Timing of Wounds

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YARALARIN MEYDANA GELİŞ ZAMANININ VE YARA YAŞININ BELİRLENMESİ

Özet

Ölümden önce ve sonra meydana gelen yaralar arasında ayırım yapılması ve yaraların gerçek yaşlarının belirlenmesi adlî tıbbın en önemli sorunlarından biridir. Yara, vücuda mekanik bir kuvvetin uygulanmasıyla dokuların anatomik devamlılığının kesintiye uğraması sonucunda meydana gelir. Canlı organizmada, yaranın meydana gelmesiyle birlikte hemen doku reaksiyonları görülmeye başlar. Bu reaksiyonlar vasküler, hemostatik ve hücresel olmak üzere 3 ana grupta toplanırlar. Vasküler reaksiyonlar, histamin ve serotonin gibi vazoaktif aminlerin etkisiyle erken dönemde kendilerini gösterirler. Hemostatik reaksiyonda, damar kontraksiyonları ve mikrotrombuslarla kanamanın durdurulması söz konusudur. Hücresel reaksiyonlarda ise, yara yerine göç eden lökositlere rastlanır; lökositlerin tipi, yaranın yaşını belirlemeye yardımcı olur. Yaralanmayı izleyen 6. günde, lökosit sayısı maksimum düzeydedir. Bu bulgular *vital reaksiyonlar* olarak adlandırılır; canlılarda meydana gelen yaralarda pozitif vital reaksiyonlar görülür, ölümden sonra oluşan yaralarda ise vital reaksiyonlar negatiftir.

Yaralanmanın ölümden önce ya da sonra olduğunu anlamak zor olmamakla birlikte, yaranın yaşını tesbit etmek oldukça güçtür. Yara yaşı kişisel (endojen) ve çevresel (ekzojen) faktörlere bağlı olarak değişir. Bu nedenle, kesin yara yaşı tanımı yerine, yaklaşık yara yaşı tanımı yapılması yeterli olmaktadır.

Yara yaşının tesbiti adlî tıp yönünden önemli olan olguların çoğunluğunda yaralanmadan sonraki yaşama süresi 8 - 10 saatten kısadır; bu nedenle hücresel reaksiyonları görmek mümkün olmayabilir, histolojik inceleme yetersiz kalabilir. Böyle vak'alarda, yara dokusunun serotonin, histamin, adenozintrifosfataz, esteraz, aminopeptidaz ve alkali fosfataz düzeyleri araştırılır.

The distinction between antemortem and postmortem injuries and their proper timing is one of the cardinal problems of forensic medicine. It helps not only to convict the guilty but also to acquit persons who are suspect but in fact not guilty. As a classic example, a person may, after death, be run over by a car. If the medical examiner does not recognize that the injuries were caused after death, innocent people may be arrested or even found guilty.

On the other hand, defective observations may lead to the acquittal of guilty persons. For example, the body of a murdered person may be put on a railroad track in order to simulate a suicide or an accident. Thus, justice in these matters often rests upon the accuracy in observation and interpretation of the injuries found.

The naked-eye appearance of wounds

It is not possible to determine exactly the age of a wound on the basis of its naked - eye appearance. This is due to the fact that the intensity of the local inflammatory reaction varies according to the circumstances. Under average conditions the edges of a wound are red and swollen after a lapse of about 12 hours. A small wound may show scab formation after approximately 24 hours. Epithelium begins to grow at the edges of a wound after about 36 hours and epithelization of small, clean wounds may be complete in four to five days. An antemortem wound is customarily identified by its profuse bleeding. However, hemorrhages, even with abundant fibrin formation, are no longer considered reliable proof of vitality. *Laiho* (1) has shown immunohistochemically, that fibrin can be formed in bruises inflicted as long as 7 hours after death.

Histological timing of wounds

Because of the uncertain and variable results of naked - eye examination of wounds, it is important to study the injuries microscopically. This is advisable both in order to distinguish between antemortem and postmortem wounds and to time wounds inflicted before death for the reconstruction of events.

A *wound* is a disruption of the anatomical continuity of tissues caused by the application of a mechanical force to the body. It is usual to distinguish between incised wounds (cuts), stab wounds (punctures), and lacerations. *Incised wounds* are caused by sharp weapons or objects such as knives, jagged portions of metal, or pieces of broken glass. *Stab wounds* are caused by long narrow instruments with blunt or pointed ends. *Lacerations* are wounds in which the tissues are torn as a result of the application of blunt force to the body. An *abrasion* is a destruction of the skin that usually involves the superficial layers of the epidermis only.

The histological estimation of the age of injuries is based on the morphology of the various stages of wound healing. The series of events in response

to the initial injury generally follow in a definite order. It is possible to divide tissue repair into three periods :

1) The inflammatory phase, which lasts 1 - 3 days after injury,

2) The proliferative phase, lasting to day 10 - 14,

3) The reorganization or remodeling phase of healing, which is of varying length, lasting at least several months. None of the phases is distinctly separate, since each blends into the next.

