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Inflammatory mediators and inflammatory cells as reliable molecular targets for assessment of wound age and vitality in rats

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Abstract

Evaluation of the wound age and vitality is a persistent problem for forensic examinations to determine when injuries occurred and whether during the antemortem or post-mortem period. This study aimed to use the inflammatory cells and mediators as a target marker to determine wound age in antemortem or post-mortem samples in rats. Wound skin samples (n=39) were collected from antemortem groups aged 30, 60, 180, and 360 min and post-mortem groups within the same periods with control samples (unwounded group). Immunohistochemical staining examined all tissue samples for two inflammatory cell markers (CD15, CD68) and 2 inflammatory mediators (IL-6, TNF-α). The results showed that CD15-positive neutrophils were expressed in all time intervals of antemortem, and the expression gradually increased from 30 to 360 min. The CD68-positive macrophages began at 30 minutes and reached the highest level at 360 minutes in the antemortem wound. Because of the negative expression of CD15 and CD68 in all post-mortem groups, they could be a marker for comparing the vital and non-vital wounds. Furthermore, the Immunoreactivity results showed that the IL-6 and TNF-α were negatively expressed in unwounded skin samples. In addition, the periods after the induced wound produced a rise in the expression area of both markers in antemortem wounds. In this study, IL-6 and TNF-α expressions were negative or low in areas far from wound margin, unwounded specimens, and post-mortem wounds. CD15, CD68, IL-6, and TNF-α may be valuable markers for assessing wound age and vitality.

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Introduction

Examining wound age and vitality is an essential topic for forensic pathologists, and it needs a vast amount of forensic experience (1-3). During a forensic examination of the wounds, it is crucial to assess the fundamental association between the death and the age of the wound and to distinguish between vital and non-vital wounds. Furthermore, if there is a vital wound, it is essential to assess how far before death it occurred (4,5). Therefore, to get an appropriate assessment of wounds' age and vitality in forensic medicine, factual, logical verification is essential as a consequence it is needed. Consequently, there is a necessity for a specific technique to estimate wound age and vitality. The immunohistochemical technique is accustomed to estimating wound age and vitality in skin samples (6,7). In this work, inflammatory mediators (interleukin 6 (IL-6), tumor necrosis factor (TNF-α)), and inflammatory cell markers (CD15 and CD68) were tested to determine the antemortem and post-mortem wound age in rats by immunohistochemical technique. The pro-inflammatory cytokines (IL-6 and TNF-α) are included in the early stage of all provocative actions in the body (8,9). The IL-6 and TNF-α play crucial functions in wound healing procedures, such as keratinocytes' motivation, immune response alteration, fibroblast proliferation, and the chemotaxis of leukocytes to the wound margin (10). The expression of many pro-inflammatory cytokines in skin wounds was tested

to wound healing determination. However, in forensic medicine, numerous immunohistochemical biomarkers were used to assess wound age and vitality more accurately and to more classical histological features of wound healing (11). The cluster of differentiation (CD) markers are some surface proteins expressed on inflammatory cells and can be efficient in wound age determination (12,13). In wounds, the most inflammatory cells present on the wound edge are neutrophils and macrophages (14). CD15 and CD68 are specific markers usually used to recognize neutrophils and macrophages, respectively (15).

This work aims to examine the expression of inflammatory cell markers (CD15 and CD68) in rat skin wounds with immunohistochemistry to evaluate the efficacy of these markers for assessing wound age and vitality depending on inflammatory cells' number and distribution. In addition, the wound age and vitality were evaluated by detecting the pro-inflammatory cytokines (IL-6 and TNF-α) at the site of the wound. Moreover, the timing of the wound was linked to the degree of expression of IL6 and TNF-α in all layers of skin samples by using of immunohistochemistry technique.

Materials and methods

Animal experiments and skin wound

Female albino Wistar rats (150-200 g body weight and 10 weeks old) were obtained from the University of Mosul, College of Veterinary Medicine. Experimental protocols were consistent with the rules of the University of Mosul, College of Veterinary Medicine (Approval no. UM.VET.2022.011). The rats were kept in separate cages and fed with a standard diet and water during the study. Initially, a mixture of xylazine 10 mg/kg and ketamine 90 mg/kg was intramuscularly injected for general anesthesia. Dorsal skin hair was clipped and shaved. Then the skin was sterilized by a tincture of iodine. The wound was made by scalpel on the dorsal skin as designated previously (16).

