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REVIEW ARTICLE

Wound Timing: Highlight the Histopathological and Molecular Methods

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ABSTRACT

Background: Although forensic pathology age estimation of a wound is difficult, it can help reconstruct murder scenes and identify potential culprits. Forensic specialists usually focus on two aspects when evaluating wound life and determining how long ago the wound occurred. Recent developments in forensic techniques, particularly in high-throughput analyses, have allowed for the simultaneous evaluation of several markers and molecular and cellular levels of material analysis.

Aim: To evaluate valuable additional information offered by each marker, summarize recent literature, and offer an update on wound-age estimation in forensic pathology. With the intention of providingsome insight for future research, prospects for evaluating wound age in forensic practice are finally examined.

Conclusions: Although forensic pathologists find it difficult to determine the age of wounds, doing so can help reconstruct crime scenes and help apprehend suspects. In circumstances where several traumas are sustained by various offenders, forensic pathologists must determine the chronology and sequence of injuries because the severity of an injury usually determines the severity of punishment. Wound tracing, photographic recording (including image analysis), biophysical approaches, and/or invasive protocols requiring wound biopsies are examples of protocols that can be used to do the assessment. We give a summary of some of the most frequently required and utilized methods in this article: (a) Preclinical and animal models, such as those involving incisions, excisions, burns, and damaged wounds; (b) techniques for assessing the rate of healing, image-based wound analysis, biophysical evaluation, histological, immunological, and biochemical assays; and techniques for assessing the healing progression.

Keywords: Forensic sciences; Forensic pathology; Wound age; Vitality; Estimation

INTRODUCTION

Any damage to the integrity of the skin, mucous membrane, or organ tissue is referred to as a wound. Complex wounds that involve damage to the muscles, nerves, and blood vessels. Trauma resulting from mechanical, thermal, chemical, or radiogenic sources can create wounds [1].

One such essential medico-legal task is forensic wound examination, which

comprises identifying the type of force used, the time elapsed between the wound's infliction and death, the manner of infliction, the relationship between the impairment and damage, etc., and differentiating between antemortem and postmortem inflictions. Among these vital jobs for the living and the dead is figuring out how old a wound is. Dating is required to confirm the duration between the wound's alleged infliction and examination. Traditionally, color changes and healing stages are examined visually to estimate the age of a wound. Abrasions and other surface injuries heal in a predictable manner [1].

If co-morbidity, foreign body impaction, or infection do not impede healing, age can be determined rather accurately. The age of the bruise is determined by color fluctuations. The individual's skin tone, the volume and intensity of the hemorrhage, and additional elements influence the color shift. Moreover, the area's vascularity, the bruise's size, the individual's general health, vascular diseases, etc., all influence how quickly a bruise heals. Incised wounds can heal with primary intent, and the healing process can be anticipated without any difficulties. However, wounds like lacerations and other deep-seated injuries are not helpful in estimating the age of the wound since they are unlikely to heal without infection and significant tissue loss. Wound healing occurs in three stages: inflammation, proliferation, and maturity. The inflammatory phase is characterized by platelet aggregation and time-dependent leucocyte infiltration, which includes neutrophils, macrophages, and T lymphocytes. During the proliferative phase, angiogenesis, granulation tissue formation, and epithelization occur. During the maturation phase, collagen is deposited, the one wound location is reorganized, and it is strengthened. Histopathological examinations are commonly performed to look at these phase-wise changes in addition to macroscopic findings in order to determine the age of a wound [1].

Methods for estimating the age of forensic wounds have been improving over traditional approaches. The first attempt at this type of investigation was conducted by Raekallio J., who used histochemistry to look at the enzyme activity in the central and peripheral zones of the wound. He came to the conclusion that the peripheral zone exhibits more detectable enzyme activity and that the infiltration of inflammatory cells observed in histological examination occurs later than the presence of enzymes such adenosine triphosphatase [2].

After immunohistochemical techniques were developed, more modern approaches to determining wound age have been studied. Wound healing has been associated with adhesion molecules, inflammatory cytokines, extracellular matrix, and various growth factors. The usefulness of these characteristics in forensic wound age estimation is thoroughly examined [3].

History of wound age estimation:

Raekallio first presented some new data in 1972 regarding the age of wounds and explained the application of a novel enzymatic histochemistry approach [4].

