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Influence of Different Storage Methods on Laser Fluorescence Values: A Two-Year Study

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Key Words
Laser fluorescence · Diagnodent · Storage methods

Abstract
The aim of this study was to assess the influence on the infrared laser fluorescence response of some storage methods commonly used in dental research. Forty extracted permanent teeth, selected from a pool of frozen teeth, were divided into four groups of 10. Three groups were stored at 4°C in 1% chloramine, 10% formalin or 0.02% thymol solution. The fourth group was stored at –20°C (no storage solution added). Fluorescence measurements were performed at 14, 77, 113, 168, 232, 486 and 737 days. After 2 years, significant decreases in fluorescence ($p < 0.01$) for the samples in formalin (–60%), chloramine (–72%) and thymol (–54%) were observed. The frozen teeth showed a slight but non-significant increase in fluorescence of 5% ($p > 0.01$). Storing solutions have a significant influence on the fluorescence yield. Samples used for in vitro purposes stored frozen do not significantly change their fluorescence response. Thus, cut-off values obtained under the latter conditions could be extrapolated to the in vivo situation.

Carious lesions fluoresce more strongly than healthy tissues at excitation wavelengths in the red and infrared part of the spectrum [Alfano and Yao, 1981; Hibst et al., 2001]. Evidence suggests that this phenomenon may be caused by the presence of bacteria and their metabolites rather than by the mineral decrease or change in scattering associated with caries [König et al., 1998, Hibst et al., 2001]. Bacteria responsible for caries would produce certain endogenous porphyrins (fluorophores) that fluoresce when excited by red laser light [König et al., 1998; Hibst et al., 2001]. The laser-fluorescence (LF)-based device for caries diagnosis is equipped with a laser diode providing the excitation light source, wavelength 655 nm, necessary for the detection of these fluorophores. In order to get the maximal sensitivities and specificities from the device and to obtain an appropriate guideline for the practitioner to interpret the results, several cut-off values were presented [Lussi et al., 2004], thus making the diagnostic decision even more difficult. In many cases, the cut-off values determined in vitro proved to be smaller than the cut-off values obtained from clinical studies [Lussi et al., 2001; Shi et al., 2001a, b; Francescut and Lussi, 2003; Lussi and Francescut, 2003]. It can be hypothesised that these differences could be due to the storage solutions used in laboratory conditions. These could promote vari-

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ations in the optical properties of the teeth [Strawn et al., 1996] and, consequently, in their LF response. The effect of storage on fluorescence readings during a certain time span needs to be clarified. The aim of this study was to assess the influence of some storage methods commonly used in dental research (formalin, thymol, chloramine and freezing) on LF during 2 years.

Materials and Methods

Pilot Study

In order to compare LF values obtained under in vivo conditions and after freezing, a preliminary pilot study was carried out. For this study, 17 enamel and dentine decayed sites on 6 permanent molars scheduled for extraction were measured in vivo and frozen at –20°C immediately after extraction. New fluorescence measurements were carried out after 7 days. To do so, the teeth were placed in single plastic containers and defrosted at room temperature (about 24°C) for 14 h. In order to ensure 100% humidity, a wet paper towel was placed at the bottom of each container. Care was taken to avoid contact between the tooth and the paper towel. After defrosting, LF measurements were performed with the LF-based device Diagnodent (Kavo, Biberach, Germany) and recorded. These teeth were afterwards discarded.

Main Study

Sample Selection

The sample comprised extracted permanent molars that had been kept frozen at –20°C from the moment of extraction and had had no contact with any storage substance. Before starting the experiment, the teeth were placed in single plastic containers and defrosted at room temperature (about 24°C) for 14 h. In order to ensure 100% humidity, a wet paper towel was placed at the bottom of each container repeating the conditions of the pilot study. Care was taken to avoid contact between the tooth and the paper towel. All teeth were then cleaned with a toothbrush and tap water for 15 s. From this pool, 40 teeth were selected and one site on each occlusal surface was chosen and marked on a drawing. Each selected site had to have an LF value ≥14 indicative of dentine caries [Lussi et al., 1999].

