) higher compared to levels estimated in PM3 specimens, but were non-significantly (p>0.05) higher compared to levels estimated in PM1 specimens with non-significant (p>0.05) difference between PM1 and PM3 specimens (Table 1, Fig. 2).

For differentiation between AM and PM specimens, there was a positive significant correlation between TEF levels of IL-1β and TNF-α with vitality of the wound, while the correlation was negative and non-significant between wound vitality and TEF levels of IL-6. On the other hand, TEF levels of IL-6 showed a positive significant correlation with time lapsed between time of death and wound inflection, while such correlation was positive but non-significant with TEF levels of IL-1β and TNF-α (Table 2).

Using ROC curve analysis, elevated TEF level of IL-1β showed high sensitivity for identification of wound inflected since 60 minutes or less after death with significant difference of AUC compared versus the null hypothesis (AUC=0.186, p=0.035) while TNF-α and IL-6 showed non-significant difference (AUC=0.334 & 0.401, p>0.05, respectively) versus the null hypothesis as sensitive predictors of similar wounds (Fig. 3).

Table (1): Mean tissue extract fluid (TEF) levels of estimated parameters in studied specimens

	AM	PM1	PM2	PM3	Statistical analysis
IL-1β	1.88±0.56 (1.22-2.6)	1.12±0.33 (0.49-1.67)	0.72±0.43 (0.2-1.84)	1.3±0.32 (0.65-1.6)	t=8.225, p_1<0.001 t=9.266, p_2<0.001 t=4.257, p_3<0.001 t=4.256, p_4<0.001 t=1.733, p_5>0.05 t=5.472, p_6<0.001
TNF-α	1.36±0.35 (0.85-2.1)	1.17±0.34 (0.4-1.6)	1.08±0.29 (0.35-1.5)	1.25±0.39 (0.55-1.8)	t=3.804, p_1=0.001 t=4.408, p_2<0.001 t=2.994, p_3=0.007 t=3.611, p_4=0.002 t=2.246, p_5=0.037 t=3.336, p_6=0.003
IL-6	2.58±0.47 (1.85-3.2)	2.63±0.65 (1.85-4.1)	3.18±0.67 (2.3-4.6)	2.35±0.41 (1.6-2.9)	t=0.491, p_1>0.001 t=3.467, p_2=0.003 t=2.621, p_3=0.001 t=2.945, p_4=0.008 t=1.975, p_5>0.05 t=4.681, p_6<0.001

Data are presented as mean±SD, ranges are in parenthesis
p_1: significance of difference between TEF levels in AM and PM1 specimens
p_2: significance of difference between TEF levels in AM and PM2 specimens
p_3: significance of difference between TEF levels in AM and PM3 specimens
p_4: significance of difference between TEF levels in PM1 and PM2 specimens
p_5: significance of difference between TEF levels in PM1 and PM3 specimens
p_6: significance of difference between TEF levels in PM2 and PM3 specimens

Table (2): Correlation between tissue extract levels of studied parameters and wound age and vitality

	Wound vitality		Wound age	
	r	p	r	P
IL-1β	0.615	<0.001	0.056	>0.05
TNF-α	0.319	=0.004	0.123	>0.05
IL-6	-0.229	=0.041	0.270	=0.015

