DOI: https://doi.org/10.53350/pjmhs2023173790

ORIGINAL ARTICLE

Forensic Analysis of Wound Healing Biomarkers for Estimating Injury Age in Medico-Legal Cases. A Cross-Sectional Study

FARHAT SULTANA[1], KHALID MAHMOOD[2], FARIHA TARIQ[3], RIASAT ALI[4], ZUBIA IQBAL[5], AATIQA ABBAS[6]
[1]*Assistant Professor Forensic Medicine Department Allam Iqbal Medical College Lahore, Pakistan*
[2]*Associate professor Forensic Medicine Department Services Institute of Medical Sciences Lahore, Pakistan*
[3,4]*Assistant Professor Forensic Medicine Department King Edward Medical University Lahore, Pakistan*
[5]*Assistant Professor Forensic Medicine Department, Ameer Ud Din Medical College / Post Graduate Medical Institute / Lahore General Hospital, Lahore, Pakistan*
[6]*Demonstrator Forensic Medicine Department King Edward Medical University Lahore, Pakistan*
Correspondence to: Farhat Sultana, Email: farhatzafar42@gmail.com

ABSTRACT

Background: Accurate wound age estimation has a very important value in forensic investigations especially in medico-legal cases. Traditional histopathological methods are subjective and can be limited by inter-individual variability. Recently there have been advances towards molecular biomarkers as an objective alternative.

Aims and Objectives: The aim of this study was to quantify wound healing biomarkers expression pattern to accurately estimate injury age in medico legal cases in Pakistan. The aims were to relate biomarker activity to known injury intervals and to develop a predictive framework for forensic applications.

Methodology: This is a cross-sectional study of n=80 medico-legal cases with documented injury to a sampling interval of 2 hours to 14 days. Histopathological evaluation and immunohistochemical analysis were performed on tissue samples obtained during autopsies or postmortem forensic examinations. The inflammatory cytokines (IL-6, IL-1β, TNF-α), growth factors (VEGF, TGF-β), and the matrix remodelling enzyme (MMP-9) were key biomarkers that we analyzed. The semi-quantitative scoring of biomarker expression was statistically correlated to injury age by Pearson's correlation analysis and one-way ANOVA.

Results: Progressive changes in cellular infiltration and tissue remodeling were demonstrated in histopathological examination of wound age groups. In early wounds, immunohistochemical findings revealed high expression of inflammatory markers, peak of growth factors in the intermediate phase, and increased MMP-9 in late wounds. The groups were significantly different ($p < 0.001$) among each other which indicates the temporal correlation between biomarker expression and injury age.

Conclusion: A multi-biomarker approach is a feasible, objective, and reliable estimation of wound age, and could be used to improve forensic investigations in Pakistan.

Keywords: Wound Age Estimation, Forensic Pathology, Biomarkers, Immunohistochemistry, Medico-Legal, Pakistan

INTRODUCTION

Forensic medicine has important applications in medico-legal cases where the time of an injury impacts legal proceedings, and wound age estimation is crucial in forensic medicine. Forensic experts in Pakistan have to face such cases of physical assault, homicide, domestic violence, and road traffic accidents where the precise age of injury has to be determined for reconstitution of events and verification of statements of the witnesses[1]. Historically, the most commonly utilized traditional forensic method for estimating the age of injury relies on histopathological examination of wound tissue where cellular changes in the wound are used to estimate the age of injury. However, these methods are prone to subjectivity, depending on individual healing variations, and are not precise as to the timing of injury[2].

Biomarker-based approaches recently introduced into forensic pathology give a more objective means of estimating wound age. Wound healing goes through three well-defined biological processes inflammatory, proliferative, and remodeling phases[3]. It is characterized by the release of cytokines such as interleukin 6 (IL 6), interleukin 1β (IL 1β), and tumor necrosis factor-alpha (TNF α) in an inflammatory phase, which occurs immediately after injury for up to 48 hours. These biomarkers are markers for early immune response and they are useful in identifying fresh injuries[4].

