

Demands on scientific studies: Vitality of wounds and wound age estimation

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Abstract

Research on vitality and wound age estimation belongs to the classic fields in forensic medicine. Despite large literature data there is still considerable demand of further research and practical transfer of knowledge and techniques to daily casework. Scientific studies must fulfil basic criteria as to appropriate methods, selection of case material, analysis of results and quality control. Nowadays, immunohistochemistry, biochemical tests and molecular biological techniques are mainly used studying questions of vitality and wound age. Investigations can be based on human tissue samples (autopsy material, vital specimens) or animal experiments. The possibilities, advantages and disadvantages of these study designs are described. Indispensable is the use of appropriate control groups or control samples and a sufficient case number which permits statistical analysis. Main strategy is to minimize variations due to methods and investigators as the unavoidable biological variation of vitality processes and wound repair is large enough.

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1. Introduction and short survey

Vitality and wound healing or wound age estimation are phenomena which are closely related to each other, in particular with regard to skin lesions—the main object of scientific research in this field. According to Oehmichen [1], there is an overlap in the early phase of repair processes following trauma: alterations within the first 30 min can be regarded as both vital reactions and signs of wound healing.

Vitality as one of the central issues in daily forensic practice deals with the question, whether injuries were caused during lifetime of an individual [2,3]. It plays a role in connection with traumatic deaths due to sharp and blunt force injuries, burns, strangulation, suffocation or drowning. With regard to the vitality of wounds it must be proven that an injury of the body was caused during lifetime and not postmortem. The most difficult question is to differentiate between wounds inflicted

shortly before and shortly after death. This coincides with processes in the late agony and the early supravital period.

Wound age describes the time interval between the infliction of a wound and the time of death [1,4]. It can be considered as the survival time of the individual following a physical injury. Forensically relevant wound age periods can vary from minutes to several months and even years. Concerning very short intervals, the wound age question is nearly identical with the issue of vitality. The forensic pathologist can be confronted with the estimation of wound age in association with murder, manslaughter, bodily harm with fatal consequences, (primarily survived) accidents and further constellations.

Vitality and wound age estimation belong to the classic research fields of forensic medicine. For many decades a huge number of scientific papers have been published (surveys: [1,4–7]). However, the majority of scientific studies in this field deal with dermal injuries due to sharp force. Studies on dermal injuries due to blunt force or other types of trauma are almost completely missing. There is a need of further investigations on the anatomical/topical dependence of dermal wound healing processes also for sharp force injuries. It should also not be overlooked, that there is a really important necessity to do more

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Table 1
Factors influencing the time course of inflammation (modified according to [8])

Local factors	
	Type and intensity of trauma
	Severity and extension of damage
	Type of tissue
	Temperature
	Circulation
Systemic factors	
	Hereditary factors
	Age
	Sex
	Nutritional state
	Diseases
	Endocrinopathy
	Metabolic disturbances
Exogenous factors	
	Drugs

research work not only on external, but on internal injuries/wounds and their age determination. Various influencing factors have to be considered (Table 1). For the future, it must be the aim to transfer the scientific knowledge to daily forensic case work in which still only a small part of potentially available techniques is used.

2. Questions to be answered

The main questions are as follows:

- Was an injury caused at lifetime and not in the supravital or postmortem period (vitality)?
- How is it possible to differentiate between vital and (early) postmortem induction?
- How long was the survival time after the infliction of an injury (wound age estimation)?

The essential question of vitality is the earliest point in time after a traumatization with a definitive morphological or biochemical alteration which can clearly be distinguished from uninjured state. The crucial issue is the so-called survival time after a traumatic event. The following issue is that of the wound age, i.e. the question whether the extent of a vital reaction can serve as a marker of (“longer”) survival intervals. It can only be answered if there are time-dependent changes of morphological or biochemical findings.

3. Appropriate method

In most of the cases, tissue samples are available for investigation purposes (skin or organ lesions). Therefore, for a long time morphologically oriented techniques have been methods of first choice and are still used:

- Routine histology with paraffin embedding of tissue samples, preparation of thin sections and staining with haematoxylin–eosin (HE). This method allows the rough differentiation of cell types and tissue structures. Immigrating granulocytes,

macrophages or fibroblasts can be frequently and easily detected. For the special differentiation of substructures (e.g. connective tissue, hemosiderophages), there exists a variety of further staining methods (e.g. elastica-van Gieson stain, E.v.G., Prussian blue stain). For scientific purposes, routine histology only serves as preliminary method for selection of appropriate cases.

