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ALA-Induced Fluorescence in the Canine Oral Cavity

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ABSTRACT

Objective: **We examined whether 5-aminolevulinic acid (ALA) could enhance the spectroscopic contrast between normal and diseased oral tissues, without prolonged photosensitivity.** ***Background Data:*** **ALA is a promising photosensitizing agent.** ***Methods:*** **A dose of 25 mg/kg of ALA was administered intravenously to five dogs with gingivitis and three dogs with oral cancer, respectively. Fluorescence was recorded from the diseased sites in the oral cavity in addition to normal sites.** ***Results:*** **ALA-induced proto-porphyrin IX fluorescence at all gingivitis sites reached a peak in 2–3 h and returned to baseline in 24 h. Fluorescence from the gingivitis site was observed earlier and was higher than the fluorescence from the normal site. For dogs with cancer, fluorescence from the cancerous sites occurred earlier in time compared to gingivitis sites and was comparatively higher in intensity.** ***Conclusion:*** **The fluorescence from the diseased sites was found to be higher than the normal site. Clinical and fluorescence data suggest that a dose of 25 mg/kg may be satisfactory for diagnostic purposes and would have minimal side effects.**

INTRODUCTION

THE PET POPULATION of the United States has been a vastly underutilized resource for cancer therapy studies.[1] Naturally occurring tumors in pets have been known to occur twice as frequently as in humans. These tumors have been found to be similar in histopathologic features and biological behavior to those found in humans.[1] However, tumors in pet populations progress at a more rapid rate than in humans. Canine malignancies—such as lymphoma, oral melanoma, and soft tissue sarcomas—are of practical use for comparative studies.[1] Spontaneous tumors in cats and dogs are appropriate and valid model tumor systems for testing cancer therapeutic agents or studying cancer biology.[2] Oral tumors, especially melanomas, are the most common canine malignant tumor in the oral cavity.[2] Metastasis is frequent.[2]

In humans, the American Cancer Society estimates up to 28,280 new oral cancer cases appeared in the United States in 2004, resulting in approximately 7,300 deaths.[3] Early diagnosis of oral tumors could increase survival from 50% to about 80% of affected persons.[4] Oral cancer is usually first recognized when symptomatic.

Fluorescence spectroscopic detection (FSD) may provide an effective approach for early detection of oral cancer. Therefore, the development of a safe photosensitizer that could enhance the spectroscopic contrast between normal and neoplastic tissue, while allowing for selective photosensitization and treatment of pre-malignant and malignant lesions in the oral cavity, is highly desired.

5-Aminolevulinic acid (ALA) is a naturally occurring precursor of heme. The photosensitization due to ALA-induced protoporphyrin IX (PpIX) is tissue specific, and is the basis for using ALA for photodynamic diagnosis and therapy. ALA could thus fulfill the need for a safe contrast agent that enhances differentiation of neoplastic tissue from normal tissue in fluorescence diagnostics. Van der Breggen et al.[5] observed that, during transformation of buccal mucosa from normal to premalignant to malignant lesion, a significant increase in both autofluorescence and ALA-induced fluorescence occurs. The fluorescence measurements were found to correlate well with histopathological assessment of tumor development. Fluorescence spectra have been measured *in vivo* in real time to detect neoplastic tissues in a variety of organ systems.[6,7] Vaidyanathan et al.[8,9] have shown that the gingivitis site (tissue) showed higher fluorescence than the normal site.[8,9]

More recently, Betz et al.[10] presented a comparative study of normal inspection and combined fluorescence diagnosis (CFD) and its two main components—autofluorescence and 5-ALA–induced PpIX fluorescence. In terms of tumor localization and delimitation properties, CFD was clearly favorable

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over either normal inspection or its two components in fluorescence imaging.

In the current study, the utility of ALA-induced fluorescence ratio (red/orange) to differentiate between normal and diseased oral tissue in the canine model is investigated. While there have been fluorescence studies conducted with ALA on small animals such as hamsters, there are few studies reported on larger animals. This study endeavors to fill that gap.

METHODS

The studies were conducted in two phases. In phase 1, dogs with non-cancerous disease (gingivitis) were studied. Phase 2 comprised studies on client dogs with naturally occurring cancer—spontaneous animal model.

