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Acceleration of ALA-Induced PpIX Fluorescence Development in the Oral Mucosa

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Background and Objectives: The development of 5-aminolevulinic acid (ALA)-induced tissue fluorescence is optimal 2–4 hours after ALA application. Goal of this work was to develop a means of accelerating oral topical ALA-induced tissue fluorescence.

Study Design/Materials and Methods: In 300 hamsters, DMBA (9,10 dimethyl-1,2-benzanthracene) cheek pouch carcinogenesis produced dysplasia in 3–5 weeks. Topical application of 20% ALA in Eucerin was followed by localized ultrasound treatment (1, 3.3 MHz) in 150 animals. In 75 animals, ALA was applied in an Oral Pluronic Lecithin Organogel (OPLO—an absorption enhancer) vehicle. Seventy-five animals received only topical ALA in Eucerin. Hamsters were sacrificed and cryosections underwent fluorescence measurements, histological evaluation, 20–180 minutes after ALA application. One-way ANOVA detected independent effects of pathology on laser-induced fluorescence (LIF). Two-way ANOVA tested for independent effect of pathology and of OPLO, ultrasound, and interaction effects.

Results: Ultrasound significantly ($P < 0.05$) accelerated tissue fluorescence development.

Conclusions: Low-frequency ultrasound can accelerate ALA-induced fluorescence development. *Lasers Surg. Med.* 32:185–188, 2003. © 2003 Wiley-Liss, Inc.

Key words: dysplasia; hamsters; laser induced fluorescence; protoporphyrin IX; ultrasound

INTRODUCTION

Claiming approximately 10,000 lives annually in the US, oral squamous cell carcinoma is usually preceded by dysplasia presenting as leukoplakia. Malignant transformation occurs unpredictably in 1–40% of patients over 5 years. Thus, there exists an urgent need for the development of a fast, reliable non-invasive modality for the clinical early detection and diagnosis of dysplasia and malignancy.

Recent studies using the hamster model have demonstrated that laser-induced fluorescence (LIF) can non-invasively identify oral dysplasia or malignancy [1,2]. Chemical agents called photosensitizers render pathologic tissues fluorescent when exposed to appropriate wavelengths of light [3,4]. While several studies have demonstrated the use of porphyrins as photosensitizers [4,5], their accumulation in skin can cause phototoxic reactions. An

alternative is to stimulate synthesis of photosensitizing agents in situ with a precursor. The photosensitizer protoporphyrin IX (PpIX) is a precursor in the biosynthetic pathway for heme. The rate of PpIX synthesis can be increased by the addition of exogenous ALA [3,6,7]. A selective accumulation of PpIX occurs in areas of increased metabolism such as tumor cells [3,4,6,7]. The resulting tissue-specific photosensitization permits photodynamic diagnosis and therapy, whereby far lower light doses are used for diagnosis. Animal studies have demonstrated stronger PpIX fluorescence after application of ALA in colon [8], bladder [9], and skin tumors [10]. ALA can induce strong PpIX fluorescence in tumors of the bronchi, skin, and mammary tissue [7,11–14]. In the oral cavity, topical ALA can be used [1,2,15]. After excitation at 405 nm, ALA-induced PpIX fluorescence emits strongly in the red spectral region with maxima at 635 and 710 nm [16].

The safety of ALA as a topical or systemic photosensitizer has been established in multiple clinical trials [7–14]. PpIX is normally present in tissues of the body. ALA-induced PpIX is cleared from the body within 24 hours, whether the route of administration is systemic or topical [7]. Thus, protection from exposure to sunlight is only necessary for 24 hours after ALA application.

In oral mucosa, ALA-induced PpIX fluorescence develops progressively earlier and to a greater intensity (405 nm excitation/635 nm detection) with increasing severity of pathology [1,2,15]. In previous studies, optimum healthy:pathological PpIX fluorescence intensity ratios occurred after 180–210 minutes of topical ALA application [1,2]. In patients, this long delay is problematic. In cell culture, a transient enhancement of tumor cell porosity to photosensitizers has been demonstrated during low-intensity ultrasound treatment [17]. The aim of this project was

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to develop a means of accelerating the development of tissue fluorescence from the 90–240 minutes currently needed to a clinically tolerable level.

MATERIALS AND METHODS

Animal Model

Using the Golden Syrian Hamster (*Mesocricetus auratus*, Harlan Sprague Dawley, San Diego, CA) cheek pouch model, thrice weekly application to the right cheek pouch of 0.5% DMBA (9,10 dimethyl-1,2-benzanthracene) (Sigma-Aldrich, St. Louis, MO) in mineral oil produced dysplastic leukoplakia after 3–4 weeks. In the control left cheek pouch of these 300 hamsters, only mineral oil (E.R. Squibb & Sons, Inc., Princeton, NJ) was applied. The animals were treated in accordance with ARC guidelines at UCI (IACUC 97-1972).

