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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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Advantage of Affinity Histochemistry Combined with Histology to Investigate Death Causes: Indications from Sample Cases*

ABSTRACT: Mast cell histochemistry has been proposed in addition to classic histological methods to estimate the course of traumatic events before and after death. We have addressed the utility of this approach on nine victims of different types of trauma. Sections of wounded skin were stained with hematoxylin and eosin and with fluorescent avidin to tag mast cells. Mast cell numbers were evaluated by both direct and digitalized counts. Intact skin was used as control. The results on mast cells implemented the findings upon hematoxylin and eosin stain and helped to put the wounds and death in chronological sequence. Digitalized morphometry allowed to reduce intra- and inter-observer variation. We conclude that combined histological and histochemical analyses can be of practical use in forensic pathology, that a preliminary setting of the reference values is needed for each laboratory, and that image analysis can be of help for the quantification of the results.

KEYWORDS: forensic science, autopsy, avidin, cell infiltrate, histochemistry, image analysis, mast cells, postmortem, time of death, wounds

The problem of the ''vitality'' of lesions, that is, whether trauma had occurred on a living victim or a deceased body, is a major one in forensic medicine and has been addressed for a long time by routine histological methods (1). Attempts to apply either enzyme histochemistry for disparate enzyme activities (2) or immunohistochemistry for disparate extracellular matrix antigens (3) have not been followed by widespread application to forensic medicine.

We have previously shown that the number of mast cells (MCs) on the border of skin lesions increases significantly between 5 min and 3 h after trauma and decreases afterward, reaching basal values between 3 and 6 h and further decreasing until 24 h (4); also, these cells increase the expression of TNF-alpha very early during wound healing (5). Oemichen et al. (6) have recently confirmed that MC degranulate at the border of skin wounds within 60 min, as estimated by enzyme histochemistry for a granule-bound esterase (7).

We have now used the 95% confidence limits, derived from our previously published data on MC counts in early stages after wounding (4,5,8), to assist in the analysis of cases of forensic pathology and see whether the consequent information could be of help to solve those cases or was irrelevant. While the previous studies dealt with skin wounds with known survival time and the variable to analyze was the number of MCs and the degree of inflammatory cell infiltration, in the present study we started from MC and inflammatory infiltration grading to address the survival time. MCs were demonstrated by fluorescent avidin staining of their granules. Avidin tags MCs specifically and with high sensitivity; the binding of the molecule is attributable to electrostatic attraction; hence, it has the same meaning as basophilia but with much higher sensitivity than conventional microscopic dyes as toluidine blue or Giemsa (9,10). This staining has the advantage of being intense hence sensitive, specific, and more simple to achieve than another sensitive method to show degranulation, that is, the enzyme histochemical demonstration of naphthol AS-D chloroacetate esterase, which furthermore labels macrophages besides MCs (7).

Methods

Nine victims were selected as representative of our experience. They were eight men and one woman aged 25–51 years, mean 35.5 years. Autopsies were performed at the Department Section of Forensic Medicine of the University of Florence (Italy). The research was approved by the Department ethical committee and was performed in the respect of the Italian law. Corpses were routinely kept at +4°C from the moment they arrived at the morgue until autopsy. The time between death and autopsy was 24–48 h. A short description of the cases is reported below.

Four victims had been found dead with severe multiple weapon wounds: three subjects had knife wounds and one had gun wounds; the analyses were directed at assessing the time sequence of the wounds and their respective role in death.

Two victims had been killed in car accidents for which there was circumstantial evidence of multiple hits: in one case, a second car had hit the victim of a first accident, who laid on the ground; in the other case, a second car had hit the victim's car, with the subject inside, which had previously collided with a fixed obstacle.

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The timing between the hits was about 20 min in the former case and unknown in the latter case. In the first case, the analyzed wounds were those that, for the position, could be attributed exclusively to the second hit, and the analyses were directed at assessing whether this hit had concurred to cause death or had affected a deceased body. In the second case, the analyses were directed at assessing which wounds could be attributed to each hit and whether the second one had hit the victim while still alive or not.

One victim had died of a railway accident and had been eventually torn to pieces; there was circumstantial evidence that the victim had been hit by more than one train in a short time range (within a few minutes). The analyses were directed at assessing whether the successive hits had concurred to cause death or had affected a deceased body.

