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Biomechanical Characteristics of Collagen Fibrils of Human Free Gingival Tissues- Atomic Force Microscope Study

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ABSTRACT

The information of biomechanical properties is crucial in the study of biological tissue and its clinical relevance. 3mm x 3mm free gingival human tissues was taken using disposable punch biopsy (Accu sharp blade, India) and stored in 0°C Freezer. The sample was sectioned to a thickness of 10µm using high profile microtome blade (Leica 818, Germany) and cryostat (Leica CM1850UV, United Kingdom). The sample was analysed using Atomic Force Microscope (Nanowizard® 3, JPK Instruments, Germany) at room atmosphere. The collagen fibrils of the free gingival tissues appeared to be stacked in basket weave like structure. The mean value of free gingival collagen fibrils width and the length of D-band were 106.71±11.18nm and 65.82 ± 3.04nm respectively. The Young's modulus of collagen fibrils for human free gingival tissue at overlap region was 212.88 ± 242.58 MPa, whereas at the gap region was 207.00 ± 230.71 MPa. Within the limitation of the study, the collagen fibrils appeared to be stacked in basket weave-like structure. The length and width of the collagen fibril were similar to the values investigated using other techniques. There was significant linear relationship between Young's modulus of overlap and gap regions.

Keywords: Collagen fibrils, Human free gingival tissues, Atomic Force Microscopy

INTRODUCTION

Periodontium is the supporting structure around the tooth, which consists of the gingiva, periodontal ligament, cementum and the alveolar bone (1). The main function of the periodontium is to provide attachment for the tooth structure to the supporting bone, acting as an absorber to withstand the masticatory force (2). Gingiva is the masticatory mucosa which surrounds the cervical portion of the teeth and covers the alveolar crest, the interdental bony septa and the coronal portion of the alveolar process to the mucogingival junction (3). It can be divided into free gingiva, attached gingiva and interdental papilla (4). Free gingiva is the coronal border with scalloped margin, extending apically to the free gingival groove, while the attached gingiva is the continuation from the free gingiva to the mucogingival junction in the apical direction. Clinically healthy free gingiva presents as coral pink in colour, dull surface and firm in consistency as shown in Fig. 1 (1).

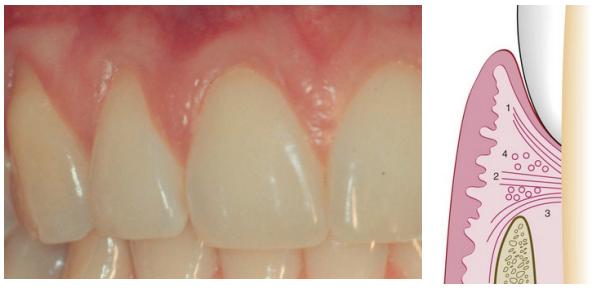

Figure 1: Free gingival tissues (5)

The lamina propria of gingiva tissues has dense network of collagen bundles and are highly vascularized (6). Collagen is the most abundant protein constituent of human tissues. Out of 20 types of collagen reported in the literature, Type I, II and III are the main types involved in the formation the large fibrillar structures. The main components of the healthy gingival tissue are Collagen Type I and III in the ratio of 7:1 (4). Each collagen fibre is made up of bundles of collagen fibrils which are arranged parallel to each other. Collagen fibril is formed by multiple tropocollagens arranged in a fixed staggered pattern and held by covalent cross-link at regular intervals, while tropocollagen is formed by the coiling of three similar polypeptide chains into a superhelix. Glycine is present at every third amino acid residue in the polypeptide chain (7).

In collagen fibrillar level, it has the “D-periodic” banding characteristic. D-periodic banding can be categorized into two, namely overlap and gap regions. Overlap region is formed by the overlaying arrangement of multiple tropocollagens, while the gap region has some voids in between the overlaying tropocollagen (Figure 2) (6, 8).

