Abstract: In this work, we utilized photoacoustic imaging (PAI) with co-registered ultrasound (US) to non-invasively assess salivary gland function in vivo. A significant increase in salivary gland oxygen saturation was observed on PAI within minutes after gustatory stimulation of healthy mice reflective of the hyperemic response associated with secretion of saliva. Good correlation was seen between PAI and Doppler sonography. Salivary adenoid cystic carcinomas showed higher oxygen saturation compared to surrounding salivary gland tissue. Our results demonstrate the potential clinical utility of PAI for visualization of salivary gland physiology and pathology.

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OCIS codes: (170.5120) Photoacoustic imaging; (170.3880) Medical and biological imaging; (170.7170) Ultrasound; (170.4940) Otolaryngology; (170.4580) Optical diagnostics for medicine.

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1. Introduction

Saliva is an integral component of oral physiology and critical for maintenance of oral health [1]. Impaired production and/or secretion of saliva can have a significant impact on speech, taste and swallowing function and lead to increased risk of dental caries and oral infection [2,3]. Several conditions ranging from inflammation, autonomic neuropathies and autoimmune disorders to neoplasms of the salivary glands can result in altered salivary secretion [4–6]. Salivary gland dysfunction is also one of the most common and severe side-effects of radiotherapy (RT) in patients with head and neck cancer [7,8]. In this regard, non-invasive imaging can enable visualization of salivary gland morphology and provide quantitative estimates of salivary gland function [9].

Photoacoustic imaging (PAI) is a promising new hybrid imaging modality which utilizes laser illumination of optical species within tissue to produce pressure waves detectable by standard ultrasound [10,11]. The ability of PAI to utilize endogenous signal from hemoglobin for relative measurements of tissue oxygenation and hemoglobin content without the use of ionizing radiation or radioactive tracers is an important clinical advantage. Consequently, there is widespread clinical interest in developing PAI as a diagnostic tool in breast, prostate, ovarian, and thyroid cancer [12–15]. In this work, we evaluated the diagnostic potential of PAI for functional salivary gland imaging in vivo. To this end, we conducted PAI studies in mice to (i) examine changes in salivary gland hemodynamics under physiologic conditions, and, (ii) assess the hemodynamic profiles of healthy and malignant salivary gland tissue.

2. Methods

2.1 Experimental setup and imaging procedures

PAI, Doppler, and B-mode ultrasound procedures were performed using the Vevo LAZR (VisualSonics Inc., Toronto) 21 Mhz linear-array transducer system. The photoacoustic parameters used were; Transducer: LZ-250, Frequency: 21 MHz, Depth: 20.00mm, Width: 23.04mm, Wavelength: 750/850nm, Threshold Hbt: 20, Acquisition: sO2/Hbt. Power Doppler sonography parameters used were; Transducer: LZ-250, Frequency: 16 MHz, Pulse Repetition Frequency: 2 kHz, Depth: 20.00 mm, Width: 23.04 mm, Beam Angle: 0°, Sensitivity: 5, Wall Filter: Medium. For in vivo experiments, mice were anesthetized using 2.5% Isoflurane (Benson Medical Industries, Markham, ON, Canada), placed on a heated platform and secured down using tape (Fig. 1(a)). B-mode ultrasound was used to visualize the salivary gland (Fig. 1(b)), and a 3-dimensional region was acquired for the entire submandibular and sublingual glands.
Fig. 1. Experimental set up for salivary gland PAI. (a) Animals were positioned supine on a heated platform and the transducer was positioned above the neck. (b) The neck region was scanned using standard B-mode ultrasound in order to locate and visualize the submandibular/sublingual salivary glands (outlined in white).