Two victims had been found hanging dead, and circumstantial evidence raised doubts whether hanging was indeed the cause of death or these were cases of hung corpses, which was addressed by the analyses.

From each skin wound, a sample was taken, which included the lesion edge; one further skin sample was obtained from an unwounded area 20 cm apart from each lesion, as internal control.

The specimens were either fixed in Bouin's fluid and embedded in paraplast or immediately embedded in freezing tissue medium (Killik; BioOptica, Milan, Italy) and frozen at −80°C; in the latter case, cryosections were postfixed in cold acetone. Sections were stained with fluoresceinated avidin (1:400, 1 h at 37°C; Sigma, Milan, Italy) to selectively tag MCs at fluorescence microscopy (4,5,8–10), or with hematoxylin and eosin to stain inflammatory cell infiltrate at light microscopy (11). The slides were placed in the microscopic stage so that the epithelium was included in the microscopic field with its surface contacting the field edge at least in one point; MC counts and infiltration grading were made in the whole dermis included in the field. The diameter of the microscopic field was 990 μm; hence, the depth of dermis analyzed was within 450 and 900 μm; the dermis occupied between 50% and 80% of the surface area of each field. Given the type of affinity staining, based on electrostatic attractions between the basic protein avidin and the polyanionic heparin in MC granules, we did not perform controls for the histochemical reaction. MCs (Fig. 1) were counted in at least seven microscopic fields of one section per specimen, at magnification ×200 (field area 0.8 mm^2). Inflammatory cell infiltration, as shown in hematoxylin and eosin stained sections, was graded on a 0–3 arbitrary scale (0 = absent; 1 = mild; 2 = moderate; 3 = severe) in at least seven microscopic fields for each fragment site at a magnification ×250. Representative pictures of each grading are given in Fig. 2.

As reference values, according to a commonly used strategy, we have accepted as values for each survival time the respective 95% confidence limits (95% CL), derived from previous studies (4,5,8) by the following equation: 95% CL = $m \pm 1.96$ s, where m is the mean value and s the corresponding standard deviation; 95% of the results for each time interval are hence assumed to fall within the range between the upper and lower values thus computed (12–14). For inflammatory cell infiltration, the upper limit was cut off at three, because the corresponding measures could not exceed this limit. Reference values are given in Table 1.

In cases of multiple wounds, we took also into account the similarity or alternatively gross divergence in the results among wounds, assuming as virtually contemporary those lesions with similar cell counts.

The aforementioned MC counts and inflammatory cell grading were all performed by the same author (S.B.).

To evaluate inter- and intra-observer variation, MC counts and inflammatory infiltrate grading were reported by two authors (S.B. and P.R.) in a set of 29 microscopic fields. The ratio between the results of the two authors for each microscopic field and the corresponding mean and standard deviation were computed and analyzed for significant divergence from 1 (i.e., equal values between