The *inflammatory phase of wound healing* is characterized by a) the vascular, b) the hemostatic, and c) the cellular *response to the injury* (Fig. 1). In the *vascular response*, the sequence of events depends on the severity rather than the nature of the injurious agent. The vascular response develops in three distinct phases. The first is *vasoconstruction*, beginning within a few seconds and lasting for a few minutes. The second is an *early vasodilatation*, accompanied by increased permeability to plasma protein, which attains a maximum response in about 10 minutes. The third, *delayed phase* requires several hours and culminates in the infiltration of the tissue with leukocytes and is accompanied by stasis and local hemorrhage. These phases are due to the release of vasoactive substances, including histamine and serotonin. In practice, the infiltration of the injured tissue by leukocytes is the most important histological characteristic of the delayed vascular response.

The *hemostatic response* means the spontaneous arrest of bleeding. Agglutinated platelets arrest hemorrhage by rapidly sealing all cut vessels larger than capillaries. The capillaries are sealed by a red cell fibrin clot. Platelet aggregation occurs at first without fibrin formation, but fibrin is laid down later with the appearance of a more massive blood clot where a fibrin network enmeshes randomly distributed platelets, erythrocytes, and leukocytes. The method of Martius scarlet blue seems to be the most useful single method for the demonstration of fibrin. Fibrin stains yellow with the Martius scarlet blues technique for the first 16 or so hours after production, and thereafter stains bright red (2).

The *cellular response* begins with the aggregation and margination of leukocytes on the vascular wall within half an hour of wounding. This in practice is difficult to detect with any certainty in human material from the usual cases of forensic interest. The early cellular response is dominated by polymorphonuclear leukocytes. At the onset of a lesion both polymorphonuclear and mononuclear cells emigrate together, but there is a predominance of

TIME COURSE OF THE EARLY EVENTS OF INFLAMMATION

1) **Vascular response**

vasoconstriction (seconds → some minutes)
vasodilation (max : 10 minutes)

increased capillary permeability

stasis

local hemorrhage

2) **Hemostatic response**

aggregation of platelets
clotting
arrest of bleeding

3) **Enzymatic response**

increase in enzyme activity
in the wound periphery (1 hr →)

4) **Cellular response**

aggregation of leukocytes

pavementing of leukocytes

extravasation of leukocytes

leucocytic infiltration (4 hr →)

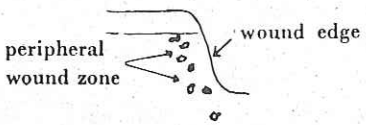

Figure 1. Time course of the early events of inflammation.

polymorphonuclear (mostly neutrophilic) cells, the ratio being 5 : 1. Later (at 16 - 24 hours) the polymorphonuclear cells move away from the site of inflammation, but the mononuclear cells remain, the ratio falling to 0.4 : 1.

The point of *the appearance of a distinct leukocytic infiltration* is still a matter of debate. According to different authors, it varies from 4 to 24 hours (3). In guinea pigs, some polymorphonuclear leukocytes migrate out of the vessels 4 hours after wounding. In the vicinity of an 8 - hour excision, polymorphs are more numerous, but they do not yet constitute a well - defined zone around the lesion. At 8 - 16 hours, it is possible to distinguish histologically two zones (Fig. 2). In the vicinity of the wound edge, a *central zone* of up to 500 µ in depth is characterized by *degenerative* changes. Karyolysis and karyorrhexis progress in the connective tissue cells, the epidermal cells maintaining their staining properties better. At some 32 hours, necrosis is

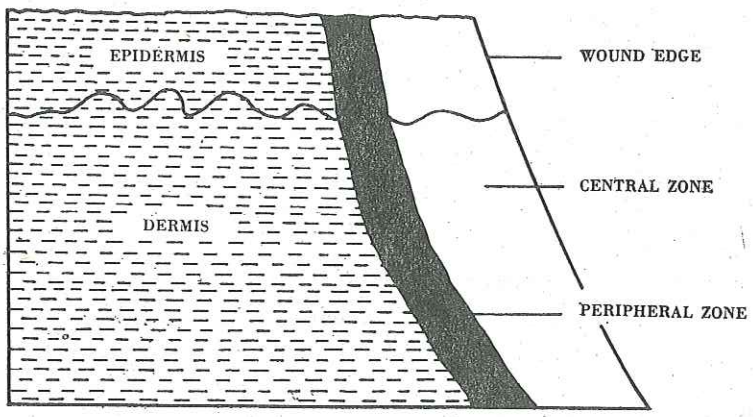

Figure 2. Schematic diagram showing the zones of a wound.

apparent in the connective tissue, the nuclei being barely stainable. Surrounding the central zone, a 100 to 200 µ deep *peripheral zone* exhibits, in addition to activated fibroblasts, mostly neutrophilic *polymorphonuclear* cells (also termed neutrophilic granulocytes by many authors). Their number increases to the maximum during day 1, remains at that level until day 2 - 3, and then decreases. This increase and decrease in granulocytes coincides with an equally prominent rise and fall in the amount of fibrin. The granulocytes are followed into the peripheral wound zone by large mononuclear cells or *macrophages* at 14 to 26 hours, reaching their maximum concentration within

approximately 48 hours. *Lymphocytes* are found in large numbers at a somewhat later stage, reaching their maximum concentration about day 6 after the injury.

The regressive phenomena in the *central* wound zone are called *negative vital reactions*, since no such local degenerative changes are observed in wounds inflicted after death. Similarly, the inflammation and other progressive phenomena in the *peripheral* wound zone may be called *positive vital reactions*.

At some 32 hours, necrosis is apparent in the central zone.

