

# Comparison of aminolevulinic-acid-induced fluorescence from normal and inflamed gingiva in the canine model

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**Abstract.** Fluorescence spectroscopic detection using 5-aminolevulinic acid (ALA) may provide an effective, noninvasive approach for early detection of oral cancer. In the present study, the use of ALA-induced fluorescence ratio (red/orange) to differentiate between normal and gingivitis-affected gingiva is investigated. Five dogs with varying degrees of gingivitis are studied. Based on previous studies, a dose of 25 mg/kg of ALA is administered intravenously to the dogs. Autofluorescence and ALA-induced fluorescence from three sites: normal gingiva, pigmented gingiva, and gingivitis, are detected with a fiber optic probe coupled to an optical multichannel analyzer. Four dogs show higher and earlier ALA-induced fluorescence from the gingivitis site as compared to the unpigmented gingiva. In two dogs, ALA-induced fluorescence peaks are seen 15 min after ALA administration. Statistical analysis using mean separation procedures reveal differences in the fluorescence from the various sites in each dog. Using a fluorescence (ratio) cutoff of 1.5, the sensitivity and specificity are found to be 92 and 80%, respectively, 1 h after administration of ALA. The indications from this study—that the characteristic protoporphyrin IX (PpIX) fluorescence is seen earlier and in higher magnitude in more vascular areas of the oral cavity—has implications for oral cancer diagnosis. © 2004 Society of Photo-Optical Instrumentation Engineers.

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## 1 Introduction

Cancers of the tongue, gingiva, buccal mucosa, lips, palate, and oropharynx now account for an estimated 30,000 cases of cancer in humans each year.<sup>1</sup> Since approximately 95% of all oral cancers are squamous cell carcinomas, the vast majority of oral cancers are diagnosed from lesions on the mucosal surfaces. The clinician's problem is to differentiate cancerous lesions from many other ill-defined lesions that occur in the oral cavity. Most oral lesions are benign, but many have an appearance that may confuse them with a malignant lesion.<sup>2</sup> Oral carcinoma in its early stage may appear as harmless induration or localized change such as erosion, erythema, or keratosis. Because of the variability in signs and symptoms among oral cancer patients, even excellent clinical judgment and experience do not preclude diagnostic errors.

Oral cancers are also common in canines. Most oral tumors arise from the gingiva. Squamous cell carcinomas are most frequently identified arising from the gingiva rostral to the canine teeth. They may be mistaken for gingivitis in the early stages and can be confused with plasmacytic-

lymphocytic gingivitis/pharyngitis, and lymphosarcoma. Invasion of surrounding bone and soft tissue is common with gingival squamous cell carcinoma; however, invasion of regional lymph nodes is uncommon.

Currently, histologic examination of tissue from a biopsy is the only definitive method of diagnosing oral cancer. Fluorescence spectroscopic detection (FSD) using 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) may provide an effective approach for early detection of oral cancer. Autofluorescence, the natural fluorescence of tissue, has been used to distinguish between normal and neoplastic tissue. However, the differentiation is not always reliable and is dependent to a large extent on the type of tissue and its properties. With the availability of photosensitizers that are more selectively retained in neoplastic tissues, the reliability of optical diagnosis could be considerably enhanced. Fluorescent dyes, such as hematoporphyrin derivative (HpD), have been used to enhance the differentiation of normal versus diseased tissues; however, their adverse side effects (cutaneous photosensitization) have limited their use. Because of its tissue-specific sensitization, ALA could thus fulfill the need for a safe contrast agent that enhances differentiation of neoplastic

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tissue from normal tissue in fluorescence diagnostics. Fluorescence spectroscopy using ALA may allow rapid, accurate identification of neoplastic tissue. Because FSD can be performed in patients who are awake and will give immediate results, it may prove a valuable adjunct for detection of oral neoplasia. The typical absorption and scattering properties of biological tissue cause light penetration to be restricted to a few millimeters of visible light, which makes fluorescence measurement especially useful for detecting superficial tumors.