At the same time, a remarkable biochemical method was discovered, which entailed detecting histamine and serotonin in the vicinity of the wound edge [5, 6]. Significant advancements in the scientific study of immunology and immunohistochemistry have been made within the next ten years. Because of the use of immunohistochemistry methods, forensic pathologists can now look into wound aging in a new field [7].

The last few decades have seen significant scientific advances due to the application of immunohistochemistry techniques and a knowledge of basic immunological principles [8-10]. The development of clinical medicine has been associated with advances in basic research. Applying the most recent fundamental research knowledge is always required to conduct forensic medicine since it is an applied branch of medicine.

Preferred samples for wound age dating:

Due to their frequent contact with forensic procedures, the most often researched tissues in reviewed studies were the surrounding skeletal muscle, brain tissue, and skin of injuries caused by an incision or contusion.

For instance, given that brain damage usually results in death and is frequently linked to violent death cases, determining the age of the wounds in cases causing brain damage has been a major area of investigation [11-13]. Most recent experimental and investigative investigations have focused on skeletal muscle and skin, respectively.

The majority of the evaluated articles were investigative and experimental investigations. While samples are typically taken at a predetermined period after wounding in experimental studies, many specimens are gathered following injury at different times in investigative investigations. Animal and autopsy specimens were used in several investigations, while samples from living human subjects were also included. One benefit of conducting controlled trials on animals is that the outcomes are more consistent and dependable. Additionally, they facilitate research on the wound-healing mechanism, a basic physiological response that both humans and animals share. Additionally, one has power over the time that passes after an injury. It's still unclear, though, how much of the findings apply to humans [14, 15]. It suggests using markers with a high degree of sequence homology since it is expected that during wound healing, human behavior will be similar. To increase the precision of injury time computations in forensic practice, human samples can also be used as a calibration standard for animal data.

The most accurate and realistic examples are autopsy specimens, especially if the age of the wound is known. However, incomplete documentation and missing data restrict the examples' usability. Moreover, the time at which the wounding happened can vary greatly and, in some circumstances, it was not determined throughout the period of interest, even when samples of wounds with established ages are obtained. A few other factors to take into account are the patient's age, the site of the wound, how long they lived, any postmortem changes (such as putrefaction, decomposition, or desiccation), and their medical history. Controlled ex vivo putrefaction ought to be used in these circumstances [16]. The trustworthiness of the results is increased when stringent restrictions are enforced for the collection criteria.

Those with skin issues, those referred to a forensic physician, and those requiring surgical resection usually offer samples from living human beings. These samples typically have exact time records and are stored until the moment of interest. Their human origin and temporal data precision are hence their two key advantages. But most of the time, these aren't healthy samples, and the in vitro preservation method impedes important functions. When an injury's macroscopic evaluation is insufficient, it may be important to determine the wound age in a living individual for medicolegal issues. The ethical implications of using tissue from living donors must also be taken into account. Actually, authorization from the regional ethics council [17, 18].Additionally, the sample size is usually insufficient because samples from wounds sustained by active human participants need to be gathered non-invasively (by swabbing), which could lead to erroneous and occasionally false-negative results [19, 20].

Methods for wound-age estimation:

Histopathological methods

There are multiple methods for determining the age of a wound. Morphological analysis has long been the most widely used method because of its objectivity, ability to evaluate marker localization, and visual or intuitive character. A bruise's age can also be ascertained visually by examining the variations in color of the bruise [21].

It continues to be invaluable and provides a plethora of useful information about wound aging. The depth, location, and skin tone of a bruise can affect its color and appearance timing, among other factors; therefore, attempts to determine the age of bruises by evaluating their hue visually have shown to be too random to be practical [16]. We are focusing on molecular approaches because the problem requires thorough experimental investigation to be solved.

As a result, a number of temporal indicators have been researched in relation to potential uses in forensic pathology. Standard histological testing (such as Berlin blue staining and hematoxylin-eosin) can detect alterations six hours following an injury [17–19], despite the limited practical applicability of it. Studies using immunohistochemistry and immunofluorescence enable the age of early-stage wounds to be estimated, as well as the location of tissue components indicative of the response stage and the identification of cell activation phases[22]. These results allow assessment of the relationship between morphology and function and provide a more thorough analysis of the interval of wound age as established by adhesion molecule and cytokine assays. Three or four markers can be found at once using an immunofluorescence multiple-staining approach, making qualitative and quantitative investigations possible. Considering that it is known that the ratios of mononuclear cells, fibroblastic cells, and polymorphonuclear neutrophils in injured areas vary with time [20, 23], these cells could help figure out just how old wounds are. Certain studies indicate a favorable relationship between marker levels and early wound stage.

