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Bone natural autofluorescence and confocal laser scanning microscopy: Preliminary results of a novel useful tool to distinguish between forensic and ancient human skeletal remains

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ABSTRACT

The fast, high-throughput distinction between palaeoanthropological/archaeological remains and recent forensic/clinical bone samples is of vital importance in the field of medico-legal science. In this paper, a novel dating method was developed using the autofluorescence of human bones and the confocal laser scanning microscope as the means to distinguish between archaeological and forensic anthropological skeletal findings.

Human bones exhibit fluorescence, typically induced by natural antibiotics that are absorbed by collagen, and provide secondary, exogenous fluorophores. However, primary natural fluorescence (or autofluorescence) caused by enigmatic endogenous fluorophores is also present as a micro-phenomenon, whose nature is still obscure. Here, we show that the endogenous fluorophores are mucopolysaccharides of the Rouget–Neumann sheath and, more relevant, that the intensity of the natural fluorescence in human bone decreases in a relationship to the antiquity of the samples. These results suggest that the autofluorescence of bone is a promising technique for the assessment of skeletal remains that may be potentially of medico-legal interest. A larger study is proposed to confirm these findings and to create a predictive model between the autofluorescence intensity and the time since death.

1. Introduction

The analysis of skeletal remains is a highly specialized field in the medico-legal science. A forensic anthropologist is usually tasked with determining the biological profile of skeletal remains (e.g., ancestry, sex, age and stature) that have been identified as human. Morphometrical features of bones and teeth can help to answer these questions [1–3]. A more difficult parameter to estimate is the time since death (TSD). As quoted from Nagy et al. [4], "dating human skeletal remains is one of the most important and yet unreliable aspects of forensic medicine. The identification of an unknown individual is a complex puzzle made up of many parts and one of the most significant of them is the dating of skeletal findings", even if the goal is only to distinguish between palaeoanthropological/archaeological remains and recent forensic/clinical bone samples.

It is common to find human remains during the construction of buildings and/or roads, and removal of earth. Moreover the accidental findings of human remains could occur in natural contexts, such as mountain or coastal routes, indicated by citizens to the judicial authorities or police. The initial concern that arises with respect to such findings is whether they are historical or recent remains and thus falls into the field of forensics. In the scientific literature, one encounters several methodological approaches and procedures covering this topic without, however, finding a reliable solution to the problem of determining the TSD. Several authors have focused their studies on different techniques of absolute dating on bones, such as X-ray diffraction, radiometrics, supersonic conductivity, Raman spectroscopy, luminol chemiluminescent reaction, or Fourier transform infrared spectroscopy,

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among others [5–19]. Other techniques are useful in distinguishing archaeological and modern skeletal remains (e.g., [18,20]). Some of them use the fluorescence of the bone to estimate the TSD in order to discriminate those with forensic relevance (e.g., [8,14,17]). However, opinions differ concerning the cause of the fluorescence, determining how to categorize the fluorescent properties, and the significance of the fluorescent characteristics in correlation with the TSD [14,17].

1.1. Fluorescence of the bone

Fluorescence is the property of certain chemical elements, called fluorophores, to emit visible light when excited by incident intense radiation. Some materials, when irradiated at a specific wavelength, are able to emit another light of a longer wavelength. This phenomenon is a short-lived luminescence, emitted simultaneously with the excitation light [21,22].

Certain biological macromolecules and structures fluoresce themselves (*primary fluorescence* or *autofluorescence*), whereas other materials that do not exhibit primary fluorescence must be impregnated with fluorescent substances (fluorochromes; *secondary fluorescence*) before they can be examined [21,23].

Primary fluorescence emission in human bone was noted many years ago in clinical samples from patients treated therapeutically with antibiotics, primarily tetracycline [24,25]. The pattern of fluorescence in tetracycline-labelled human bone was also observed in ancient human bones from archaeological contexts [23,26]. In some cases, the fluorescence was attributed to the possible use of natural antibiotics in ancient societies: in a Sudanese Nubian population dated to 350–550 CE, the autofluorescence of human bones was related to the use of stored grains contaminated by tetracycline-producing *Streptomyces* [27]. In the Roman population from Herculaneum, dated to 79 CE, the intense fluorescence of bone was related to the frequent (and possibly deliberate) use of tetracycline produced by *Streptomyces* artificially cultivated on a pomegranate substrate, as described by ancient Roman physicians [28]. However, many studies reported the presence of an autofluorescence phenomenon in bone samples probably due to the organic matrix, mainly collagen, of bone tissue [25,29,30], although the nature of this autofluorescence is not well defined currently. In this sense, it is necessary to understand the origin of the bone autofluorescence in order to explain the relationship between its intensity and bone dating.

