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ORIGINAL ARTICLE

The determination of firing distance applying a microscopic quantitative method and confocal laser scanning microscopy for detection of gunshot residue particles

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Abstract In this study, we applied a microscopic quantitative method based on the use of sodium rhodizonate to verify the presence of residues and their distribution on the cutis of gunshot wounds. A total of 250 skin samples were selected from cases in which the manner of death (accidental, suicide, and homicide) and the shooting distance could be reliably determined. The samples were examined under a light microscope, in transmitted bright field illumination and phase contrast mode, and with confocal laser scanning microscopy. In all skin specimens the area of each histological section was directly measured by an image analysis system. Both the number and the size of powder particles were measured. The distribution of gunshot residues (GSR) in the epidermal and subepidermal layers was also analyzed. The evaluation of the microscopic entrance wounds demonstrated different findings related to the range of fire. The data derived from the evaluation of both macroscopic and microscopic features demonstrated that the amount and the spatial distribution of GSR deposits in the skin surrounding entrance wounds strictly correlate with shooting distance.

Keywords Histology · Gunshot wound · Gunshot residues · Sodium rhodizonate · Quantitative morphometry · Confocal laser scanning microscope

Introduction

The evaluation of gunshot wounds and the analysis of the shooting distance are still based mainly on gross examination and on variables described in literature, such as the presence of visible soot or of powder tattooing [1]. Certain kinds of observations are typically encountered with near-contact and contact shots, such as the singeing of hairs or textile fibers. The examination of gunshot wounds during autopsy may also reveal distinctive defects, such as the splitting of tissue [2–4]. Even when gunshot residue (GSR) patterns are poorly visible (e.g., when present on a dark substrate), various techniques may be applied for visualizing residue patterns [5].

The analysis of GSR is considered one of the most reliable methods for establishing the shooting distance [6–10]. GSR particles are produced from the primer, propellant, metals contained in the bullet, bullet jacket, cartridge case, and gun barrel when a gun is fired [11]. Detection and identification of GSR can be performed by several methods, which involve chemical tests; X-ray fluorescence analyses; and scanning electron microscopy (SEM) with energy-dispersive X-ray analysis, with wavelength-dispersive X-ray analysis, or with energy-dispersive spectroscopy [2, 12, 13]. Although these methods are all relatively sensitive and specific, some are costly and require highly specialized equipment.

In this study, we analyzed 250 samples of gunshot wounds using a quantitative microscopic method to investigate the powder soot pattern according to firing distance.

Materials and methods

The files of the Department of Forensic Pathology of the University of Foggia (Italy) were checked for gunshot-related autopsies (n=108 from 2002–2005). Full metal jacket projectiles fired by 7.65- and 9-mm caliber pistols

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were used in all cases. A total of 250 skin samples were selected from cases in which the manner of death (accidental, suicide, and homicide) and the shooting distance was reliably determined on the basis of detailed scene report, examination of the clothes, police report, postmortem findings, and the report of the firearms examiner. In all cases we selected naked skin's samples. For the histological investigation, the external shot perforations were excised together with at least 1–2 cm of surrounding tissue and were removed together with the subcutaneous tissue. The skin samples consisted in 187 entrance and 63 exit wounds, which were identified on the basis of macroscopic appearance, and routine (hematoxylin and eosin) histological examination. Staining was performed on paraffin sections using the reaction with sodium rhodizonate. Deparaffinized and rehydrated paraffin sections were coated with a solution of sodium rhodizonate (rhodizonic acid and disodium salt at 97%; Sigma-Aldrich, Basel, CH, Switzerland) at 0.3 g in 100 ml of distilled water and tartaric acid (acid L-tartaric 99.5%, Sigma-Aldrich) at 1 g in 100 ml of distilled water with a ratio 5:1 (five drops of rhodizonate solution and one drop of tartaric acid solution); the reaction time was 1 min. The sections were contrasted with hematoxylin for 30 s, washed, dehydrated, and mounted in Eukit [14].

For the negative controls 50 specimens of normal epidermis were used. The specimens were examined under a light microscope (DM 4000 B Leica, Cambridge, UK), connected with a photo camera computer system (DC 480 Leica) in transmitted bright field illumination and phase contrast mode.

The GSR-positive samples were also examined under a confocal microscope and a three-dimensional reconstruction was performed (True Confocal Scanner, Leica TCS SP2).

Histological evaluation and quantitative analysis

Using the sodium rhodizonate reaction, GSR appear as light orange deposits (Figs. 1, 2 and 3). On positive skin samples a morphometric study using an image analysis system (IM100 Leica) was carried out to determine (1) the number of powder particles, (2) the size of the powder particles, and (3) the distribution of GSR in epidermal and subepidermal layers.

All the measurements were performed with a magnification of ×10.