Prior to sacrifice, 1 g of 20% ALA (5-aminolevulinic acid HCl, Sigma-Aldrich, St. Louis, MO) in Eucerin (Beiersdorf, Inc., Norwalk, CT) adjusted to a pH of 5.5 using 1 N sodium hydroxide was applied to both cheek pouches. In 150 animals, ultrasound at 1 or 3.3 MHz with a total intensity of $\sim 0.3 \text{ W/cm}^2$ (Dynatron 150 Plus, Dynatronics Corporation, Salt Lake City, Utah) was applied to external cheek surfaces for 60 seconds prior to sacrifice 20–180 minutes later. Energy conduction was enhanced using a water-filled pad between the ultrasound device head and the external cheek surface. In another 75 animals, freshly prepared 20% ALA in Oral Pluronic Lecithin Organogel (OPLO—an absorption enhancer) was applied topically to the internal surface of the cheek pouches prior to sacrifice 20–180 minutes later. In the control group, another 75 animals, freshly prepared 20% ALA in Eucerin only was applied to the internal surface of the cheek pouch prior to sacrifice 20–180 minutes later. Directly after sacrifice, cheek pouch tissues were excised, then frozen in liquid nitrogen. Routine 6- μm cryosection were used for fluorescence measurements and histological evaluations to determine optimum acceleration modality and any potential effects of this modality on diagnostic sensitivity and specificity.

Fluorescence Measurements

The low-light level tissue fluorescence microscopy (LLFM) fluorescence microscopy system was comprised of a slow-scan cooled CCD camera (576×384 pixels) with 16-bit per-pixel dynamic range of data acquisition interfaced with Power Macintosh 8600/300 attached to a Zeiss Axiovert 10 inverted microscope. Excitation was provided by a 100 W Hg-lamp. Both bright field, phase contrast and fluorescence images were acquired with this system, permitting a direct quantification of tissue fluorescence characteristics and comparison with histological status for each slide. Excitation was at 405 nm; detection at 635 nm. For the purposes of analysis, fluorescence values for each slide were quantified as follows. First, a pre-standardized investigator blinded to fluorescence levels selected sites with a typical histological appearance (healthy and dysplasia), then a second pre-standardized investigator performed five fluorescence measurements in each of these

areas. Fluorescence measurements were performed in a total of 25 areas of each histological appearance (healthy and dysplasia) present in each animal.

Histological Evaluation

After fluorescence evaluation, slides underwent routine hemosin & eosin (H & E) staining. Detailed histological evaluation of each section was quantified by the same blinded, pre-standardized investigator according to the semi-quantitative criteria established by Macdonald [18].

The following numerical grading system was used for each site: 0, healthy; 1, dysplasia. The criteria for Oral Epithelial Dysplasia are as follows [18,19].

- (1) Drop-shaped rete ridges;
- (2) Irregular epithelial stratification;
- (3) Keratinization of cells below keratinized layer;
- (4) Basal cell hyperplasia;
- (5) Loss of intercellular adherence;
- (6) Loss of polarity;
- (7) Hyperchromatic nuclei;
- (8) Increased nucleocytoplasmic ratio;
- (9) Anisocytosis and anisonucleosis;
- (10) Pleomorphic cells and nuclei;
- (11) Level of mitotic activity; and
- (12) Presence of bizarre mitoses.

Each fluorescence measurement site was assessed for each of these characteristics at a level of either none (0), slight (1), or marked (2).

Statistical Analysis

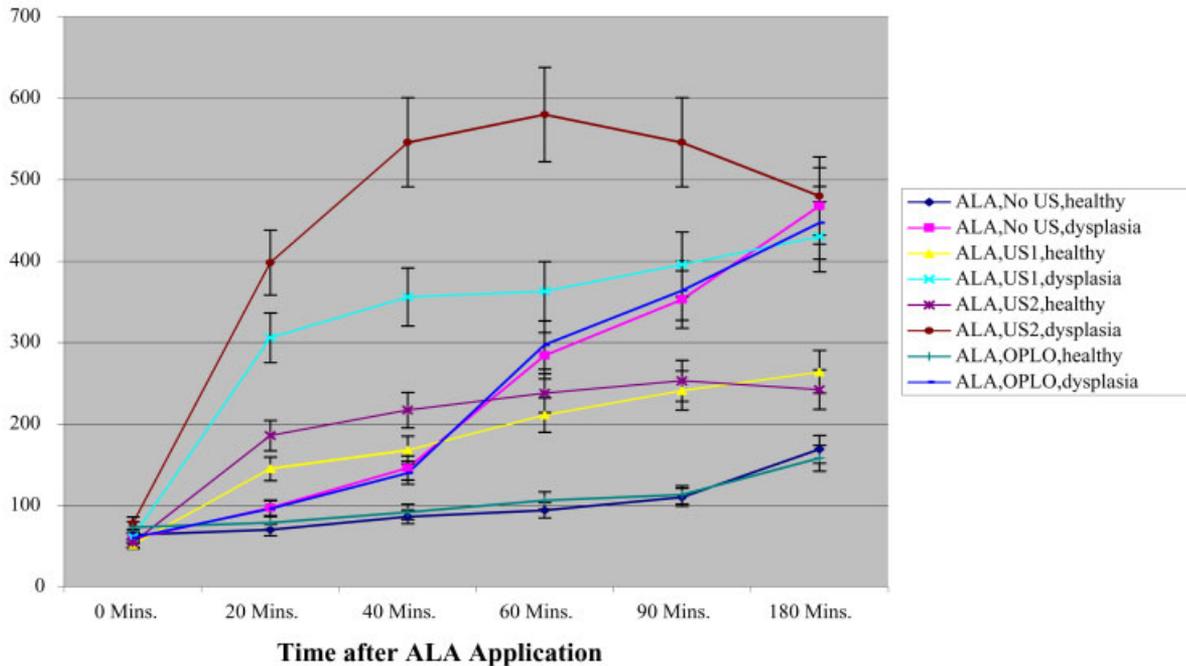
One-way ANOVA was used to detect independent effects of pathology on LIF. A two-way ANOVA model was used to test simultaneously for independent effect of pathology and of ultrasound treatment modality, and interaction effects. We used non-parametric models and exact methods appropriate for small samples to compare the different ultrasound parameters.

