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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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Forensic Applications of Sodium Rhodizonate and Hydrochloric Acid: A New Histological Technique for Detection of Gunshot Residues

ABSTRACT: Demonstration of the presence of lead residues deriving from gunshot in skin and underlying tissues is essential for the correct forensic analysis of numerous legal cases. Optical microscopy remains the fastest, cheapest diagnostic technique, even though its sensitivity and specificity are poor because of the scarce quantity of histological tissue that can be examined and possible environmental lead pollution. To confirm the presence of lead from gunshot residues, we applied to histological sections of human skin a technique proposed by Owens and George in 1991 for macroscopic detection of lead on the clothing of shooting victims, involving a reaction with sodium rhodizonate and subsequent confirmation by color change on application of HCl. Our results demonstrate the technical possibility of using this macroscopic technique even on histological samples and support the need for further studies on a larger series of cases correlated with the type of ammunition and firing distance.

KEYWORDS: forensic science, forensic pathology, skin histology, gunshot residues, sodium rhodizonate, hydrochloric acid, lead

The demonstration of gunshot residues (GSR) in firearm injuries is of primary importance in forensic science. Both an understanding of the injury's true nature and information on the direction of the shot are essential for the expert witness' analysis and must be clearly demonstrated in the courtroom to illustrate the shot's effects. Finding simple, reliable methods to achieve these goals, especially when working with organic materials that have already begun to decompose, is thus a constant objective.

The numerous histochemical studies of these problems have produced varying results. Several of these have examined the use of sodium rhodizonate (NaR), with a varying degree of reliability in identifying lead residues in GSR (1–5). To maximize the clarity of results and the robustness of the evidence offered by any findings, we applied a technique proposed by Owens and George for the study of the clothing of shooting victims (6) to histological preparations of human tissues. This technique (NaR-HCl) is based on the treatment of samples with acetic acid, followed by a solution of NaR and a pH 2.8 buffer solution, followed by a solution of HCl 5%. Positive samples are then tested using the modified Griess test for nitrite residues.

The aim of our pilot study is to verify the technical practicality of this macroscopic technique even to histological section of human skin fixed in formalin and paraffin embedded.

Materials and Methods

Six cases of homicide occurring over a 4-year period and subjected to autopsy at the University of Milan's Institute of Forensic Medicine were taken into consideration. All cases involved multiple close-range gunshots from a single-shot firearm on nude skin, with GSR macroscopic visible (Fig. 1); death occurred not more than 48 h before the time of autopsy. Several perforating wounds whose pathological reconstruction demonstrated the entry point and, where possible, the path and exit point of the projectile were selected for each of these cases by harvesting of the tissue in various areas of each wound. Table 1 reports the tissue sites harvested and the number of histological samples prepared. A total of 88 samples from different organs from the six cases under study were examined. Three sections were prepared for each of these: one stained with hematoxylin–eosin, one using the NaR technique, and one with the NaR-HCl 5% technique described by Owens and George.

Tissue samples 5–10 mm thick were fixed in neutral-buffered formalin 10% for 48 h. The blocks were then dehydrated in an automatic processor (process duration: 24–36 h) and embedded in paraffin with a high melting point (60°C). Sections of 5–7 μm thickness were then cut by microtome; after drying, the sections were stained by deparaffination and then rehydrated with distilled water.

One to two hours before use, a NaR solution (rhodizonic acid disodium salt 97%®; Sigma-Aldrich, Basel, CH) was prepared by dissolving 0.3 g in 100 mL of distilled water and a tartaric acid solution (L-tartaric acid 99.5%®; Sigma-Aldrich) was prepared by dissolving 1 g in 100 mL of distilled water. These solutions cannot be stored (7).

The histological sections were then stained by wetting them with a solution consisting of five drops of NaR and one drop of tartaric acid until completely covered. The reaction time was 1 min, after which sections presented a weak, light brown background color. They were then rinsed in distilled water until the background color disappeared. At this point, the first Pb—NaR reaction was revealed,

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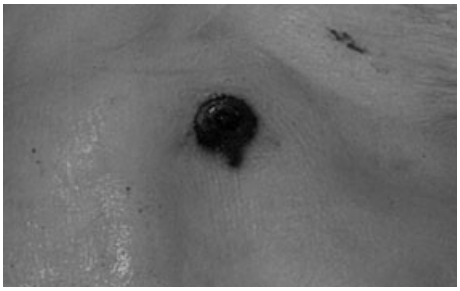

FIG. 1—*Entrance wound, case no. 2 (corresponding histological sections in Fig. 2).*

TABLE 1—*Number of histological samples prepared per harvesting site per case.*

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Skin, entrance wound		4	4	4		2
Skin, exit wound		3			2	
Cranial bone					5	
Brain	4			17	15	
Cerebellum		3				
Dura mater	2	1			2	
Lung			5			
Palate					2	
Stomach			2			
Heart			3			
Kidney			2			
Tongue						6

presenting a reddish-brown color. The slides could now be dried and mounted.