- Enzyme histochemical methods tried do detect enzymes of fibroblasts at their location in the tissue section (e.g. esterase, acid phosphatase, ATPase). Formerly, they were used to define wounds with an age of about several hours. However, the methods proved to be too unreliable and showed a high rate of negative cases even after intervals of hours. Therefore, they did not find large dissemination and seem to be obsolete nowadays.
- Immunohistochemistry is the method of choice in modern investigations on vitality and wound age. This method serves for the identification of special substructures within the tissue. Basic principle is the immunological reaction between an antigen in the tissue section and an antibody applied. There is a huge variety of antibodies against cellular, superficial or matrix-associated antigens. The antibody can be monoclonal or polyclonal which has consequences for its specificity and the selection of the detection system. As a first step, it must be checked, whether an antibody can be used on formalin-fixed and paraffin-embedded tissue sections, or whether native frozen material is required. A further critical step is the dilution of antibodies and reagents which usually must be tested (the recommended dilution is often not suited). Then, the investigator has to make a decision concerning the detection system which is to make the reaction visible between antigen and (primary) antibody. There are well-established systems such as ABC, APAAP and LSAB [9,10]. The methods have been established for many years, details are permanently improved. Numerous parameters have been tested with respect to their time-dependent appearance in wound repair (extract in Table 2 and Fig. 1). Most of these factors are expressed within days after traumatization. Recently, the existing gap concerning the intervals of minutes to hours could be closed by the immunohistochemical detection of cytokines and adhesion molecules [4,11].

Apart from morphological methods, there were numerous attempts to establish biochemical methods, in particular for the evaluation of vitality (time intervals up to approximately 30 min). Such quantitative analyses can be performed in wound fluids or tissue extracts of wounds. Modern techniques are frequently based on immunological tests such as enzyme-linked immunosorbent assay (ELISA). Due to considerable methodological effort and high standard deviations of results, biochemical techniques have missed to achieve an essential breakthrough up to now. However, there are good own experiences with the analysis of proinflammatory cytokines [12].

Whereas the methods discussed so far serve for the detection of vitality and wound markers on the protein level, it is also possible to analyse the earlier stage of a reaction on the mRNA level. For this purpose there are morphological methods such as in situ hybridization and molecular biological techniques such

Table 2
Immunohistochemical detection of parameters in dependence on the survival time (human skin wounds)

Antigen/Marker	Earliest detection	Regular detection or marked reaction	Longest detection
TGF- β 1	(several) minutes	30–60 min	
TGF- α	ca. 10 min	30–60 min	
Fibronectin	ca. 10–20 min	>4 h	Months
IL-1 β	15 min	30–60 min	
IL-6	20 min	60–90 min	
TNF- α	15 min	60–90 min	
ICAM-1	50 min	>2 h	
VCAM-1	30 min	>1.5 h	
E-selectin	30 min	>1–1.5 h	
L-selectin	30 min	>1.5 h	
Tenascin	2–3 days	From 5 days	Months
Collagen III	2–3 days	From 6 days	Months
Collagen V	3 days	From 6 to 7 days	Months
Collagen VI	3 days	From 6 to 7 days	Months
Collagen I	4–6 days	From 7 days	Months
Myofibroblasts			
Laminin	ca. 1.5 days		Months
HSPG	ca. 1.5 days		Months
Collagen IV	4 days		Months
SMC-actin	5 days		Months
Marker of macrophages			
RM 3/1	7 days		Months
25 F 9	11 days		Months
G 16/1	12 days		Months
Epithelial basal membrane			
Fragments (Lm, HSPG, K IV, K VII)	4 days	From 13 days	ca. 21 days
Complete restitution	8 days	From 21 days	
Keratin 5			
Complete staining of basal layer	13 days	From 23 days	

as RT-PCR. As these methods are not so commonly used in forensic context, it can be referred to special literature.

It depends on the aim and experience of the investigator to select the appropriate method and to achieve the best results for his/her purposes (Table 3).

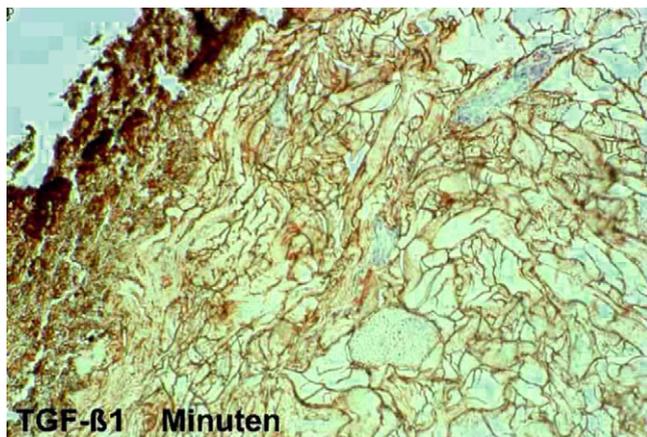


Fig. 1. Network-like immunohistochemical detection of transforming growth factor (TGF) β 1 at the margin of a stab wound, survival time of several minutes (LSAB, 200 \times).

4. Case material

It is possible to use (a) animal experiments, (b) samples from living human subjects or (c) autopsy specimens:

- (a) Animal experiments have the advantage of controlled conditions. It is easily possible to evaluate various posttraumatic intervals (survival times) under standardized circumstances. Sequential biopsies can be taken. The assessment of influencing factors (age, wound location, cause of death, etc.) is also no problem. However, using animal experiments, it always raises the question, whether

Table 3
Essential methods for studying vitality and wound repair on different levels

Cellular structures
Routine histology
Special staining methods
Protein/antigen
Immunohistochemistry
Quantitative biochemical methods
mRNA
In-situ hybridisation
RT-PCR

the results can be transferred to human conditions. Therefore, the output is sometimes of limited value.