During the proliferative phase (48 hours to 7 days), when healing progresses, fibroblasts and endothelial cells are activated, angiogenesis, and collagen deposition occur. Vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β) are key biomarkers in this stage and are involved in tissue repair[5]. Later stages include remodeling, for which extracellular matrix reorganization occurs, enzymes such as matrix metalloproteinase 9 (MMP9) help in tissue restructuring. This sequential expression of the biomarkers allows for a more accurate estimation of wound age than historical methods of histology[6].

Forensic investigations in Pakistan are fraught with lack of resources, delayed medical examination and variability in wound healing due to malnutrition, diabetes, infectious diseases such as tuberculosis and hepatitis. There are also reasons to develop a reliable, standardized way of estimating wound age. Forensic pathologists can use expression patterns of wound healing biomarkers to establish objective criteria of injury dating and improve the accuracy of medico-legal assessments[7].

The objective of this study was to evaluate the forensic potential of wound healing biomarkers in estimating the age of injury in medico-legal cases in Pakistan. Current study aimed to determine a timeline of biomarker expression through immunohistochemical analysis of forensic tissue samples and apply it in forensic practice. The findings of this study may help to develop standardized forensic protocols that will enhance the reliability of injury age estimation in the country's medicolegal system[8].

MATERIALS AND METHODS

Study Design and Setting: This was a cross-sectional study carried out in collaboration with Forensic Medicine Department of Mayo Hospital Lahore and Jinnah Hospital Lahore, Pakistan from January 2022 till January 2023. The main aim was to examine the expression patterns of wound healing biomarkers to predict the age of injury in medico-legal cases. Cases with known injury to sampling intervals of 2 hours to 14 days were sampled. Ethical approval was obtained from the institutional review boards at the participating centers, and all procedures were conducted in accordance with national forensic research and medicolegal guidelines. The case information was kept confidential during the study and all data were anonymized before analysis.

Sample Collection: Autopsies or forensic examinations of n=80 medico-legal cases revealed wound tissue samples. To be included in the study the cases had to have a reliably documented time of injury, confirmed by forensic and medical records and the subjects were between 18 and 65 years of age. They included cases of common types of trauma such as blunt force trauma, sharp force injuries, and firearm-related injuries. Samples were also only collected when tissue could be taken under sterile conditions. However, cases were excluded if there was evidence of

severe infection, large amounts of contamination in the wound tissue, existing pathological conditions (for example advanced diabetes mellitus, chronic inflammatory diseases, malignancies), or if the tissue was extensively decomposed or otherwise unsuitable for histopathological or immunohistochemical analysis. Individuals aged 36 ± 12 years 62.5% male and 37.5% female were a part of the study population. Forensic and medical reports were also used to collect data on the cause of injury and the location of the incident.

Histopathological Examination: Tissue sections were cut at 4 µm thickness, formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin (H&E). This procedure enabled the assessment of important histopathological features related to wound healing including inflammatory cell infiltration, fibroblast proliferation, neovascularization, and extracellular matrix remodeling. Based on these morphological changes, wounds were preliminarily classified into three wound age groups: early (less than 24 hours, n = 30), intermediate (between 24 and 72 hours, n = 25), and late (older than 72 hours, n = 25).