All 40 samples were then randomly divided into four groups of 10 and frozen again in the refrigerator (–20°C). After 2 days, the teeth were defrosted and stored as explained above and the first LF measurements were performed (baseline measurement, t = 0) with the LF-based device. Once the measurements were accomplished, all the teeth were frozen again for 7 days in the refrigerator at –20°C. After this time, LF measurements were carried out after defrosting the teeth as described above. Finally, the samples were stored as follows: samples in group 1 were immersed in 10% formalin solution, group 2 was stored in 1% chloramine solution while group 3 was immersed in 0.02% thymol solution (all the solutions adjusted to pH 7). The storage temperature was 4°C for all three groups. No solution was added to group 4 which was frozen again in the refrigerator at –20°C. Measurements were repeated at 14, 77, 113, 168, 232, 486 and 737 days. Before every measurement, all groups were allowed to reach room temperature for 14 h. During this time, the frozen teeth (group 4) were kept at 100% humidity as previously described, while the samples of groups 1, 2 and 3 were kept immersed in their solutions and then rinsed with tap water for about 3 min in order to remove remnants of the chemical agent immediately before the measurements were carried out. After each measurement, the teeth in groups 1, 2 and 3 were stored again in fresh solutions while group 4 was frozen in the refrigerator at –20°C. All specimens were stored in the refrigerator in darkness. The fluorescence measurements were taken with the same device throughout the study.

Measurements with LF

Prior to each measurement, the LF device was calibrated according to the manufacturer's instructions. Afterwards, each tooth was dried with air and left exposed at room temperature for 5 min. After this time, and before performing the measurements, a standardization of the fluorescence registrations was carried out by registering the fluorescence value on a sound spot. This recording was then subtracted from the LF value of the site to be measured. Four consecutive measurements were performed on each site. The mean value of these 4 measurements was calculated and used for analysis.

Data Analysis

For the pilot study, the interrater reproducibility between the fluorescence values obtained before and after extraction was tested by means of Cohen's κ. Likewise, mean values and their standard deviations were calculated in both cases.

In the main study, in order to allow comparisons between individual LF measurements with time, the baseline was set at a value of 100 and the following registrations were normalised. The areas under the curves time/LF were calculated. QQ plots showed a normal distribution of the LF values. Repeated-measures analysis of variance was carried out (SPSS 11.0.3, Chicago, Ill., USA). To compare daily measurements in each group and areas under the curves, Student's paired t test was applied. The level of significance was set at <0.01.

Results

In the pilot study, mean fluorescence values of 62.4 (SD 34.4) before extraction and 56.3 (SD 35.2) after extraction and 7 days storage were found. The intrarater reproducibility between both measurements was 0.82 (= excellent agreement [Rosner, 1986]).

In the main study, one sample in group 4 was discarded because its LF showed an unexpected increase of a factor 3 which did not fit the behaviour of the whole group. All curves dropped dramatically immediately after storage in any of the solutions (between days 7 and 14; fig. 1). LF yield decreased 39, 48 and 33% in teeth stored in formalin, chloramine and thymol, respectively. This difference in LF was statistically significant ($p < 0.01$). After the 14th day, a slight and steady decrease in LF values was registered for all three groups. The total LF decreases after 2 years were –60, –72 and –54% for the sam-

