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Determination of Postmortem Interval from Old Skeletal Remains by Image Analysis of Luminol Test Results*

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ABSTRACT: The luminol test is routinely used in forensic serology to locate blood traces and identify blood stains not visible to the naked eye; its sensitivity is reported as ranging from 1:100.000 to 1:5.000.000. To evaluate the possibility of correlating the postmortem interval with blood remnants in bone tissue, the luminol test was performed on 80 femurs with a known time of death, grouped in five classes. Powdered bone (30 mg) was recovered from compact tissue of the mid-shaft of each femur and was treated with 0.1 mL of Luminol solution (Sirchie Finger Print Laboratories, Inc.). The reactions were observed in a dark room and filmed by a TV camera equipped with a recording tape. An intense chemiluminescence was observed after a few seconds in all 20 femurs with a PMI ranging from 1 month to 3 years. On the 20 femurs with a PMI ranging from 10–15 years, a clear chemiluminescence was visible with the naked eye in 80% of the sample. Among the 20 femurs with a PMI ranging from 25 to 35 years, a weaker chemiluminescence appeared in 7 femurs (33% of the sample). In the 10 femurs with a PMI ranging from 50 to 60 years, a faint reaction was observed only in a single femur. In none of the ten femurs with a PMI over 80 years was chemiluminescence observed. The image of each reaction was computerized and analyzed for gray scale. The results of image analysis show a possible quantitative relationship between the PMI and luminol chemiluminescence in powdered bone.

KEYWORDS: forensic science, time since death, postmortem interval, skeletal remains, luminol test, image analysis

The forensic pathologist is frequently requested to examine human skeletal remains. The investigating authorities usually wish to know the cause and manner of death only if the remains are recent enough to warrant any further inquiries. If the bones are ancient, or indeed older than 50–80 years, they lose any forensic interest (1).

The postmortem interval is generally determined from skeletal remains by analyzing morphological appearances, by estimating the related-time deterioration of other elements recovered on the site of the discovery (clothing, personal objects, etc.), or by using chemical and physical methods (2).

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The method involving assessment of the external changes of the bones is probably more reliable than the others, even though it is less objective (3). The bones themselves can give an idea of the elapsed time since death according to the presence or absence of ligamentous attachments and the rates of leaching out of fats and other organic matter (4). Several authors have focused their studies on the chemical and physical changes of bones after death and on the numerous variables influencing the diagenetic process, such as the characteristics of the burial environment (temperature, ground moisture, oxygen tension, soil pH, availability of ions in the groundwater, and degree of microbial activity) and the type of bone tissue itself (surface area and volume ratio, degree of fragmentation, and porosity of skeleton) (5–7).

Various techniques have improved the testing of chemical-physical bone changes, that may be related to the weathering of skeletal remains: consistency and weight of the specimen, radiographic structure analysis, histological examination (8–10), staining with Nile Blue and dichloroindophenol (11), reaction with mineral acid, reaction with benzidine, nitrogen loss (12), amino acid content of proteins, serological protein determination (13–15), degradation of lipids, remnants of fat-transgression (16–17), UV-fluorescence, radiocarbon method (18), and strontium-90 content (19).

As to the benzidine reaction, Knight (20) stated that positive benzidine testing on bone surface and powdered usually vanishes after 50 years, but may occasionally be observed even after 150 years. Facchini and Pettener (21) concluded that within the period from 0 to 350 years the trend of the intensity and distribution of reactions on sectional surfaces of bone shaft can supply indications for dating human skeletal remains.

In the present paper we have evaluated the possibility of correlating the time since death with blood remnants in bone tissue. The chemical tests for blood currently in use depend upon the detection of hemoglobin or one of its derivatives. Hemoglobin has several properties that make it suitable: the most important is the peroxidase activity of the heme portion (22–23) which forms the bases of the different reagents most commonly employed for the identification of blood stains (benzidine, leuco-malachite green, phenolphthalein, and luminol reactions).