In the peripheral zone, macrophages are principally responsible for the increase in the number of cells in the dermis. Only occasional mitotic figures are seen within the connective tissue. The epithelial cells of the peripheral zone are enlarged. A few isolated figures are seen in the stratum basale.

At 64 hours, there is advanced necrosis in the central zone. In the peripheral zone, macrophages and activated fibroblasts dominate. Some mitoses are also seen in the dermis, but mitotic figures are more numerous in the lower layers of the thickened epidermis.

In *human wounds*, the inflammation proceeds slower than in laboratory animals. In forensic autopsy materials, some neutrophilic granulocytes are seen perivascularly at 4 hours. A definite peripheral wound zone, consisting of infiltrating granulocytes and activated fibroblasts, is recognizable at 8 - 16 hours (4 - 7). There are, however, authenticated cases in which a distinct leukocytic infiltration has taken still longer to become apparent (7).

Among the inflammatory cells, the *neutrophils* are essential in the control of infection at the wound site. Further, they are capable of engulfing and digesting nonmicrobial material. Large numbers of degenerated neutrophils can be seen during the first 24 hours. The lysis of neutrophils, with resultant release of the stores of hydrolytic enzymes, represents an important function of this cell.

Many names have been given to the large mononuclear cell that plays so important a part in the later stages of acute inflammation. The word *macrophage* means «large eater» and suitably designates a large phagocytic cell. It can ingest bacteria and also debris that results from the breakdown of cells or their products. Macrophages are termed histiocytes or clasmatocytes by some authors.

Morphologically, there are small (diameter of 7 to 8 μ) and large (around 12 μ) *lymphocytes*. As is well known, the rounded nucleus of a lymphocyte is its most prominent feature. There is very little cytoplasm around the nucleus. The essential function of the small lymphocyte is to mediate the immune response. Together with its close relative, the *plasma cell*, the lymphocyte constitutes the so-called «small round cell infiltration» that dominates the picture in the late stages of acute inflammation. The plasma cell is larger than the lymphocyte, with a more abundant cytoplasm and an eccentric, spherical nucleus. The latter contains coarse, angular, densely staining flakes of chromatin that sometimes are arranged like the spokes of a wheel. The plasma cells are a very important source of circulating antibodies.

During acute inflammation, *mast cells* degenerate and liberate *histamine*, *heparin*, and *hydrolytic* enzymes into their surroundings. Mast cell histamine may participate as an edema - producing substance in the early phase of inflammation (cf. vascular response). Mast cell granules, which are the chief distinguishing feature of this cell, do not show up in hematoxylin - eosin sections.

The origin of the new mononuclear cells in the healing wound is still a matter of debate. Some authors assume that these cells are derived from preexisting fibroblasts, others regard them as migrating from the blood.

The *proliferative phase* of wound healing is characterized by epithelial and connective tissue repair. The earliest detectable change in the epidermis is a thickening of the epidermis remaining at the wound margin. This thickening appears to result from an increase in the volume of the epidermal cells adjacent to the wound.

The epithelial growth in human wounds commences in the interval between 24 and 48 hours. The advancing epidermis appears to follow a plane defined by a fibrin net, which in turn is enclosed by serous exudate containing inflammatory cells. This plane lies deep to the wound crust. Epithelization of small wounds and abrasions is invariably complete 3 days, and often 2 days, after the injury.

After wound closure the newly regenerated epidermis becomes highly stratified and thicker than the normal surrounding epidermis. Epidermal thickness decreases to nearly normal by day 5 - 7 after the injury.

Healing by primary intention occurs when epithelial cells bridge across the wound under the dry superficial clot in a clean incision. With healing by

secondary intention, when the defect is too large for epithelial cells to grow across, the cells of granulation tissue gradually invade and replace the dry fibrin covering. In this case the wound closure relies on epithelization, granulation tissue, and contracture. Healing by secondary intention is typical in deep wounds in which there is loss of the dermis and epidermis. These injuries are of the type more closely related to those encountered in forensic practice.

Fibroblasts are the cells most actively concerned wit the *connective tissue repair*. These cells, often spindle - shaped, can be seen within the wound in the first 48 - 72 hours. The *fibroblasts* are polymorphic. Some cells are elongated with elongated nuclei, others are oval or irregular in shape (8).

One or two days after the appearance of fibroblasts, there is a profuse ingrowth of new *capillaries* to restore the circulation. The invasion of capillary buds causes the granulated appearance of the wound surface. At the surviving vascular border, sprouts appear from preexisting vessels in the form of syncytia or solid cords that later become canalized.

The *reorganization or remodeling phase* of wound healing is characterized by decreasing cellular activity and regressing fibroplasia. Usually at about 12 days a definite stage of regression of cellular activity occurs in both epidermal and dermal tissue. Fibroplasia reaches its peak at 14 days and thereafter occurs a gradual shrinkage and maturation of connective tissue in the wound. Histologically, the blood vessels gradually disappear, and the number of fibroblasts in relation to collagen fibers rapidly falls. Some of the fibroblasts, having done their job of synthesis, must remain as resting fibrocytes, but the fate of the remaining fibroblasts and other cells, such as phagocytes, is not precisely known. There are no obvious signs of necrosis or degeneration. In a strictly quantitative sense, the phenomenon of contraction makes the biggest contribution to the covering of a raw area. Contraction starts about 5 - 7 days after wounding and has just about stopped at 6 weeks.