Immunohistochemical Analysis: Specific wound healing biomarkers were assessed by immunohistochemistry (IHC) on the paraffin-embedded sections. The inflammatory cytokines (IL-6, IL-1β, and TNF-α) were chosen to represent the early phase of wound healing; whereas the growth factors (VEGF and TGF-β) were chosen as markers of the proliferative phase. The later phase of healing was indicated by using the matrix remodeling enzyme MMP-9. Tissue sections were deparaffinized in xylene and rehydrated to a series of grad ethanol solutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and antigen retrieval was performed on endogenous antigen using a citrate buffer (pH 6.0) under heat-induced conditions. The incubations overnight at 4°C with the primary antibodies that were specific to the selected biomarkers were performed on the sections. The antibody-antigen complexes were then visualized using diaminobenzidine (DAB) as the chromogen after incubation. The sections were then counterstained with hematoxylin, dehydrated, and mounted. Two independent forensic pathologists evaluated the expression of biomarkers semi-quantitatively and were blinded to the clinical and injury timing data. Staining intensity and percentage of cells positive were combined on a scale of 0 (negative with fewer than 5% positive cells) to 3+ (strong with more than 50% positive cells) for scoring.

Statistical Analysis: Finally, statistical analyses were performed using SPSS v 26.0. Demographic characteristics, type of injuries, and the distribution of cases across the wound age groups were summarized using descriptive statistics. Mean immunohistochemical scores of the biomarkers were compared against the three wound age groups using one-way ANOVA with $p \leq 0.05$ considered significant. The relationships between biomarker expression levels and the time elapsed since injury were examined by Pearson's correlation analysis. Also, multivariate regression analysis was conducted to determine whether the measurement of the biomarkers could provide a combined predictive value for estimating wound age.

RESULTS

Demographic Data and Sample Characteristics: The study population consisted of 80 medico-legal cases with documented intervals of wound age ranging from 2 hours to 14 days. The age of the individuals varied from 18 to 65 years of age with a mean age of 36 ± 12 years. The cases were constituted by 62.5% (n = 50) males and 37.5% (n = 30) females. Also, it recorded other relevant demographic variables such as the cause of injury and the location where the incident took place based on forensic and medical reports. Most of the cases were caused by blunt force trauma (45%), sharp force injury (35%), and firearm-related injury (20%). A summary of the demographic data and cases by injury type is given in Table 1.

Distribution of Wound Age Groups: The injury time was divided into three groups, early (< 24 hours), intermediate (24–72 hours), and late (> 72 hours), and the cases were classified accordingly. The group of 30 cases was early, the group of 25 cases intermediate, and the group of 25 cases late. The early group had a mean injury time of 12 ± 6 hours, the intermediate group of 48 ± 12 hours, and the late group of 120 ± 30 hours. The distribution of cases by wound age group is presented in Table 2.

Table 1: Demographic Characteristics and Injury Type Distribution

Characteristic	Value
Total number of cases	80
Age (years)	18 – 65; Mean ± SD: 36 ± 12
Sex	Male: 50 (62.5%); Female: 30 (37.5%)
Cause of Injury	Blunt force: 36 (45%) Sharp force: 28 (35%) Firearm: 16 (20%)
Injury Location	Urban: 60 (75%) Rural: 20 (25%)

Table 2: Distribution of Cases by Wound Age Group

Wound Age Group	n	Mean Time (hrs)
Early (<24 hrs)	30	12 ± 6
Intermediate (24–72 hrs)	25	48 ± 12
Late (>72 hrs)	25	120 ± 30

Histopathological Findings: Histopathological examination of H&E-stained sections showed distinctive morphological features correlating with wound age. Early wounds showed prominent inflammatory infiltrate, mostly neutrophils, and edema. In intermediate wounds, acute inflammatory cells decreased and fibroblast proliferation and neovascularization began. Organized collagen deposition, diminished inflammatory activity, and pronounced extracellular matrix remodeling were seen in late wounds. The histological observations were a qualitative baseline complementing subsequent immunohistochemical analysis of wound healing biomarkers.

Immunohistochemical Analysis of Wound Healing Biomarkers: To evaluate expression of selected wound healing biomarkers of the different phases of the healing process, immunohistochemical (IHC) analysis was performed. Primary inflammatory cytokines (IL-6, IL-1beta, TNF-alpha) were expressed early and growth factors (VEGF and TGF-beta) were expressed late during intermediate phase. On the other hand, early wounds expressed MMP 9 at very low levels and levels of MMP 9 in late wounds were very high.

