Section A -Research paper

Methods of Estimating Wound Age in Forensic Medicine

Amira A. M. Said [1], Ghadeer M. M. Abdelaal [1], Bothina H. F. Omran [1], Sarah E. Y. Elreffaey [1]

[1]Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Zagazig University, Egypt

Corresponding author: Amira Ahmed Mohamed Said

Email: amiraazmy1295@gmail.com , **Mobile:** +201000199131

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Abstract:

Background: In forensic practice, examining wounds is one of the most important tasks of a forensic pathologist. It is necessary to estimate how much time has passed since each wound was sustained, which is known as wound age estimation. The age of a wound refers to the length of time a person continues to live following an injury. It helps with criminal investigations by verifying the occurrence and development of violent incidents and pinpointing possible criminal suspects. A diversity of methods for estimating wound age have been established, and the recent developments in forensic methods allow for the simultaneous examination of wounds at both the cellular and molecular levels, in addition to assessing different markers.

Aim: The current work provides a review of the different methods applied for wound age estimation in the field of forensics.

Conclusion: Researchers have pointed out many techniques for estimating the age of a wound including regular histopathological analysis, immunohistochemical staining, biochemical examination and reverse transcription polymerase chain reaction (RT-PCR). Immunohistochemistry is the preferred technique in forensic pathology due to its simplicity and dependability. Advancements in molecular biology have enabled the utilization of mRNA for forensic purposes. The mRNA levels of wound healing factors and inflammatory markers can accurately determine the age of wounds in their early stages. In the future, there is a need to establish a system for estimating the age of wounds at both the molecular and protein levels.

Keywords: Wound Age; Wound Dating; Methods; Injury; Forensic.

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Introduction:

Estimating the age of wounds has consistently been a significant focus of research in the field of forensic medicine. In certain injuries, both new and older lesions may be present together, making it crucial to determine the timing of lesions to clarify claims in a criminal case involving multiple parties. Multiple parameters have been investigated with regard to time-dependent appearance in wound healing, and numerous studies have been carried out to assess wound vitality and estimate wound ages over several decades **[1].**

A wound is described as a morphological functional disturbance that interrupts the continuity of a tissue structure **[2].**

When the force applied to the body is greater than the tissue's ability to resist, a wound is created. The tissue's ability to adjust to or withstand the force relies on factors such as mass, velocity, the specific area of the weapon in contact with the body, tissue structure, and the manner in which the force is applied, including compression, traction, torsion, tangential stress, and leverage. The type of tissue damage is determined by how much kinetic energy is transferred from the weapon and/or body in motion **[3].**

Medico legal importance of wound age estimation:

Establishing the age of a wound is difficult in the field of forensic pathology, yet it plays a crucial role in reconstructing crime scenes and apprehending the perpetrators. Forensic experts typically concentrate on assessing the vitality of wounds and establishing the post-injury time frame. Advancements in forensic methods now allow for the analysis of injuries at both the cellular and molecular levels, along with the simultaneous examination of several indicators **[4].**

After examining the wound, the key questions to address are **[5]**:

- Was the injury sustained during the individual's lifetime rather than after death (vitality)?
- How is it possible to differentiate between vital and (early) postmortem induction?
- What was the duration of survival following the injury? (Estimation of the age of a wound)

The primary focus of vitality is the initial moment following a trauma where a distinct physical or chemical change is evident and can be distinguished from the state prior to injury. The critical factor is the survival period following a traumatic incident. The topic at hand is the age of the wound, specifically regarding whether the level of a vital response can indicate longer survival times. The question can be addressed only if there are temporal alterations in morphological or biochemical observations **[6]**.

Doctors witness the impact of applying mechanical forces to the body on a daily basis. These injuries can happen through homicide/assault, suicide, or accident. It is important to provide

precise details and descriptions of all wounds, especially in legal proceedings related to these injuries **[7]**.

Based on the autopsy results obtained during the examination, a conclusion is provided indicating the classification of the injury, the kind of weapon that caused the wounds, the age of the injuries, and if they were self-inflicted or caused by someone else. The doctor signs the report and places his seal impression before sending it in a sealed envelope to the investigating officer. The doctor also keeps copies of medical documents for future use **[3]**.