Animal grouping

In the antemortem group, the rats were divided into 5 groups, four antemortem incised wound groups (wound samples are collected at 30, 60, 180, and 360 min after injury) and 1 unwounded control group (each group contains 3 mice). After that, the skin tissues were collected, and the rats were euthanized. In the post-mortem group, after euthanization, the rats were divided into 8 groups, four post-mortem incised wound groups (wound samples are collected at 30, 60, 180, and 360 min after death) and 4 unwounded control groups (normal skin samples are collected at 30, 60, 180, 360 min after death) (each group contains 3 mice). After that, the skin tissues were collected. The skin samples from antemortem and post-mortem groups were collected and stored in 10% neutral buffer formalin (pH 7.0), and then the samples were paraffin-embedded.

Immunohistochemistry

Paraffin-embedded skin sections (4μm) were deparaffinized on charged slides. After that, sections were rehydrated in a series of ethanols and washed in PBS. The slides were incubated in 3% hydrogen peroxide to deactivate endogenous peroxidase for 30 min, then washed in PBS, and blocked with blocking solution for 1 hour at room temperature. After that, the slides were incubated overnight with primary antibodies at 4°C. The antibodies used were CD15 Rabbit Monoclonal (dilution 1:50; LifeScience), CD68 Rabbit Polyclonal (dilution 1:30; Elabscience), IL6 Rabbit Polyclonal (dilution 1:100; Elabscience) and TNF-α Rabbit Polyclonal (dilution 1:100 Elabscience). After three times washing, the slides were incubated in poly-HRP Goat Anti-Rabbit IgG (Wuhan Fine Biotech, China) at 1:100 dilutions for 1 hour at room temperature. After PBS washing, slides were treated with diaminobenzidine (DAB) chromogenic substrate. Then, the slides were counterstained with haematoxylin and washed with distal water. Finally, slides were dehydrated in ethanol and xylene and cover-slipped. Slides were inspected using a microscope and photographed using a digital camera to assess the degree of gene expressions (17-19).

Immunohistochemical analysis

The positive expression of stained cells and their deliveries were seen under a microscope. For inflammatory cells, the number of neutrophils and macrophages stained with CD15 and CD68 antibodies, respectively, in the wound area were scored using a four-point scale: (0= negative staining), (1=mild <10), (2=moderate <30), (3= strong >30) (20). For inflammatory mediators, the number of inflammatory cells and the degree of intensity expression of the epidermis stained with IL-6 and TNF-α antibodies in the wound area were scored using a four-point scale: (0=negative staining), (1=mild staining), (2= moderate staining), (3= intense staining) (21).

Results

The Immunohistochemical study of the vital wound in the control group of the antemortem group (without wound) showed that there were no CD15-positive staining cells (Figure 1a). However, after 30 min from injury, CD15-positive cells (neutrophils) were detected inside the blood vessels in the wound margin (Figure 1b). Additionally, within 180 min from injury, CD15-positive cells (neutrophils) were detected outside the blood vessels in the wound margin (Figure 1c), and after 360 min from injury, a large number of CD15-positive staining cells were detected with extensive spreading throughout the dermis to the deep subcutaneous tissue in wound margin (Figure 1d). However, in the post-mortem groups, there were no staining cells by CD15 marker in all-time wound intervals (Figure 1e and f).

CD68 is used as a marker of macrophages and monocytes cells. An Immunohistochemical study of the antemortem wound showed that there were no positive cells for this marker in the control group (without wound) (Figure 2a). After 30 min from induced injury, there were very few numbers of CD68 positive macrophages seen near the blood vessels at the wound edge (Figure 2b). In 180 min from induced injury, there were an increasing number of CD68 positive macrophages seen in the tissue section at the wound edge (Figure 2c). By 360 min from injury, there were severe infiltration of CD68 positive macrophages at the wound margin and spread to the dermal skin layers (Figure 2d). in the post-mortem groups, CD68 positive macrophages was not expressed at any period in skin tissue sections (Figure 2e and f).