RESULTS

Oral Pluronic Lecithin Organogel (OPLO) consisting of lecithin compounds in organic solvents can assist the transdermal transport of drugs, increasing the transport rates of small molecules such as ALA by approximately one order magnitude [17]. Due to its high viscosity, it adhered well to the cheek pouches. Dissolving ALA in OPLO prior to its application to the cheek pouches did not significantly ($P < 0.05$) affect time-based fluorescence development in healthy and dysplastic tissues (Fig. 1). Maximum fluorescence levels in all tissue types also did not differ significantly between control and OPLO groups ($P < 0.05$) (Fig. 1).

Application of ultrasound at 1 and 3.3 MHz to cheek pouch tissues with a total intensity of $\sim 0.3 \text{ W/cm}^2$ for a duration of 60 seconds significantly ($P < 0.05$) accelerated time-based fluorescence development in healthy and dysplastic tissues (Fig. 1). As soon as 20 minutes after application of ALA and ultrasound, strong PpIX fluorescence was visible, compared with the 180–240 minutes

Time-Based PpIX Fluorescence



U1: 3.3 MHz, 50% duty cycle, 1J, 60 sec.

U2: 1 MHz, 50% duty cycle, 1J, 60 sec.

Fig. 1. Time-based protoporphyrin IX (PpIX) fluorescence intensity (excitation 405 nm; detection 635 nm) 0–180 minutes after topical 5-aminolevulinic acid (ALA) application to the hamster cheek pouch. [Figure can be viewed in color online via www.interscience.wiley.com].

currently needed to achieve comparable fluorescence development. Using lower-frequency ultrasound (1 MHz) in healthy tissues, PpIX fluorescence levels at 20 minutes were comparable to those achieved in tissues without ultrasound at 180 minutes. In dysplastic tissues, the effect was similar, with fluorescence intensities in ultrasound-treated tissues at 20 minutes comparable to those in tissues without ultrasound treatment at 180 minutes. Thus, a significant ($P < 0.05$) acceleration effect was seen in healthy and dysplastic tissues. Although a significant ($P < 0.05$) acceleration effect was also achieved using ultrasound at 3.3 MHz, this frequency was associated with a significantly ($P < 0.05$) smaller acceleration effect than the ultrasound at 1 MHz frequency. Moreover, there appeared to be a consistent and significant ($P < 0.05$) reduction in the fluorescence maximum reached over time as compared to the non-ultrasound and the 1 MHz ultrasound groups.

DISCUSSION

Possibly the lack of accelerating effect of OPLO on ALA-induced fluorescence development may be linked to interactions between the OPLO and the ALA or an altered state

of the OPLO at the low pH levels used to maintain ALA stability. Locally applied low-frequency ultrasound application greatly accelerated the development of ALA-induced PpIX fluorescence. These results confirm other studies demonstrating effects of ultrasound on transdermal penetration of drugs, although the reported results range from very strong to minimal acceleration, to actual hindrance, depending on the drug and ultrasound parameters used [20]. Contributing mechanisms postulated include disruption of membrane lipid bilayers and enhanced convection to generation of transient pores in cell membranes [20]. As in those studies, the effects of ultrasound were shown to vary depending on the parameters used, more work is needed to identify optimal ultrasound parameters and techniques, and to ensure that diagnostic sensitivity and specificity are not affected by ultrasound.

CONCLUSION

Low-frequency ultrasound can significantly accelerate ALA-induced fluorescence development, whereby a frequency of 1 MHz was shown to be more effective than 3.3 MHz.

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