Among these the luminol test is unique because its reaction with blood produces light rather than colorimetric evidence (24–25). Luminol is an alkaline solution that, with the addition of hydrogen peroxide, produces a bluish-white light that may be viewed in the dark and photographed or videotaped with the aid of night vision attachments. The luminol test is routinely used in forensic serology to locate blood traces and identify blood stains which are not visi-

ble to the naked eye (26). Its sensitivity is reported as ranging from 1:100.000 to 1:5.000.000 depending upon the prior treatment of the suspected bloodstain and the composition of the reagent. It has been reported that old blood reacts better and more rapidly with luminol than fresh blood (27–28).

On these bases we have performed a research to evaluate the relationship between the time since death and the intensity and distribution of the chemiluminescence obtained by the luminol test in bone tissue.

Materials and Methods

The luminol reagent was tested on eighty non pathological femurs (40 male and 40 female), grouped in five classes according to the known time of death (Table 1). The gaps of PMI between the groups were deliberately rather large to eliminate the possibility of overlaps in the positive results of the tests.

We collected 30 mg of bone powder from the compact tissue of the mid-shaft of each femur. According to the technique suggested by Colonna e coll. (29), only the inner compact tissue was recovered from each bone to avoid a possible influence of external contaminant agents. Moreover, compact bone was selected because it is more likely to be recovered during site excavation and is far less susceptible to diagenetic effects, being considerably more durable in the burial environment.

The luminol powder was supplied by Sirchie Finger Print Laboratories Inc. as they suggested in the "technical information," we heated all the samples to 100°C for a period of 5 min prior to the reaction with luminol solution and to avoid the possible influence of plant peroxidases.

The luminol solution was prepared using Weber's method (30). Three solutions were made up:

- solution A (0.354g luminol + 62.5 mL sodium hydroxide 0.4N + 437.5 mL of distilled water);
- solution B (8 g sodium hydroxide + 500 mL distilled water);
- solution C (10 mL 30% hydrogen peroxide + 490 mL distilled water).

The final and operative solution (100 mL) was obtained by mixing 10 mL of each solution (A,B,C) with 70 mL of distilled water.

Each sample of bone powder was tested with 0.1 mL of the final solution; all the reactions were observed in a dark room and filmed by a video-camera (SONY Digital VCR V-200E) equipped with VCR (Panasonic NV-F77).

The effects of all the reactions were recorded for the first 3 min. The frame corresponding to the 30th second of the reaction was computerized using a work station (Olivetti PC PRO 486/33 MHz-8MbRAM). Each frame was analyzed for gray scale classification using software (Optimas 5.1 for Microsoft MS-DOS for Windows 3.11—Optimas Corporation, Edmonds, WA) which calculated the intensity and distribution of chemiluminescence and displayed a corresponding histogram.

The X-axis of the histogram reported the light intensity scale. By default the histogram spanned the range from black (minimum value of the luminance range) to white (maximum value). The gray value of white was dependent upon the bit depth of the image. The Y-axis of the histogram reported the number of pixels found for each gray value in the range. We chose to consider the logarithmic rather than linear scales to enhance the differences between the low values in the gray scale classification. Possible values for this classification range from 1 to 255.

The histograms of each image analyzed were shown with statistical data calculated by software: the weighted average luminance (mean), the standard deviation of the pixel count around the mean, the statistical variance of the pixel count around the mean; the dimmest pixel luminance (min); the brightest pixel luminance (max); a count of all pixels within the range of the image; the number of bins in which the luminance values are distributed.

Results

Using luminol on compact tissue bone powder, a different intensity of chemiluminescence was recorded only in three groups and in one femur of the fourth group (PMI ranging from 50 to 60 years); it was constantly negative in group number five corresponding to the oldest bone powder. The results are reported in Table 2.