Phase 1

Five dogs with varying degrees of gum inflammation (gingivitis) were identified for participation in the study. The dogs were of medium size (average weight, 22 kg) and ranged in age from 3 to 5 years. The dogs were clinically evaluated for severity of gingivitis—by visual observation and palpation.[8] The gingivitis sites were classified as mild, moderate, and severe, with the following characteristics for each category[8]:

Mild: Some tartar present, minimal inflammation seen
Moderate: Some tartar present, noticeable inflammation and hyperemia
Severe: Marked hyperemia and inflammation

A bifurcated fiber optic probe (Jobin Yvon Inc.) was used to deliver blue light at 415 nm and collect the emitted fluorescence (in the red spectrum). The probe comprised a central 400-μm-excitation fiber, surrounded by eight smaller 200-μm emission collection fibers, housed in a hollow, metal sheath. The autofluorescence and the ALA-induced fluorescence from the sites were recorded with an Optical Multichannel Analyzer (PMA-11, Hamamatsu, Inc.). The OMA was connected to a laptop that contained the OMA software and LabVIEW software. The light source was a 300-W xenon lamp, filtered to provide blue light for excitation (KV415, Omega Opticals). A 550-nm long-pass filter was coupled to the spectrograph at the detection end to reject the reflectance of the excitation light (Fig. 1).

Previous studies on clinical and fluorescence data from normal dogs[8] suggested that a dose of 25 mg/kg of ALA would be satisfactory for diagnostic purposes and would have minimal side effects such as vomiting. It was also found that use of 25 mg/kg of ALA did not cause an increase in liver enzymes and did not produce any toxicity in the animals.[8] Based on these findings, it was decided to use a dose of 25 mg/kg body weight of ALA in the current study. ALA was administered intravenously for the following reasons: (1) consistency with previous studies[8,9]; (2) to eliminate the effect of sedatives or anesthesia on the metabolism and hence, synthesis of ALA, the animals were not sedated. Hence, the animals could have licked off the topically applied ALA. The ALA was dissolved in phosphate buffered saline at a concentration of 80 mg/mL and administered intravenously through the cephalic vein. All the fluorescence data was recorded as a ratio of intensities observed at red (636 nm) and orange spectrum (594.8 nm)—i.e., R/O. Fluorescence data were collected from the target areas at 1, 2, 3, 4, 5.5, and 24 h, respectively, after administration of ALA. hysical conditions of the dogs (temperature, respiration and heart rate) were monitored on a daily basis. nimals were housed in a darkened kennel for 24 h after ALA administration to prevent cutaneous phototoxicity.

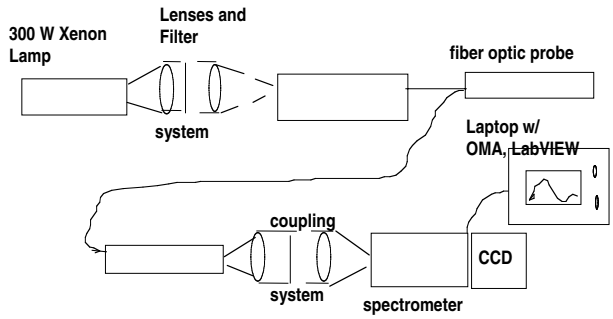

FIG. 1. Experimental set-up for measurement of 5-aminolevulinic acid (ALA)–induced protoporphyrin IX (PpIX) fluorescence from canine oral cavity.

The fluorescence data was collected in two forms—raw spectral data and fluorescence ratios (R/O). The fluorescence spectra was collected in the inherent format of the OMA and converted to an MS Excel file. The Excel file was then used as an input to LabVIEW (Laboratory Virtual Instrument Engineering Workbench). LabVIEW is a powerful and flexible instrumentation and analysis software development application that uses a graphical programming language to create programs relying on graphic symbols to describe programming actions.[12] The LabVIEW VI for spectral analysis is shown in Figure 2. The VI shows spectral data being read as an excel file and converted to a graphical output on LabVIEW. This process of capturing a temporal spectral response and displaying it in LabVIEW can be accomplished in real-time.