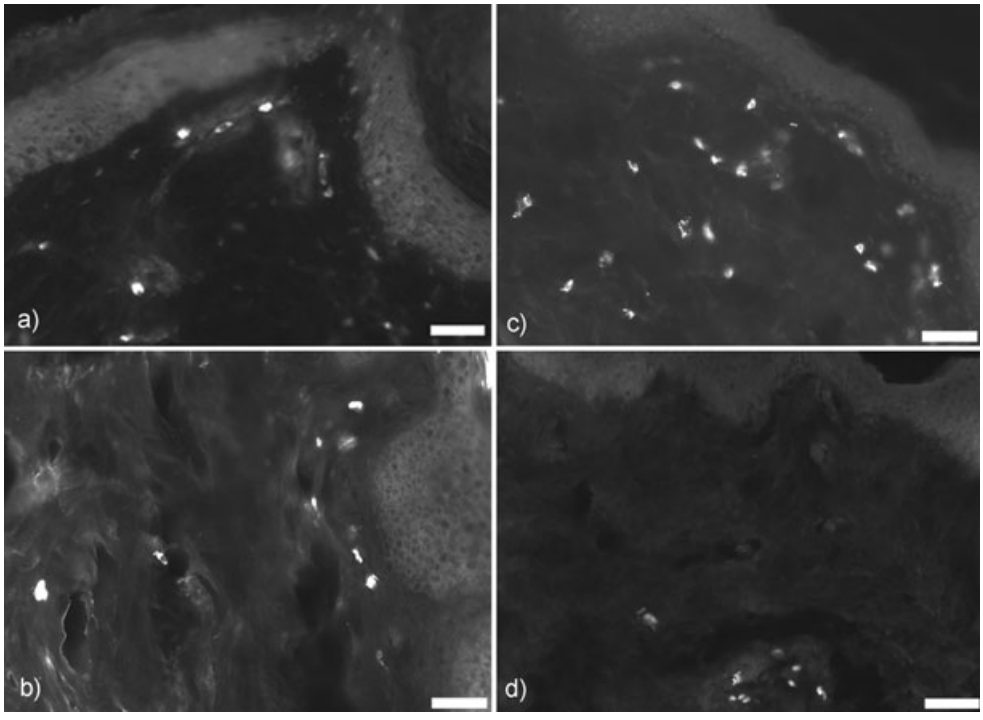

FIG. 1—*Mast cells tagged by fluorescent avidin. (a) Unwounded skin (control); (b) mast cells in a wound where their number was similar to controls; (c) mast cells in a wound where their number was increased over controls; and (d) mast cells in a wound where their number was decreased below controls. Fluorescence microscopy; bar = 20 μm.*

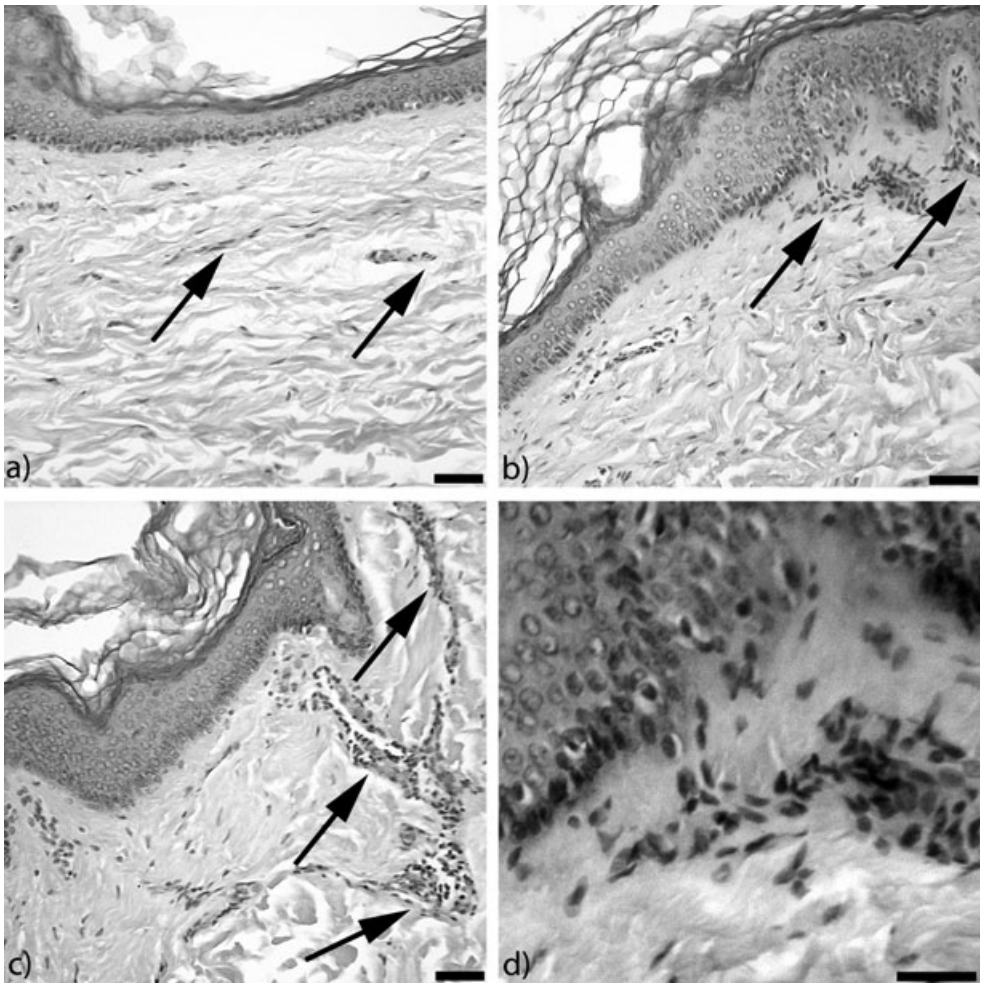

FIG. 2—*Cell infiltrate in skin wounds, as shown by hematoxylin and eosin stain. (a) grade 1 (mild infiltrate); (b) grade 2 (moderate); (c) grade 3 (severe); and (d) details of infiltrate, showing mononuclear cells. Light microscopy; bar = 20 μm.*