Schema for histological estimation of the age of open skin wounds and abrasions

In order to make the histological timing of open skin wounds and abrasions easier, the following schema is given. It must be emphasized that all the points of time listed in the schema are approximate. So many factors are concerned in both the inflicting and healing of wounds that the exact deter-

mination of the age of a wound is impossible. The following data correspond to the experience of the present author and of many others.

1. Survival period : *less than* 4 *hours* after the injury :
   - — no distinct histological signs of inflammation
   - — histological distinction between antemortem and postmortem skin wounds and abrasions is not possible during this period

2. Survival period : 4 - 12 *hours* :
   - — at 4 hours : some neutrophilic granulocytes perivascularly
   - — at 8 - 12 hours : granulocytes, macrophages and activated fibroblasts form a distinct peripheral wound zone
   - — granulocytes dominate macrophages in the ratio 5 : 1
   - — imminent necrosis in the central zone

3. Survival period : 12 - 48 *hours* :
   - — at 16 - 24 hours : the relative number of macrophages increases, the ratio (granulocytes : macrophages) falling to 0.4 : 1
   - — after 16 hours : « o l d e r » fibrin stains bright red with Martius scarlet blue, whereas before 16 hours « n e w e r » fibrin stains yellow
   - — at 24 hours : the number of granulocytes and the amoung of fibrin increase to a maximum (they remain at this level until 2 - 3 days)
   - — the cut edge of the epidermis shows cytoplasmic processes
   - — at 24 - 48 hours : the epidermis migrates from the incised edge toward the center of the wound
   - — at 32 hours (and thereafter) : necrosis is apparent in the central wound zone
   - — at 48 hours : macrophages reach their maximum concentration in the peripheral zone

4. Survival period : 2 - 4 *days* :
   - — at 2 - 4 days : fibroblasts migrate from the nearby connective tissue to the wound periphery
   - — at 3 days : epithelization of small wounds and abrasions is complete; thereafter the regenerated epidermis becomes highly stratified and thicker than the normal, surrounding epidermis
   - — at 3 - 4 days : capillary buds appear

5. Survival period : 4 - 8 *days* :
   - — at 4 days : the first new collagen fibers are seen
   - — at 4 - 5 days : profuse ingrowth of new capillaries; the capillaries continue to proliferate until the eighth day
   - — at 5 - 7 days : epidermal thickness decreases to nearly normal in the epithelialized small wounds
   - — at 6 days : lymphocytes reach their maximum concentration in the wound periphery

6. Survival period : 8 - 12 *days* :
   - — at 8 - 12 days : decrease in the number of inflammatory cells, fibroblasts, and capillaries, increase in the number and size of collagen fibers

7. Survival period : *over* 12 *days* :
   - — over 12 days : definite stage of regression of cellular activity in both epidermis and dermis. The vascularity of the dermis diminishes. Collagen fibers are restored. Epithelium shows a stainable basement membrane
   - — at 14 days : fibroplasia reaches its peak : thereafter there occurs a gradual shrinkage and maturation of connective tissue in the wound.

Enzyme histochemical timing of wounds

Histological investigations have contributed substantially to our knowledge of the estimation of the age of injuries. The appearance of a distinct leukocytic infiltration is the most trustworthy histological criterion for the distinction between antemortem and postmortem wounds. There is, however, the disadvantage that no definite leukocytic zone becomes visible until 8 - 10 hours after the injury (7). This latent phase is far too long to satisfy the demands of a forensic - examination, because in the majority of cases of medicolegal interest the survival time is shorter than 8 - 10 hours after the injury.

It is now known that morphological alterations are preceded by functional changes and these moreover, often correlate with the actions of *enzymes* (3, 9). Thus, it is to be expected that the demonstration of enzymes and other substances causing important changes could reveal earlier reactions than could the visualization of the resulting, morphologically (histologically) demonstrable alterations. Enzymes and other substances, being essential in the

inflammatory response to injury, can be demonstrated histochemically and biochemically.

For *enzyme histochemical examination*, the wound is excised along with its surroundings, about half an inch in each direction. The tissue block is then divided (Fig. 3). One half of the block is fixed in 10% neutral formalin for 24 hours, automatically processed and cut in the form of 5 to 6μ paraffin sections. The sections are routinely stained by hematoxylin and eosin for histological study.

For *histochemical study*, the other half, is freshly frozen without any fixation between two blocks of dry ice (solid carbon dioxide). The frozen specimen is then attached, with some drops of water, to the previously chilled, metallic chuck of the cryostat microtome. Thereafter, sections are cut at 15 to 16μ in a cryostat at —20°C. For the demonstration of certain enzymes, like phosphatases, the frozen sections are fixed for 5 minutes in 10% neutral formalin at +4°C, i.e. at refrigerator temperature. For the demonstration of other enzymes, such as aminopeptidase and esterase, no fixation is used. Instead the frozen sections are picked from the cryostat microtome knife by placing a «warm», i.e. room temperature, glass slide on the cold knife. The sections on the cold knife thaw and adhere to the warm glass slide.