Immunohistochemical examination of the antemortem wounds for IL-6 expression showed that there were no IL-6 positive stained epidermal cells in the control group (without wound) (Figure 3a). On the other hand, results of antemortem wound age groups showed increased expression in the staining pattern with increasing time intervals regarding epidermal layers, sub-epidermal cells and inflammatory cells (Figure 3b, c and d). In 30 min wound time, IL-6 expression indicated increased reactivity at the earliest release in epidermal cells and gradually increased after 180 min and 360min. However, in post-mortem wound groups, the immunoreactivity of positive cells with IL-6 was detected in low intensity in the epidermal layer at 30 min wound time (Figure 3e). and disappeared at 360 min (Figure 3f). Similar changes were noticeable with TNF-α (Figure 4).

The scoring system showed that wound age and vitality assessment depends on the increasing number of CD15 and CD68 positive cells. The number of stained cells with CD15 (cells/field) in 30, 180, and 360 min antemortem wounds were 5-9, 23-28, and 50-70, respectively, with no positive cells in post-mortem in all time intervals. The number of stained cells with CD68 (cells/field) in 30, 180, and 360min antemortem wounds were 2-3, 7-9, and 15-20, respectively, with no positive cells in post-mortem in all time intervals. The staining intensity of IL-6 and TNF-α were mild staining in the antemortem group within 30 min of the antemortem group. However, 180 min revealed higher expression than 30 min. Whereas within 360 min showed notably higher expression than 30 and 180 min. On the other hand, the post-mortem groups showed low or negative expression in all time intervals (Table 1).