ALA is a naturally occurring precursor of heme. The native compound is not a photosensitizer, but in certain types of cells and tissues, it is metabolized to the photosensitizer PpIX in the body.<sup>3</sup> When excited with suitable wavelength of light (~410 nm), PpIX emits characteristic fluorescence in the red spectrum (636 nm). ALA-induced PpIX is a promising photosensitizer that could be used in noninvasive diagnosis and photodynamic therapy of oral cancers. Vaidyanathan et al.<sup>4</sup> investigated the *in vivo* kinetics of ALA-induced fluorescence in the canine oral cavity for different doses at various sites. It was seen that higher doses of ALA produced higher peak fluorescence at a later time. It was also observed that the gums and the tongue showed the highest peak fluorescence. Fluorescence and clinical data suggested that a dose of 25 mg/kg of ALA would be satisfactory for diagnostic purposes and would have minimal side effects. Clinical work by Leunig et al.<sup>5</sup> presents quantitative fluorescence measurements and results in the visualization of cancerous oral mucosa with 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX (PpIX). Fluorescence images in the red and green spectral range from the tumor tissue were recorded with a charge-coupled device (CCD) camera. After topical application of 0.4% 5-ALA and incubation for 1 to 2.5 h, all patients revealed higher intensities of red fluorescence in neoplastic tissue compared with the surrounding normal tissue. Maximum contrast was reached after 1.5 h of incubation.

Betz et al.<sup>6</sup> presented a comparative study of normal inspection, combined fluorescence diagnosis (CFD), and its two main components, autofluorescence and 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX (PpIX) fluorescence. Biopsy-controlled fluorescence imaging and spectral analysis were performed on a total of 85 patients with suspected or histologically proven oral carcinoma both before and after topical administration of 5-ALA. In terms of tumor localization and delimitation properties, CFD was clearly favorable over either normal inspection or its two components in fluorescence imaging. Spectral analysis revealed that cancerous tissue showed significantly higher PpIX fluorescence intensities and lower autofluorescence intensities than normal mucosa. Leunig et al.<sup>7</sup> performed semiquantitative fluorescence measurements following topical application of 5-aminolevulinic acid (5-ALA) in 16 patients with neoplastic lesions of the oral cavity. Protoporphyrin IX 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 to 2 h after application, allowing a demarcation of tumor tissue even with the naked eye. Zheng et al.<sup>8</sup> built a 5-ALA mediated digitized fluorescence endoscopic imaging system to enable the on-line image acquisition, analysis, and fluorescence quantification for the early detection of neoplasms in the oral

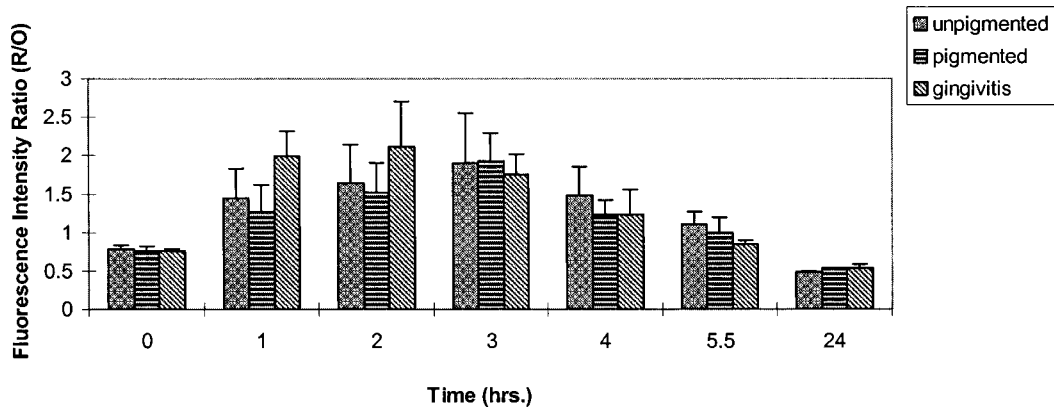
cavity. PpIX fluorescence endoscopy and fluorescence image quantification were performed on 16 patients with known or suspected premalignant or malignant lesions in the oral cavity. Preliminary data from the head and neck clinical trials showed that the red-to-blue intensity ratio of malignant tissue is larger than that of benign tissue. They also showed that suspicious lesions display bright reddish fluorescence, while normal mucosae exhibit a blue color background in the fluorescence images.<sup>9</sup> The red-to-blue and red-to-green intensity ratios of malignant tissues were larger than those of benign tissues. 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.

