RESEARCH ARTICLE

Discovering a new anatomy: exploration of oral mucosa with ultra-high frequency ultrasound

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Objectives: Ultra-high frequency ultrasound (UHFUS) is a recently developed diagnostic technique involving the use of ultrasound frequencies up to 70 MHz, allowing to obtain 30 µm resolution of targets located within 1 cm from the surface. Oral mucosa can be affected by diverse pathological conditions, which are currently investigated by means of clinical examination. In this scenario, intraoral UHFUS can provide additional information and support clinical assessment of oral mucosa. In this preliminary study, typical features of normal oral mucosa are described, in order to set a benchmark for the future identification of oral soft tissue alterations.

Methods: Twenty healthy subjects (10 males and 10 females, mean age 30 years) were enrolled and underwent intraoral UHFUS examination. In all the subjects, tongue, buccal mucosa, gingiva, lip mucosa, and palate were scanned, and images acquired. Intraoral UHFUS scan included Brightness-mode and Doppler mode acquisitions performed with a standardized protocol. UHFUS images were postprocessed and analyzed using a dedicated software. UHFUS-based biomarkers (epithelial thickness, echogenicity, and vascularization) were employed for image description.

Results: Normal oral anatomy of the different sites analyzed was described. For all the sites, UHFUS biomarkers were characterized, and information on typical aspect of oral mucosa was retrieved.

Conclusions: In this explorative study, we suggest a potential role for intraoral UHFUS in the study of oral mucosa, giving insights into the possibility to improve the assessment, diagnosis, and management of the conditions involving oral mucosa. UHFUS seems a promising tool, which could potentially support clinical examination in daily oral medicine practice.

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Introduction

Oral diseases are currently diagnosed by means of clinical examination and supported by surgical biopsy procedures. In particular, daily oral medicine practice is

lacking the use of a routine diagnostic support to the visualization of anatomical structures located beyond the mucosal surface. Considering the growing interest in minimally invasive diagnosis, the possibility of having an imaging technique dedicated to the investigation of oral soft tissues and their alterations may be instrumental

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Figure 1 The Vevo MD equipment employed in the study (A) and the 70 MHz probe UHF 70 (B).

to support the clinical diagnosis. Therefore, attempts to introduce conventional ultrasonography (US) to the diagnostic work-up of pathological conditions of the head and neck have been done, exploiting the unique features of this technique in terms of limited invasiveness, repeatability, and cost efficiency.

In the literature, both extraoral and intraoral applications of US have been reported. Extraoral applications mainly focus on the characterization of oral and maxillofacial swellings of various origin, while intraoral applications mostly involve the study of malignant lesions in terms of tumor thickness and depth of invasion.¹⁻⁴ Considering extraoral applications, Chandak *et al*¹ described the US appearance of inflammatory swellings (cellulitis and abscesses), cystic swellings, benign neoplasms (hemangioma, lymphangioma, lipoma, and fibroma), and malignant neoplasms (squamous cell carcinoma, melanoma, fibrosarcoma, and carcinoma ex pleomorphic adenoma), and described each lesion according to shape, margins, echogenicity, homogeneity, presence of necrosis/calcifications, and posterior echo. Wu *et al*² applied US to the study and characterization of parotid masses, in order to discriminate between benign and malignant diseases, considering also dimensions and vascularization of the mass. Yamamoto *et al*³ applied 12 MHz frequencies to the intraoral study of tongue carcinoma, and combined Brightness-mode (B-mode) parameters, such as the boundary line of the invasion front, with power Doppler information on vascularization. Sugawara *et al*⁴ employed both linear (12.5 MHz) and convex (6.5 MHz) probes to investigate various types of lesions prior to surgery, analyzing borders, size, location, depth, presence of a capsule, and vascularity of the mass.