The *histochemical demonstration of an enzyme* depends upon its action on a specific substrate, i.e. on the substance upon which the enzyme acts. Under appropriated cirumstances the reaction product resulting from enzyme activity forms an insoluble deposit at the site of the enzyme action. If this deposit is not already coloured it is rendered visible by the use of suitable chemicals:

$$\textit{substrate} \xrightarrow[\text{reaction}]{\text{enzyme}} \textit{dye precursor} \xrightarrow[\text{reaction}]{\text{capture}} \textit{final dye}$$

The enzyme histochemical methods are published in detail elsewhere (10).

To summarize *the results of the enzyme histochemical studies*, *two zones* can also be distinguished histochemically around the antemortem wound (Figs. 2, 4, and 5). The 100 - 200μ deep *peripheral zone* shows *increase in enzyme activity*, beginning as little as one hour after injury. The increases in enzyme activity in the peripheral wound zone may be called *positive vital reactions*. In the most immediate vicinity of the wound edge, the 200 to 500μ deep central zone exhibits a progressive loss of enzyme activities. These regressive phenomena are called *negative vital reactions*. They are demonstrable as a diminishing stainability and become visible 1 - 4 hours after

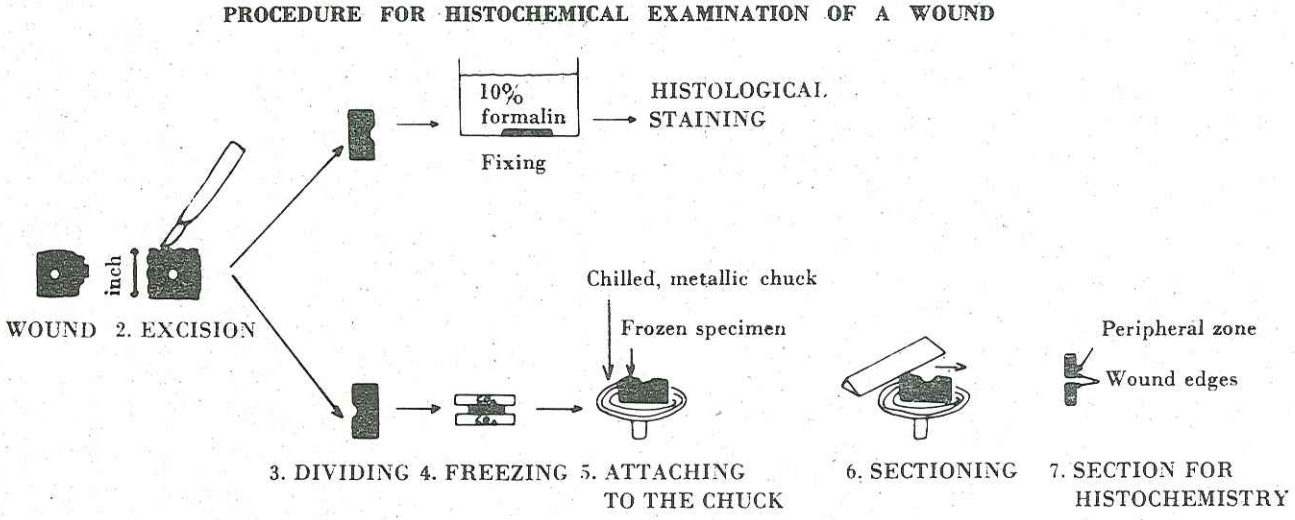

Figure 3. Procedure for histochemical and histological examination of a wound.

wounding. They are early signs of imminent necrosis in the most damaged area at the centre.

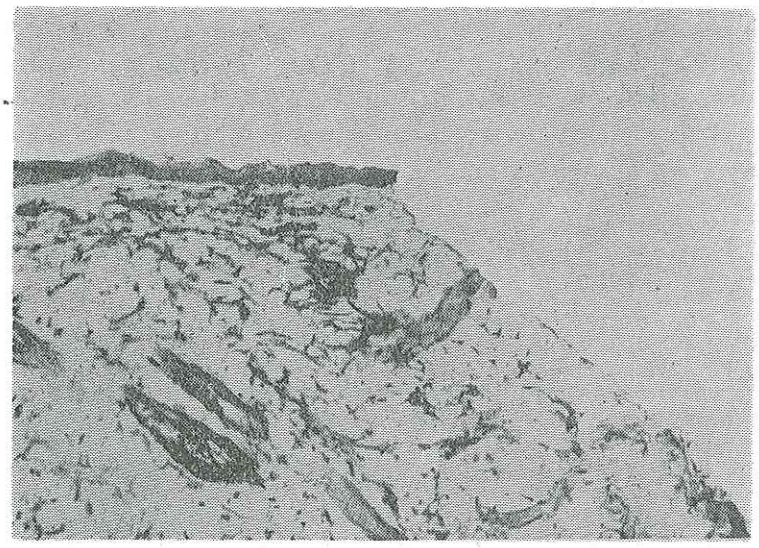

Figure 4. *Adenosine triphosphatase* activity in an incised wound. The victim lived 1.5 hours after injury. The sample was taken 18 hours after death (150 ×).

