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Abstract

Objective: A preliminary study to determine collagen fibril diameter (CF-ED) distribution on medial and lateral sides of cleft lip (CL).

Material and Methods: Tissue samples from medial and lateral sides of CL were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in Araldite CY212 resin for transmission electron microscopy. The analysis of CF-ED was performed using the ImageJ program. To characterize the packaging of collagen fibrils (CFs) in the two tissues, we estimated the collagen number density (CF-ND) and fibril-area-fraction (FAF). Differences in measurements across the two sides were calculated using Wilcoxon signed rank test.

Results: The CF-ED was statistically significantly ($p<0.001$) smaller on the medial side (45.69 ± 7.89 nm) than on the lateral side (54.18 ± 7.62 nm). The medial side had a higher CF-ND and a higher percentage of FAF than the lateral side.

Conclusion: Our finding of a smaller CF-ED and higher CF-ND and FAF for the medial side suggest possible differences in size and distribution of CFs between medial and lateral sides of CL. This finding provides knowledge towards underlying tissue biomechanics that may help reconstruction of perioral tissue scaffolds, ultimately resulting in better treatment of patients with oral clefts.

Introduction

Cleft lip with or without palate (CL/P) is one of the most frequent congenital anomalies worldwide, with a prevalence among live births of 1 in 700 (Dixon, Marazita, Beaty, & Murray, 2011). Approximately, 70-80% of CL/P cases are non-syndromic (Leslie et al., 2016). Labial architecture is dramatically altered in CL/P cases that may be attributed to the impairment in the muscle forces (Barlow, Trotman, Chu, & Lee, 2012; Trotman, Barlow, & Faraway, 2007). CL/P clinically can occur as a unilateral or bilateral gap between the medial and the lateral upper lip structures (Carroll & Mossey, 2012).

The development of the upper lip involves co-ordination among complex series of events which requires cell migration, differentiation and apoptosis (Mossey, Little, Munger, Dixon, & Shaw, 2009), and particularly growth and fusion of the paired medial nasal process (MNP) and maxillary process (MxP) (Jiang, Bush, & Lidral, 2006). The MNP and MxP form the medial and lateral aspects of the upper lip structure, respectively (Dixon et al., 2011). Any cellular and morphological changes affecting the growth or fusion of the MNP and/or MxP may result in orofacial clefting involving the upper lip (Walker & Podda, 2018).

A fine modulation in the extracellular matrix in the orofacial region during development is essential for the cells to interact and respond to the remodeling or change in mechanical properties of the extracellular matrix (McDaniel et al., 2007; Gagliano et al., 2010), which is likely critical to cellular migration, differentiation (Badylak, 2005), and etiology of orofacial cleft (Smane-Filipova, Pilmane, & Akota., 2016; Mansell et al., 2000). Studies in experimental models of fibroblast cells obtained from CL/P cases have suggested molecular mechanisms involved in phenotypic variation among fibroblasts (Bosi et al., 1999; Bodo et al., 1999). These mechanisms are thought to be regulated by the collagen degradation pathway via matrix metalloproteinases and their endogenous tissue inhibitors; or by regulation of collagen cross-linking (Gagliano et al., 2010). Mechanical loading of the tissues

can influence metalloproteinases expression, production and activity causing an imbalance between metalloproteinases and their inhibitors that further remodel the tissue extracellular matrix component (Nagase, Visse, & Murphy, 2006). Relatively little attention has been given to the structure of the extracellular matrix in CL/P tissues.

The collagen fibrils (CFs) and proteoglycans are the two major components that make up the extracellular matrix of oral tissue structure (Levine, 2011), with fibrillar shaped collagen being the predominant component. This fibrillar collagens form a rope like structure, which shows characteristic banding pattern, and appears rounded on a transverse section under electron microscopy (Arseni et al., 2018). Several ultrastructural studies on healthy and pathological oral connective tissues have been reported (Chavier, Couble, Magloire, & Grimaud, 1984; Pêgo et al., 2016; Agrawal, Rai, & Jain, 2011); these studies have shown different CF arrangement in different oral tissues, with variation in CF distribution pattern and fibril diameter (Craig, Birtles, Conway, & Parry, 1989; Xu, Ohsaki, Nagata, & Kurisu, 1993) and exposure to biomechanical forces (Craig, Eikenberry & Parry, 1987).