In the current study, the utility of ALA-induced fluorescence ratio (red/orange) to differentiate between normal and inflamed (gingivitis) gingiva in the canine model is investigated. It is important to understand the behavior of ALA-induced PpIX fluorescence obtained from noncancerous, diseased gingival tissue as compared to normal tissue. Fluorescence data and statistical analysis are presented to facilitate the differentiation between normal and inflamed gingiva. A fluorescence cutoff is used to evaluate the sensitivity, specificity, and positive and negative predictive values.

## 2 Materials and Methods

This study was approved by an institutional review board to allow fluorescence studies to be performed on research dogs with non-neoplastic disease. Five dogs with varying degrees of gum inflammation (gingivitis) were identified for participation in the study. The dogs were of medium size and ranged in age from 3 to 5 years. The basic elements of the fluorescence detection system were similar to the one used in a previous study on normal dogs. A bifurcated fiber optic probe (NV002, Instruments S.A., Incorporated, Edison, New Jersey) was used to deliver excitation (blue light, 415 nm) and collect the emitted fluorescence (in the red spectrum). The probe comprised a central 400- $\mu\text{m}$ -excitation fiber, surrounded by eight smaller 200- $\mu\text{m}$  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 (OMA 4, Model 1530, EG&G Instruments, Princeton Applied Research). The modular detector head contains the CCD array, driver circuit, and signal amplifier. Digital information is transmitted from the electronics enclosure to the controller board over a high-speed fiber optic link that provides immunity to laboratory noise. The detector is liquid cooled and has an excellent quantum efficiency in the 400- to 1100-nm range. The light source was a 100-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.

Clinical and fluorescence data from a previous study on normal dogs<sup>4</sup> 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.



**Fig. 1** ALA-induced PpIX fluorescence from the gingiva of dog 1. Fluorescence peak occurred earlier in time and was higher in magnitude in comparison with unpigmented normal gingiva. Fluorescence peak in pigmented gingiva occurred later in time.

Based on these findings, it was decided to use a dose of 25 mg/kg body weight of ALA in this study.

Autofluorescence of the tissue was measured from the sites of interest, prior to administration of ALA. Sites of interest were: gingivitis area, pigmented gingiva, and unpigmented normal gingiva. The probe was placed in contact with the site and perpendicular to it during measurements. It was cleaned after measurement at each site. Five readings were obtained from each site. The fluorescence measurements were made in a darkened laboratory. A catheter was inserted into the cephalic vein to inject ALA.  $\delta$ -aminolevulinic acid hydrochloride (ALA, Sigma Chemical Company, Saint Louis, Missouri, purity 98%) was dissolved in sterile phosphate buffered saline at a concentration of 80 mg/ml and administered within 10 min (average) after preparation, through the catheter over 5 min. The PpIX fluorescence was measured from the same sites after administration of ALA. All the fluorescence data were recorded as a ratio of intensities observed at red (636 nm) and orange spectrum (594.8 nm), i.e., R/O. Some dogs that were restive during the course of the experiment were administered acepromazine (0.025 mg/lb.) intravenously. Fluorescence data were collected from the target areas at 1, 2, 3, 4, 5.5, and 24 h, respectively, after administration of ALA. Physical conditions of the dogs (temperature, respiration, and heart rate)

were monitored on a daily basis. Animals were housed in a darkened kennel for 24 hours after ALA administration to prevent cutaneous phototoxicity.