For the treatment with HCl, sections already stained with NaR were washed with distilled water, immersed in HCl 5% for 30–60 sec, shaken dry, and then dried with a hairdryer. They were then cleared in xylol or a similar product and mounted as normal.

Drying after the HCl treatment and before clarification is an important step, which must be performed with a normal hairdryer: oven-drying at 60° and dehydration using a conventional alcohol series have been found to cause the loss of the lead grains' blue-violet coloration and must be avoided.

Results

Table 2 reports the positive results, indicating the sample site and the number of positive samples with the different techniques against the total samples examined.

The GSRs are stained pink-brown with the NaR technique and turn to a more obvious blue-violet with the HCl treatment. With both techniques, the residues manifest as essentially regular fine grains of a variable size (Figs 2–4). In more concentrated areas, where there is a heavier buildup of residue, the features of individual grains can only be seen at the edges of the deposit.

Only one case tested positive with NaR and negative with NaR-HCl. In two cases, positive results were similar for both NaR and NaR-HCl in terms of number of samples and residue site. In three cases, reactivity with NaR-HCl was found in a lower number of preparations, even though the residue site corresponded.

It has to be pointed out that after 6 months from the staining, the slides were still evaluable but showed a noticeable discoloration. In forensic cases, we always suggest to take photographic samples of the results a short time after the staining.

TABLE 2—*Positive samples, comparison of staining with sodium rhodizonate (NaR) and NaR-HCl.*

Case No.	Positive Site for Gunshot Residues	NaR (positive samples/all samples)	NaR-HCl (positive samples/all samples)
1	Dura mater	1/2	Negative
2	Skin, entrance wound	4/4	3/4
	Dura mater	1/1	1/1
3	Skin, entrance wound	4/4	3/4
4	Skin, entrance wound	1/2	1/2
5	Palate	2/2	1/2
6	Tongue	1/6	1/6

Discussion

In 1991, Owens and George described a fast method to detect the presence of lead in GSR on clothing by reaction with NaR and HCl. Their samples were treated with acetic acid 15% followed by application of a NaR solution and pH 2.8 buffer solution. Areas containing lead residues presented a pink-brown color. Subsequent treatment with HCl 5% confirmed the presence of lead by color change to blue-violet. Cases testing positive then underwent the modified Griess test for nitrites (6). The reactive chemistry of NaR and NaR + HCl has been widely discussed in the literature (7). These results led us to test the technique on human tissue for medical and forensic purposes.

Sodium rhodizonate has been tested and proposed for histochemical detection of GSR in human tissues in a number of forensic pathological studies, concerning diagnosis of firearm injuries, shooting distance, and differential diagnosis between the entry and exit wounds of perforating injuries. Importantly, the technique has also proved useful when applied to tissues in an advanced state of decomposition (1–5). NaR is known to have a relatively low specificity for lead and is thus not sufficient in itself to provide robust courtroom evidence. In fact, it also reacts positively to barium, cadmium, tin, silver (8), and strontium salts, as well as to the keratinous structures of hair follicles and lingual papillae (9). A confirmation technique providing a clearer demonstration would therefore be a useful forensic tool.

Our study has demonstrated that dilute HCl is an easy-to-use technique that is quickly prepared and can be applied to human tissues without any significant effort. The fixation and paraffin-embedding phase is similar to that in use in most laboratories equipped with automated embedders: prolonged fixation of tissues in buffered formalin does not affect the reaction results, even after several years. As already noted, the drying of the histological sections after treatment with HCl and before clarification with xylol or similar is highly important: use of a normal hairdryer avoids the need for use of a conventional alcohol series or oven-drying at 60°C, which have caused the loss of the lead grains' blue-violet color.

Rather than a stain providing highly intense contrast of connective tissue, a stain giving weak or even no contrast at all is preferable: in fact, in the case of scarce or small areas of residue (especially when the projectile has passed through other objects before reaching human tissue), the contrast color can mask or change the color of the grains, providing an intermediate tone because of the overlap between the color of the NaR and the contrast agent.

For diagnostic purposes, the application of the two techniques (NaR and NaR + HCl) proved to be extremely useful in the confirmation and morphological demonstration of the presence and area

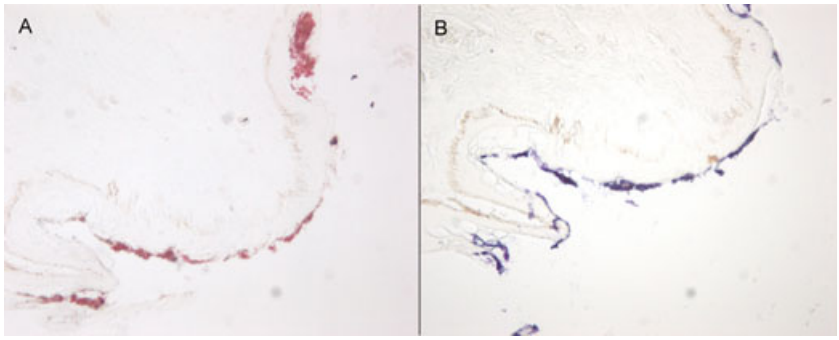

FIG. 2—*A: lead residue stained red with sodium rhodizonate (NaR) on the skin surface at the edge of the projectile's entry hole. B: confirmation of the presence of lead following color change to blue with hydrochloric acid (A: NaR; B: NaR-HCl 200×).*