Methods of wound age estimation:

The timing of an injury can vary greatly when assessed based on a subjective visual examination. Hence, examining the injuries microscopically is crucial **[8].** Various techniques have been developed to estimate the age of wounds, including regular histopathological analysis, immunohistochemical staining, and reverse transcription polymerase chain reaction (RT-PCR) **[2].** Recent progress in forensic methods allows the analysis of a wound at both the cellular and molecular levels while also examining different markers **[9]**:

Wound dating through histopathological examination:

Estimating the age of a wound is intricately linked to the process of skin wound healing. Currently, the sequential histopathological alterations during various stages of wound healing are used to estimate the age of a wound **[10].**

Numerous researchers have applied regular histological standards to identify the age of a wound by assessing its healing process over time **(Table 1).** In addition, the release of various mediators (such as coagulation factors, cytokines, growth factors, etc.) by damaged tissue could provide a hopeful perspective in pinpointing key markers **[11]**. Key histological observations that help determine the vitality of a wound consist of red blood cell infiltration, inflammatory reactions, and the existence of fibroblasts, macrophages, and migrating granulocytes, along with tissue alterations **[12].**

Histopathological changes in chronological order can help identify different stages of wound healing and estimate the wound's age. The severity of the injury also plays a crucial role in determining the body's cellular repair response. Neutrophils are the first to be attracted to the site of injury, followed by macrophages, depending on the time frame after the injury. Overall, a range of biological processes are essential for the proper healing of wounds **[13]:**

- Degenerative changes to injured cells (e.g. muscle cells)
- Local reaction of the blood circulation
- Reactions of non-injured parenchyma

- Presence of necrosis or fatty degeneration of damaged cells
- Appearance of polymorphic nucleated leukocytes
- Appearance of iron pigment
- Changes in the size of nuclei
- Mitoses in parenchymatous and connective tissue cells in proximity to the injury, indicating proliferation
- Appearance of newly formed mucopolysaccharides, collagen, and elastic fibers

Table (1): Chronology of injury healing **[13]**

Time following injury	Histological findings and enzyme histochemical reactions
<20 min–1hr	**Hemorrhage with destroyed tissue and cells, but with no cellular reaction, in particular no signs of granulocytic invasion**
<1hr	**Neutrophil granulocytes, partly marginalized at the inner vascular wall, partly amoeboid migration into the tissue**
1hr	**Fresh hemorrhage, tissue edema, local acidosis, single polymorphonuclear leukocytes, evidence of Adenosine Triphosphatase (ATPase), unspecific esterase, aminopeptidase, increased histamine, serotonin, α –esterases**
2hr	**Degranulation of mast cells, infiltration of polymorphonuclear leukocytes, fiber necrosis, ground substance segregation, extracellular activation of fermentation – glucosidase, monoaminoxidase**
2-4hr	**Invasion of monocytic cells, phagocytic reactions**
4-6hr	**Peripherally increasing reactive hyperemia, fibrin deposition, peripheral formation of a leukocyte wall, also involving granulocytes**
6-8hr	**Necrobiosis of cells and tissue, pronounced inflammatory demarcation, increasing phagocytosis**
8-12hr	**Increase in and further activation of mononuclear cells and histiocytes, invasion of single macrophages, evidence of alkaline phosphatase, cytochrome oxidase, and phosphorylases**
12-16hr	**Gradually, mononuclear cells predominate, leukocyte decomposition**
16-32hr	**Mobilized histiocytic cell elements, formation of collagen fibers with fibroblasts and fibrocytes, angioneogenesis with first branched capillary blood vessels**
32-72hr	**Formation of granulation tissue with collagen fiber tissue and capillary blood vessels, embedded macrophages (siderophages, lipophages)**
3–4 days	**Ground substance formation, denser collagen fiber tissue, potential decrease in number of macrophages, new formation of mast cells, possibly polynuclear foreign-body giant cells**
4–10 days	**Decrease of histochemical reactions in collagen fiber tissue, densification of scar tissue, decrease in the number of leukocytes and macrophages, possible persistence of siderophages**
>10 days	**Denser scar tissue with fewer cells, decreasing vascularization, potential persistence of siderin deposits; after a very long time, basophilic calcium salt deposits are also possible**

Wound dating through immunohistochemical examination:

Immunohistochemical methods are commonly used in forensic pathology to differentiate diagnoses, estimate wound age, and more, and showing that they are dependable and practical **[10].** Immunohistochemical staining confirms the histological results and enhances the objectivity of observations and interpretations **[8].**

It relies on an immune response involving an antigen and an antibody. The most commonly utilized immunostains detect cytokines (Inerleukin-1 (IL-1), Inerleukin-6 (IL-6), Tumor necrosis factor (TNF), etc.), inflammatory cells (mast cells, myofibroblast cells, etc.), and other molecules associated with tissue damage. Furthermore, researchers discovered that certain molecules' levels vary over time, making them useful for estimating the age of a wound **[12].**

Immunohistochemical techniques are capable of identifying various substances released in the inflammatory phase including esterase and adenosine triphosphatase levels rising one hour post-injury, aminopeptidase peaking at approximately two hours, acid phosphatase at four hours, and alkaline phosphatase at eight hours. Additionally, examining the levels of fibronectin, Cluster of Differentiation 62p (CD62p), and factor VIII, which are indicators linked to coagulation and inflammation mechanisms. This results in a notable rise in injuries created shortly before dying (15-30 minutes old). Furthermore, the quantity of macrophages with matrix metalloproteinase- 2+ also rises in correlation with the age of the wound as shown in **Table 2 [14].**