A SAS code was written, incorporating hypotheses test procedures to verify differences between fluorescence observed at various sites in the gingiva. This was done for all dogs at the times (1, 2 h, etc.) that fluorescence data were collected. The program was then modified to include Student-Newman-Keul's (SNK) mean separation procedure to categorize the means ( $\alpha=0.05$ ).

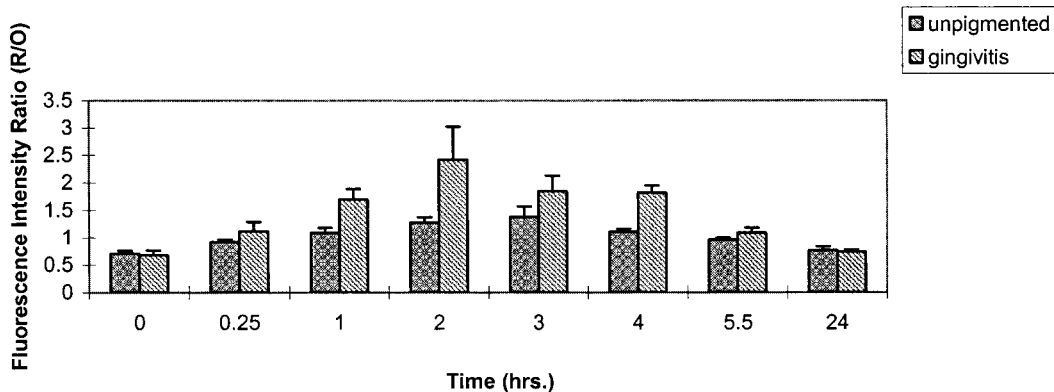
The dogs were clinically evaluated for extent of gingivitis, by visual observation and palpation. Three categories were used to classify the gingivitis sites: mild, moderate, and severe. The categories were defined as follows.

Mild: some tartar present, minimal inflammation seen.

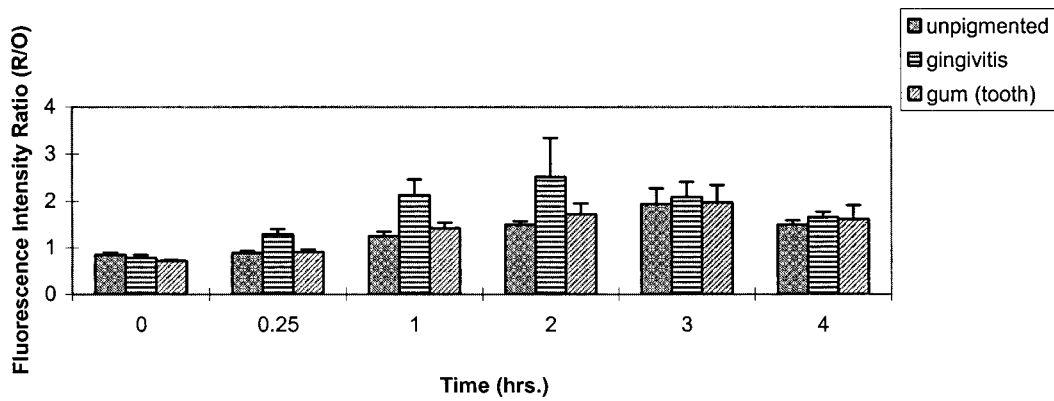
Moderate: some tartar present, noticeable inflammation and hyperemia.

Severe: marked hyperemia and inflammation.

Based on these classifications, dogs 1, 2, 4, and 5 were found to have moderate gingivitis, whereas dog 3 was found to have a mild case of gingivitis.



**Fig. 2** ALA-induced PpIX fluorescence from the gingiva of dog 4. Fluorescence peak occurred earlier in time and was higher in magnitude in comparison with unpigmented normal gingiva.



