“Evaluation of macroscopic changes and the efficiency of DNA profiling from burnt teeth”

Short title: Macroscopic and molecular changes in burnt teeth

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- Abstract

Identification of human remains subjected to incineration is extremely challenging. Our study evaluates the macroscopic changes and efficiency of DNA profiling in burnt teeth under controlled temperature and time conditions. 28 teeth were exposed to temperatures between 100-700°C for a duration of 1-15 minutes. Two non-burnt teeth were used as control. Macroscopic changes were evaluated and recorded. DNA was extracted using a silica-based methodology. Efficiency of DNA profiling was assessed through Quantitative PCR for STRs. Burnt teeth reached chalky white appearance at 400°C 5 min and fractures were observed from 300°C 10 min. Amplification of STRs was very low from 300°C and 1 or 5 min. In contrast, the housekeeping gene, GAPDH, was amplified in all combinations of temperatures and times. Although it is possible to amplify the housekeeping gene at high temperature, DNA profiling is difficult to obtain, probably due to small size of these regions making them more prone to degradation.

- Keywords: forensic odontology, burnt teeth, macroscopic changes, DNA isolation, STR profiling, housekeeping genes

- Introduction

Identification of a deceased individual is important from the viewpoint of the relatives and it is a vital factor in facilitating criminal investigation, for example at international criminal tribunals [1]. Mass disasters, aircraft or car accidents and accidental deaths can involve fire. In some instances, a fire may have been set in order to destroy forensic evidence in criminal cases, for example: to prevent the identification of the deceased [2,3]. The identification of human remains subjected to incineration depends on the degree of destruction of the remains, which is affected by the intensity and duration of the fire. For that reason, the multidisciplinary team effort of forensic pathologists, anthropologists and odontologists is required in order to facilitate the correct identification of the victim.

During the cremation process, at a macroscopic level, fire and heat modify and destroy the bone structure in size, color and shape. At microscopic level, there are different changes in chemical and crystalline structures. In fact, bone loses water with increasing temperatures, promoting the combustion of the organic components from temperatures of 250°C, leading to the expansion of the crystal size within the bone structure as temperature increases [4-6].

The color of burned bone is affected by the temperature and duration of exposure, ranging from brown, black and grey, to the most burnt white color [7,2]. The same macroscopic changes are found in teeth [8]. One of the approaches for identification of burned remains is genetic identification. The genetic approach is usually unproblematic in cases of fire victims with conserved internal organs. However, extremely charred bodies frequently render highly degraded DNA, hampering STR (Short Tandem Repeat) analysis [2]. Also, few studies focus on the possibility of amplifying nuclear DNA from burnt remains [2,7,9-11]. As of yet, no consensus has been

reached regarding the degree of cremation at which bone will still yield nuclear DNA signals [7]. Although previous studies have used similar temperature ranges (between 100°C to 1000°C), the duration of fire exposure differs between them; and not all studies evaluated direct contact of the fire flame. In the studies developed on whole teeth and pulp DNA, [9,12,10], the same problem is encountered: the temperatures were similar to those used in the bone studies and the durations of exposure changed. Therefore more research is needed to evaluate the possibility of extracting DNA from burned remains.

Teeth are the hardest structures in the human body. The pulp is well protected by the dentin, which, in turn, is protected by the enamel and cementum. As a result it is possible that dentin can be protected during a fire and can be suitable for DNA extraction and isolation. Thus, the aims of this study are to analyze the macroscopic changes presented in burnt teeth and the efficiency of human DNA isolation, evaluating the achievement of complete identification at different temperatures and times.

-Material and Methods

Sample preparation

Twenty-eight healthy erupted third molars, from individuals aged 20-70, were collected from dental clinics. The Smithsonian ethical committee has approved all procedures related with manipulation of human samples. These teeth were divided into 7 groups of 4 teeth per group, exposed to different temperatures: 100, 200, 300, 400, 500, 600, 700 °C. Each group was exposed to these temperatures for 1 min, 5 min, 10 min and 15 min, removing one tooth after each time period. Two non-burnt teeth were used as controls. Control and burnt teeth were then mechanically ground and submitted to DNA extraction and quantification.

Macroscopic evaluation

Color changes and fractures observed in roots and crowns were recorded for the 28 studied teeth with a 5 megapixel camera.

DNA extraction, quantification, amplification and visualization of PCR products

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's protocol. DNA was quantified using the Qubit system (Life Technologies), according to the manufacturer's protocol. DNA was amplified looking for AMEL (Amelogenin) gen as control by conventional PCR as previously described [13].