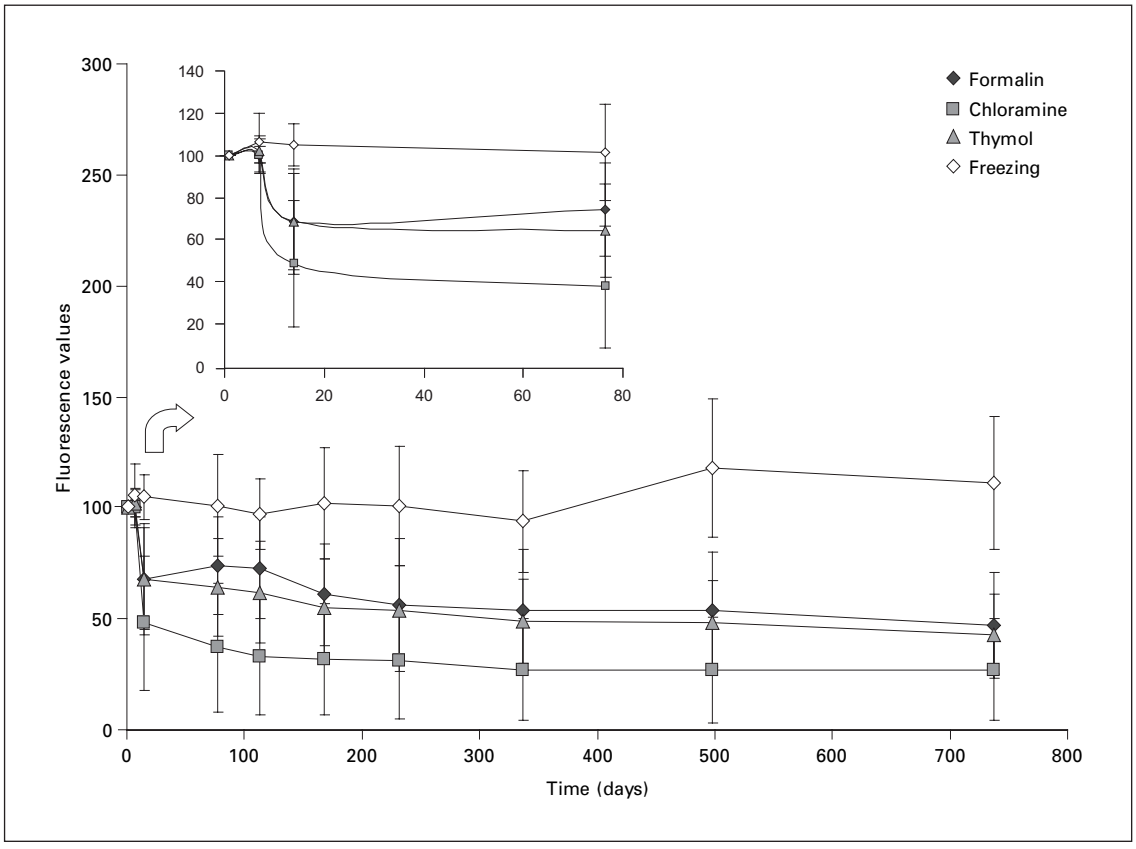

Fig. 1. Daily mean and standard deviation of the mean of the fluorescence for teeth stored in formalin, chloramine, thymol and for frozen teeth (values normalised). The upper graph shows an expanded view of the first 4 measurements (days 0, 7, 14 and 77). Filled symbols are significantly different from the baseline measurement ($p < 0.01$).

Table 1. Areas under the curves of figure 1 and changes in fluorescence after 2 years for each storage method

Storage method	Change in LF, %		Area under the curve (LF × day), units
	immediately	after 2 years	
Formalin	–39	–60	4.604×10^4 b
Chloramine	–48	–72	2.669×10^4 b
Thymol	–33	–54	4.249×10^4 b
Freezing	+1	+5	7.905×10^4 a

Values with different letters show statistically significant differences ($p < 0.01$).

ples stored in formalin, chloramine and thymol, respectively ($p < 0.01$). A different behaviour was observed in the frozen teeth. The curve exhibited a slight upward trend with a 5% difference after 2 years, but no statistically significant differences were recorded during the whole period of the study ($p > 0.01$). The highest area under the curve corresponded to the frozen teeth with 7.9×10^4 units, followed by formalin, thymol and chloramine with areas of 4.6×10^4, 4.25×10^4 and 2.67×10^4 units, respectively (table 1). The area under the curve for the frozen teeth was statistically significantly greater than those for the formalin, chloramine and thymol groups ($p < 0.01$). For these three groups, no statistical differences were detected between each of their areas.