Immunohistochemical staining was semi-quantitatively scored and the mean scores of IL-6, IL-1β, and TNF-α in the early group were 2.8 ± 0.4, 2.7 ± 0.5, and 2.9 ± 0.3, respectively. In the intermediate group, these values were reduced to 1.2 ± 0.3, 1.3 ± 0.4, and 1.1 ± 0.4 respectively, and further to 0.4 ± 0.2, 0.5 ± 0.2, and 0.6 ± 0.3 in the late group. On the other hand, the mean scores for VEGF and TGF-β were low at 1.0 ± 0.3 and 0.8 ± 0.2, respectively, peaked at 2.3 ± 0.4 and 2.5 ± 0.4 in the intermediate group, and slightly decreased again at 1.5 ± 0.3 and 2.0 ± 0.3 in the late group. MMP-9 expression increased progressively from 0.5 ± 0.2 in early wounds to 1.8 ± 0.3 in intermediate wounds and finally to 2.7 ± 0.4 in late wounds. The mean IHC scores for each biomarker among the 3 wound age groups are summarized in Table 3.

Table 3: Mean Immunohistochemical Scores of Wound Healing Biomarkers by Wound Age Group

Biomarker	Early (n = 30) Mean ± SD	Intermediate (n = 25) Mean ± SD	Late (n = 25) Mean ± SD
IL-6	2.8 ± 0.4	1.2 ± 0.3	0.4 ± 0.2
IL-1β	2.7 ± 0.5	1.3 ± 0.4	0.5 ± 0.2
TNF-α	2.9 ± 0.3	1.1 ± 0.4	0.6 ± 0.3
VEGF	1.0 ± 0.3	2.3 ± 0.4	1.5 ± 0.3
TGF-β	0.8 ± 0.2	2.5 ± 0.4	2.0 ± 0.3
MMP-9	0.5 ± 0.2	1.8 ± 0.3	2.7 ± 0.4

Differences in biomarker expression among the three wound age groups were assessed using one-way ANOVA. All biomarkers (F values ranging from 31.1 to 50.4, p values always < 0.001) were significant according to the ANOVA results. The results confirmed that the expression of inflammatory cytokines (IL-6, IL1β, and TNF-α) was significantly reduced as wound age increased; and the expression of VEGF, TGF-β, and MMP-9 was expected for the proliferative and remodeling phases. Further Pearson's correlation analysis showed a strong negative correlation between inflammatory cytokine expression with wound age and a strong positive correlation between growth factor expression with MMP-9 with wound age. The ANOVA results for each biomarker are summarized in Table 4.

Table 4: ANOVA Results for Biomarker Expression Across Wound Age Groups

Biomarker	F-value	p-value
IL-6	45.2	< 0.001
IL-1β	42.3	< 0.001
TNF-α	48.7	< 0.001
VEGF	31.1	< 0.001
TGF-β	35.6	< 0.001
MMP-9	50.4	< 0.001

The results of the statistical analyses are consistent with the idea that the temporal changes in biomarker expression are highly correlated with the time since injury and, thus, the use of these markers as forensic wound age estimators.

The demographic analysis of the 80 cases showed a preponderance of males of a wide age range (range 8 to 81 years) with a mean age of 36 ± 12 years. The immunohistochemical profiles of the selected biomarkers reflected distinct phases of wound healing that were histopathologically observed. Early wounds had significantly higher inflammatory markers which decreased with time, growth factors were highest during the intermediate phase, and matrix remodeling enzyme MMP-9 increased progressively and peaked in late wounds. The massive variations observed via ANOVA and the strong correlation of biomarker expression with wound age, suggest a potential role of multi biomarker approach in objectively assessing wound age in medico-legal cases in Pakistan.