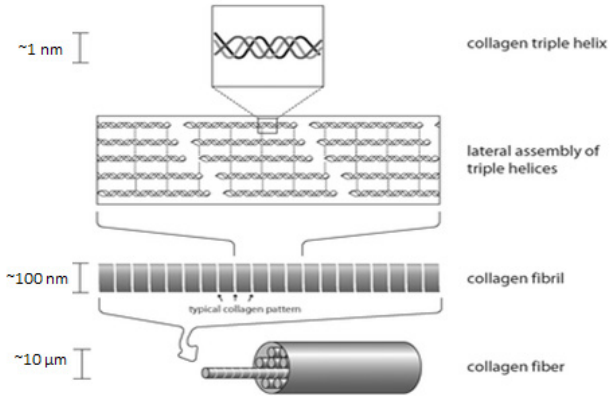

Figure 2: The hierarchical level of collagen (8)

The advent of Atomic Force Microscope (AFM), allows the tissue to be studied at nanometer scale. Atomic Force Microscope (AFM) was first introduced in 1986 (9-10). It is a device which uses a sharp tip, known as cantilever, to probe the sample surface and provide the three dimensional information on the surface topography as well as the mechanical properties of the samples (10). This device is increasingly popular in the study of biomaterials due to its capability to acquire data on structure and force interactions at different hierarchical level. The main working principle of AFM is the deflection of cantilever, without destroying the sample, as the tip approaches the surface (10). A position-sensitive photodetector (PSPD) is used in AFM to detect the deflection of the cantilever. It then reconstructs the topography of the sample (11-12).

Until recently, we are not aware of any reported information on the biomechanical properties of the gingival tissue. This could be due to the difficulty in getting the bulk tissue sample of the human gingival for a standard universal mechanical testing that typically requires 1.5 inches in length and 0.25 inches in width. However, this information can be made possible with the advent of atomic force microscope (AFM), which only requires sample with small dimension. Hence, the aims of this study were to (i) evaluate the structure and biomechanical properties of human free gingiva collagen fibrils, and (ii) relationship between the structure and its Young Modulus using atomic force microscope.

MATERIALS AND METHOD

Ethics application was approved prior to the conduct of the study from Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DF CD1509/0065(U)). Inclusion criteria includes: age 20-50 years and medically fit patient. Exclusion criteria includes cancerous tissue and tissue which has any signs and symptoms of inflammation. Suitable candidates were offered to be included in the study. Upon agreement, consent was taken from the patient.

The excised tissue was taken from patient with 3mm x 3mm disposable punch biopsy (Accu sharp blade, India). The punched tissue was placed in the aluminum foil and stored in the dry ice immediately. Subsequently, the tissue was washed with Phosphate Buffer Solution (PBS). (Figure 3 a) The sample was sectioned to a thickness of 10µm using high profile microtome blade (Leica 818, Germany) and cryostat (Leica CM1850UV, United Kingdom) (Figure 3 b), then mounted on the Superfrost Excell Microscope Slides (Fisherbrand, United States of America). The slides were then stored in 0°C Freezer. Each sample was thawed for approximately an hour prior to the scanning.

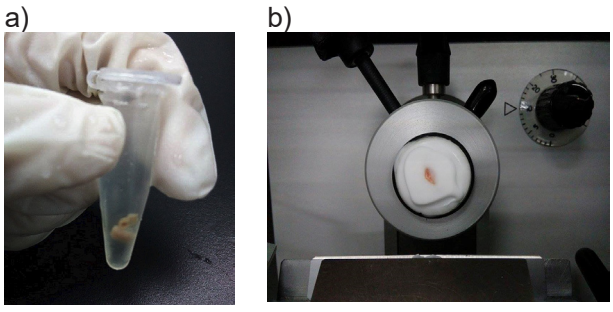

Figure 3 (a): Sample was washed with PBS (b) Mounting of the sample using cryostat.