However, the extreme variability of the employed frequencies and acquisition protocols hinders the direct comparison between current and previous studies. In

the literature, US frequencies ranging between 10 and 15 MHz have been reported for the intraoral study of oral lesions, with wide differences in terms of resolution and depth of penetration of the US beam. Ten MHz frequencies can reach up to 60 mm below the surface with a 0.15 mm axial resolution, while 15 MHz frequencies just arrive at 40 mm with a 0.10 mm axial resolution.⁵

In fact, it is well-known that an increase in US frequencies leads to lower depth of penetration, but also provides higher resolution imaging. Therefore, in this scenario, ultra-high frequency ultrasound (UHFUS) could become a valuable instrument for the study of oral superficial structures. UHFUS is a recently introduced ultrasonographic technique involving the use of ultrasound frequencies between 30 and 100 MHz, providing high spatial resolution (pixel size as small as 30 μ m) at the expense of the depth of penetration, which is limited to 10 mm from the surface when using frequencies of 70 MHz. Initially introduced in the pre-clinical setting and mainly employed in the study of animal models,⁶⁻⁸ UHFUS has proven to allow correlation between US images and histology with high specific value, in particular being able to discriminate healthy and pathological tissues, with accuracy comparable to biopsy.^{9,10} The possibility of obtaining high-resolution images has thus expanded UHFUS applications to human studies in several medical fields.¹¹⁻¹⁶

The intraoral application of UHFUS has been previously explored in the diagnosis of several oral pathological conditions, with promising results.¹⁷⁻¹⁹

In this preliminary evaluation, we suggest a potential role for intraoral UHFUS and provide a description of normal anatomical structures of the oral mucosa, in order to identify the typical features of normal oral mucosa as evaluated by means of UHFUS, and to establish the ground for further studies investigating UHFUS applications to oral diseases.

Methods and materials

Patient flow

UHFUS scan of 20 healthy subjects (10 males and 10 females, mean age 30 years) was performed with a 70 MHz probe (Vevo[®] MD, VisualSonics, Toronto, Canada), available in our institute since 2016 and representing one of the first UHFUS systems for clinical use (Figure 1). The study protocol was approved by the local Ethics Committee, and all subjects gave written consent to undergo UHFUS examination. The study was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects.

UHFUS acquisition protocol

UHFUS scans were performed alternatively by a radiologist expert in US (A) and by an oral surgeon expert in dentomaxillofacial radiology (B). They also served

as the examiners of the UHFUS images. Prior to the beginning of the study, training and calibration were conducted. The two examiners were calibrated in terms of UHFUS scan performance, image acquisition, and image analysis on images that were not part of the research. When high concordance (Cohen's κ value >0.70) was obtained between operators, data collection and patients' enrollment in the study began.

Intraoral UHFUS scan was carried out on five anatomical sites, namely tongue, buccal mucosa, gingiva, lip mucosa, and palate. Each site was scanned accordingly to the following protocol:

The buccal mucosa was bilaterally scanned from the upper buccal fornix to the lower buccal fornix and from the retrocommissural area to the retromolar trigone;

The tongue was evaluated both in its dorsal and in its ventral aspect on both sides;

The gingiva was studied in the buccal aspect of the upper and lower dental arch bilaterally, and the scan was extended to the second premolar;

The upper and lower lip were scanned entirely on both sides;

The palate was evaluated from the incisive papilla to the limit between hard and soft palates, on both sides.

For the management of cross-infection, a disposable sterile probe cover was employed to prevent contact between oral mucosa and the probe. Ultrasound gel was employed as couplant material and was applied both on the probe in a thin layer prior to putting the cover and on the mucosal surface.

Each site was scanned twice in B-mode and once in Doppler mode. Doppler mode allows to perform the modal qualitative evaluation of intensity and direction of the blood flow in the selected box on B-mode images.

B-mode and Doppler mode acquisition were performed for each anatomical site, using a standardized preset and keeping the parameters constant (gain, time gain compensation, dynamic range, mechanical index, and thermal index). Scan depth and focus position were adjusted depending on the site investigated to optimize the scan. Axial and longitudinal acquisitions of B-mode images were performed for all the mucosal sites. The degree of vascularization of each site was assessed using Doppler mode with a setting scale of ± 1.9 cm/s and low pulse repetition frequencies, in order to standardize image acquisition and detect blood vessels with low flow rate in the interval between ± 1.9 cm/s.

Figure 2 Example of epithelial thickness measurement. Epithelial thickness is measured as the distance between the mucosal surface and the lower margin of the submucosa.

Image post-processing and analysis of UHFUS-based biomarkers

DICOM format images were processed using Horos software (<https://horosproject.org/>, Nimble Co LLC d/b/a Purview, Annapolis, MD, USA). Three parameters were considered as UHFUS-based biomarkers employed to describe the characteristics of each site analyzed.

Total epithelial thickness was measured as the distance between epithelial surface and the lower margin of submucosa (Figure 2). Mean thickness values were reported for each site. Three measurements were carried out for each subject, and a mean value was obtained for each subject in each site. The final value reported was the mean thickness encountered in the 20 subjects analyzed.