Moreover, immunohistochemical techniques have shown to be more valuable than other methods due to their simplicity, high reliability, and, most importantly, their capability to determine the location of molecules of interest **[15].**

Wound dating through biochemical examination:

Biochemical methods use chemical and physical techniques to their advantage. Microspectrophotometry, microfluorimetry, and spectrophotometry have been employed to assess concentrations of vasoactive amines, despite conflicting findings, while atomic absorption spectrometry has been utilized to examine the diagnostic importance of individual ions and ion ratios in skin injuries. Certain researchers discovered higher levels of iron in skin and muscle tissue that was injured before death; however, they did not observe any variations in zinc and magnesium levels. Additionally, a decrease in the potassium/sodium ratio was observed in muscle samples taken before death, while no change was found in skin samples **[15].**

In this region, biochemical methods such as enzyme-linked immunosorbent assays (ELISA) have also shown promising outcomes. Some studies suggest that analyzing cytokine levels in skin tissues can help assess wound healing progress, as these markers are found in higher concentrations in early-stage wounds compared to healthy skin **[16].**

Table (3): Immunohistochemical markers and earliest appearance **[13]**

Marker	Earliest appearance after infliction
Cluster of Differentiation 14 (CD 14)	**1–5 days**
Collagen I	**4–6 days**
Collagen III	**2–3 days**
Collagen IV	**4 days**
Collagen V	**3 days**
Collagen VI	**3 days**
Collagen VII	**4 days**
Endothelial Progenitor Cells (EPCs)	**Average number >20 = 7–12 days Average number <15 = 14–21 days**
Laminin in myofibroblasts	**1.5–4 days**
Heparan Sulfate Proteoglycans (HSPG) in myofibroblasts	**1.5–4 days**
Fibronectin	**10–20 min**
α-Actin in myofibroblasts	**5 days**
Laminin—basement membrane Components	**4–8 days**
Matrix metalloproteinase (MMP-2^{+}) macrophages	**Semiquantitative >20 = 7–12 days**
MMP-9^{+} macrophages	**Semiquantitative >30 = 3–14 days**
P-selectin	**Several minutes–7 h**
E-selectin	**1 h–17 days**
Intercellular Adhesion Molecule-1 (ICAM-1)	**1.5 h–3 days**
Vascular Cell Adhesion Molecule-1 (VCAM-1)	**3 h–3.5 days**
Transforming Growth Factor alpha (TGF-α)	**Circa 10 min**
TGF-β1	**Several minutes**
Smooth muscle cells-actin (SMC-actin)	**5 days**
Keratin 5—complete staining of basal cell layer	**13 days**

- **Wound dating through molecular biological analysis:**

Recently, real-time (polymerase chain reaction) PCR has been utilized more often in forensic medicine, showing that using real-time PCR to detect messenger RiboNucleic Acid (mRNA) is a better method for estimating the age of early wounds **[17].**

Following an injury, the mRNA levels of cytokines and enzymes tend to change quicker than the protein levels and the histomorphology. Therefore, evaluations using mRNA are suitable for determining the age of wounds in their early stages. Despite being less stable than protein, RNA has still been found in a well-preserved sample. Biological stains that are several months or even years old can yield sufficient quality and quantity of total RNA. Therefore, the expression levels of inflammatory cytokines and wound-healing factors in mRNA are analyzed through real-time (PCR) in order to assess the age of the wound **[4].**

Real-time PCR is able to identify the mRNA amounts of inflammatory cytokines and wound-healing factors such as caspase level 3, 8, and 9 expressions in tissues; VEGF and TGFβ1 mRNA demonstrate elevated levels from the beginning, peaking at day 7 **[14].**

Summary: In summary, researchers have highlighted many methods that can be used for wound age estimation. Advancements in forensic wound age estimation methods have progressed beyond traditional techniques such as conventional histopathological examination. With the advent of immunohistochemical techniques, they have shown to be more valuable than other methods due to their simplicity and high reliability. The focus of forensic pathologists has now shifted to study mRNA expression of various markers using RT-PCR. The significance of wound age estimation as well as its medicolegal importance has been frequently emphasized by researchers. In the future, a system for the estimation of wound age needs to be established at the molecular as well as protein level.

Key Points:

- Estimating the age of wounds is a crucial aspect of forensic medicine research, particularly in criminal cases involving multiple parties.
- Forensic experts focus on assessing wound vitality and post-injury time frames, utilizing advancements in forensic methods.
- Techniques such as histopathological analysis, immunohistochemical staining, biochemical examination and RT-PCR are used to estimate wound age.
- The emphasis for forensic pathologists has now changed to analyzing mRNA expression of different markers using RT-PCR.
- Forensic methods now allow for analysis of wounds at cellular and molecular levels, identifying various markers to determine wound vitality and healing stages.

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