The image scanning and force spectroscopy were carried out using Atomic Force Microscope (Nanowizard® 3, JPK Instruments, Germany) at ambient atmosphere. The sample was initially observed under the microscope (Top View, JPK Instruments, Germany) attached to the AFM to select the area of interest, and later cantilever was placed directly above the area. Contact mode cantilever of resonant frequency of 13 kHz and 0.2 N/m spring constant with tip radius of 0.01 nm were utilized in the imaging with constant force of 10 nN in air. The samples were scanned at the scale of 100µm x 100µm to identify the collagen fibrils then rescanned at 10µm x 10µm and finally at 1µm x 1µm. The characteristic of D-periodic banding was determined by measuring the length of gap and overlap regions of the collagen fibrils. Thus, D-periodic banding, including the length of the gap and overlap regions, the width of the collagen fibrils were measured from the 1µm image. The D-banding was measured along the longitudinal axis of the collagen fibrils, and the distance between the valleys of ten full width waves from the cross section profile was measured and divided by ten to obtain the D-banding. The width of single collagen fibril was measured perpendicular to the longitudinal axis of the fibrils.

The Young's modulus of the collagen fibrils was derived from fitting the contact region of the force curve using Hertzian model. The cantilever obeys Hooke's Law, F=kx, where F is the force exerted on the sample, k is the spring constant of the cantilever and x is its deflection. Considering the use of 0.01 nm paraboloid tip in this experiment, determination of Young's modulus for each sample was computed by AFM using the following equation:

$$z = \left[\frac{3.k.(d-d_0).(1-v^2)}{4.E.\sqrt{R}}\right]^{2/3} + (d-d_0) + z_0$$

where, z are the corresponding values of the cantilever deflection and the z-piezo extension at the contact point, *E* is the Young modulus, *v* is the Poisson ratio and *R* is the tip radius.

All images and force spectroscopy data were processed and analyzed using JPK SPM Data Processing software (JPK Instruments, Germany). The relationship between the measured parameters and the Young modulus was analysed using Spearman correlation test using SPSS version 22. The workflow is summarized in Figure 4.

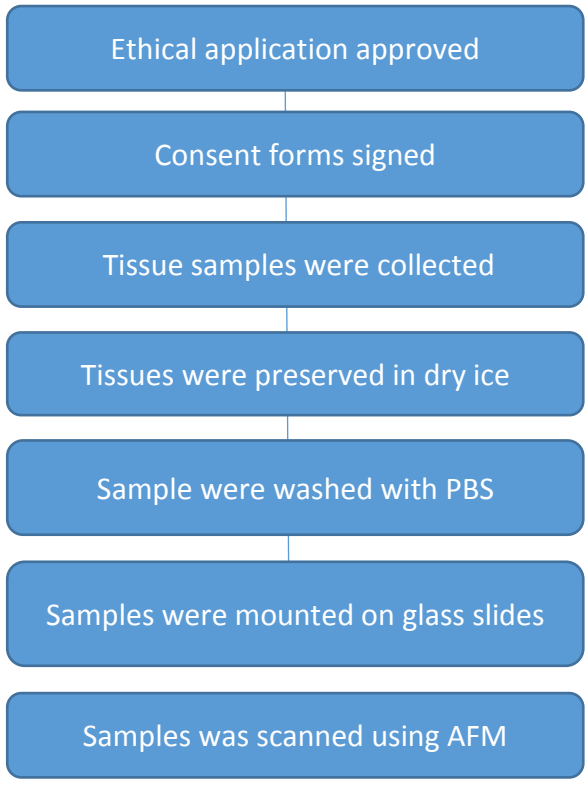

Figure 4: Summary of the work flow.

RESULTS

(1) Structure of free gingival collagen fibrils

The collagen fibrils of the free gingival tissues appeared to be stacked in basket weave-like structure as shown in Figure 4 (a1 & a2). At a higher magnification, the collagen fibrils emerged as rope-like structure with each D-band segments presented as dark and light banding as revealed in Figure 4 (b1 & b2).