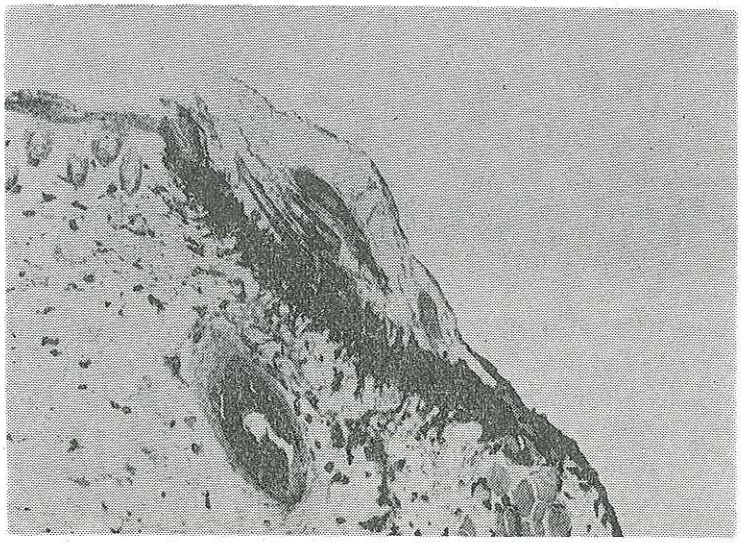

Figure 5. *Alkaline phosphatase* activity in an incised wound inflicted eight hours before death. The sample was removed 24 hours post mortem (150 ×).

The activity of adenosine triphosphatases (Fig. 4) and esterases in the peripheral zone increases as early as about 1 hour after the injury, that of aminopeptidases at 2 hours, of acid phosphatases at 4 hours, and of alkaline phosphatases at 8 hours (Fig. 5). The increase in enzyme activity in the peripheral zone may be called a positive vital reaction since there are no such changes in postmortem wounds. It is important, in practice, that the histochemical vital reactions are recognizable several (up to 5) days after death.

The *consecutive appearance of enzymes*, demonstrable by the various methods of enzyme histochemistry, allows the construction of a *biological timetable* (Fig. 6) which is useful in the rough timing of antemortem wounds.

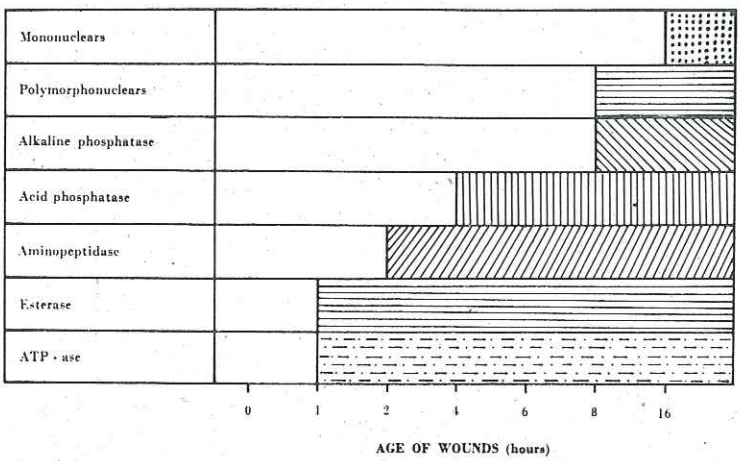

Figure 6. Schematic diagram showing the consecutive appearance of enzymes and migrating cells in the peripheral wound zone.

The following case illustrates the *practical use* of the enzyme histochemical timetable in forensic practice. A jealous man believed that he had killed his fiancée when he hit her head with a stone. He carried the body to a fenced railway track in order to simulate a suicide. More than two hours later, a train arrived and caused fatal injuries. A medicolegal autopsy was performed, and the wounds were examined enzyme histochemically.

In a laceration on the head the activity of the following enzymes was increased: adenosine triphosphatase, esterase, and aminopeptidase. By

contrast, there was no increase in the activities of acid and alkaline phosphatase. The results (cf. Fig. 6) indicated that the laceration on the head was produced at least two hours before the final fatal wounds caused by the train. The girl had obviously been unconscious after the initial head injury. The police again confronted the man with the facts, and a confession was forthcoming, with the young murderer asking : «how did you know» of the investigators.

There are, however, certain *exceptions in the biological timetable* when it is applied to human autopsy material. According to *Raekallio* (11), conditions such as far-advanced senility, cachexia, and very severe and multiple injuries may impair the local reaction of the wounded skin. The enzymatic response to injury is quantitatively influenced by such factors as blood loss, coldness, and alcohol intake. However, the increase in the activity of various enzymes appear according to the general biological timetable in spite of the presence of these endogenous and exogenous factors.

The methods of enzyme histochemistry thus permit the recognition of tissue reaction to injury at an earlier phase than is possible by the standard techniques of classical histology. In practice, the *methods of enzyme histochemistry act as guide to the approximate age of the wounds*, especially those *inflicted* 1 - 12 *hours before death*. Histochemical methods thus are *useful supplements to the histological study*, which still is absolutely indispensable in the evaluation of wounds for forensic purposes.

Biochemical timing of wounds

Although enzyme histochemistry has shortened the «latent» period to about one hour, it still leaves that very last hour before death as a challenge for further study.

As mentioned before, the first period of inflammation, the *vascular response*, from 0 to 1.5 h, is dominated by serotonin and histamine. This observation prompted us and others to study the histamine content of skin injuries under controlled conditions. At first experimental investigations on animals were performed (12, 13). The maximal increase in the *histamine* content occurred within 20 - 30 minutes after wounding. The *serotonin* content started to increase immediately and reached its maximum within 10 minutes after wounding. Further, histamine and serotonin contents have been studied in human autopsy material (14, 15). Recently *Raekallio* (16) made an additional control study on the serotonin and histamine contents in an autopsy material

consisting of a) 120 corpses of injured persons who died after a known post - traumatic period and of b) 120 corpses of injured victims of violent death whose death occurred after an unknown survival time.