Confocal microscopy

A confocal microscope illuminates and detects the scattered or fluorescent light from the same volume within the specimen. There are two enhancements of the imaging characteristics of a confocal microscope compared with standard light microscopes: (1) enhanced lateral resolution and (2) enhanced axial resolution. The increased axial resolution results in a strong rejection of light from regions outside of the focal plane. The main advantage of a confocal microscope is its ability to optically section thick specimens [15].

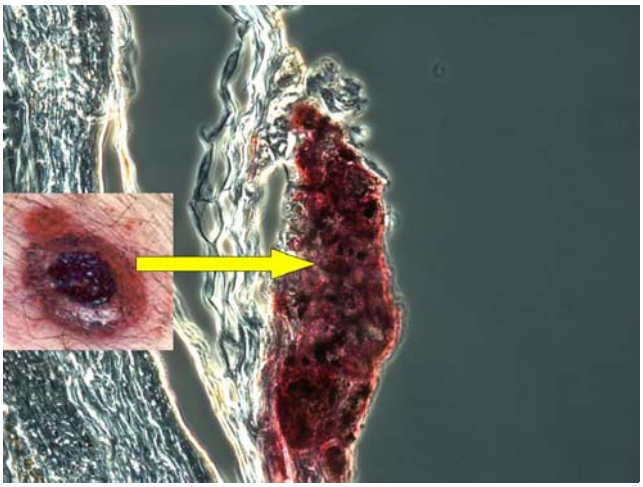

Fig. 1 Loose contact wound from a 9-mm caliber bullet (*insert*: macroscopic view) with soot deposits in zone around entrance (sodium rhodizonate, phase contrast, ×100)

Results

Rhodizonic acid exists as needle-shaped crystals of a dark green color and forms a sodium salt, which reacts with heavy metal ions (barium, antimony, lead, and tin) contained in GSR with a red or light orange precipitate.

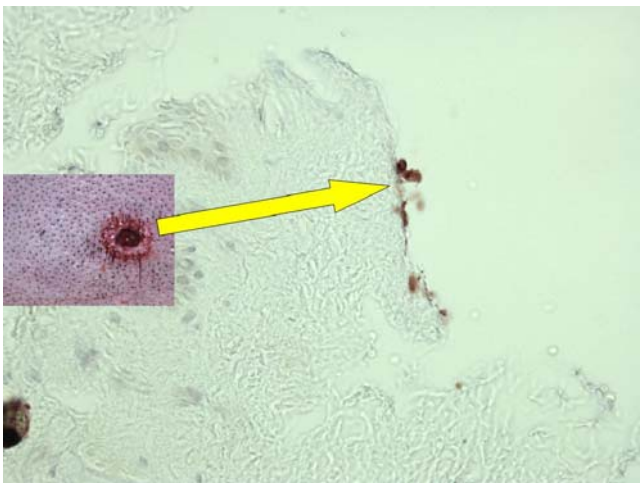

Fig. 2 Intermediate range wound from a 7.65-mm caliber bullet (approximately 40 cm, see *insert* for macroscopic appearance) with few deposits of gunpowder residues on epidermal surface (sodium rhodizonate, ×40)

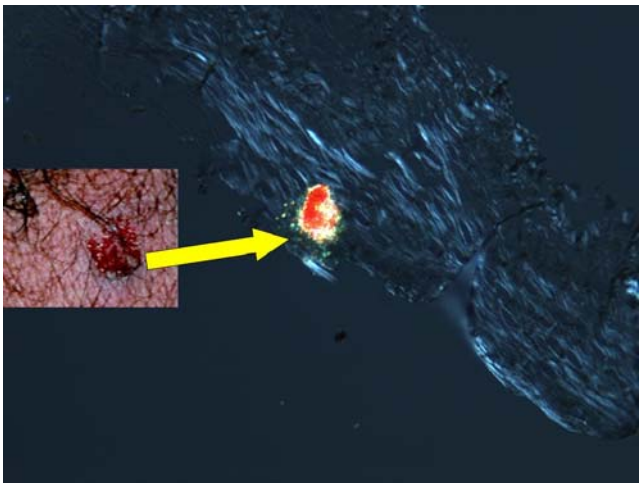

Fig. 3 Microscopic view with polarized light in intermediate range wound from a 7.65-mm caliber bullet (approximately 40 cm, see *insert* for macroscopic view) (sodium rhodizonate, ×100)

Although sodium rhodizonate is capable of reacting with other metals, it was described to be relatively specific for demonstrating the presence of lead [16]. On histological tissue sections, sodium rhodizonate reacts with heavy metal particles from the primer by generating a finely granular red or light orange pattern.

In the majority of the 187 skin samples taken from macroscopically identified entrance wounds, the sodium rhodizonate reaction was positive, showing red or light orange deposits with very different patterns of distribution (Figs. 4 and 5).