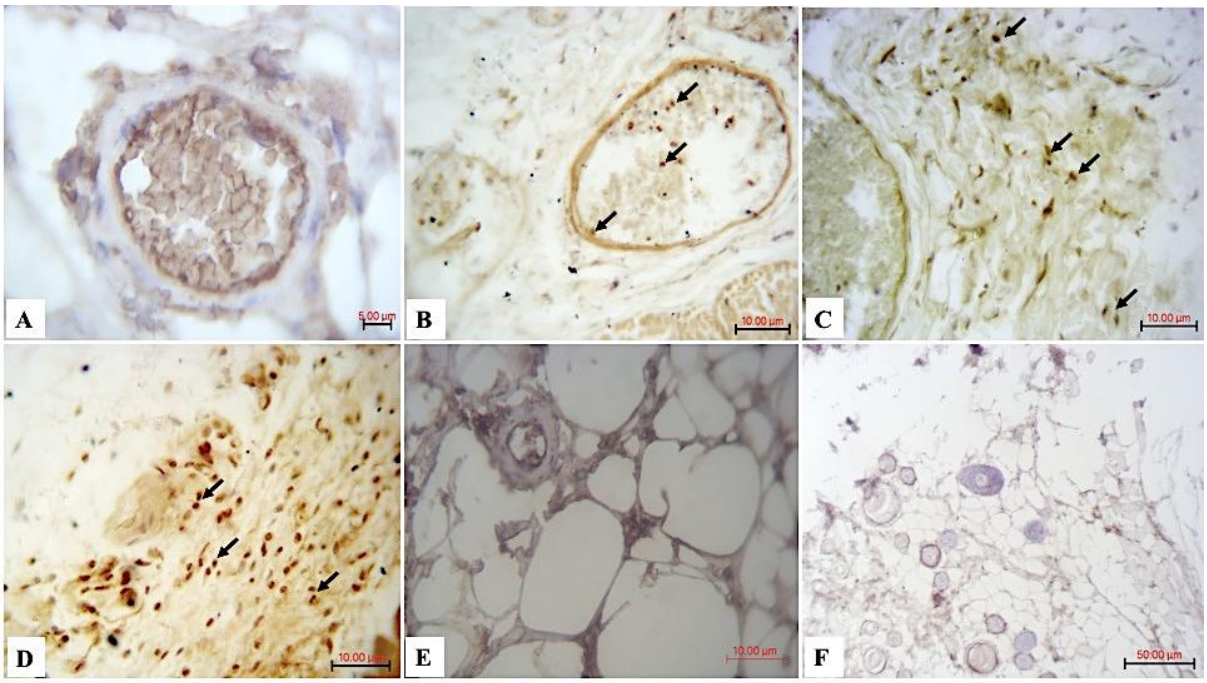

Figure 1: Immunohistochemical examination for CD15 expression in antemortem and post-mortem skin wounds in rats. A. Negative CD15 gene expression around the blood vessels in the dermal layer in the control group of antemortem (without wound). IHC. 5µm. B. Few intravascular and marginated endovascular neutrophils strained for CD15 within 30 min in an antemortem incision (arrows). IHC. 10µm. C. Increasing numbers of neutrophils around the blood vessels stained for CD15 within 180 min in an antemortem incision (arrows). IHC. 10µm. D. Several neutrophils were stained for CD15 within 360 min in an antemortem incision infiltrated throughout the dermis to the deep subcutaneous tissue in the wound margin (arrows). IHC. 10µm. E. Negative CD15 gene expression in the post-mortem wounds within 30 min. IHC. 10µm. F. Negative CD15 gene expression in the post-mortem wounds within 360 min. IHC. 10µm.

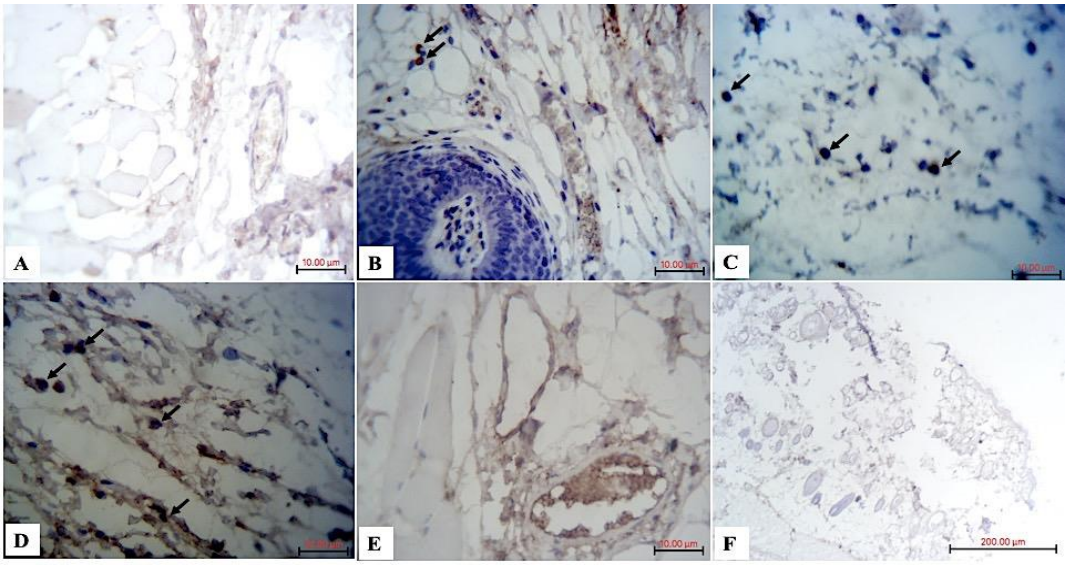

Figure 2: Immunohistochemical examination for CD68 expression in antemortem and post-mortem skin wounds in rats. A. Negative CD68 gene expression around the blood vessels in the dermal layer in the control group of antemortem (without wound). IHC. 10µm. B. Few numbers of macrophages staining with CD68 within 30 min in an antemortem incision (arrows). IHC. 10µm. C. Several macrophages staining for CD68 within 180 min in an antemortem incision infiltrated the tissue section at the wound edge (arrows). IHC. 10µm. D. Severe CD68 positive macrophages proliferation in the wound area within 360 min in an antemortem incision at the wound margin (arrows). IHC. 10µm. E. Negative CD68 gene expression in the post-mortem wounds within 30 min. IHC. 10µm. E. Negative CD68 gene expression in the post-mortem wounds within 360 min. IHC. 10µm.