An intense chemiluminescence was observed in all 20 femurs of the first group, in which time since death ranged from 1 month to 3 years. In the second group (PMI ranging from 10 to 15 years), a clear chemiluminescence was visible with the naked eye in 16 femurs (80% of the sample), of which only 7 femurs showed a more intense reaction. In the third group (PMI ranging from 25 to 35 years), a weak chemiluminescence appeared in 7 femurs (33%), whereas in 67% no reaction was observed. Only one femur (10%) of the fourth group (PMI ranging from 50 to 60 years) revealed a very faint light-reaction.

Focusing only on the maximum gray level obtained for each luminol test by using image analysis, we performed descriptive tests; the range, mean, variance, standard deviation and standard error of the mean are reported for each group in the Table 3.

The results of the image analysis show that intense chemiluminescence is obtained when luminol reacts with powdered bone indicating a short postmortem period: the threshold of the highest values of luminance is at the 138th gray level, while the average is

TABLE 1—*The PMI of the sample and the number of femurs examined.*

Group	Tot. Femura	PMI
1	20	1 month–3 years
2	20	10–15 years
3	20	25–35 years
4	10	50–60 years
5	10	>80 years

PMI = postmortem interval.

TABLE 2—*Results of luminol test in each sample group.*

Group	PMI	Luminescence	Femura* Lumin/Tot	%
1	<3 years	++++	20/20	100
2	10–15 years	+++	16/20	80
3	25–35 years	++	7/20	33
4	50–60 years	+	1/10	10
5	>80 years	—	0/10	0

NOTE: ++++ = intense; +++ = clear; ++ = weak; + = faint.
* = number of positive tests.

TABLE 3—*Statistical data on the brightest gray levels of the images in each group.*

Group	PMI	Tests	Range	Mean	Variance	SD	SEM
1	<3 years	20/20	138–155	146.9	20.8	4.56	1.02
2	10–15 years	16/20	99–115	106.9	20.68	4.54	1.13
3	25–35 years	7/20	69–79	72.7	20.7	4.55	1.72
4	50–60 years	1/10	34	—	—	—	—
5	>80 years	0/10	—	—	—	—	—

SD = standard deviation; SEM = standard error of the mean.

around the 147th. The brightness of the images recorded from the second cluster is grouped near the 100th value of the gray scale. The weaker luminance observed in powdered bone of the third group corresponds to lower gray levels than the other two groups: maximum values ranged between the 69th and the 79th point of gray scale (mean value 72). The single positive result of the fourth group had a mean gray level - 34 - close to the black limit.

Discussion and Conclusions

The present study shows that the luminol test gives interesting results when performed in bones. It is always positive and very intense in bones from subjects with a recent time of death. After the tenth year since death, the percentage of positive tests diminishes and there is also a gradual reduction in the brightness of the reaction. The negative tests increase (up to 70% of cases) after 25 years from death: this probably corresponds to a significant loss of the hemoglobin protein to react with the luminol. Bones with a postmortem interval of half a century respond to the test in 10% of cases, but with a very weak luminance. The luminol test is ineffective when it is performed on older bone.

These results suggest a good correlation between different levels of intensity in the reactions and the different PMI of bones. The luminol test could therefore be very useful to determine the time since death from skeletal remains, especially when only isolated or fragmented bones are collected.

The second step is to obtain an objective method for evaluating the luminance-reaction and thus to eliminate observer bias. For this purpose, image analysis procedures enabled us to classify the intensity and distribution of the chemiluminescence phenomena. In the present study we utilized the gray-level classification of the image; this common procedure is available in the menu of all image enhancement or analysis softwares. Thus the luminance of each luminol reaction may be quantified and numerical data obtained representing the phenomena observed. The quantitive data in the present paper constitute a preliminary attempt to classify the correlation between the chemiluminescence obtained with luminol in bones and the PMI. We believe luminol should be tested on a wider sample of bones with a closer PMI range to establish its statistical significance.

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