Echogenicity was described both qualitatively and quantitatively. A qualitative description of each area was performed in terms of echogenicity of each layer encountered in the anatomical site described. Considering the quantitative analysis, Horos software automatically samples gray-level distribution inside a selected region of interest (ROI), assigning to each pixel a variable value coded with a number between 0 and 256. We placed a ROI of fixed area at the interface between mucosa and submucosa, and retrieved data on gray-level distribution pattern (mean, standard deviation (SD), min and max) (Figure 3). As evaluated for thickness values, mean echogenicity was reported for each site. Three measurements were carried out for each subject, and a mean value was obtained for each subject in each site. The final value reported was the mean echogenicity encountered in the 20 subjects analyzed.

Figure 3 Example of ROI positioning. A ROI of fixed area is placed at the interface between mucosa and submucosa.

Finally, vascularization was visually categorized as low, moderate, and intense. Spectral analysis was not performed due to the lack of the specific software. The evaluation of vascularity was partially hindered by the limited performance of Doppler mode on the equipment employed, which appeared to be extremely sensitive to movement.

Figure 5 UHFUS scan of the tongue. The images report the acquisition of the dorsal area of the tongue. The superficial hypoechoic layer corresponds to mucosa. Below mucosa, submucosa can be seen as a hyperechoic stratum. Deeper in the epithelium muscular fibers can be observed. (A) B-mode image of the tongue; (B) scheme reporting the anatomical structures: intrinsic muscles can be identified due to muscular fiber orientation; (C) Doppler mode image of the tongue, showing intense vascularization.

Results

UHFUS findings: description of normal oral anatomy

Tongue (Figures 4–6): Epithelial thickness: mean thickness of mucosa 0.4 mm; mean thickness of submucosa 0.5 mm.

Echogenicity: At UHFUS, tongue shows a hypoechoic superficial layer corresponding to the dorsal mucosa. The underlying muscular layer is formed by extrinsic and intrinsic muscles, which have the appearance of a thick inhomogeneous layer at UHFUS scan. On the tongue dorsum, it is possible to highlight button-like structures, representing filiform papillae (Figure 6A). Moreover, tongue septum can be clearly identified in the median area of the tongue as a markedly hyperechoic linear structure (Figure 6B).

Gray value distribution: mean 116.18; SD 29.81; min 35; max 200.

Figure 4 Probe placement inside the mouth to image tongue surface. The tongue is gently stretched out of the mouth by means of a sterile gauze and kept still to avoid movement artifacts.

Figure 6 A) Close-up on the structure of the filiform papillae on the anterior dorsal part of the tongue (mean dimensions 0.039 mm); B) Tongue septum, which can be seen as a markedly hyperechoic linear area (black arrow).

Vascularization: intense vascularization. Several blood vessels are visible as hypo/anechoic band-like or circular structures.

Buccal mucosa (Figures 7–9): Epithelial thickness: mean thickness of mucosa 0.5 mm; mean thickness of submucosa 1.2 mm; mean thickness of muscular layer 1.0 mm.

Echogenicity: UHFUS of the internal lining of the cheek shows a superficial, narrow, hypoechoic layer

Figure 7 Probe placement inside the mouth to image the buccal mucosa. Patient's mouth is open and the head slightly turned towards the operator (imaging of the right buccal mucosa). No additional tissue divarication is required.

corresponding to the mucosa, and an underlying hyperechoic thicker layer corresponding to the submucosa. Under mucosa and submucosa, several lobular hypoechoic structures representing the adipose tissue surrounded by connective tissue can be detected. In this stratum, it is possible to identify muscular tissue, visible as an inhomogeneous, hypoechoic linear structure with internal hyperechoic fibrils.

Gray value distribution: mean 133.9; SD 26.32; min 73; max 199.

Vascularization: moderate vascularization. Below the muscle, it is possible to identify the facial artery as a linear anechoic structure. In Figure 9, pulsed wave Doppler mode of the facial artery is shown.

Gingiva (Figures 10 and 11): Epithelial thickness: mean thickness 1.2 mm.

Echogenicity: UHFUS examination shows a thin superficial layer (mucosa) in continuity with the underlying submucosa; mucosa surrounds and adheres to the alveolar bone. In some cases, blood vessels can be observed as roundish structures in the thickness of the mucosa.