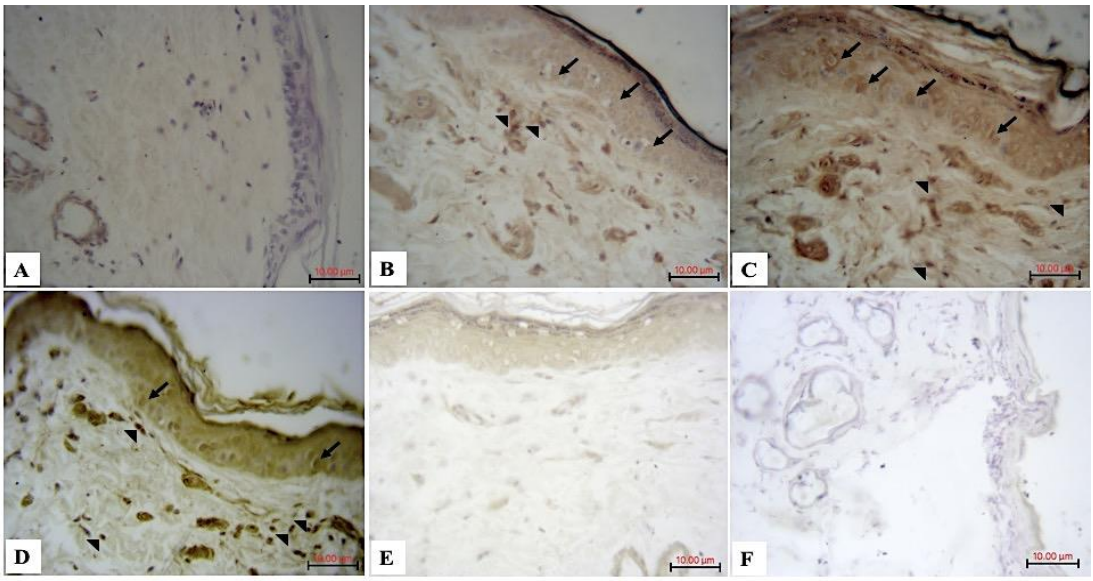

Figure 3: Immunohistochemical examination for IL-6 expression in antemortem and post-mortem skin wounds in rats. A. Negative IL-6 gene expression in the cytoplasm of epidermal cells in the control group of antemortem (without wound). IHC. 10µm. B. Moderate expression in the cytoplasm of epidermal cells (arrows) and inflammatory cells (arrowhead) after 30 min in an antemortem incision. IHC. 10µm. C. Increase expression in the cytoplasm of epidermal cells (arrows) and inflammatory cells (arrowhead) after 180 min in an antemortem incision. IHC. 10µm. D. Strong expression in the cytoplasm of epidermal cells (arrows) and inflammatory cells (arrowhead) after 360 min in an antemortem incision. IHC. 10µm. E. Moderate expression in the cytoplasm of epidermal cells after 30 min in a post-mortem incision. IHC. 10µm. F. Negative IL-6 gene expression in the cytoplasm of epidermal cells after 360 min in a post-mortem incision. IHC. 10µm.