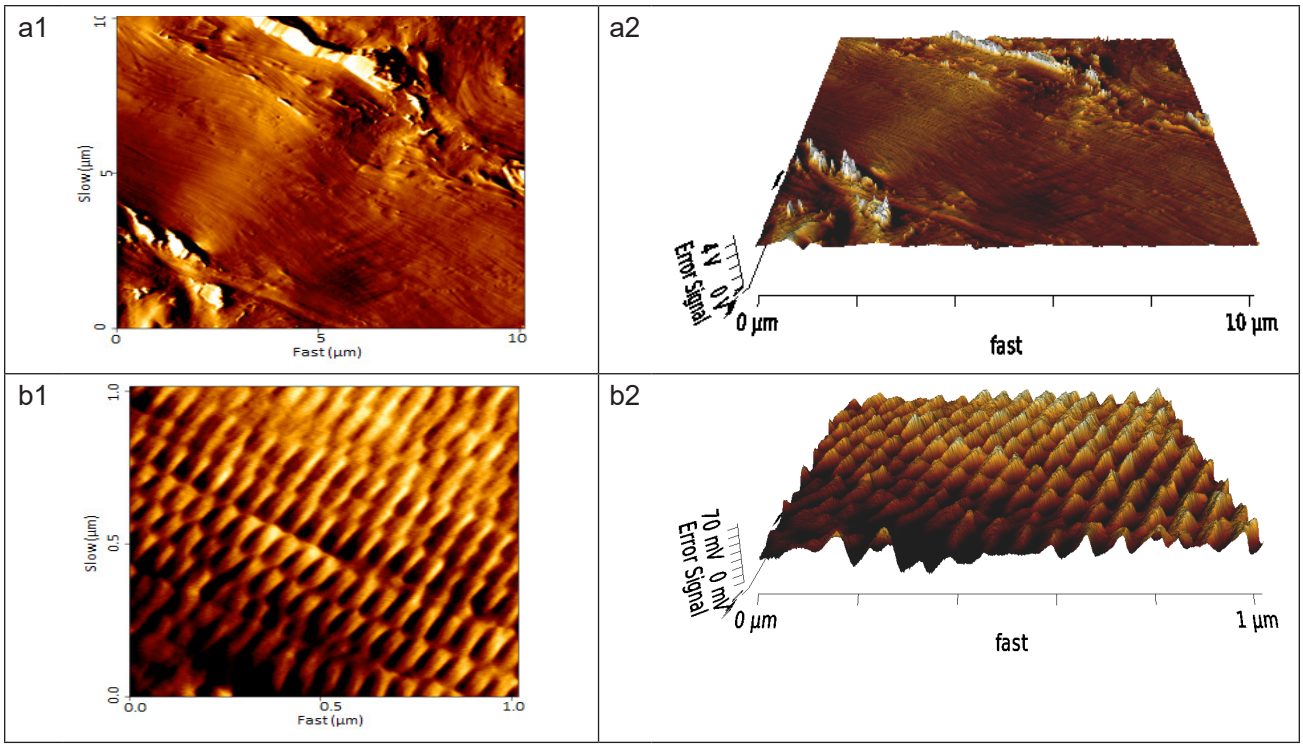

Figure 4 (a1 & a2): Basket-weave like structure of collagen fibrils; Figure 4 (b1 & b2): Rope-like structure of collagen fibrils

(2) Gap and overlap region, width and D-band of free gingival collagen fibrils

Figure 5 (a) shows the typical length of overlap and gap regions of collagen fibrils. The distance between the line A and B corresponds to the gap region while the distance between line B and C is corresponds to the overlap region.