Phase 2

ALA-induced PpIX fluorescence was collected from three dogs with cancer—spontaneous animal models. The experimental set-up was same as the one used in phase 1. Fluorescence data (R/O) was collected over a 5-h period from cancerous sites and from gingivitis sites in the oral cavity. Oral biopsy was performed after fluorescence data collection to determine if the site was cancerous. For dog 1, fluorescence measurements were taken every hour after administration of ALA. For dogs 2 and 3, fluorescence measurements were taken at 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, and 5 h after administration of ALA. This was done in order to ensure that early fluorescence peaks were not missed. The fluorescence data was collected using the OMA software (phase 1) and converted to an excel file, which was then input to LabVIEW for subsequent analysis and processing.

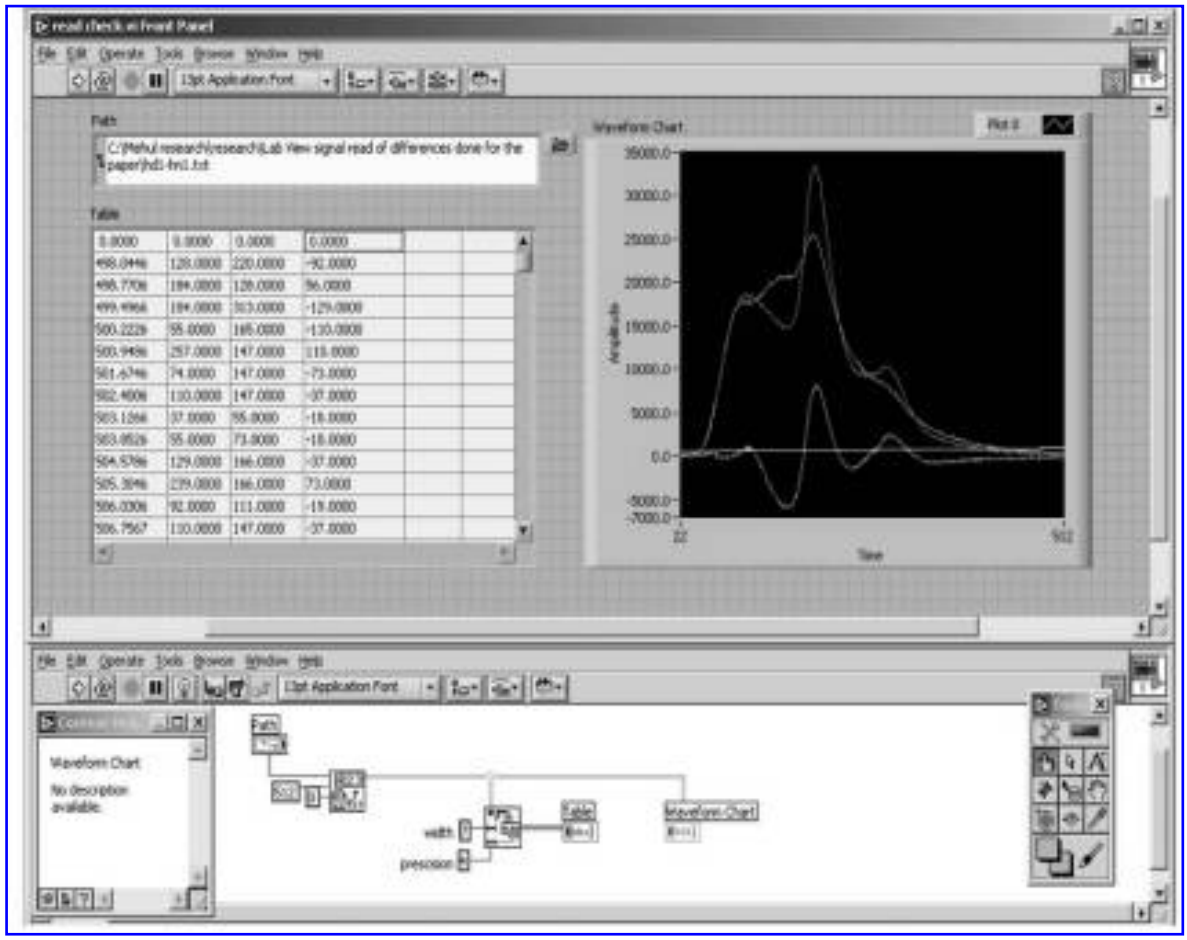

FIG. 2. The LabVIEW VI for reading spectral data in MS Excel format. The red, green, and blue lines represent spectra from gingivitis site, normal site, and the difference between the two, respectively.