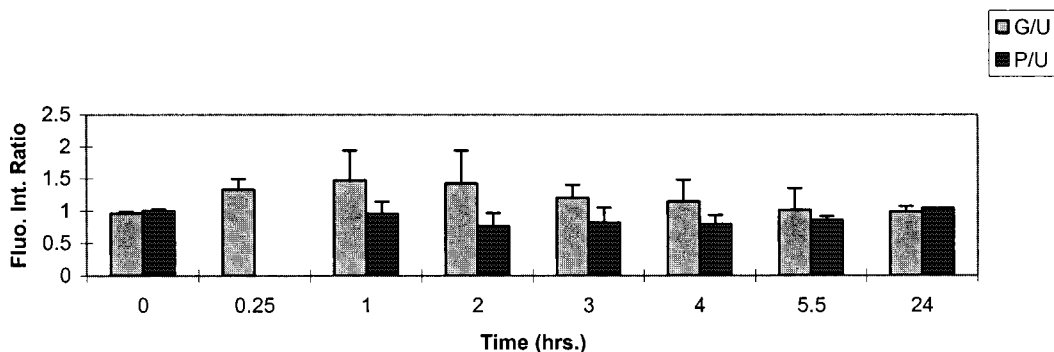
**Fig. 3** ALA-induced PpIX fluorescence from the gingiva of dog 5. Fluorescence peak in the gingivitis site occurred earlier in time and was higher in magnitude in comparison with unpigmented normal gingiva. Fluorescence peak in the gums occurred later in time compared to the gingivitis site.

### 3 Results

Dogs used in this study showed varying degrees of pigmentation of the gingiva. Some dogs had a pinkish, unpigmented gingiva (dogs 4 and 5), whereas others had darker, pigmented gingiva (dogs 1 and 3). The autofluorescence ratios (R/O) for the pigmented and unpigmented gingiva and the gingivitis (inflamed) site did not indicate 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 between unpigmented, pigmented, and inflamed gingiva, depending on the hours at which the measurement was made. Peak fluorescence at all sites of interest was observed 2 to 3 h after administration of ALA, and the fluorescence returned to baseline in 24 h. The PpIX fluorescence obtained from dogs 1, 4, and 5 are shown in Figs. 1, 2, and 3, respectively. For dog 1 (Fig. 1), peak fluorescence from the unpigmented gingiva was observed 3 h after administration of ALA, as opposed to 2 h for the gingivitis site. Peak fluorescence in the pigmented site was seen an hour later. Peak fluorescence from the sites of interest in dog 4 (Fig. 2) followed a similar pattern as in dog 1. For dog 5 (Fig. 3), the fluorescence from the unpigmented areas peaked 2 h after administration of ALA, as did the fluorescence from the pigmented site. Peak fluorescence from the gingivitis site was observed 3 h after administration of ALA. For dogs 4 and 5, peak fluorescence from the gingivitis site was observed 2 h after administration of ALA, as opposed to 3 h for the unpig-

mented gingiva. Four dogs showed higher PpIX fluorescence from the gingivitis site as compared to the unpigmented gingival site. Fluorescence from the pigmented gingiva was found to be lowest. For dogs 4 and 5, PpIX fluorescence peaks were observed in the spectrum as early as 15 min after ALA administration.

For each dog, the fluorescence data were analyzed statistically using mean separation procedures (SNK) to test for differences between the means at different sites of fluorescence measurement, for various hours of measurement. For dog 1, no significant differences were observed ( $\alpha = 0.05$ ) between the fluorescence at various sites, for all hours of measurement. For dog 2, significant differences in the fluorescence were observed up to 3 h after administration of ALA, with the fluorescence from the gingivitis site being higher than the fluorescence from the unpigmented and pigmented sites. For dog 3, fluorescence from the normal, unpigmented site was found to be higher and significantly different from fluorescence at the pigmented and gingivitis sites up to 3 h after administration of ALA. Dogs 1 and 3 had darker gingiva and showed pigmentation at the gingivitis sites also. Dog 3 was classified under the mild category after clinical evaluation. For dog 4, fluorescence from the gingivitis site was significantly higher than the fluorescence from the unpigmented site, up to 5.5 h after administration of ALA. For dog 5, the fluorescence from the gingivitis site was significantly higher than