TABLE 1—*Reference values for mast cell number (MC/per square of skin tissue section) and score for inflammatory cell infiltration (in arbitrary units) in autoptic skin specimens, depending on time between wounding and death.*

		MC/mm^2		Cell Infiltrate (Arbitrary Units)*	
	Hours	Mean	95% CL	Mean	95% CL
Skin wounds survival time after wounding	12–24 h	04.60	1.29–7.91	02.73	1.79–3.00
	6–12 h	03.91	0.82–7.00	02.33	1.71–2.95
	3–6 h	07.02	4.63–9.41	01.81	1.36–2.26
	1–3 h	14.71	8.36–21.06	00.89	0.52–1.26
	5 min–1 h	11.06	5.95–16.17	00.84	0.49–1.19
	0–5 min	07.45	1.96–11.98	00.68	0.45–0.91
Unwounded skin		04.88	1.56–7.99	00.42	0.36–0.73
Postmortal wounded skin		02.55	1.24–3.86	00.30	0.08–0.52

*Cell aggregates in the dermis, surrounding small blood vessels (capillaries or venules), were considered as made of inflammatory cells, which were predominantly mononuclear.

The figures are based on the means resulted from previous studies [Bonelli et al. (4,8); Bacci et al. (5)].

observers) by Student's *t*-test. For the grading of infiltration, the difference between the results of the two observers was analyzed by Student's *t*-test for paired values. $p < 0.05$ was assumed as significant for both comparisons. This same set of figures was used to explore the possible advantage of computerized instead of visual counts for MCs, using ImageJ 1.44c software (NIH, Bethesda, MD). The background gray level (in arbitrary units; 0 = black, i.e., absence of signal, 255 = white) was measured in an area with only background labeling at visual inspection (usually in the epithelium); the threshold was then set at 1.5 times the background, and the structures in the dermis that were brighter (i.e., more intensely fluorescent) than the threshold and with area above 1.25 μm^2 on the tissue (to avoid possible noise) were counted by the software. In this case, each of the two authors (S.B. and P.R.) repeated the counts twice; Student's *t*-test for paired values was used to evaluate intra- and inter-observer agreement, assuming $p < 0.05$ as significant.

Results

Estimation of the Sequence of Injuries

The observations for this part of the study were all made by the same author (S.B.). The number of MCs and the intensity of infiltration at the wound border and in control skin from the same body region, 20 cm far from each wound, are given in Table 2 and commented below. The cell infiltrate was composed mainly of mononuclear leukocytes (Fig. 2).

TABLE 2—*Number of mast cells per square millimeter of skin tissue section and score of cell infiltration (arbitrary units) in the wounds and their controls.**