RESULTS

Phase 1

The autofluorescence ratios (R/O) for the normal gingiva and the gingivitis (inflamed) site did not show significant differences ($\alpha = 0.05$). The PpIX fluorescence (R/O) measured from the sites of interest after administration of 25 mg/kg of ALA showed significant differences ($\alpha = 0.05$) between normal and inflamed gingiva, depending on the time at which the measurement was made. Peak fluorescence at all sites of interest was observed 2–3 h after administration of ALA, and the fluorescence returned to baseline in 24 h. Figure 3 encapsulates the average fluorescence response (R/O) from the five dogs. Fluorescence from the gingivitis sites is higher than the fluorescence collected from the normal gingival. Also, the fluorescence peaks occurred earlier in time in the gingivitis site as compared to the normal site.

Phase 2

ALA induced fluorescence was collected from three client dogs with cancer over a five hour period. The fluorescence data was recorded as R/O ratio. For dog 1 (a Sheltie), no significant difference was observed between the normal and diseased site. For dogs 2 and 3, the variation in fluorescence data with time is shown in Figures 4 and 5, respectively. The fluorescence ratios for dog 1 are shown in Figure 6. For dogs 2 and 3, the fluorescence ratios showed a peak earlier in time (30 and 45 min after administration of ALA) compared to adjacent inflamed tissue and normal tissue.

Oral biopsy results

Dog 1. A section of the gingival revealed that the surface epithelium is hyperplastic and covers a focally protruding gingival nodule. A moderate increase in submucosal connective tissue and mild edema was found. No neoplasia or infectious organisms were seen. Diagnosis was consistent with gingival hyperplasia and the prognosis was considered to be good.

Dog 2. The dog had a soft, round elevated area near the dorsal manibular symphysis region. Histopathological results revealed a keratinizing amelobastoma. Microscopic description showed a gingival neoplasm composed of irregular islands and trabeculae of odontogenic epithelial cells embedded in periodontal ligament stroma. The neoplastic cells were found to be polyhedral and have prominent intercellular bridges. Also, palisading of cells in the periphery of islands and trabeculae

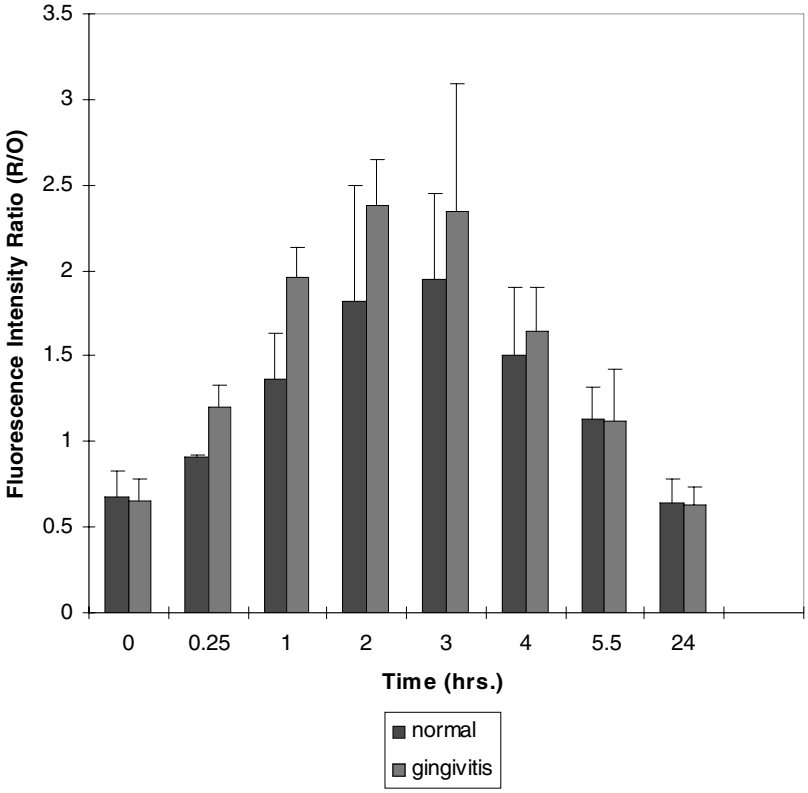

FIG. 3. Average R/O ratio for normal and gingivitis sites, recorded over a period of 24 h from the canine oral cavity. Fluorescence from the gingivitis site occurred earlier in time and was higher in intensity compared to the fluorescence from normal site.

was found. Mitotic activity was found to be minimal. On the basis of histopathological results, the prognosis for the patient was fair to guarded.