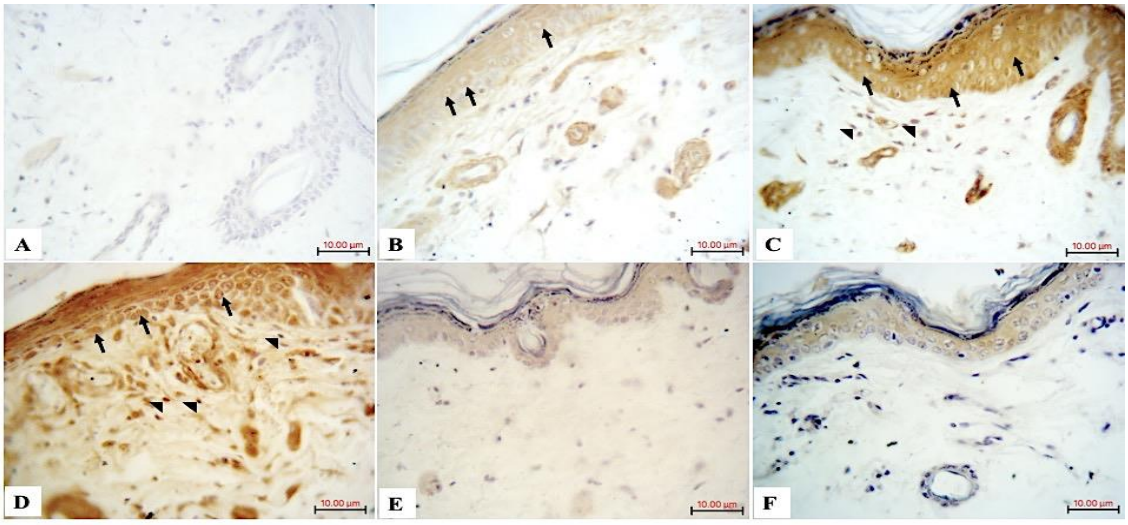

Figure 4: Immunohistochemical examination for TNF-α expression in antemortem and post-mortem skin wounds in rats. A. Negative TNF-α gene expression in the cytoplasm of epidermal cells in the control group of antemortem (without wound). IHC. 10µm. B. Moderate expression in the cytoplasm of epidermal cells (arrows) after 30 min in an antemortem incision. IHC. 10µm. C. Increase expression in the cytoplasm of epidermal cells (arrows) and inflammatory cells (arrowhead) after 180 min in an antemortem incision. IHC. 10µm. D. Strong expression in the cytoplasm of epidermal cells (arrows) and inflammatory cells (arrowhead) after 360 min in an antemortem incision. IHC. 10µm. E. Moderate expression in the cytoplasm of epidermal cells after 30 min in a post-mortem incision. IHC. 10µm. F. Few TNF-α gene expressions in the cytoplasm of epidermal cells after 360 min in a post-mortem incision. IHC. 10µm.

Table 1: Scoring evaluation of CD15, CD68, IL-6, and TNF-α expression in antemortem and post-mortem skin wounds

Marker	Time	Ant-mortem	Post-mortem
CD15	30 min	1	0
	18 0min	2	Data not shown
	360 min	3	0
CD68	30 min	1	0
	180 min	1	Data not shown
	360 min	2	0
IL-6	30 min	1	1
	180 min	2	Data not shown
	360 min	3	0
TNF-α	30 min	1	1
	180 min	2	Data not shown
	360 min	3	1

CD15 and CD68 (0=negative staining), (1=mild <10), (2=moderate <30), (3= strong >30). IL-6 and TNF-α (0=negative staining), (1=mild staining), (2= moderate staining), (3= strong staining).

Discussion

Estimating wound age and vitality is one of the essential topics in forensic research (22,23). Many studies have recognized histopathology, molecular pathology, and immunohistochemistry as the techniques to resolve this problem in forensic medicine (2,24). The most commonly used technique is immunohistochemistry due to its simple application on paraffin-embedded tissue and its ability to detect the antigens' location and degree of expression (1). This work aims to show the correlation between the degree of expression of inflammatory cell markers (CD15, CD68) and inflammatory mediators' marker (IL-6, TNF-α) and the times of wounds in antemortem and post-mortem in rats.

Typically, the CD15 marker is a specific and sensitive marker of neutrophils and monocytes in the endothelial surface during the start of inflammation (25-27). It has been stated that CD15 is expressed during cell infiltration in case of brain trauma and suicidal hanging (28,29). We know no report about CD15 comparing wound age and vitality between an antemortem and a post-mortem has been studied. Our results showed that the number of stained cells with CD15 was considerably expressed in all time intervals of antemortem compared with post-mortem wounds. CD15-positive neutrophils were expressed in the antemortem wound with a minimum interval of 30 min.

Consequently, it can verify to be a valuable marker in the identification of antemortem skin wounds prior to neutrophils can be recognized in the injury by histopathological examination. In addition, the ratio of CD15-positive neutrophils after 360 min from injury considerably exceeded those after 30 and 180 min from injury. This result specified that CD15 might be a valuable marker for detecting wound ages and vitality.

CD68 marker is generally expressed in macrophages and monocytes, and its glycoproteins belong to a lysosomal-associated membrane family (30-32). Although the CD68 function is unclear, it may be associated with cell-cell interactions (33,34). The proportion of CD68-positive macrophages after 360 min in antemortem wounds considerably exceeded those after 180 min from injury. Our result presented that CD68 might be an indicator of wound age at least 360 min after antemortem wound infliction. This result disagrees with the prior findings that the macrophages were seen after day 3 from injury (35-37). Because of the negative expression of CD68 in all post-mortem groups, CD68 could be a marker for comparing the ant-mortem and post-mortem wounds.