The biochemical timing of wounds depends on the measurements of the serotonin and histamine contents of the *injured skin*. These are *compared* to the serotonin and histamine contents of a *control sample*, removed from the the neighbouring, intact skin of the same person. This is necessary, because there are great individual and regional differences in the contents of these amines.

At the autopsy, a sample of the injured skin (a 5 cm long piece of the hanging groove or an approximately 1— to 2— cm wide rim surrounding a wound) is removed and trimmed from the subcutaneous fat. In addition, a *control sample* of equal size is removed from the neighbouring intact skin. The wet weight of the skin samples should be at least 2 g to allow determination of both histamine and serotonin contents. If the samples are not studied at the institute at which the autopsy was performed, they may be put into tightly closed plastic tubes (without any fixative!) and air-mailed to a *laboratory, familiar with the biochemical methods*. At the laboratory, the skin samples are excised as very small pieces with scissors. The *extraction* of free histamine from skin is performed with the so - called Tyrode solution. The histamine content is measured according to a spectrofluorimetrical method. The serotonin is extracted from the skin samples with 0.1 N hydrochloric acid. The serotonin *content is measured spectrofluorimetrically*. The methods are published in detail elsewhere (10). The determination of the histamine and serotonin contents may be performed several days after the autopsy.

On the basis of autopsy studies, the *increase in the serotonin content of the wounded tissue must be at least twofold, and that in the histamine content* 1.5— *fold or more* (*as compared to the respective contents of the control samples*), in order to indicate with certainty that *the wound was inflicted before death.* There is *no increase in the serotonin and histamine contents in the wounds inflicted after death* (11, 14).

The results of biochemical serotonin and histamine determinations have been compared with each other (Fig. 7). *In wounds inflicted immediately before death*, there is usually *a great increase in the serotonin content* and often even a slight decrease in the free histamine content. *In wounds, inflicted* 5 - 15 *minutes* before death, there is a *relatively higher increase in histamine* than in serotonin, while *in wounds inflicted* 15 - 60 *minutes before death the reverse is true* (14, 15).

The following cases illustrate the *application of biochemical serotonin and histamine determinations to the study of violent deaths.*

A farmer killed his wife by stuffing the mouth and throat with cloth and other soft material causing suffocation. In an attempt to conceal the crime by simulating suicide, he later hung the dead body by the neck. He then telephoned the police, telling the sad story that his wife had killed herself. A medicolegal autopsy was performed as very few asphyxial petechiae were visible (these petechiae are frequently seen after suicidal hanging). A sample of the *ligature mark* (*hanging groove*) and a control sample of the neighbouring, uninjured skin were removed. In this case a difference in the serotonin and histamine contents of the two samples, as would have been expected in the victim of a suicidal hanging, could not be shown. The police confronted the husband with an accusation that he had hung the body after having murdered his wife, and he at once broke down and confessed.

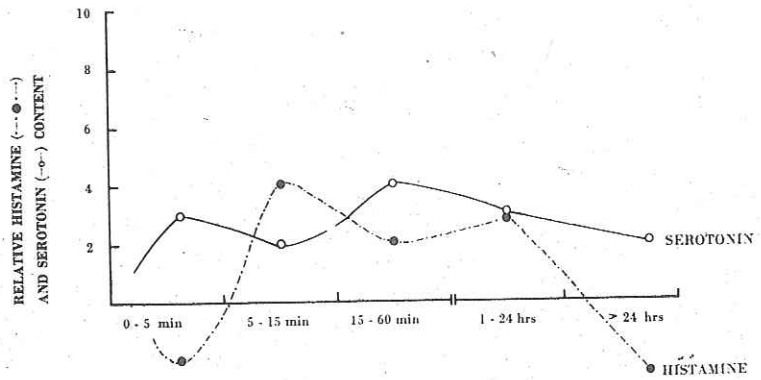

Figure 7. Relative increase in wound *serotonin* and *histamine* contents as a function of time.

We have had several *cases of road death* in which we have shown that the driver was dead before the crash. In these cases there have been signs of coronary artery disease and the cause of death had been, accordingly, natural. In these cases there have been no changes in the serotonin and histamine contents of the wounds, inflicted after death.

The naked body of a young woman was found in a storage cellar. No signs of violence were visible at the external examination of the body. Sperm was found in her vagina. At autopsy, the organs were cyanotic and congested.

The blood in the heart was dark and fluid. Small hemorrhages were found in the lungs, pericardium, pleura and larynx. In addition to these signs of asphyxia, there were no visible marks on the skin and no injuries to the deeper structures of the neck. Skin samples were excised from the front part of the neck. The serotonin content was twofold and the histamine content about 1.5—fold as compared to those in the control samples taken from the subclavicular skin. There were thus *biochemical signs of violence* in the front part of the neck. Bearing in mind the general signs of asphyxia stated at autopsy, the suspicion of *strangulation* was confirmed. The police confronted the owner of the cellar with an accusation that he had *raped* and strangled the woman found. The man broke down and confessed that he had, indeed, compelled the woman, by threats of killing her, to have intercourse with him. In order to prevent her from screaming he had pressed his hands on the front part of her neck. Suddenly the woman had ceased to breathe.