It has been reported that the subjective definition of positive standards by operators can have an impact on the stability and accuracy of data acquired from quantitative immunohistochemical assays. The application of immunohistochemistry in clinical settings is restricted by these issues [24]. By recognizing and assessing various sample sections, digital slice-scanning technologies automatically remove subjectivity and make it easier to determine the separation between inflammatory cells and free vasculature.

In the early stages of wound healing, adhesion molecules are crucial. Both the migration of leucocytes and their interactions with endothelial cells depend on them. Therefore, the age evaluation of wounds that are inflicted within a few hours is studied using these markers. According to Drebler J et al., P-selectin exhibits immunopositive reactivity as soon as the wound is inflicted and remains positive for up to seven hours. Positive responses to other adhesion molecules, including VCAM (vascular cell adhesion molecule), ICAM (intercellular cell adhesion molecule), and E-selectin are also seen within 4-6 hours. Betz and associates have investigated the function of collagen in the assessment of wound healing. They discovered that while collagen V and VI are expressed in wounds lasting longer than three days, collagen III expression may be examined in wounds that are two to three days old. In wounds that have been open for more than four days, basement membrane collagens are visible. Lesions greater than five days after the injuries were discovered to contain collagen I. Additionally, Betz and colleagues had investigated the use of fibronectin in wound age estimate. Fibronectin plays a part in fibroblast cell adhesion and migration. Fibronectin expression was detected as early as 10 to 20 minutes, peaking at about 4 hours [25].

Different inflammatory cells release cytokines, which are markers of inflammation. They play a multisystemic role. Pro-inflammatory mediators include cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α). Japanese researchers found that these cytokines first had a positive interaction with neutrophils in their mouse study, and then they also reacted favorably with macrophages and fibroblasts. TNF-α and IL1β peaked three hours after the injury, but IL-1α and IL-6 peaked six and twelve hours after the injury, respectively. For every cytokine, there was an extra peak at about 72 hours. Consequently, it was shown that these cytokines are involved in both the early phases of inflammation and the subsequent remodeling of the wound. Chemotactic cytokines, also known as chemokines, facilitate cell motility. MCP-1 (monocyte chemoattractant protein-1), MIP-1α (macrophage inflammatory proteins-1α), and IL-8 (interleukin-8) are significant chemotactic cytokines for neutrophils, monocytes, and macrophages. Kondo T et al. investigated these indicators' immunoexpression[26].

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have a major impact on angiogenesis. In their studies on animals, Takamiya et al. proved the usefulness of VEGF and bFGF for estimating wound age. [27]. Human subjects in the study by Hayashi et al. found that VEGF-positive ratio was expressed on fibroblasts and macrophages in human skin wounds with ages ranging from 7 to 21 days [28]. Collagen is deposited by TGF-β1 (transforming growth factor beta 1), whereas epidermal cell proliferation is facilitated by TGF-α (transforming growth factor alpha). These indications showed enhanced immunopositivity in the first hour after the injury was inflicted, suggesting that they may be useful in assessing the age of wounds. Ubiquitin is the term for heat shock protein (Ub). After examining its role in roughly estimating the duration of wounds, researchers found that wounds that were 7 to 14 days old had a positive rate of more than 30% [29].

A study that looked at the expression of the p53 gene, a marker of cellular apoptosis, found that wounds that were healed one to two days after the injury had elevated p53-positive fibroblast expression [30]. Additionally, an attempt has been made to use the neutrophil migration distance to assess wound age [31].

Molecular biological methods:

Histological analysis usually isn't able to identify whether a wound was inflicted before or after death in the initial minutes or hours following wound infliction[15]. Nevertheless, following damage, cytokine and enzyme mRNA levels typically change before protein levels and histomorphology do [30-32].

Therefore, mRNA-based tests can be used to identify the age of early-stage wounds. RNA (Ribonucleic acid) has been found in a sample that has been preserved for a long time, despite being less stable than protein[23]. Biological stains that are months or even years old can be used to extract total RNA of sufficient quality and quantity [33]. Therefore, the real-time polymerase chain reaction (PCR) is used to assess the mRNA levels of inflammatory cytokines and wound-healing factors in order to establish wound age.