In all the 63 skin samples taken from grossly identified exit wounds and in all the 50 control cases, the sodium rhodizonate reaction was negative.

The results obtained from the morphometric analysis performed on microscopic slides are listed in Table 1 regarding disposition of powder residues and in Table 2 regarding size of powder residues (Fig. 6).

Discussion

Although test-firing the gun gives the best estimate of the range of fire, the pattern of soot and/or gunpowder deposited around the entrance wound is frequently a valuable tool in assessing the muzzle-to-target distance [17–19]. Smoke stain and interspersions of gunpowder are important histological findings that can be visualized quite well in histological sections by their own coloration without special treatment [18]. After the experimental study carried out by Marty et al. [14] who demonstrated that differentiating between the different range shots is possible with the assessment of the distribution of sodium rhodizonate-positive primer particles on and in tissue, we compared the different pattern of sodium rhodizonate-positive deposits with the shooting distance, reliably known by macroscopic and histological characteristics of entrance wounds.

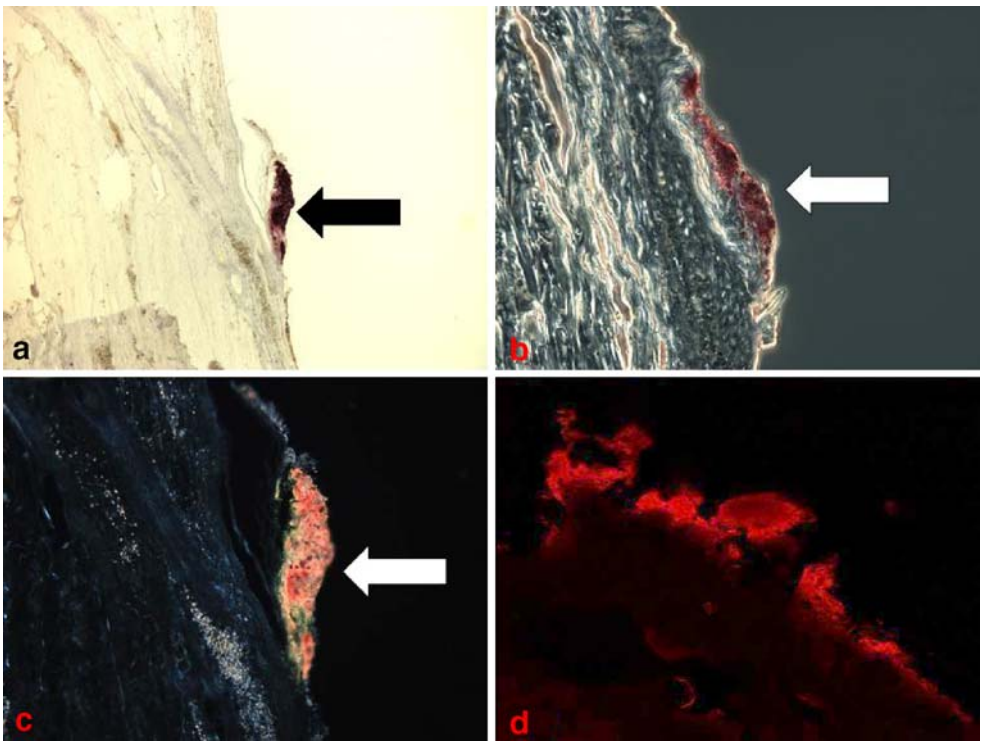

Fig. 4 Loose contact wound. **a** Sodium rhodizonate technique (×20), **b** sodium rhodizonate technique and microscopic view with phase contrast (×40), **c** sodium rhodizonate technique and microscopic view with polarized light (×40), and **d** confocal microscopy: 3D visualization of the gunpowder residues on epidermal surface