Quantitative PCR

The efficiency of STR profiling was estimated using real-time PCR by analysis of different STRs: D7S820, D13S317, D5S818, CSF0, TPOX, TH01, VWA, D16S539, FES/FPS. To perform this study, Perfecta SYBR Green FastMix, Low Rox (Quanta Biosciences, Gaithersburg, MD) was used in an Applied Biosystems ViiA 7 (Applied Biosystems, Foster City, CA) as previously described [14]. STRs primers were obtained from the general identification database. GAPDH (Glyceraldehyde-3-Phosphate dehydrogenase) was used as the housekeeping gene.

Statistical Analysis

Statistical analysis to compare the efficiency of DNA profiling after exposed teeth to different temperatures and times was performed using Statistical Package for Social Science (SPSS) version 15. Statistical significance was accepted when $p < 0.05$.

- Results and Discussion

Macroscopic changes

The changes in color observed turned from brown, black, grey/blue and finally chalky white; which corresponds to an incinerated stage of burning [15] (Fig. 1). Calcination in the root was observed from 400°C 5 min of fire exposure, while calcination in the crown was observed from 500°C 10 min. Calcination of the surface of

crown and root, inner layers (dentin, pulp chamber and root canal) showed a lower degree of alteration by the fire, due to the protection of the more affected outer layers (Supplementary material TableS1 and TableS2)[16]. These color changes observed in the studied teeth are similar in order of appearance of those observed in bone [13, 25].

Fractures due to fire were observed from 300°C and 10 min of fire exposure in the crown and 400°C and 10 min of fire exposure in the root (Supplementary material TableS3). Crown fragmentation and separation from the root was observed from 300°C and 15 min of fire exposure. Fractures observed in the root surface were basically transverse fractures. Root separation was observed at 700°C and 15 min of fire exposure. The results obtained are consistent with those reported by other authors [17,18].

However, we have to take into account that this study was done in nude teeth, normally maxillary and mandibular bones as well as facial tissues protect them from external insults, thus the results can be slightly different at least in the first exposed temperatures. In fact, Ellingham et al. [5] studied this factor in bone, demonstrating that fleshed bones exposed to temperatures below 400°C showed less damage than defleshed bones, since soft tissue acted as a barrier against heat. In contrast, temperatures from 400°C to 700°C induced the same damage analyzed by FTIR in both fleshed and defleshed bones, due to the combustion of soft tissue. Consequently, it is expected from our study that at higher temperatures our results will be similar to teeth protected by tissue.

DNA quantification

As shown in table 1, it was possible to extract DNA from almost all samples. Only 400°C at 10 and 15 min; 500°C at 15 min; 600°C at 5 and 15 min; 700°C at 5, 10 and 15 min gave DNA values lower than the detection limit of the quantification assay. The first

two treated samples (100°C 1 and 2 min) showed similar quantification results to the control teeth. Values below 1µg/ml were found in the rest of the temperatures and times. The study of Williams et al [1] treated deciduous teeth with temperatures between 100°C to 500°C for 15 min, finding that DNA quantification was unreliable for the majority of the samples. This research used the same DNA extraction kit as used in the present study, however DNA was extracted from the pulp. In the present study, the whole tooth was ground and submitted for DNA extraction, letting the rest of the tissues like dentin, cementum and enamel increase the DNA yield.

STRs amplification

The evaluation of STRs amplification performed in teeth showed DNA concentration higher than detection limit. The results were similar for all STRs tested (Fig 2, 3 and supplementary material FigureS1). In the first temperatures and times (100°C and 200°C 1 and 5 min), it was possible to get amplification similar to the controls, sometimes better, like FES/FPS. However, in the majority of STRs the amplification was very low from 300°C and 1 or 5 min. In order to analyze these results, statistical analysis was carried out. After the failure of normality and variance homogeneity hypotheses, U-Mann-Whitney test was performed. The results of these test pointed out that in the first temperatures significant differences were not found between the control teeth and the treated teeth ($p>0.05$) in FES/FPS and VWA. In TH01 and TPOX significant differences were found in the first temperatures due to the amplification increase with the first temperatures (particularly 100-200°C). The same happens with the other genes (Figure S1). However, from 300°C approximately, significant differences were found in all genes with respect to the control ($p<0.05$), these differences were more patent in the highest temperatures.

There are not so many studies in the literature that analyze the amplification profile efficiency in teeth after temperature exposure. Particularly the study of Álvarez-García et al. [9] analyzed the amplification of HLA DQA1, D1S80, HUMTH01, HUMFES/FPS treated at temperatures ranging from 75°C to 500°C for 1 and 2 min, finding that from temperatures of 400°C the amplification efficiency of specific STRs (HUMTH01 and HUMFES/FPS) was lower than previous temperatures. In the present study, TH01 amplification was almost undetectable from 300°C 1 min. In contrast, it was possible to find high amplification of FES/FPS at temperatures and times higher than 300°C 1 min, probably due to increase in DNA template.