The very sensitive technique known as real-time PCR (qPCR) may identify even the tiniest variations in gene expression between samples. So care must be taken at every step of the procedure, including data processing [34]. Data normalization through the use of reference genes is crucial for effective data analysis and the identification of unavoidable experimental modifications, particularly variations in the amount of sample loading. The issue with some housekeeping genes having higher expression after damage is that, in order to properly normalize the condition, it is essential to select a housekeeping gene that consistently expresses itself after harm [35, 36].

High-throughput methods, like real-time PCR, allow many genes to be analyzed at once thanks to high-throughput sequencing, gene chip analysis, and the 384 microplate system, greatly reducing testing costs and yielding highly repeatable and stable results. These methods allow for the identification of markers that are used to measure wound age, and it is likely that future studies will employ them to determine the differential expression of mRNAs after injury. In the interim, a plethora of studies on humans and animals are actively exploring the role of additional novel markers, including FoxO1 expression and IL–33. Immunohistochemistry is less sensitive than Western blotting and enzyme-linked immunosorbent tests when assessing wound vitality and protein levels. Furthermore, proteomics can offer details on the signal transduction pathways that have a direct impact on life's metabolic processes, in contrast to transcriptomics and genomes. The difficulties of maintaining environmental control and the complexity of the procedures still limit the ability to compare results between laboratories. A sensitive high-throughput technique that allows the simultaneous detection of several protein analytes in a single sample is protein microarray technology[37].

Other methods:

Mao et al. [38] developed a novel, rapid technique based on electric impedance spectroscopy for determining the age of a wound. Zhang et al. [39] employed liquid chromatography-mass spectrometry in conjunction with an isobaric tag for both relative and absolute quantifications to identify differentially expressed proteins as trustworthy indicators of widespread axonal damage. While not yet often utilized in the field of legal medicine, these methods exhibit promise.

Every tactic has benefits and drawbacks. In traditional histology, red blood cell extravasation is studied and considered an indicator of critical responses. Nevertheless, it is not a valid indicator of wound life because it may potentially reveal postmortem[15]. Therefore, in order to minimize inaccuracy when estimating the time at which a wound was inflicted, morphological and molecular data should be combined [22, 24]. Applied to mRNA or proteins, high-throughput analysis is an important methodological development.

Problems and future perspectives:

The estimation of wound ages has been the subject of studies conducted in the last few decades. Especially at the beginning, the pathologist's experience has a significant impact on establishing the age of the wound. One learns more about the various facets of wound-age assessment, including the deceased's age and gender, the reason of death, and the degree of damage, the longer they practice forensics. However, an animal model with wounds that accounts for the time between death and the postmortem, the age of the wound, the degree of damage, the age of the deceased, the many seasons and their environmental fluctuations, and the storage conditions would satisfy even the most seasoned forensic pathologist. Skin sample data, standardized and controlled conditions, and animal models are required to generate trustworthy results, as autopsy sample data is frequently insufficient or incomplete. Using different markers improves the accuracy and reliability of establishing a wound's age. Advancements in technology, namely in high-throughput analysis, have made it possible to examine several mRNAs and proteins simultaneously in a single sample. Thus far, a wide range of wound healing indicators have been studied. Furthermore, a strategy for selecting pertinent markers must be applied. To obtain objective results, morphological and molecular approaches like proteomics, metabolomics, and genomes will probably need to be combined [38].

Assessing the findings' applicability to individuals and their usefulness in determining the age of wounds is crucial. Although weather forecasting and other complex problems have benefited from mathematical modeling, the possible interactions between these characteristics and those needed for wound dating are unknown. Metcalf et al. [40] created a mathematical model that can be used to determine the postmortem time period, and encouraging outcomes were seen. Any mathematical model should be based on data from large-scale animal trials, with results from human autopsy samples being used for calibration, as wound-age estimation is influenced by numerous factors.

CONCLUSIONS

It is evident that in the past few years, advances have been achieved in the estimation of wound ages. Rapid technological advancements have facilitated data availability, and numerous time-dependent features have been investigated. While numerous marker combinations have drawn a lot of critical attention, no method or model to employ such markers for wound aging has been offered as of yet. The problem at hand is interpreting and applying the gathered data to real-world scenarios.

Conflict of Interest: None

Financial Disclosure: None

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