- (b) Samples from living human subjects can be gained during surgery. Skin tissue or other specimens are frequently removed in the normal course of an operation (see chapter ethical issues). In this way the investigator obtains vital tissue samples with a well defined wound age (time after start of surgery) under controlled and standardized conditions. The advantages are therefore partly similar to those of animal experiments without the disadvantage of non-human tissue. However, the influencing factors are not comparable to reality, as the tissue donor does not die or suffer from severe life-threatening events such as hemorrhagic shock or respiratory problems.
- (c) Autopsy samples reflect the “reality” best. The tissue donor underwent severe life-threatening events which finally lead to his death. Insofar the effects of survival times under conditions of stress, respiratory failure and especially hemorrhagic shock can be studied very well. The main problem of postmortem studies – apart from possible autolysis and putrefaction – is associated with the frequently not exactly known survival time (wound age). Therefore, in the sense of optimal results, strictly selected case material is required with well documented times of infliction of wounds and known times of death. Influencing factors cannot be quantitatively considered.

Independent of the type of case material it is always necessary to collect an adequate number of cases with different survival times. The required number depends on the heterogeneity of influencing factors and the statistical evaluation aimed at.

Furthermore, it is essential to perform the same investigations in at least one control group (e.g. samples without injuries) under comparable conditions. Dependent on the design of the study, it can be highly recommendable to compare wound samples with contralateral uninjured specimens, e.g. in the research on cytokines and adhesion molecules [11,12]. For vitality studies, it may be necessary to investigate a sufficient number of samples including agonal injuries (e.g. by emergency measures) and (early) postmortem wounds.

5. Analysis of results

Histological and immunohistochemical studies can be evaluated (a) qualitatively, (b) semi-quantitatively or (c) quantitatively:

- (a) Qualitative detection means presence or non-presence of special features or markers.
- (b) Semi-quantitative detection requires a scale for the (relative) intensity of a histological phenomenon or an immunohistochemical staining, e.g. the distribution of positive epidermal cells can be assessed semi-quantitatively in the separate epidermal layers on a percent range (<10%, 10–25%, 25–50%, >50%). The degree of reactivity of other histological structures (corium, vessels, sweat glands, subepidermal cells) may be classified as negative, slightly positive, moderately positive and strongly positive.

- (c) Quantitative studies include cell counts or counts of special signals, mainly in the sense of counts per microscopic field. For example, 10 or 15 fields are counted and then the average value is calculated for one field.

Biochemical studies must strictly follow the test protocol for the appropriate marker. The abovementioned ELISA requires standard solutions with known concentrations and the establishment of a standard curve. Statistical evaluation completes the biochemical (and morphological) analyses.

Typical for studies on vitality and wound age estimation is the underlying high variability of biological phenomena and time-dependent alterations. Forensically usable are therefore in particular the minimum time limits of the detection of special parameters, i.e. the earliest detection of a positive finding in the sense of a minimum wound age. In contrast, regular detection of a special parameter is only seldom possible. Negative results should therefore not be interpreted. The maximum time of detection is not so expressive either.

6. Ethical issues

Nowadays, most scientific journals demand a statement on compliance with ethical regulations prior to publication of a study. As a rule, investigations with human beings must have been reviewed by a local ethics committee and have been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki. In studies with material of living persons, informed consent must be obtained; normally, only tissue samples come into question which are to be resected anyway. Material from autopsies can be used if specimens belong to the routine asservation. Otherwise consent of prosecution authorities and relatives must be obtained.

In animal experiments, the “Principles of laboratory animal care” must be followed and specific national law must be observed. Exclusively postmortem forensic experiments are possible if animal experiments with final killing were allowed within the scope of a clinical study.

7. Quality control

It is absolutely necessary to include appropriate control groups or control samples as described in chapter 4. With regard to methodical aspects standardized study design and approach is indispensable. Using immunohistochemical methods, it is necessary to carry negative and positive controls along with the wound samples. As a negative control the primary antibody is replaced by a non-reactive immune serum with the same concentration of immunoglobulins. Positive controls are samples which contain the antigen and, therefore, must show positive signals. The intensity of the staining quality must be adapted by experimentation with the dilutions of primary/secondary antibodies and detection system, so that on the one hand positive controls react positively and on the other hand no unspecific background staining is present. Skin wounds and control tissues must be stained in the same way to avoid artefacts.

Only on the basis of correct control results and optimal establishment of the test procedure, the results on wound samples can be used. It may be advisable to evaluate microscopic slides by at least two independent investigators in order to raise the quality of a scientific study. Moreover, it makes sense, that the examiner does not know the wound age.

Biochemical techniques require the establishment of correct standard curves, double measurements and positive and negative controls.

Every scientist working on vitality should be highly aware of the possible postmortem induction of putative vital parameters. The validity of a vital parameter increases with the duration of its manifestation time. In cases with “simple” vital reactions within short manifestation times supravital induction is possible. This could be proven for fibronectin and leukocyte immigration under certain circumstances [13,14]. Complementary experiments should be undertaken where it seems necessary.

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