For combined PAI and Doppler studies, a series of single slice and 3-dimensional (3D range: 4.95 mm, step size: 0.248 mm) images were acquired. PAI and Doppler data sets were acquired before and following gustatory stimulation (up to 10 mins) using 10% citric acid applied to the dorsum of the tongue. Animals were removed from the imaging platform after completion of the imaging procedure and monitored to ensure full recovery. All imaging data sets were analyzed using the Vevo LAB (Ver 1.7.2) workstation software. PAI based measurements of salivary gland oxygen saturation were calculated using the two-wavelength approach (750/850 nm) based on a previously reported algorithm [16–18]. A region of interest (ROI) was traced around the salivary gland for a single, central slice of the submandibular/sublingual salivary gland. Calculated oxygen saturation values are reported as %O$_{2}$ average or %O$_{2}$ total. The %O$_{2}$ average (reported in Fig. 2) represents the average value calculated from pixels with an oxygen saturation estimate. The %O$_{2}$ total values total (reported in Figs. 3 and 4) represent the average value calculated from all pixels with the ROI of the entire salivary gland (including zero/estimate-void pixels). For combined PAI and Doppler studies, an ROI was traced for the entire 3-dimensional region (4.95 mm) of the salivary gland. Comparisons of PAI and Doppler are based on measurements from the same 3D region. Doppler values are reported as percent vascularity (PV) based on the percent of Doppler signal detected within the whole gland. The relative percent change in PAI and Doppler signal following stimulation was calculated using the formula % change = [(Post-stim – Pre-stim)/Pre-stim]*100. For salivary gland cancer studies, reported %O$_{2}$ levels are representative of a single slice of the salivary gland located at the central slice of the tumor. Statistical analysis and graphical display of data was performed using Graphpad software (Version 5.00 for Windows, GraphPad Software, SanDiego California USA, http://www.graphpad.com). Error bars represent the standard error of the mean.

2.2 In vivo studies

All experimental studies were performed under protocols approved by the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute (RPCI). Male athymic nude mice (NCr-nu/nu) purchased from Harlan Strague-Dawley (Indianapolis, IN) were housed in microisolator cages, provided standard chow/water and maintained on 12-h light/dark cycles in a HEPA-filtered environment. In the initial salivary gland stimulation studies mice (n = 5) were imaged with PAI alone. In the second study mice (n = 8) were imaged with both PAI and Doppler. For the salivary gland cancer model, patient-derived adenoid cystic carcinoma (ACC) xenografts were surgically implanted into the salivary gland of naïve mice (n = 4) under US guidance. PAI was performed prior to tumor implantation and 40 days following implantation.
3. Results

3.1 Temporal changes in salivary gland hemodynamics in response to gustatory stimulation

We first examined temporal changes in oxygen saturation of salivary glands in naive mice before and after citric acid stimulation (Fig. 2). PAI was able to detect %sO$_2$ of salivary glands at baseline (pre-stim) (Fig. 2(a)). A minute following citric acid stimulation (post-stim) a marked increase in PAI %sO$_2$ signal was observed (Fig. 2(b)). This increase in signal was sustained even at 10 minutes post stimulation (Fig. 2(c)). Quantification of %sO$_2$ average levels revealed a significant increase at one minute post-stimulation (54.8 ± 19.2%, $p < 0.05$) compared to baseline estimates (39.3 ± 9.9%) which was sustained for 10 minutes following stimulation (61.3 ± 8.7%, $p < 0.01$).

![Fig. 2. PAI of salivary gland hemodynamics following gustatory stimulation. (a) Dynamic series of PA oxygen saturation maps of salivary glands before (a; pre-stim), 1 min (b) and 10 mins (c) after citric acid stimulation. (d) A significant increase in salivary gland %sO$_2$ was observed post stimulation. Reported %sO$_2$ average values represent the average value calculated from pixels with an oxygen saturation estimate.](image)

3.2 Comparative evaluation of PAI and Doppler sonography of salivary gland vascularity

We next performed studies comparing PAI and Doppler sonography in order to validate PAI measured changes in salivary gland hemodynamics following stimulation. Figure 3 shows representative PA and Doppler images overlaid on B-mode US images of the mouse salivary gland (Fig. 3(a)). Gustatory stimulation resulted in an increase in salivary gland hemodynamics that was observed with both PAI and Doppler. To enable accurate comparison of PAI and Doppler data, the relative change in PAI and Doppler signal was calculated for the 3D ROI covering the entire gland. As a result, the values of oxygen saturation (%sO$_2$ total) measured for the whole gland were lower than the values reported in Fig. 2. Nevertheless, consistent with the initial observations, a significant increase in %sO$_2$ levels (13.7 ± 6.8% to 20.1 ± 7.9%, $p < 0.001$) was observed with PAI following citric acid stimulation (Fig. 3(b)). In agreement with the PAI data, Doppler sonography also showed an increase in percent vascularity (from 23.7 ± 5.2% to 33.8 ± 11.3%, $p < 0.01$) following stimulation (Fig. 3(c)). A good correlation ($r = 0.8168$) was observed when comparing the relative percent increase in PAI %sO$_2$ levels and Doppler percent vascularity (Fig. 3(d)).
Fig. 3. Comparative PAI and Doppler sonography in mouse salivary gland. (a) Spatially-coregistered PA (upper panel) and Doppler images (lower panel) before and after gustatory stimulation. Within 1 minute follow stimulation an increase in both PAI and Doppler signal was observed. Stimulation resulted in a significant increase in total %sO2 levels (PAI; b) and percent vascularity (Doppler; c). (d) The relative percent change in PAI and Doppler signal following stimulation revealed a good correlation between the two data sets.