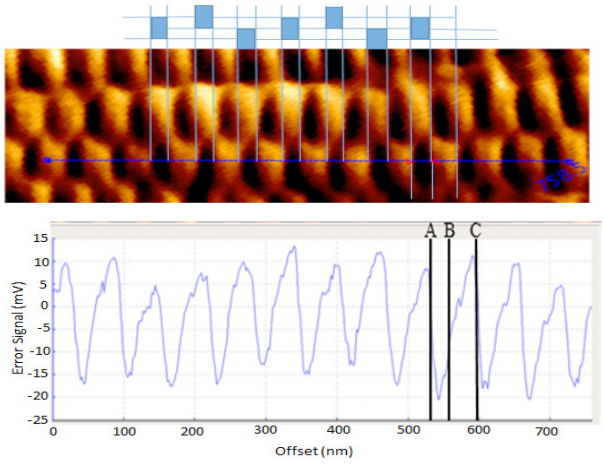

Figure 5 (a): AFM images of the overlap and gap regions of free gingival collagen fibrils (length).

Figure 5 (b) shows the typical measurements of width of collagen fibrils. The value of gap and overlap region, width and D-band of free gingival collagen fibrils is summarized in Figure 5. The length of collagen fibrils for human free gingival at overlap region was 32.53 (± 2.32) nm, whereas at gap region was 33.33 (±2.29) nm. The ratio of length of the gap and overlap is near to 1:1 with slightly longer gap region however it was not statistically significant. The mean value of free gingival collagen fibrils width and the length of D-band were 106.71±11.18nm and 65.82 ± 3.04nm respectively. The values of gap and overlap region, width and D-band of free gingival collagen fibrils are summarized in Figure 6.

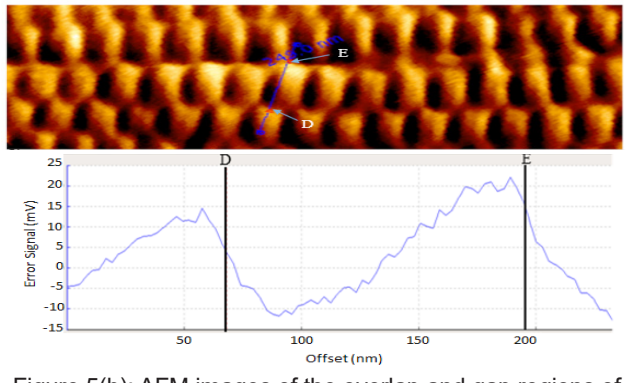

Figure 5(b): AFM images of the overlap and gap regions of free gingival collagen fibrils (width).

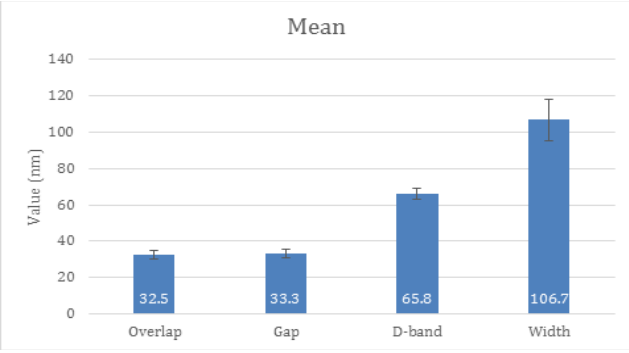

Figure 6: The summary of gap and overlap region, width and D-band of free gingival collagen fibrils values.

(3) Young's Modulus of free gingival collagen fibrils

Figure 7 (a) displayed the typical tip-distance curve obtained from the force spectroscope. One hundred curves were generated from the force spectroscopy for each of the collagen fibrils from all the free gingival samples. The Young's modulus of collagen fibrils for human free gingival tissue at overlap region was 212.88 ± 242.58 MPa, whereas at the gap region was 207.00 ± 230.71 MPa as summarized in Figure 7 (b).

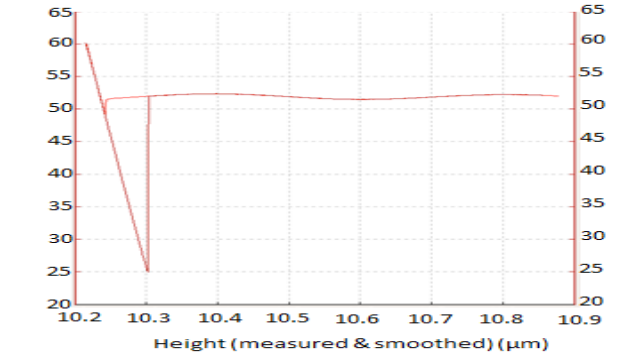

Figure 7(a): Typical tip-distance curve obtained from the force spectroscope.