		Wound			Unwounded	
Cases and Wound Type	Lesion	Location	Mast Cells/mm^2	Cell Infiltrate (Arbitrary Units)	Mast Cells/mm^2	Cell Infiltrate (Arbitrary Units)
1. Knife wounds	1	Sternum/clavicle	11.31	0.8	5.94	0.44
	2	Hand palm	11.09	0.84	4.97	0.41
	3	Hand dorsum	11.89	0.86	5.84	0.49
	4	Sternum body	11.2	0.84	4.78	0.47
	5	Sternum body	11.09	0.87	5	0.44
	6	Sternum body	11.43	0.81	6	0.36
	7	Chest	11.2	0.8	5.15	0.47
	8	Hip	10.8	0.83	4.89	0.4
	9	Arm	7.2	0.66	5	0.39
	10	Knee	7.09	0.67	5.15	0.42
	11	Scapula	2.97	0.3	4.89	0.45
	12	Thigh	2.86	0.37	5	0.49
2. Knife wounds	1	Forehead	11.09	0.86	5.37	0.4
	2	Eyebrow	11.31	0.8	5.26	0.43
	3	Neck	11.2	0.83	4.57	0.5
	4	Clavicle	10.86	0.89	5.6	0.49
	5	Breast	11.09	0.84	4.57	0.46
	6	Neck	7.09	0.69	4.69	0.4
	7	Jugular fossa	6.97	0.67	5.6	0.5
	8	Areola	7.02	0.66	4.91	0.43
3. Knife wounds	1	Left wrist	10.8	0.83	0.4	0.49
	2	Right wrist	11.2	0.8	0.39	0.5
	3	Chest	10.95	0.81	0.41	0.46
	4	Neck	10.74	0.84	0.45	0.57
4. Gun shots	1	Right thigh	7.2	0.71	5.83	0.44
	2	Back	7.09	0.69	5.6	0.46
	3	Left leg	7.31	0.67	5.49	0.41
	4	Forearm	7.2	0.69	5.37	0.43
5. Double-hit car accident	1	Head	11.2	0.94	5.71	0.4
suffered by a pedestrian	2	Calf	10.86	0.89	5.57	0.44
	3	Axilla	10.74	0.84	5.43	0.41
	4	Pubis	2.86	0.27	5.29	0.43
	5	Thigh	2.51	0.33	5.43	0.4
	6	Thigh	2.4	0.31	4.47	0.39
	7	Thigh	2.4	0.33	4.71	0.41
6. Double-hit car accident	1	Knee	2.74	0.34	5.6	0.44
suffered by a driver	2	Abdomen	2.63	0.33	5.2	0.41
	3	Lower limb	2.51	0.34	4.8	0.4
	4	Chest	2.4	0.34	5.3	0.42
	5	Hand	2.67	0.3	4.9	0.47
7. Railway accident	1	Shoulder	11.2	0.8	4.71	0.47
	2	Forehead	10.97	0.87	5	0.39
	3	Back	10.86	0.89	5.29	0.4
	4	Neck	10.8	0.83	5.14	0.49
	5	Thigh	2.74	0.33	4.71	0.53
	6	Leg	2.63	0.34	5.86	0.54
	7	Thigh	2.51	0.36	5.43	0.47
	8	Thorax	2.4	0.37	4.86	0.49
	9	Back	2.4	0.33	5	0.51
	10	Pelvis	2.4	0.31	4.86	0.44
8. Ligature furrow	1	Neck (groove)	7.2	0.7	5.43	0.43
9. Ligature furrow	1	Neck (groove)	11.09	0.8	5.45	0.42

*Control tissue was sampled from the same body region, 20 cm from the wound.

Cases 1, 2, and 3 (knife wounds): Following the criteria illustrated in material and methods, in case 1, one could recognize that wounds 1–8 had occurred first and close in time to each other and wounds 9–10 later than the former ones while the victim was still alive, whereas wounds 11–12 had occurred after death. In case 2, one could recognize that wounds 1–5 had occurred first and close in time to each other and wounds 6–8 later than the former ones but while the victim was still alive. In case 3, one could recognize that all the wounds had been inflicted within a short time while the victim was alive.

Case 4 (gun wounds): The coherent results of MC histochemistry and conventional histology gave confidence that all the wounds had been inflicted within a short time while the victim was alive.

Cases 5, 6, and 7 (multiple-hit accidents): In case 5, wounds 1–3 had occurred while the subject was alive and close in time to each other and wounds 4–7 had occurred after death. The wounds analyzed in case 6 were only those that from circumstantial evidence could be attributed to the second hit. The results convinced that in case 6, all the studied wounds had occurred after death. In case 7, wounds 1–4 had occurred within a short time and while the subject

was alive, while all the other wounds had to be attributed to further hits occurred after death. Therefore, in these three cases, the successive hits were on a deceased body and had not concurred to cause death.

Cases 8 and 9 (hanging): In both cases, the coherent results of MC histochemistry and conventional microscopy gave confidence that the neck lesion had occurred while the subjects were alive, so the cases could be considered true hanging.

Inter-observer Variation of Noncomputer-assisted Measures

For MC counts, the overall mean ratio between the counts of two authors (S.B. and P.R.) for each sample was 1.88 (standard deviation 0.92, $N = 29$); the inter-observer ratio for each sample was comprised between 1.17 and 6, which indicated a systematic bias between the observers ($p < 0.05$). Milder yet appreciable differences were found also between successive counts of a same observer.