Furthermore, the Immunoreactivity findings of the current study exposed that inflammatory mediators (IL-6 and TNF-α) were negatively expressed in unwounded skin samples, and the periods after the induced wound produced a growth in the expression area of both markers. The staining intensity altered notably in antemortem wounds regarding sub-epidermal cells and inflammatory cells in the wound margins. The earliest expression of both inflammatory mediators was noticed in 30 min antemortem wound time, whereas the reactivity increased at 180 min and peaked at 360min. Our results agree with the previous findings where it was observed that increased expression of the IL-6 and TNF-α markers is related to the time of injury (38,4,39-41). In this study, the expression of inflammatory mediators was negative or low in areas far from wound margin in antemortem wounds, unwounded specimens, and post-mortem wounds.

Conclusion

To sum up, this study assessed the probable ability of CD15, CD68, IL-6, and TNF-α to be tested as markers for wound age and vitality assessment. The inflammatory cell markers and mediators can work as suitable markers for assessing wound age and vitality, specifically in the first minutes of wounds before starting neutrophil reactions. Verification studies in larger cohorts and independent samples are still required to approve its accuracy in forensic medicine.

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Conflicting of interests

The author(s) declared no potential conflicts of interest concerning this article's research, authorship, and/or publication.

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الواسطات الالتهابية والخلايا الالتهابية كأهداف جزيئية موثوقة لتقييم عمر الجروح وحيويتها في الجرذان

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الخلاصة

يعد تقدير عمر الجروح وحيويتها مشكلة مستمرة لفحوصات الطب العدلي لتحديد وقت حدوث الإصابات وما إذا كان ذلك أثناء الفترة ما قبل الموت أو بعد الموت. هدفت هذه الدراسة إلى استخدام الخلايا الالتهابية والواسطات الالتهابية كعلامة مهمة لتحديد عمر الجروح في عينات ما قبل الموت أو بعد الموت في الفئران. تم جمع عينات جلد الجروح (ن =٣٩) من مجموعات ما قبل الموت في أعمار ٣٠، ٦٠، ١٨٠، ٣٦٠ دقيقة ومن مجموعات ما بعد الموت خلال نفس الفترات مع عينات السيطرة (مجموعة بدون جرح). تم فحص جميع عينات الأنسجة لاثنين من علامات الخلايا الالتهابية (عنقود التمايز ١٥، عنقود التمايز ٦٨) واثنين من الواسطات التهابية (الانترلوكين ٦، عامل نخر الورم الفا) باستخدام التلوين الكيميائي النسجي المناعي. أظهرت النتائج أن التعبير للعدلات الإيجابية لـ عنقود التمايز ١٥ كانت موجودة في جميع الفترات الزمنية قبل الموت وزاد التعبير تدريجيا من ٣٠ إلى ٣٦٠ دقيقة. بدأت البلعمات الإيجابية لعنقود التمايز ٦٨ في ٣٠ دقيقة ووصلت إلى أعلى مستوى عند ٣٦٠ دقيقة في جرح ما قبل الموت. نظرا للتعبير السلبي عن عنقود التمايز ١٥ وعنقود التمايز ٦٨ في جميع مجموعات ما بعد الموت، فقد تكون علامة للمقارنة بين الجروح الحيوية وغير الحيوية. علاوة على ذلك، كشفت نتائج نشاط المناعة أن الانترلوكين ٦ وعامل نخر الورم الفا قد تم التعبير عنها سلبا في عينات الجلد غير المصابة، وأنتجت الفترات التي أعقبت الجروح زيادة في منطقة التعبير لكلا الواسمتين في جروح ما قبل الموت. في هذه الدراسة، كان التعبير عن الانترلوكين ٦ وعامل نخر الورم الفا سلبيا أو منخفضا في مناطق بعيدة عن حافات الجروح، والعينات غير المصابة وجروح ما بعد الموت. يمكن الاستنتاج أن عنقود التمايز ١٥ وعنقود التمايز ٦٨ والانترلوكين ٦ وعامل نخر الورم الفا علامات قيمة لتقدير عمر الجرح وحيويته.