DISCUSSION

In the present study, the expression pattern of wound healing biomarkers was investigated and used to estimate the injury age in medico-legal cases in Pakistan. We find that inflammatory cytokines, growth factors, and matrix remodeling enzymes are expressed sequentially and in correlation with the elapsed time since injury[9]. Specifically, high levels of IL-6, IL-1β, and TNF-α in the early phase of wound healing, then a peak in VEGF and TGF-β in the middle phase, and finally an increasing MMP-9 expression in the later phase confirm that wound healing is a dynamic process that can be tracked using molecular markers. These findings are consistent with existing literature that a biomarker-based approach may offer a more objective and reproducible means to estimate wound age than histopathological analysis alone[10, 11].

The demographic data and clinical variables such as age, sex, cause of injury, and injury location were also considered so that the study population was representative of the medico-legal cases that are encountered in Pakistan. In addition, the observed variability in wound healing secondary to underlying health conditions and environmental factors, only further emphasizes the importance of a standardized protocol in forensic pathology[12, 13]. However, due to the cross-sectional nature of the study, causality cannot be established and different healing responses between individuals may lead to variability in biomarker expression. In addition, the sample size was sufficiently large to be able to demonstrate significant trends, but larger cohorts and diverse collaborations are needed to validate and refine the predictive model for wound age estimation[14].

Several advantages are presented by the integration of immunohistochemical analysis into forensic practice. So, it is an objective method to quantify molecular changes in the wound healing process and thus it decreases the reliance on subjective histopathological evaluations[15, 16]. Furthermore, instead of using a single indicator, a panel of biomarkers is used to increase the accuracy and reliability of estimating wound age. Nevertheless, the use of such sophisticated molecular techniques in regular forensic investigations in Pakistan may be hindered by resource limitations and the requirement for costly equipment and trained personnel. Future studies can be devoted to the choice of the biomarker panel and the development of inexpensive protocols that can be used in every forensic laboratory in the country[17, 18].

CONCLUSION

It is demonstrated in this study that immunohistochemical analysis of biomarkers is a promising method for accurately estimating wound age in medico-legal cases. As with the wound healing timeline, there is a good correlation between the dynamic expression of inflammatory cytokines, growth factors, and matrix remodeling enzymes. Despite these limitations (inter-individual variability and sample size), the results provide a strong basis for standardizing protocols in forensic pathology. The reliability of this approach still needs to be enhanced and this approach needs to be integrated into routine forensic practice in Pakistan through further research in larger populations and longitudinal designs.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments: We would like to thank the forensic departments of the participating hospitals for their invaluable support during the sample collection and data acquisition process. We also appreciate the technical assistance provided by the laboratory staff and the contributions of the forensic pathologists who participated in the histopathological and immunohistochemical analyses. Their commitment and expertise were critical in completing this study.

Authors' Contributions: All authors contributed significantly to the study design, data collection, analysis, and interpretation of the results. The manuscript was drafted collaboratively, with each author reviewing and approving the final version. Specific contributions include the conceptualization and methodology design, sample collection and laboratory analyses, statistical analysis, and manuscript preparation. All authors agree to be accountable for all aspects of the work and confirm that all those who contributed substantially to the study have been included as authors.

REFERENCES

1. FRCPATH SCMF. SCIENTIFIC SESSION 1: IMAGING IN FORENSICS CT SCANNING AS A ROUTINE PROCEDURE IN MEDICO-LEGAL DEATH INVESTIGATION. Int J Legal Med. 2012;126(1):S5-S21.
2. Eldesoky MM, Zayed AA, Gaballah IF, ElSebaie AMM, Dayem OAE, Mekdad AA. Cell free DNA as a biomarker in medicolegal assessment in burn patients. International journal of health sciences. 2022;6(S8):566-75.
3. Tattoli L, Dell'Erba A, Ferorelli D, Gasbarro A, Solarino B. Sepsis and nosocomial infections: the role of medico-legal experts in Italy. Antibiotics. 2019;8(4):199.
4. Ferrara SD, Cecchetto G, Cecchi R, Favretto D, Grabherr S, Ishikawa T, et al. Back to the Future-Part 2. Post-mortem assessment and evolutionary role of the bio-medicolegal sciences. International journal of legal medicine. 2017;131:1085-101.
5. Cattaneo C, Gibelli D, De Angelis D, Grandi M. Clinical Forensic Pathologists and the Assessment of Refugees for Signs of Maltreatment and Torture: Italian First Steps. INTERNATIONAL JOURNAL OF LEGAL MEDICINE. 2012;126(suppl 1):101-.