Dog 3. The sample submitted was a section of a neoplasm from the maxilla composed of pleomorphic polygonal and spindle cells arranged in sheets and trabeculae. The cells were found to have granular cytoplasm, enlarged nuclei, variable nuclei and moderate mitotic activity. Numerous spicules of neoplastic osteoid were scattered throughout the mass. The neoplasm was diagnosed as osteosarcoma with a prognosis of guarded to poor.

DISCUSSION

Under normal conditions, the rate of synthesis of PpIX is controlled by the rate of synthesis of heme and the demand for heme in the biosynthetic pathway.[13] The presence of excess amounts of ALA, bypasses the ALA feedback control mechanism. The low dose of ALA used in this study (25 mg/kg) may not inhibit the enzymatic reactions as extensively as higher doses, resulting in faster production and clearance of PpIX in tissues.[8] In the present study, it was found that the gingivitis site in the oral cavity showed higher fluorescence peaks as compared to the normal gums. Kennedy et al.[13] have stated that

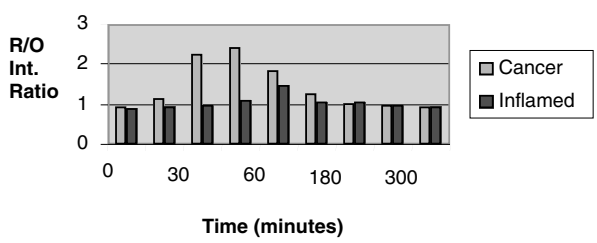

FIG. 4. Fluorescence ratios recorded from inflamed and cancerous sites in the oral cavity of dog 2. Fluorescence from the cancerous site occurred much earlier compared to that from the inflamed gingiva.

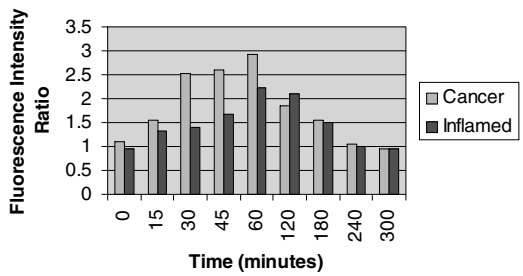

FIG. 5. Fluorescence ratios recorded from inflamed and cancerous sites in the oral cavity of dog 3. Fluorescence from the cancerous site occurred much earlier compared to that from the inflamed gingiva.

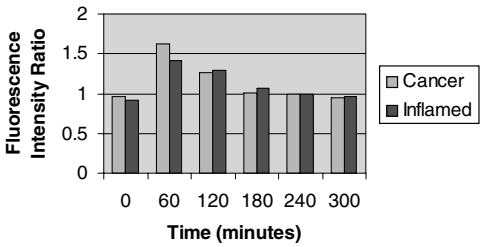

FIG. 6. Fluorescence ratios recorded from inflamed and cancerous sites in the oral cavity of dog 1. No significant difference was observed between the two sets of data.

different cells and tissues have different capacities to synthesize PpIX and heme. The difference in the capacity to synthesize PpIX could lead to a corresponding variation in fluorescence from site to site. Gingivitis is the inflammation of the gingiva. When tissue injury occurs, multiple substances that cause dramatic secondary changes are released by the injured tissues.[14] The secondary changes are called inflammation. Inflammation is characterized by (1) vasodilation of the local blood vessels with consequent excess local blood flow and (2) increased permeability of the capillaries with leakage of large quantities of fluid into the interstitial spaces.[14] The faster exchange of ALA in the vascular gingivitis site and consequently earlier uptake, could be contributing to the higher fluorescence from the gingivitis site than that from the normal gums. In a previous study on oral cancer in a hamster model, van der Breggen et al.[4] reported higher fluorescence peaks in transformed tissue, compared to normal tissue. It was also reported that the fluorescence peaks occurred earlier in time (2–3 h after administration of 40 mg/kg of ALA) compared to normal tissue (4 h after administration of 40 mg/kg of ALA). Our data is consistent with the findings reported by van der Breggen at al.[4] in the hamster cheek pouch model. An important factor to be considered is the variation in tissue optical properties from site to site. Wavelength-dependent absorption and scattering properties of tissue could affect the depth of the probing site and also the re-absorption of emitted light. The optical property of the tissue in turn could be influenced by tissue vascularization. The use of a non-dimensional fluorescence spectroscopic function (R/O) ensures that the measurements made are independent of such uncontrollable variations as distance variations and light source fluctuations.