Discussion

When working under laboratory conditions, many variables like room temperature, humidity or storing solutions are to be considered. The differences in cut-off values found in different studies [Lussi et al., 2001; Shi

et al., 2001a, b; Francescut and Lussi, 2003; Lussi and Francescut, 2003] led us to investigate the influence of one of these variables, namely storage. Most of the substances used as antiseptics, disinfectants or storage agent act as 'protoplasmic poisons' for the bacteria. Cold temperatures, however, have a bacteriostatic effect. In fact, temperatures below –20°C are commonly used at the laboratory to preserve cultures of certain micro-organisms [Nolte, 1982]. Some of them will be progressively affected by extremely low temperatures, but many other cells will survive to permit the propagation of the culture [Nolte, 1982]. In the present study, teeth stored in formalin, chloramine and thymol showed a statistically significant decline in LF intensity over 2 years. On the other hand, the frozen teeth experienced no statistically significant changes throughout the whole time of the experiment. The storage substances might reduce the concentration of fluorophores in the sample by dilution and therefore decrease the fluorescence signal. It could also be due to a chemical modification of the fluorophores or even to a change in the optical properties like dispersion of the hard tissues over long storage periods. More investigations on the solubility of these fluorophores in different storing solutions are required. On the other hand, it is less probable that the colony or its endogenous subproducts (fluorophores) become either altered or eliminated if no solutions come into contact with them, like in frozen teeth. Moreover, some bacteria might either succeed in surviving under low temperature conditions, retaining the fluorophores responsible for the fluorescence emission or the fluorophores themselves might not be affected by low temperatures and continue emitting a signal. Further studies are required to clarify this issue. Furthermore, the LF intensity might not be affected by time. The slight LF increase recorded in the frozen samples was not statistically significant.

The excellent agreement obtained in the preliminary pilot experiment between LF measurements in vivo and in frozen teeth allows the assumption that frozen teeth can be compared to the in vivo situation. Only a few studies were found in the literature dealing with the issue of the influence of storage on LF response. In one of these studies, ultraviolet, visible and near-infrared spectral ranges were applied to determine the effects of storage solutions on dentine [Strawn et al., 1996]. It was confirmed that changes in the optical properties of dentine did take place as a function of storage and time. Contrary to our findings, another study detected an increase in fluorescence after formalin fixation [Shi et al., 2001b]. However, in that experiment the samples had first been kept in thymol before being stored in formalin while the specimens in the present study had had no contact with any chemical. Thus, comparison of the two studies is not possible. On the other hand, a 77 and 20% fluorescence decrease was detected in groups of samples which, after being immersed in isotonic saline solution, were stored either in sodium hypochlorite or isotonic NaCl again. The sudden fluorescence decrease took place immediately after immersion [Mendes et al., 2004a] as observed in our study. Similarly, a recent study [Zhang et al., 2005] also showed a decline in fluorescence readings after 3 days of storage in thymol-saturated saline immediately after extraction. These readings remained stable after 3 months. In order to establish the appropriate drying time for the specimens, a previous testing was accomplished. The recordings obtained after drying were systematically higher than those obtained before drying, similarly as in previous studies [Mendes et al., 2004b; Lussi et al., 2005]. Even though much shorter dehydration times (15 s) have been recommended [Mendes et al., 2004b; Pretty et al., 2004], in the present study the most stable readings were obtained 5 min after drying. Immediately after drying and after 10, 15 and 20 min of dehydration, more variations between individual measurements were registered. The influence of dehydration time on fluorescence shows that changes in fluorescence might not only be due to the microbial endogenous porphyrins. There may also be an influence of the absorption coefficient of the water present in the lesion, and on scattering of incident and fluorescent light [Lussi et al., 2005]. The fluorescence detected on teeth stored at low temperatures and without contact with any storing solution is similar to that found in the clinical situation. Therefore, an extrapolation of both cut-offs could be possible.

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