Gray value distribution: mean 162.95; SD 31.74; min 79; max 253.

Vascularization: moderate vascularization. Several blood vessels can be observed in Doppler mode, mainly characterized by low velocity.

Lip (Figures 12 and 13): Epithelial thickness: mean thickness of mucosa 0.3 mm; mean thickness of submucosa (excluding minor salivary glands) 0.9 mm.

Echogenicity: UHFUS of the lip shows a superficial hypoechoic layer corresponding to the mucosa and an underlying hyperechoic layer corresponding to the submucosa. Inside the submucosa, salivary glands can be detected as round, hypoechoic, homogeneous structures. Beneath the submucosa, muscular fibers can be seen as thick hypoechoic structures with hyperechoic fine fibrils. Deeper in the epithelium, a thick hyperechoic connective tissue with several blood vessels is present.

Gray value distribution: mean 112.45; SD 28.41; min 53; max 202.

Vascularization: moderate vascularization. Increase in vascularity can be observed around minor salivary glands. Occasionally, terminal branches of labial arteries can be observed as roundish pulsing structures.

Palate (Figures 14 and 15): Epithelial thickness: mean epithelial thickness 2.5 mm.

Echogenicity: UHFUS shows a superficial narrow hypoechoic layer corresponding to the mucosa, with an underlying thick hyperechoic layer corresponding to fibrous submucosa. The markedly hyperechoic band-like structure with posterior acoustic shadowing which can be seen beneath the submucosa is the cortical bone of the hard palate.

Gray value distribution: mean 197.25; SD 29.41; min 92; max 254.

Figure 8 UHFUS scan of the buccal mucosa. The images report the acquisition of the central area of the buccal mucosa. Superficial hypoechoic structure corresponds to mucosa, while submucosa can be recognized as a hyperechoic stratum. In the submucosa, facial artery can be observed as a linear structure, characterized by intense blood flow (A) B-mode image of the buccal mucosa; (B) scheme reporting the anatomical structures: the facial artery can be clearly identified, and the buccinator muscle can be seen below the artery; (C) Doppler mode of the buccal mucosa, showing intense blood flow in correspondence with the facial artery.

Vascularization: low vascularization. Vascularization is scarce and present in the deeper layers of the epithelium.

Discussion

In oral medicine and surgery, diagnosis of oral soft tissue lesions is predominantly clinical, with the support of surgical biopsy to obtain histological confirmation. The complexity of oral anatomy and the variability of presentation of oral diseases can make diagnosis equivocal, thus highlighting the need for non-invasive diagnostic imaging to support clinical examination. Defining characteristics of oral mucosa and delineating oral anatomy preoperatively could improve the performance of surgical biopsy, both in terms of sampling and preserving vascular and nervous structures during surgery. Technological advancement with the introduction of UHFUS apparatuses for clinical use may therefore provide a solution to the need for diagnostic imaging of oral cavity in the diagnostic and surgical phases of the treatment.

Although some studies are present in literature, intra-oral US is not routinely employed in clinical practice. A first attempt to study the anatomy of the tongue and floor of the mouth was carried out by Shawker et al,²⁰ who evaluated the *in vivo* extraoral application of US and the *ex vivo* direct US application on tongue surface by means of a maximum of 7.5 MHz probe. Several studies investigated the role of US in the study of tongue diseases, with a particular focus on malignancies of the tongue.^{4,21–26}

Sugawara et al⁴ applied intraoral US of the tongue to the study of various lesions, selectively excluding OSCC and leukoplakia. Shintani et al²¹ employed a 7 MHz probe and aimed at evaluating tumor thickness and depth of invasion. Songra et al²² compared preoperative and intraoperative evaluation of tongue tumor thickness by means of a 5–10 MHz probe, in order to verify the presence of post-surgical clear resection margins.

Figure 9 Pulsed wave Doppler mode image of the facial artery. Doppler waveform is typical of a high resistance artery.

Figure 10 Probe placement inside the mouth to image the gingiva. Patient's mouth is partially open, in order to reduce the traction on surrounding soft tissues and to favor probe placement.

Figure 11 UHFUS scan of the gingiva. The images report the acquisition of the upper right canine area (tooth 1.3). Gingiva is characterized by a thin superficial mucosal layer and dense submucosa firmly attached to the underlying alveolar cortical bone. The presence of mineralized tissue (bone and teeth) causes posterior acoustic shadowing. Vascularity is predominantly present in the submucosal layer. (A) B-mode image of the gingiva; (B) scheme reporting the anatomical structures: the profile of the alveolar bone and of tooth 1.3 can be detected as a continuous white line hindering ultrasound penetration; (C) Doppler mode of the gingiva.