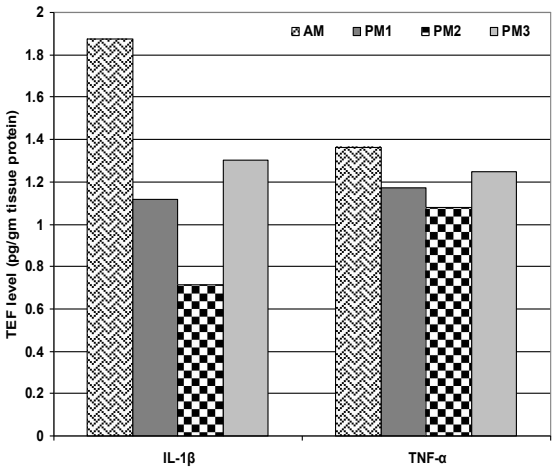

Fig. (1): Mean TEF levels of IL-1β and TNF-α estimated in studied specimens

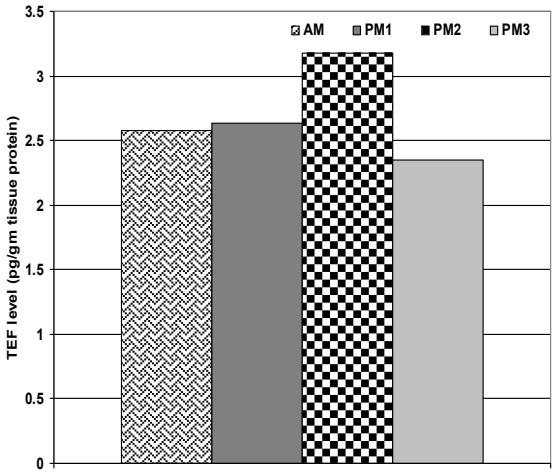

Fig. (2): Mean TEF levels of IL-6 estimated in studied specimens

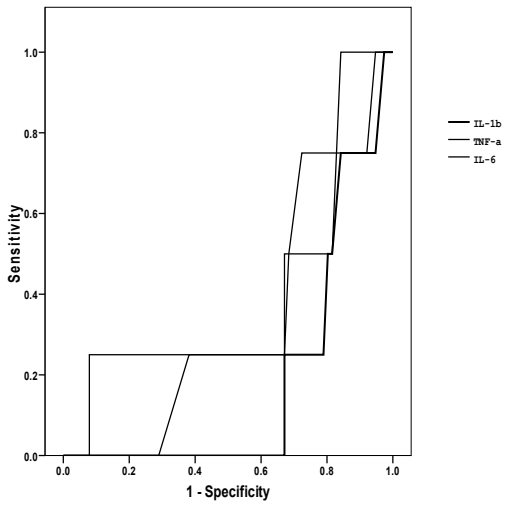

Fig. (3): ROC curve analysis of estimated parameters as predictors of vitality

4. Discussion

Skin wound healing is a primitive but well orchestrated biological phenomena consisting of three sequential phases, inflammation, proliferation, and maturation. Wound examination is indispensable in forensic practice; it is always necessary to determine wound vitality or wound age to correctly evaluate the relationship between death and any wounds. With the development of immunohistochemistry and chemical analyses, the scientific field of wound age determination has advanced progressively during recent years. In particular, it has been demonstrated that collagens, cytokines, and growth factors are useful candidates and markers for the determination of wound vitality or age **(Braiman-Wiksman *et al.*, 2007, Cecchi *et al.*, 2012)**.

The current study aimed to evaluate the ability of estimation of TEF levels of certain inflammatory cytokines for differentiation between antemortem and postmortem wounds and as a trial for rough determination of time lapsed since death till wound inflection. In line with the methodology applied regarding estimation of TEF cytokines levels and not in serum, **Carvalho *et al.* (2008, 2012)** found no correlation between wound exudates levels of IL-6, IL-1β and TNF-α and other cytokines and their serum levels in patients undergoing surgical procedure and concluded that the lack of significant correlations between wound and serum levels emphasizes the importance of determining site-specific release.

In line with the target of the work, **Guler *et al.*(2011)** tried to determine the importance of ubiquitin and tenascin in wound age and found that tenascin and ubiquitin together was useful in determining wound age semiquantitatively. **Ishida *et al.* (2012)**, examined the expression of cyclooxygenase-2 (COX-2) using 60 human skin wounds of different ages and reported that collectively, COX-2 would be a useful marker for the determination of early wound age

Mean AM specimens TEF levels of IL-1β and TNF-α were significantly higher compared to their levels estimated in the three PM specimens with significantly higher levels in PM1 and PM3 specimens compared to PM2 specimens. Mean PM2 specimens TEF levels of IL-6 were significantly higher compared to AM, PM1 and PM3 specimens with significantly higher levels in AM specimens compared to PM3 specimens.