6. Li N, Li C, Li D, Dang LH, Ren K, Du QX, et al. Identifying biomarkers for evaluating wound extent and age in the contused muscle of rats using microarray analysis: a pilot study. PeerJ. 2021;9:e12709.
7. Ros AC, Bacci S, Luna A, Legaz I. Forensic Impact of the Omics Science Involved in the Wound: A Systematic Review. Frontiers in Medicine. 2022;8.
8. Li N, Du Q, Bai R, Sun J. Vitality and wound-age estimation in forensic pathology: review and future prospects. Forensic Sci Res. 2020;5(1):15-24.
9. Ren K, Wang L, Wang Y, An G, Du Q, Cao J, et al. Wound age estimation based on next-generation sequencing: Fitting the optimal index system using machine learning. Forensic Science International: Genetics. 2022;59:102722.
10. Casse J-M, Martrille L, Vignaud J-M, Gauchotte G. Skin wounds vitality markers in forensic pathology: an updated review. Medicine, Science and the Law. 2016;56(2):128-37.
11. Fronczek J, Lulf R, Korkmaz HI, Witte BI, van de Goot FR, Begieneman MP, et al. Analysis of inflammatory cells and mediators in skin wound biopsies to determine wound age in living subjects in forensic medicine. Forensic science international. 2015;247:7-13.
12. Wang L-L, Zhao R, Liu C-S, Liu M, Li S-S, Li J-Y, et al. A fundamental study on the dynamics of multiple biomarkers in mouse excisional wounds for wound age estimation. Journal of forensic and legal medicine. 2016;39:138-46.
13. Zhang J, Niu F, Dong H, Liu L, Li J, Li S. Characterization of protein alterations in damaged axons in the brainstem following traumatic brain injury using fourier transform infrared microspectroscopy: a preliminary study. Journal of forensic sciences. 2015;60(3):759-63.
14. Ishida Y, Kimura A, Nosaka M, Kuninaka Y, Shimada E, Yamamoto H, et al. Detection of endothelial progenitor cells in human skin wounds and its application for wound age determination. International journal of legal medicine. 2015;129:1049-54.
15. Mao S, Fu F, Dong X, Wang Z. Supplementary pathway for vitality of wounds and wound age estimation in bruises using the electric impedance spectroscopy technique. Journal of forensic sciences. 2011;56(4):925-9.
16. Fronczek J, Lulf R, Korkmaz HI, Witte BI, van de Goot FR, Begieneman MP, et al. Analysis of morphological characteristics and expression levels of extracellular matrix proteins in skin wounds to determine wound age in living subjects in forensic medicine. Forensic Science International. 2015;246:86-91.
17. Takamiya M, Fujita S, Saigusa K, Aoki Y. Simultaneous detections of 27 cytokines during cerebral wound healing by multiplexed bead-based immunoassay for wound age estimation. Journal of neurotrauma. 2007;24(12):1833-44.
18. Gaballah MH, Horita T, Takamiya M, Yokoji K, Fukuta M, Kato H, et al. Time-dependent changes in local and serum levels of inflammatory cytokines as markers for incised wound aging of skeletal muscles. The Tohoku journal of experimental medicine. 2018;245(1):29-35.