The article of Schwark et al [2] analyzed the efficiency of DNA profiling in bones exposed to different temperatures and times. The analysis of blue-grey and blue-grey-white burnt bones frequently resulted in negative results. These results are in agreement with our study since grey teeth color started with 300°C 10-15 min, the temperatures and times at which it was not possible to amplify the STRs.

The amplification of the amelogenin gene by conventional PCR (Fig 3) pointed out to similar results, although it was possible to see slight bands until exposed to temperatures of 300°C for 15 min. These results, particularly related to identification of the sex, are in agreement with previous studies. Williams et al [1] demonstrated the successful identification of human biological sex from deciduous teeth exposed to incineration temperatures of 200°C or below, as it was possible to determine in our study. With respect to permanent teeth, the study of Álvarez García et al [9] reported success in sex determination at 200°C for 10 min and 300°C, 400°C and 500°C for 2 min. In the present research, higher temperatures (400°C and 500°C) did not show Amelogenin bands, although as with the study of Urbani et al [10] at 300°C for 15 min slight banding was possible to discern.

Another important issue is the difference of amplification between STRs, the ones located on p arm [19] and in an intron to a specific gene (like vWA, TH01 and TPOX, fig 2) showed almost undetectable amplification from 200°C 15 min. In contrast, the ones located on q arm [19] (like FES/FPS, fig 2 and supplementary material FigureS1) showed higher amplification even in temperatures and times where with the previous STRs it was not possible to get amplification. The outliers were CSF1PO (Fig 3), located on 5q33.1 and in 6th intron of c-fms proto-oncogene [19], whose amplification efficiency can be placed between these two groups; and D5S818 (supplementary material FigureS1), although it is located on q arm, its amplification efficiency was similar to the first group located in p arm.

In spite of these results with STRs, the analysis of amplification of the housekeeping gene used for the QPCR quantification, GAPDH, showed that DNA for this region was amplified in all combinations of temperatures and times (Fig 3), finding highest Ct (Cycle Threshold) (meaning the least amplification) with the increase in temperatures and times. In fact, One-way ANOVA performed after passed hypothesis of normality and variance homogeneity did not find significance differences between control teeth and teeth treated in the first temperatures (mainly 100°C) ($p > 0.05$); in contrast, significant differences were found from this temperature ($p < 0.05$). This indicates that even after exposure to high temperature, it is still possible to amplify DNA, at least the housekeeping DNA. However, a STR profile would be difficult to obtain probably due to the small size of these regions, which makes them more prone to degradation. Fredericks et al. [20,21] discussed the possibility of amplifying STRs or SNPs on burnt bones depending on the degradation of DNA. In our study, due to the amplification of this housekeeping gene, the possibility of developing DNA identification using big amplicon size is indicated, like the oldest methods of identification [22-24].

Limitations, recommendations and suggestions for future studies

This research provides a qualitative study of the response of the different dental structures to fire exposure. Additionally, it provides a quantitative study for the achievement of obtaining a DNA profile from burnt teeth. However, one of the limitations of this study that must be taken into account is that teeth are surrounded and as a result, protected, by maxillary and mandibular bones as well as facial soft tissues. This structural protection would increase the resistance of teeth to fire. Thus, future studies may be able to expand these results, analyzing macroscopic changes and DNA profiling in teeth protected by bone and tissue. Furthermore, and based on DNA results, our future plan is to analyze other potential DNA regions for identification under the same conditions.

- Figure and Table Legends

Figure 1. Color changes due to temperature exposure at different times. Temperature in Celsius degrees and time in minutes.

Table 1. DNA extraction efficiency at different temperatures and times. DNA concentration was measured in µg/ml.

Figure 2. Relative Amplification of FES/FPS, vWA, TH01 and TPOX. Temperature in Celsius degrees and time in minutes.

Figure 3. a) Relative Amplification of CSFO STR. b) Representative GAPDH Amplification, showing Ct. c) Results of Amel gen amplification by conventional PCR.

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Table 1

Sample		DNA concentration
Temperature (°C)	Time (minutes)	µg/ml
Control 1	RT	32.8
Control 2	RT	60
100	1	31.8
100	5	19
100	10	0.257
100	15	0.231
200	1	0.828
200	5	0.387
200	10	0.589
200	15	0.346
300	1	0.665
300	5	0.447
300	10	0.336
300	15	0.130
400	1	0.230
400	5	0.104
400	10	<0.05
400	15	<0.05
500	1	0.169
500	5	0.068
500	10	0.1
500	15	<0.05
600	1	0.055
600	5	<0.05
600	10	0.102
600	15	<0.05
700	1	0.142
700	5	<0.05
700	10	<0.05
700	15	<0.05

Figure 1

Figure 2

Figure 3