In line with these data, **Grellner *et al.* (2002)** found IL-1β, IL-6 and TNF-α were weakly expressed in normal human skin, however, the staining pattern changed significantly in vital wounds concerning epidermal layers, subepidermal cells,

vessels and sweat glands and showed enhanced expression after 15 and 20 min at the earliest increase of epidermal reactivity and after 30-60 for IL-1β and 60-90 min for IL-6 and TNF-α, marked expression was observed, persisted over several hours and then decreased to basal levels again and concluded that proinflammatory cytokines can serve as a useful tool for the estimation of vitality and wound age, in particular in the early post-traumatic interval prior to leukocyte reaction. **Wang & Ding, (2003)** explored the relationship between the change of cytokines levels and the wound age during the healing process of rats skin wound using ELISA assay performed on intravital skin wounds after incision 0.5-168 h to detect their dynamics expression and reported that the level of IL-2 and TNF-α increased at 30 min after wounding, got to a peak at 3 hrs for IL-2 and 1 h for TNF-α after injury, rebound of levels were shown at 48 h after wounding, and levels were inclined thereafter.

Bai *et al.* (2008) reported time-dependent expression of IL-1beta, COX-2, MCP-1 mRNA after incised wounds in rabbit skin using real-time fluorescent quantitative PCR and concluded that the significant increase of these cytokines and enzyme at different early wound ages implied that the combined investigation could make wound age determination more objective and accurate and the three markers could also be used to distinguish the supravital injuries. **Takamiya *et al.* (2008 & 2009)** performed quantification of IL-2, 4, 6, 8 and 10, IFN-γ and TNF-α in human dermal wounds for wound age estimation and found that among the cytokines analyzed IL 6, IL 8, IFN-γ, and TNF-α were strongly expressed and suggested that multiplex cytokine analysis at the wound site can be useful for useful in daily forensic practice for wound age estimation.

The significantly higher levels of the estimated cytokines in AM specimens compared to PM3 specimen indicated the fact that the transudation of the tissue with blood components (soluble cytokines in the serum, additional release from cellular elements such as monocytes) may contribute to the rapid quantitative increase of proinflammatory mediators and the negative results in PM wound point to the fact that intact circulation is required and that sole passive transudation occurring in postmortem injury is not sufficient to raise tissue cytokine levels, **(Grellner *et al.*, 2000)**. However, the significantly higher levels of IL-6 in PM2 wound specimens; i.e., wound inflected postmortem could be attributed to the fact that multipotent keratinocytes, mast cells, sweat glands and partly macrophages contain higher amounts of various cytokines especially IL-6 and are stored there as inactive precursors or in the active form thus acting as a continuous source for this cytokine for a longer duration, **(Holzheimer & Steinmertz, 2000)**.

There was a positive significant correlation between TEF levels of IL-1β and TNF-α with wound vitality, while TEF levels of IL-6 showed a positive significant correlation with time lapsed since wound inflection. ROC curve analysis defined elevated TEF level of IL-1β as a sensitive indicator of vitality during wound inflection and assured wound age of ≤60 minutes. This finding could be attributed to the fact IL-6 production requires the expression and release of IL-1 so as long as the tissues are viable there is more IL-1 and subsequent increase of IL-6 production. In line with this attribution **Sugawara *et al.* (2001)** reported that constitutive keratinocyte-derived IL-1 is a stimulus for IL-6 production in wounded epidermis and the response involves nuclear factor (NF) kappa B and NF-IL-6 transcription factors. As another explanation, **Kagawa *et al.* (2009)** reported that each gene of examined cytokines had differentially expressional patterns with time-course in wounded skin, and this could serve to aid in the accurate diagnosis of wound age.

The obtained results and review of literature allowed concluding that estimation of TEF levels of IL-1β, TNF-α and IL-6 could differentiate between wounds inflected while victim was alive and PM wound. However, estimation TEF level of IL-1β works better for such differentiation and could determine wound inflected within 60 minutes with high sensitivity.

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12/29/2012