3.3 Hemodynamics of salivary gland cancer

Fig. 4. Influence of focal salivary gland malignancy on hemodynamics. (a) B-mode ultrasound (left) and PA (right) images of mouse salivary gland prior to (baseline) and 40 days (d40i) following implantation of adenoid cystic carcinoma xenografts. At d40i the tumor was clearly visualized on B-mode ultrasound images (outlined in yellow). (b) Sub region analysis of the tumor bearing salivary gland into peritumoral (outlined in red) and distant salivary gland regions (outlined in white) showed lower %sO2 levels in the peritumoral region compared to distant salivary gland tissue and the tumor.

We examined changes in salivary gland hemodynamics associated with tumor growth in mice bearing salivary adenoid cystic carcinomas. A series of PA images were acquired prior to tumor implantation to determine the %sO2 levels in the naïve salivary gland (Fig. 4(a) Top).
By 40 days post implantation the tumor was detected growing in the salivary gland (Fig. 4(a) Bottom). PA images revealed a drastic reduction in salivary gland $\%sO_2$ levels in the the region surrounding the tumor while distant salivary gland tissue showed comparable levels of oxygen saturation (Fig. 4(b)).

4. Discussion and conclusion

In this work, we have demonstrated the ability of PAI to non-invasively assess hemodynamic changes in salivary glands under physiologic (gustatory stimulation) and pathologic (cancer) conditions in vivo. Studies in preclinical models have previously highlighted the relationship between salivary blood flow and secretion [19–22]. In our studies, citric acid stimulation resulted in a marked increase in salivary gland oxygenation levels as measured with PAI that persisted out to 10 minutes. Co-registered Doppler sonography validated our PAI observations. Doppler sonography is a clinical imaging technique that has been used previously for imaging the vascularity of salivary glands in vivo [23,24]. Stimulation of the salivary gland resulted in an increase in both PAI $\%sO_2$ (55.1% increase) and Doppler percent vascularity (40.7% increase). A good correlation was observed comparing the relative percent increase in PAI $\%sO_2$ levels and Doppler percent vascularity following stimulation. Consistent with clinical observations [25] in salivary gland malignancies, adenoid cystic carcinomas exhibited higher $\%sO_2$ levels compared to neighboring salivary gland tissue. Interestingly a reduction in $\%sO_2$ was seen in the region of the salivary gland surrounding the tumor, while distant areas were less affected. This may be due to the shunting of blood supply to support the growth and development of the tumor, as $\%sO_2$ levels were highest in the tumor.

There are limitations to our study. The anatomy of the salivary glands in mice is unique with the submandibular and sub-lingual glands forming a joint complex separated by connective tissue in the cervical region. In our studies, we did not attempt to distinguish the PA signal from the two glands. Another limitation is the sample size since our studies were designed to examine the feasibility for salivary gland imaging. Future studies will focus on examining the hemodynamic differences between healthy and malignant salivary glands using larger experimental cohorts to validate our preliminary observations. Such studies could also assess the response of salivary gland cancers to conventional and novel targeted therapies.

In conclusion, we have demonstrated for the first time, the utility of PAI for assessment of salivary gland physiology and pathology in vivo. Our findings have several important clinical implications. First, while Doppler can provide information on blood flow, PAI can simultaneously provide information on hemodynamics (hemoglobin concentration) and oxygenation of tissues. This is particularly important in the context of malignancies in which blood flow measurements alone may not accurately reflect the tumor microenvironment. Our results suggest that PAI can potentially be applied for examination of neoplastic and inflammatory diseases of salivary glands (e.g. Sjogren’s syndrome). Second, mucositis and xerostomia are two of the major side effects associated with radiation-induced damage to the salivary glands. Given the influence of oxygenation on radiotherapeutic efficacy, PAI could be utilized for early evaluation of radiation-induced salivary gland injury in patients with head and neck cancer. PAI could also enable longitudinal monitoring of salivary gland function and efficacy of ‘radioprotective’ agents.

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