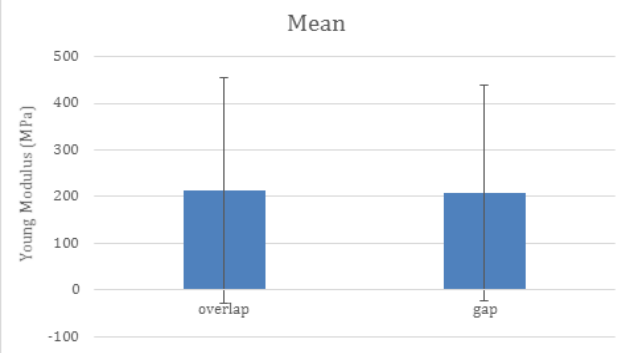

Figure 7(b): The summary of the Young's modulus of free gingival collagen fibrils values.

(4) Relationship between Gap and overlap region, width, D-band and Young's Modulus of free gingival collagen fibrils

Figure 8 (a) shows the the relationship between Young's modulus (E) of the overlap and gap regions using Spearman correlation. From Spearman test, there is a significant correlation between Young's modulus of overlap and gap ($p<0.01$). The strength of correlation is 0.964, shows that the strength of correlation is very good to perfect correlation. There is significant linear relationship between Young's modulus of overlap and gap regions. The Young's modulus of Overlap region increases proportionately with the Young's modulus of gap region. Table 1 summarized the shows the relationship between length of overlap and gap regions; the relationship between length and Young's modulus of overlap region and the relationship between length and Young's modulus of gap region using Spearman correlation.

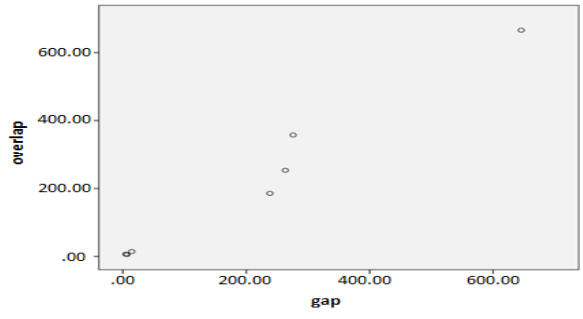

Figure 8 (a): The relationship between Young's modulus of the overlap and gap regions using Spearman correlation.

Table 1: The relationship between length of overlap and gap regions; the relationship between length and Young's modulus

Parameter	Spearman test	Significance
length of overlap vs gap regions	p=0.337	NS
length vs Young's modulus of overlap region	p=1.000	NS
length and Young's modulus of gap region	p=0.119	NS

*NS: Non Statistically significant

DISCUSSION

Free gingiva is a unique keratinized tissue of mucosa. It is the coronal portion with scalloped margin and the interdental papilla, extending until the free gingival groove. Basic periodontal examination is done as a routine screening and clinical baseline

for every patient using periodontal probe to measure the periodontal pocket depth in millimeters (1) which is the distance measured from the free gingival margin to the base of the sulcus (1-2). It is recorded as 1-3mm in healthy periodontium. However, when inflammation occurs, the periodontal pocket depth increases. Simultaneously, free gingiva changes in terms of size, colour, surface texture and consistency. Thus, the characteristic of free gingiva is clinically significant. Its significance is demonstrated in periodontal patients, whereas patient may have loss of gingiva tissue, also known as recession, leading to the exposure of the root of the teeth. Root exposed surface of teeth may cause dentine hypersensitivity to the patient (5, 13). Nevertheless, gingiva is difficult to be regenerated. Therefore, we would have to understand the biomechanical properties of the gingiva before the replacement or regeneration of gingiva.