For infiltration grading, identical results were obtained between the observers for 21 of 29 samples, and there was a difference of one grade for the other samples, without any evidence of systematic bias; the inter-observer difference was not significant.

Intra- and Inter-observer Variation of Computer-assisted Cell Counts

Image analysis was performed only for MC counts. Every count was repeated twice by two authors (S.B. and P.R.), independent of each other. Identical results between the counts of the same author and between authors were obtained only in a minority of cases. The second round of counts was performed upon improving the inter-observer agreement in the selection of background. This led to a marked reduction in the inter-observer variation, which became insignificant but was not entirely abolished. In the latter round of counts, the overall mean ratio between the counts of the two authors for each sample was 1.05, and the standard deviation was 0.22; the inter-observer ratio for each sample was comprised between 0.53 and 1.75.

Discussion

The results presented here show that the histochemical detection of MCs in skin wounds may add to other techniques of analysis to estimate the sequence of injuries in respect to death in cases of forensic relevance (11,15–19).

We are aware that the finding of multiple wounds in a short or respectively a long period of time may raise special legal issues in single cases, especially of voluntary lesions. However, the timing of lesions with respect to each other and to death is a specific question posed to the forensic medicine expert, at least in Italy. Obviously, the microscopic findings need to be evaluated in the whole context of available data (crime scene, witnesses, and so on); it would be deceiving to use them alone to solve complex cases. This caution is more important, because MC numbers after 6 h from lesion decrease at or below control values, so the data need to be interpreted at the light of all the available information.

The results also suggest that MCs are among the first cells to appear in a wound. Most probably, they are not the starter of the inflammatory process, because nerve fibers, platelets, and damaged cells are there first, but in the tissues examined here, MCs were found earlier than granulocytes.

Detection of MCs by fluorescent avidin has the advantage of being nondestructive and can be used in conjunction with other types of analysis, including conventional microscopy to appreciate the extent of inflammatory cell infiltration, as shown here; it is an easier, and at least as sensitive as, and may be a more specific method than enzyme histochemistry for unspecific esterase (7) to appreciate MC response to injury. Combination of MC count and semi-quantitative evaluation of cell infiltration gave more confidence about the sequence of multiple lesions than either technique alone.

We should like to draw attention on the following points: (i) MC histochemistry was helpful to discriminate vital from postmortal lesions and the sequence of multiple lesions by a technique independent of that used to evaluate leukocyte infiltration; on the contrary, MCs are not susceptible to identification by conventional microscopy; (ii) the study was conducted on routinely fixed and stored tissue samples and did not require dedicated procedures, which—if applied—should be carefully planned and performed to offer reliable results and therefore would be difficult to propose as routine; and (iii) labeled avidin is relatively cheap, and the procedure can be performed in any forensic pathology laboratory equipped with a fluorescence microscope.

Unexpected findings were the presence of inflammatory cell infiltration above control samples in wounds, which, on account of firm circumstantial evidence and coherently with MC counts, had occurred less than 1 h before death, and the virtual lack of granulocytes from these infiltrates. We have no clear explanation for either finding, which however prompts to reconsider the usual time schedule of cell infiltration, which may become the object of further research.

An important caveat is that the reference values (i.e., the number of MCs per unit section surface area and the degree of inflammatory cell infiltration at different time points) need to be assessed for each laboratory and operator, because the techniques in use may cause different degrees of tissue shrinkage and, most important, the measurements are somewhat subjective; therefore, it is recommended that either they are performed always by the same person or the people in charge of the assay coordinate their judgment criteria by practicing on a sufficiently large set of reference slides. In this study, we found only minor differences between the observers for infiltration grading, which were insignificant for the ranking of the samples. On the contrary, a systematic inter-observer bias was found for MC counts, which was expected on the basis of previously published experience (20) and showed the importance of computing the reference values for each observer. Quantitative digitized analysis led to appreciable reduction in the inter-observer differences; however, a degree of subjectivity was not totally eliminated. Critical consideration of these differences indicated that they depended on the background value, which varied, to a small extent but enough to influence the results, depending on the individual area selected for its measurement. Therefore, this method should be recommended but should be implemented by detailed setting of the laboratory rules for estimating background. Provided such preliminary reference settings are performed, we propose combined histochemical and histological methods, with results evaluated on the basis of basic knowledge on the histological response to tissue injury, as more valuable tools than either alone to solve issues in forensic pathology.

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