For this reason, we examined samples of ancient bones from different archaeological periods in comparison with samples of recent human bones to (i) define the real nature of natural autofluorescence (NA) and (ii) delineate a possible relationship between NA intensity and bones dating.

2. Material and methods

2.1. Sample collection and preparation of histological thin sections

In our study, human bone samples were selected from the mid shaft of the femurs from 13 adult individuals from Italian archaeological sites and ossuaries ranging from the X–VIII century BCE to modern periods [31–34] (Table 1). The human remains from the necropoles of Comino and Opi were dated through archaeological data [32,33]; the skeletons from the ossuary of Castel di Sangro were dated through historical documents [34]; the recent bone samples from Sicily came from the cemetery of Palermo and belongs to identified individuals; the human remains from the necropolis of Monte D'Argento were dated with radiocarbon method [31].

For these skeletal samples, no possible sources of natural antibiotics had been documented or identified. The pattern

Table 1
Collection of human bone samples.

Population	Sample	Age at death	Sex	Antiquity
Comino	#25	20–25	♂	X–VIII BCE
Comino	#18	45–50	♂	X–VIII BCE
Comino	#45	35–40	♀	X–VIII BCE
Opi	#187	35–40	♂	VI–III BCE
Opi	#198	45–50	♂	VI–III BCE
Opi	#173	30–35	♀	VI–III BCE
M. d'Argento[a]	#32	30–35	♀	1034–1470 CE
M. d'Argento[a]	#70	25–30	♀	1034–1470 CE
M. d'Argento[a]	#35F	Adult	♀?	1034–1470 CE
Castel di Sangro	#Ind.1	Adult	?	First half of XX CE
Castel di Sangro	#Ind.2	Adult	?	First half of XX CE
Sicily	#1	87	♂	Second half of XX CE
Sicily	#2	44	♀	Second half of XX CE

[a] Radiocarbon dating (^{14}C) (Ref. [31]).

distribution of the fluorescence in the examined histological sections is quite different from the one observed in the bone tissue of individuals who swallowed natural or synthetized antibiotics [24–26]. In particular, the fluorescence produced by natural antibiotics (e.g., tetracycline) is well evident in correspondence of the circular lamellar bone around the Haversian canal, whereas the fluorescence of our bone sections is not linked to histological structure and is homogeneously distributed in the microscopic field.

With a circular blade diamond-edged (Leica 1600), three thin sections of thickness of 150 μm were obtained of each sample. Each section consists of non-decalcified, macerated compact bone including the *linea aspera*. Next, the samples were prepared using two procedures according to the different goals of the study. Fig. 1 shows a brief summary of the methodological procedures.

2.1.1. Procedure 1. Origin of the natural fluorescence

The aims of this procedure are (i) to determine the chemical of the endogenous fluorophores and (ii) to test if the natural autofluorescence (NA) was influenced by the mineral components or organic matrix of the tissue. For this purpose, two thin sections from the contemporary bone sample Sicily #2 (second half of XX century CE) received different treatments before dehydration and subsequent analysis.

Section #1: thin section of bone incubated in a solution of EDTA (0.05 M, pH 8) for four days to remove the mineral component of bone.

Section #2: thin section of bone incubated in a solution containing bacterial collagenase (1 mg/ml of the lyophilized enzyme in Hank's balanced salt solution) for 24 h at room temperature to digest the collagen.

2.1.2. Procedure 2. Dating of bone samples

To test empirically that (i) the observed NA trend of a decrease of the fluorescence intensity in relation to the bone samples dating and (ii) to quantify the fluorescence levels representative of each sample, we analysed a collection of selected human bone samples, coming from vary antiquity periods (Table 1).

Thin untreated sections were prepared from all of the bone samples from archaeological sites and modern ossuaries, to quantify the NA intensity and its relationship with the antiquity of the bone samples.

Section #3: thin section of untreated bone.

All of the histological thin sections (*Sections #1,#2* and *#3*) were dehydrated using alcohol with an increasing gradation (from 70° to 100°, with alternating washes of 15 min) and incubated with Histolemon (at least 15 min). Next, the thin sections were mounted on a microscope glass slide with Canada balsam and a 0.17 mm