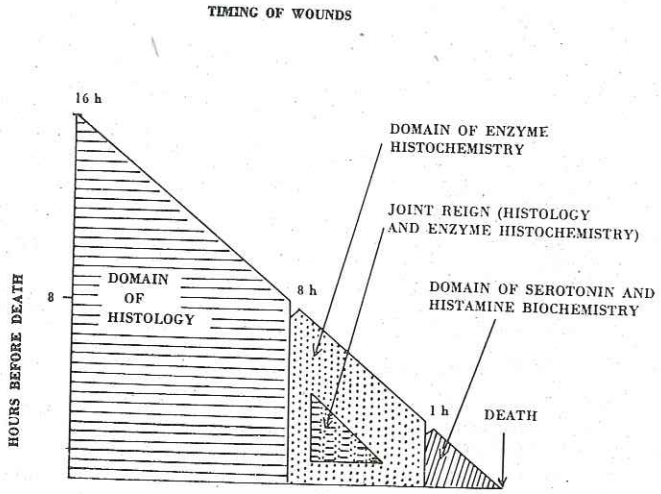

Figure 8. Schematic diagram summarizing timing of wounds.

To *conclude* (Fig. 8) a more accurate timing of wounds is now applicable. *The distinction between antemortem and postmortem wounds* is possible, by using *biochemical serotonin and histamine methods*, after a survival time of as little as a few *seconds or minutes*. The methods of *enzyme histochemistry* act as a *supplementary guide to the timing of wounds, especially of those inflicted 1 - 8 hours before death*. If a wound had been inflicted eight hours or more

before, death it may be timed *histologically*. The *histological study is still an essential part of the timing of wounds*. In addition to histology and enzyme histochemistry, the biochemical determinations of serotonin and histamine also are applicable to forensic autopsy material.

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Corrected TLC-Data of 61 Benzodiazepines and Metabolites In Two Systems*

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Dedicated to Prof. Dr. Dr. h.c. D. RINGLEB, Dean of the Medicine Faculty of the Justus - Liebig - University Gießen, in recognition of his merits in establishing the partnership between Istanbul (Cerrahpaşa Medicine Faculty) and Gießen (Fachbereich Humanmedizin)

61 BENZODİAZEPİN VE METABOLİTLERİNİN İKİ SİSTEMDE, DÜZELTİLMİŞ İNCE TABAKA KROMATOGRAFİSİ VERİLERİ

Özet

Alman Araştırma Kurumu'nun (Deutsche Forschungsgemeinschaft) ince tabaka kromatografisi ile ilgili veri bankasını genişletmek amacıyla yapılan bu araştırmada 61 benzodiazepin ve türevi ile çalışıldı. Bu maddelerin önce metanol'de, daha sonra etilasetat, metanol, % 25 amonyak (85 : 10 : 5) karışımındaki R_f değerleri belirlendi. Elde edilen bulgular *Franke* ve *De Zeeuw* tarafından önerilen formüle göre düzeltildi. Bu formülün uygulanması ile, ince tabaka kromatografisinde elde edilen değerlerin, nem, sıcaklık, çözücü ve dolgu maddelerinin saflığı gibi faktörlerden etkilenmedikleri sonucuna varıldı.

Summary

Since the discovery of chlordiazepoxide (*Librium*) in 1957 the benzodiazepines have had a tremendous impact on the treatment of disorders of nervous origin. These drugs have become among the most widely prescribed in medicine and referred to in the analytical literature. Many other benzodiazepines have also been introduced. In this work we have determined the thin - layer chromatographic properties of 61 important 1,4– and 1,5– benzodiazepines respectively and metabolites on the base of corrected R_f - values which are calculated according to the procedure reported by *Franke* and *De Zeeuw*.

Key words : *Benzodiazepines - Metabolites - Analytical Data - Corrected R_f - values.*

*) Part of thesis of *W. - R. Schneider*.

INTRODUCTION

In the working groups «Analytik» and «Dokumentation» of the «Senatskommission für Klinisch - toxikologische Analytik*) der Deutschen Forschungsgemeinschaft» (DFG; German Research Foundation) it was recently decided to extend the data bank for TLC on the base of corrected R_f - values.

It was the task of our laboratory to determine the TLC - data of all available benzodiazepines and metabolites (see (1) for details of this class of compounds).

The TLC - systems were chosen according to proposals of *Franke* and *De Zeeuw* (2, 3) and described as follows :

Description of TLC-Systems

Thin layer plates with silicagel and fluorescence indicator are used. About 5 µL of the substance in a proper solvent e.g. chloroform at a concentration of ±1 mg/mL is spotted 1.5 cm from the bottom of the plate. The distance between 2 substances is 1.5 to 2 cm. On each plate 5 µL of a mixture of reference substances at a concentration of 2 mg/mL is spotted, but not near either side of the plate.

The plates are developed over a distance of 10 cm (from start to front).

System A

Solvent : methanol

Chamber : unsaturated; solvent is poured into the tank just before putting in the plate; the tank is closed properly.

Reference substances with corrected R_f - values × 100 :

codeine	: 20
flurazepam	: 52
papaverine	: 74

*) Supervision : Prof. Dr. Dr. *M. Geldmacher - von Mallinckrodt* (Erlangen).