Other authors investigated the role of US in tongue carcinoma surgical planning, revealing also its potential role in predicting the presence of neck metastases.^{23–26}

The main variable between all the mentioned studies is related to the use of a wide variety of frequencies, which makes literature difficultly comparable. Moreover, to the best of our knowledge, we are the first to explore and report on the ultrasonographic appearance of normal oral anatomy by means of intraoral UHFUS.

In this scenario, UHFUS appears as an effective tool for the investigation of the oral mucosa. Ultra-high

Figure 12 Probe placement inside the mouth to image the lip mucosa. Patient's mouth is closed, in order to reduce the traction on the lip muscles. Gentle divarication is performed to facilitate the probe positioning on the inner side of the lip.

Figure 13 UHFUS scan of the lip mucosa. The images report the acquisition of the lower lip in the central area (coronal view). In the lip submucosa, groups of roundish structures can be observed, corresponding to minor salivary glands. Vascularity is distributed in the superficial layer of the mucosa and around the glands. (A) B-mode image of the lip mucosa; (B) scheme reporting the anatomical structures: in the submucosa, there are several minor salivary glands, appearing as multiple roundish structures with regular echogenicity corresponding to glandular pattern; below, the orbicularis oris muscle can be seen; (C) Doppler mode of the lip mucosa.

frequencies overcome the limitations of conventional US by allowing to image the first centimeter of the epithelium at a high resolution. UHFUS-based description of oral anatomy allows to set a benchmark for further investigation of pathological conditions affecting the oral cavity, as the knowledge of normal structure of oral mucosa is the first step before delineating the alterations that occur in course of disease.

Figure 14 Probe placement inside the mouth to image the palate. The patient's mouth is wide open, and the probe is inserted diagonally to reach distal part of the palate. No additional divarication on the surrounding tissues is required.

Figure 15 UHFUS scan of the palate. The images report the acquisition of the palate in the right lateral area, in correspondence with the palatal aspect of the premolars. the mucosa can be observed as a narrow hypoechoic layer. Submucosa appears as a strongly hyperechoic stratum adherent to the cortical bone of the hard palate. The presence of the bone causes posterior acoustic shadowing. (A) B-mode image of the palate; (B) scheme reporting the anatomical structures: a thick submucosal layer can be observed, corresponding to the fibrous submucosa tightly adherent to the underlying cortical bone; (C) Doppler mode of the palate.

However, some limitations have been encountered in the performance of this preliminary assessment, mostly related to the absence of a dedicated probe for intraoral UHFUS investigation. In particular, the size of the probe may affect the performance of intraoral UHFUS examination due to a difficulty in the accessibility to the posterior sites of the oral cavity. Moreover, in most cases floor of the mouth appears not accessible due to the shape of the probe. Considering probe angulation when performing UHFUS scan, the palate requires an oblique positioning of the probe due to the presence of teeth, which can potentially lead to artificially increased measurements for mucosal thickness, and make it difficult to perform Doppler mode and even to detect bone. In this sense, the implementation of a dedicated probe may improve the quality of UHFUS examination on a larger number of intraoral sites.

In this preliminary study, we aimed to describe the structure of the most common sites of localization of

oral lesions, in order to delineate which structures are normally present in absence of disease. Although this is a preliminary evaluation, the study of oral mucosa by means of UHFUS seems extremely promising, suggesting a potential role in supporting the clinician in the everyday clinical practice of oral medicine.

Conclusion

UHFUS could represent the missing jigsaw in the evaluation of oral mucosa, hopefully becoming a diagnostic support in the management of oral soft tissue lesions, in terms of diagnosis, surgical procedure, post-op discomfort reduction, and prevention/early detection of malignant transformation. Further studies are needed to explore the full possibilities of the technique and to validate intraoral UHFUS application and its potential impact on diagnosis and therapy.

Informed consent:

Informed consent was obtained from all individual participants included in the study.

Compliance with ethical standards

Research involving Human Participants and/or Animals: the study involves human subjects. The study has been conducted in respect of the declaration of Helsinki, and the study protocol has been approved by local ethics committee (CEAVNO).

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