**Fig. 4** Mean double ratios (fluorescence) to indicate contrast between sites in the oral cavity.

**Table 1** Weighted area under curve (AUC) for different sites in the oral cavity.

Dogs	Normal	Pigmented	Gingivitis
1	0.95	0.9	0.9
2	1.34	1.25	1.94
3	1.27	0.94	1.08
4	0.9	-	1.09
5	1.68	-	1.99

the fluorescence from the unpigmented site up to 2 h after administration of ALA.

To develop another test statistic to distinguish the fluorescence from the gingivitis and normal sites, a double fluorescence ratio was obtained by dividing the fluorescence (R/O) in the gingivitis (G), and pigmented (P) sites, respectively, by the fluorescence from the unpigmented (N) site i.e., G/N and P/N. Average double ratios are shown in Fig. 4. The double ratios were then analyzed statistically by modifying a SAS code that incorporated mean separation procedures. The double ratio for the gingivitis site was found to be significantly different ( $\alpha=0.05$ ) from that for the pigmented site, 1 h after administration of ALA.

The area under the curve Area Under the Curve (AUC) is an approach to take summary statistics directly from the observed data. In this study, the AUC was calculated by adding the areas under the curve between each pair of consecutive observations, using the trapezium rule.<sup>10</sup> The AUC was expressed as a weighted average by dividing it by 24 h, which was the time frame of data collection. The weighted AUCs for the five dogs are shown in Table 1. With the exception of dogs 1 and 3, the weighted AUCs from the gingivitis sites were

**Table 2** Fluorescence rates of rise (RR) and rate of decline (RD) in five dogs for various sites in the oral cavity.

Rates	Normal	Pigmented	Gingivitis
Dog 1: RR	0.38	0.39	0.68
RD	-0.32	-0.38	-0.36
Dog 2: RR	0.40	0.31	1.11
RD	-0.26	-0.16	-0.34
Dog 3: RR	1.24	0.59	1.04
RD	-0.46	-0.21	-0.47
Dog 4: RR	0.22	-	0.87
RD	-0.17	-	-0.38
Dog 5: RR	0.36	-	0.87
RD	-0.44	-	-0.44

**Table 3** Efficiency of fluorescence measurement in distinguishing gingivitis from a normal site in the oral cavity, after ALA administration, for a fluorescence cutoff=1.5.

Hours after ALA administration	Sensitivity	Specificity	Positive predictive value	Negative predictive value
1	0.92	0.8	0.81	0.91
2	0.93	0.5	0.64	0.92
3	0.93	0.33	0.56	0.9
4	0.77	0.5	0.57	0.69

higher than those for the unpigmented gingiva. Dogs 4 and 5 did not show pigmentation of gingiva.

Another summary statistic that enables researchers to distinguish between data is evaluation of the rise and decline rates, respectively. The rise and decline rates correspond to the positive and negative slopes, respectively, in the fluorescence kinetics data obtained from various sites in the oral cavity. Table 2 shows the rise and decline rates for the fluorescence data obtained from the dogs.

To use the ALA-induced PpIX fluorescence (R/O) as a tool for diagnosis, the questions of sensitivity and specificity must be addressed. Sensitivity may be defined as the proportion of true positives that are correctly identified by the test, whereas specificity is defined as the proportion of true negatives that are correctly identified by the test.<sup>10</sup> There are two other discriminators that yield better information on the probability of a test giving the correct diagnosis, whether it is positive or negative. The positive predictive value is the proportion of patients with positive test results who are correctly diagnosed, whereas the negative predictive value is the proportion of patients with negative test results who are correctly diagnosed.<sup>10</sup> The proportions defined earlier were calculated on the basis of a cutoff value of the fluorescence ratio (R/O). The choice of the cutoff value is not a statistical decision. The sensitivity, specificity, and the positive and negative prediction values for cutoff fluorescence ratios of 1.5 and 1.8 are shown in Tables 3 and 4 for up to 4 h after administration of ALA. Fluorescence ratios greater than or equal to the cutoff were classified as positive for gingivitis. Fluorescence ratios less than the cutoff were classified as negative for gingivitis. The sensitivities for

**Table 4** Efficiency of fluorescence measurement in distinguishing gingivitis from a normal site in the oral cavity, after ALA administration, for a fluorescence cutoff=1.8.