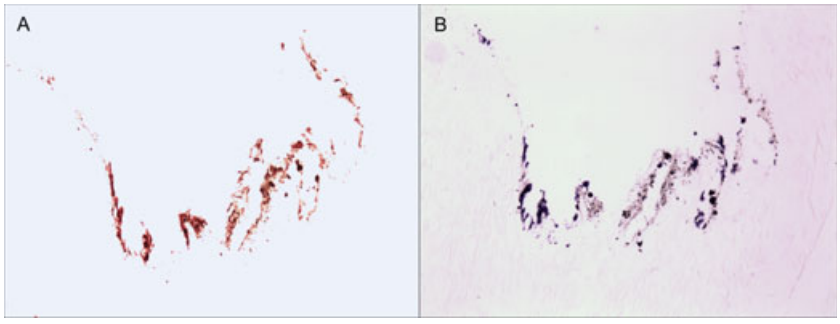

FIG. 3—*Lead residue along the projectile's path through the derma/subcutaneous adipose tissue (A: sodium rhodizonate [NaR]; B: NaR-HCl 100×).*

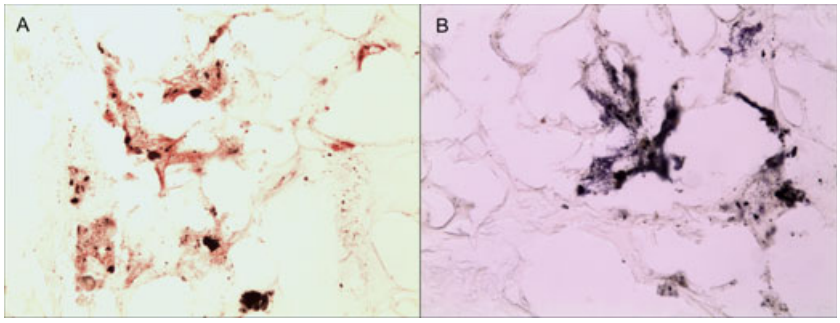

FIG. 4—*Lead residues in subcutaneous adipose tissue; the quantitative difference between the residues is because of the loss of material after the preparation of the section series (A: sodium rhodizonate [NaR]; B: NaR-HCl 200×).*

of lead residues. In one case of a close-range injury to the head, we found a positive reaction to lead in the entry wound and in the dura mater. When a series of adjacent sections are tested for GSR, the two techniques provide an excellent three-dimensional qualitative and quantitative view. We therefore consider this to be a useful screening technique, which in some cases may be definitive and in other circumstances could confirm the need to proceed with more precise—but also more costly—demonstrative methods (chemical analysis or SEM—EDS) (10,11).

In this study, the correspondence between the reactivity of NaR and of NaR-HCl was excellent in five of the six cases, identifying an identical residue morphology, although the clearest and best identification—and thus most useful for medicolegal purposes—was provided by NaR-HCl.

In three of five cases, fewer samples were positive with NaR-HCl. This is probably attributable to the lack of reactive material after preparation of a multiple series of sections from the paraffin block. In fact, as the preparation protocol consisted of a numbered series of sections stained first with hematoxylin–eosin, then with NaR, and finally with NaR-HCl, it is likely that the residues had become exhausted by the time of the final preparations. This would also apply to the only case positive with NaR and negative with NaR-HCl.

In contrast with NaR-HCl, hematoxylin–eosin staining is not specific for GSR, which appears as generic blackish deposits of a variable size and shape. This is therefore not a valid control method.

In conclusion, Owens and George's NaR-HCl macroscopic technique has proven to be applicable even to histological sections of human tissue and, according to the authors (6), can confirm the presence of lead in GSR. The technique is simple, low cost, and accessible for every histological laboratory as a screening test, so that more specific and sensitive techniques (SEM, chemical

analysis) can be used in histological negative cases. Moreover, the use of NaR-HCl technique improved the results of macroscopic skin view and simple hematoxylin–eosin stain: in fact, hematoxylin–eosin stain can only demonstrate black residues on the skin without allowing the evaluation of lead particles, which needs a special technique to be found.

However, because every histological and ultrastructural technique (including SEM) suffers from possible misinterpretation owing to the scarcity of material samples that can be examined or environmental pollution, our results support the need for further studies on a larger series of cases correlated with the type of ammunition, firing distance, and compared with SEM analysis to confirm the usefulness of this histological technique in routine forensic cases.

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