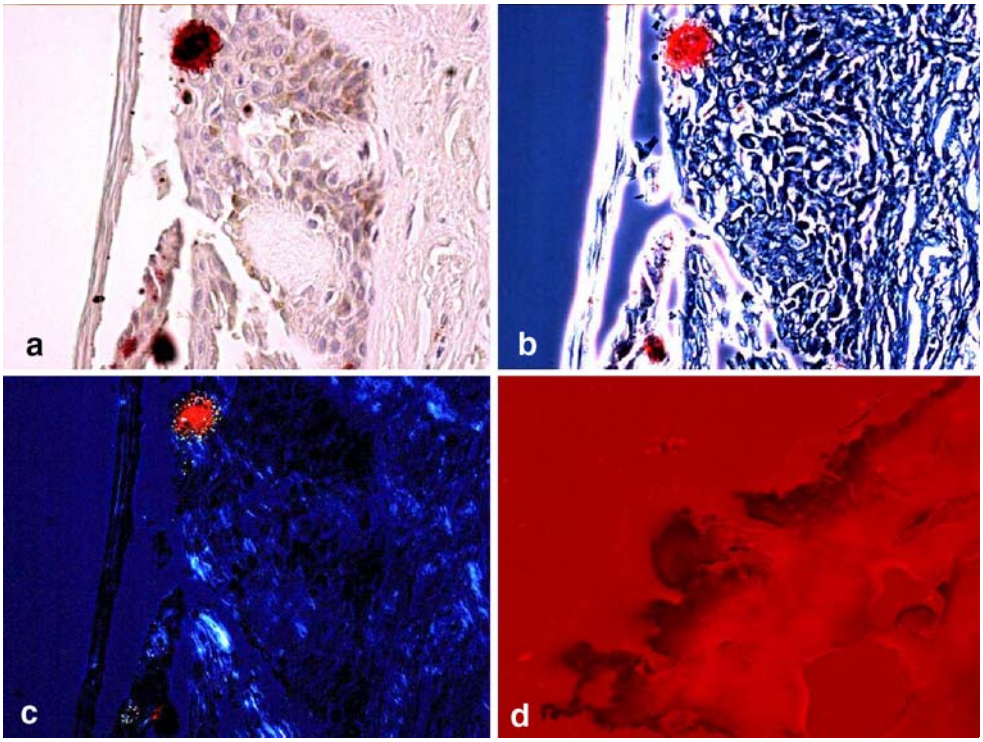

Fig. 5 Intermediate-range wound (approximately 30 cm). **a** Sodium rhodizonate technique (×40), **b** sodium rhodizonate technique and microscopic view with phase contrast (×40), **c** sodium rhodizonate technique and microscopic view with polarized light (×40), and **d** confocal microscopy: 3D visualization of the gunpowder residues on epidermal surface

The histological sections were also analyzed with a confocal laser scanning microscope. Confocal microscopy primarily offers the possibility of removing unwanted background in thick sections. Removal of this out-of-focus haze is achieved by optically cutting a section from the specimen. A sequence of these sections collected along the *z*-axis can be used to render and quantitatively analyze the structure three-dimensionally [20].

The microscopic evaluation of the entrance wounds demonstrate different findings related to the range of fire. A pattern of sodium rhodizonate-positive residues centered very tightly around the entrance wound with a contiguous distribution is characteristic of close-range shots. In these samples we observed the greater quantity (number) of GSR and the dimensions of the single deposits were bigger than in the other specimens. When the firing distance increases, we could demonstrate a lower quantity of small, scattered sodium rhodizonate-positive residues. As the shooting distance increased we observed dot-shaped, finely coarsely granulated, and noncontiguous sodium rhodizonate-positive residues and when the shooting distance exceeded 40–50 cm, the sodium rhodizonate reaction was negative.

Elicitation of cases involving the discharge of a firearm frequently requires information about the range of firing [21, 22]. Based on the observation that the way in which GSR are deposited around the entrance hole varies partly with the distance of firing, experiments can be conducted under controlled conditions, i.e., firing from known distances, to assist in evaluating the distance from which a GSR pattern in question was shot [23]. Such examinations are essentially based on a visual inspection during which parameters such as the size and density of GSR patterns are compared [5].

The combined utilization of morphometric analysis and confocal microscopy using the rhodizonate staining technique for the analysis and the quantification of GSR deposits present on skin sections is a simple and inexpensive method, which can be used in routine practice in forensic science laboratories. Moreover, this method has the potential to provide quantifiable, measurable, and comparable evidence for estimation of the shooting distances in

Table 1 Disposition of sodium rhodizonate-positive powder residues

Firing distance	Contiguous Deposits	Scattered Deposits	Dotted Deposits
Hard contact	+	−	−
Loose contact	+	−/+	−
<15 cm	−	+	−/+
15–40 cm	−	−	+
>40–50 cm	−	−	−

Table 2 Sizes of sodium rhodizonate-positive particles

Firing distance	Sizes (mean μm2)
Contact	2,399.4
<15 cm	649.8
15–40 cm	79.37
>40–50 cm	–

Fig. 6 Quantitative morphometry: the numbers of sodium rhodizonate-positive residues and the size of the same residues measured with the computerized image analysis system in an intermediate-range wound case

the reconstruction of events in gunshot fatalities [16, 24, 25].

Our contribution confirmed the previous results obtained in experimental scenarios [14] showing that the amount and the spatial distribution of GSR deposits in the skin surrounding entrance wounds strictly correlate with shooting distance.

The information obtained utilizing microscopic quantitative analysis through examination of skin entrance wounds contributes definitively to the documentation of shooting distance. Histopathological examination is a component of gunshot wound investigation that may be pivotal to thorough and scientific case resolution [14, 18, 23].

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