Hours after ALA administration	Sensitivity	Specificity	Positive predictive value	Negative predictive value
1	0.67	0.84	0.8	0.72
2	0.88	0.63	0.7	0.83
3	0.79	0.48	0.58	0.72
4	0.45	0.69	0.58	0.57

cutoff=1.5 were greater than those for cutoff=1.8. The specificities for both cutoff values at the first hour of measurement were not different. Higher negative predictive values were obtained with a cutoff fluorescence ratio of 1.5.

#### 4 Discussion and Conclusions

Based on their studies, Kennedy and Pottier<sup>3</sup> have stated that different cells and tissues have different capacities to synthesize PpIX and heme. The difference in the capacity to synthesize PpIX could be responsible for a corresponding variation in fluorescence from site to site. In this study, the gingivitis sites showed a higher fluorescence peak earlier in time than normal gingiva. Gingivitis is the inflammation of the gingiva. When tissue injury occurs, whether bacteria, trauma, or other reasons cause it, multiple substances that cause dramatic secondary changes are released by the injured tissues.<sup>11</sup> 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.<sup>11</sup> The faster exchange of ALA in the vascular gingivitis site, and consequently earlier uptake, could be contributing to the earlier and higher fluorescence from the gingivitis site, as opposed to the normal gingiva. In this study, fluorescence peaks from the gingivitis site were seen 15 min after administration of ALA in two dogs. Subsequent statistical analysis of data showed a significant difference between gingivitis and normal sites up to 1 h after administration of ALA. The fluorescence from the gingivitis site was approximately 1.5 times greater than the fluorescence from the normal site at 1 h after ALA administration.

The results obtained from this study suggest that the gingivitis site takes up ALA faster than the normal or pigmented gingiva. Also, the fluorescence decline rate is slower for the gingivitis site, suggesting a comparatively longer retention of PpIX in diseased tissue as opposed to normal tissue. In dogs with darker and pigmented gingiva, the distinction between the normal and gingivitis sites seems difficult using the fluorescence test (R/O) as a yardstick. Melanin in the pigments shows good absorption of light in the visible range.<sup>12</sup> The reabsorption of the emitted light would cause a decrease in the overall fluorescence detected by the probe. 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 reabsorption of emitted light. The optical property of the tissue could be influenced by tissue vascularization. The use of a nondimensional fluorescence spectroscopic function (R/O) ensures that the measurements made are independent of such uncontrollable variations as distance variations and light source fluctuations.

The current study attempts to highlight differences between PpIX fluorescence obtained from normal sites and sites affected by gingivitis. A closer examination of Table 2 reveals that in general, for all dogs except dog 3, the rise rate is higher for the gingivitis site as compared to the pigmented

and unpigmented sites. The decline rate data suggests that PpIX is cleared slowly in the gingivitis site as opposed to the normal site.

Diagnosis is an essential part of clinical practice, and much medical research is being carried out to try to improve methods of diagnosis. Fluorescence spectroscopic detection (FSD) using ALA-induced PpIX could enhance the spectroscopic contrast between tissues and provide an effective, noninvasive method for diagnosis of oral cancer. 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 noncancerous, diseased gingival tissue. This study attempts to bridge that gap. Fluorescence monitoring up to 2 h after ALA administration could help distinguish normal tissue from diseased tissue. A fluorescence ratio of 1.5 could be used as the guideline for fluorescence guided biopsy. FSD allied with imaging techniques has the potential to provide real-time diagnosis of oral cancer in canines and humans.

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