In this study, the collagen fibrils appeared as wavy-like structure in bundles intervening with extracellular matrix at lower magnification. At higher magnification, the collagen fibrils emerged as basket weave like structure, running parallel to each other. The orientation of the collagens fibrils are found to be similar with the orientation of fibrils in skin. However, qualitatively, it appeared to be more densed than what was reported in skin tissues (14). However, direct comparison cannot be made as all the studied tissues using AFM were derived from animal tissues.

The striation of D-banding emerged as rope-like structure with each D-band segments present as dark and light pattern with 65.82 nm periodicity. The bands was found to be similar with the values obtained from other studies using electron microscope (15), X-ray diffraction (16) and AFM (17). In our study, the width of collagen fibrils is 106.7 ± 11.2 nm. It has been reported that the width of the Collagen type I fibril is 157 ± 46nm whereas the width of Type V collagen 144± 39 nm (18). Further studies need to be carried out to ascertain the composition collagen fibers in free gingival tissues.

The length of collagen fibrils for human free gingival at overlap region was 32.53 (± 2.32) nm, whereas at gap region was 33.33 (±2.29)nm. The ratio of length of the gap and overlap is near to 1:1 with longer gap region is in agreement with the present study (14). However, structural difference and physical variation between individual collagen fibrils can contribute to the variation in length of gap and overlap region of different samples.

The information on the biomechanical properties of gingival tissue is scarce. Currently, only the study carried out by the Oklahoma group that compared the biomechanical properties of oral soft tissue by Goktas *et al.* (19). In their study the biomechanical properties was carried out using tensile test of the porcine tissues. They found that the keratinized gingiva to have increased tensile strength (3.94 – 1.19 MPa) and stiffness (Young modulus of 19.75 – 6.20 MPa) relative to non-keratinized mucosal regions, where densely arranged elastin fibers contribute to a tissue with increased viscoelastic properties (19). However, in this study, free gingival was not tested. In our study, the Young modulus was calculated from the tip-distance curves where the modulus at overlap region and gap region were 212.88 ± 242.58 MPa and 207.00 ± 230.71 MPa respectively. This findings could be due to the collagen type I that forms the bulk of the lamina propria and provides the tensile strength to the gingival tissue. This densely packed collagen bundles contribute to the stability of connective tissue attachment. Direct comparison cannot be made as Goktas *et al.* and coworkers were using animal tissues and employed different testing method (19). The large standard deviation for Young's modulus is expected in the biological studies as highlighted by Wenger *et al.* (2007). It could be attributed to the variation between individual fibrils and regions. Wenger et al. reported that the mechanical properties of collagen fibrils are dependent on the direction of force used during testing. Collagen fibrils are isotropic in cross sectional area, but anisotropic in longitudinal area (20). The load will be equal in all directions when indentation of collagen in the cross section is applied, however the force will be varied in different directions when indentation is made at the longitudinal plane. The orientation of collagen fibrils varies from one sample to another, but the orientation of the cantilever tip remains the same. Care has been taken in our study to ensure the direction of force and testing surface are standardized.

Our findings would be able to provide baseline information for biomechanical properties of the free gingival tissue. This information is very useful in understanding the difference between the free gingival and the attached gingiva that could present with thin or thick biotype. In clinical setting, the average measurement of gingival thickness is 1.56 +0.39mm (21). The information could be useful in treating gingival recession, peri-implantitis, black triangle, and fabrication of new regenerative materials for gingival tissues.

CONCLUSION

Within the limitation of the study, the collagen fibrils appeared to be stacked in basket weave like-structure. The length and width of the collagen fibril were similar to the values investigated using other techniques. There was significant linear relationship

between Young's modulus of overlap and gap regions. The Young's modulus of Overlap region increased propotionally with the Young's modulus of gap region.

DECLARATION OF INTEREST

The authors alone are responsible for the content of this article, and report no conflicts of interest. This research was supported by High Impact Research MoE Grant UM.C/625/1/HIR/MoE/Dent21 from the Ministry of Education, Malaysia.

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