Our studies on three dogs with cancer have yielded encouraging results. Fluorescence data from the first dog, a female Sheltie, did not show significant differences between cancerous and normal sites. Data was collected every hour after administration of ALA. The reason for this could be that the fluorescence reading was taken at intervals of 1 h post-administration of ALA. In doing so, the early occurrence of the fluorescence peak in the cancerous site may have been missed. The dog was very small in size and its metabolism could have played a role in the rapid synthesis of PpIX. The dogs used in the gingivitis study (mentioned earlier) were hound dogs and were heavier and bigger in size. Fluorescence data from dogs 2 and 3—both Labradors—included readings taken 15, 30, and 45 min after administration of ALA, in addition to the hourly readings. In both dogs (dog 2 and dog 3), higher fluorescence (red/orange) peaks were seen, earlier in time, as compared to the adjacent inflamed area. The fluorescence peaks occurred earlier in time (30 and 45 min after administration of ALA) as compared to the non-cancerous site.

These results corroborate recent findings using ALA as the photosensitizing agent. Betz et al.[9] showed that in terms of tumor localization and delimitation properties, CFD using ALA was clearly a favorable detection technique. Their spectral analysis revealed that cancerous tissue showed significantly higher PpIX fluorescence intensities and lower autofluorescence intensities than normal mucosa. Leunig et al.[15] performed semiquantitative fluorescence measurements following topical application of 5-ALA in 16 patients with neoplastic lesions of the oral cavity. PpIX in neoplastic tissue was seen to accumulate earlier in comparison with the surrounding normal tissue. The maximum fluorescence contrast of 10:1 between tumor and host tissue was generally seen 1–2 h after application, allowing a demarcation of tumor tissue even with the naked eye. Zheng et al.[10,11] used PpIX fluorescence endoscopy and fluorescence image quantification on 16 patients with known or suspected premalignant or malignant lesions in the oral cavity. Preliminary data showed that the red-to-blue intensity ratio of malignant tissue is larger than that of benign tissue. They also showed that suspicious lesions display bright reddish fluorescence, while normal mucosas exhibit blue color background in the fluorescence images.[11] Combining the two ratio diagnostic algorithms yielded a sensitivity and specificity of 95% and 97%, respectively, exceeding each diagnostic algorithm alone for discriminating malignant tissue from benign tissue.

The results presented in this study and recent findings from other studies point to fluorescence spectroscopic detection, allied with imaging as a potent combination in the diagnosis of oral cancer. More investigation is required on client dogs with naturally occurring cancer. An ALA dose of 25 mg/kg seems adequate for diagnostic purposes.

CONCLUSION

In this study, we conducted in vivo fluorescence spectroscopic detection in the canine model—both non-cancerous disease and cancerous disease. The ALA dose was well tolerated by the animals without any side effects. ALA induced fluorescence was seen earlier in time and had higher intensities at the cancerous site than the fluorescence seen from the non-cancerous site in the same animal.

Fluorescence spectroscopic detection using ALA-induced PpIX could enhance the spectroscopic contrast between tissues and provide an effective, non-invasive method for diagnosis of oral cancer. In order to make a definitive diagnosis of oral cancer using FSD, it is imperative to understand the differences in PpIX fluorescence between various tissue types in the oral cavity. It is important to understand the behavior of ALA-induced PpIX fluorescence obtained from non-cancerous, diseased gingival tissue. Fluorescence monitoring up to 2 h after ALA administration could potentially help distinguish non-cancerous tissue from diseased tissue. An analytical tool such as LabVIEW allied with fluorescence spectroscopic detection could potentially be used initially for optically guided biopsies prior

to a full-fledged, stand-alone non-invasive diagnosis of oral cancer.

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