The few studies that have investigated the relationship between structural and biomechanical properties in clefting have generally focused on proteoglycans and their involvement in cleft palate (Brinkley & Morris-Wiman, 1987a), with evidence of hyaluronic acid proteoglycan content regulating the hydration, and allowing elevation of palatal shelves (Brinkley & Morris-Wiman, 1987b). However, knowledge of the variations in structural organization of CFs, which is pivotal in determining the biomechanical properties of the oral soft connective tissue (Cornelissen, Stoop, Von den Hoff, Maltha, & Kuijpers-Jagtman, 2000) has been little investigated in research on orofacial clefts, particularly CL.

The tensile strength of the extracellular matrix and the mass average diameter of the constituent CFs are positively correlated, with CF diameter reflecting the mechanical properties of a tissue (Parry, Barnes, & Craig, 1978). Tissues with small diameter CFs can withstand high mechanical load (Parry, 1988), determined by unimodality or bimodality-mainly represented in fibril frequency histogram (Williams, Elder, Horstemeyer, & Harbarger, 2008). Additional studies in a mouse model have showed the structural-functional relationship between CFs area fraction to be correlated to strength and stiffness of a tissue (Robinson, Lin, Jawad, Iozzo, & Soslowsky, 2004).

The size distribution of CFs is mainly determined by intra-fibrillar covalent cross-linking provided by enzymes of the Lysyl-oxidase (LOX) family (Herchenhan et al., 2015). The deletion of *LOXL*3 gene has been shown to impair collagen assembly and cross-linking leading to smaller size CFs during palate development in a mouse model (Zhang et al., 2015). Additionally, our group has recently found a variant in this gene to be associated with non-syndromic cleft palate (Khan et al., 2018). The morphogenesis of lip and palate fusion involves highly regulated sharing of signaling molecules, and so it is possible that lip fusion promotes palate fusion (Smane-Filipova et al., 2016). Therefore, improper fusion of the lip may secondarily affect palate fusion (Meng, Bian, Torensma, & Von den Hoff, 2009). This inter-relationship demonstrates the importance of investigating CFs distribution to gain insight into the contribution of extracellular matrix component at a stage prior to palate development, i.e. upper lip fusion, which to our knowledge, remains largely unknown.

There are many approaches to studying the involvement of extracellular matrix in CL/P morphogenesis such as investigation of metalloproteinases and their inhibitors and collagen turnover, and/or the genes encoding them, as previously described. Each approach emphasizes different aspects of the extracellular matrix structure. However, to our knowledge no studies to date have investigated the organizational diversity of CFs, as revealed by electron microscopy, in medial and lateral tissues of CL cases. This might in future provide possible useful avenues underlying tissue biomechanics and help towards reconstruction of perioral tissue scaffolds.

Materials and Methods

Tissue samples

Tissue samples from the medial and lateral sides of CL were collected from four non-syndromic CL/P cases (two with CL and two with cleft lip and palate [CLP], each with the cleft on the left side). The average age of cases at the time of first lip surgery was eight months (95% CI 4.6-11.4). The samples were collected at the Regional Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy in the framework of the PENTACLEFT project (Khan et al., 2018), which was approved by local IRB (prot. N.08-2011). Written informed consent from one or both parents for case enrolment was obtained.

Tissue fixation and processing for transmission electron microscopy

The medial and lateral CL tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 overnight. They were then washed three times in 0.1 M phosphate-buffer (15 min ×3). Tissues were post-fixed in 1% osmium tetroxide for 1 hour, washed thrice (15 min ×3) with buffer and dehydrated with graded series of ethanol (50-100%) for 30 min in each. Tissues were infiltrated in toluene and Araldite CY212, and embedded in Araldite resin. Four blocks for each specimen (two for medial and two for lateral tissues) were prepared and polymerised in Araldite CY212 at 60°C for 72 hours. Ultrathin sections (70 nm thickness) were cut (from different regions of the tissues in the block) using Leica ultramicrotome UC7 and collected on 200 mesh copper grids. Each grid with ultrathin sections were stained with 2% uranyl acetate (10 min) and lead citrate (10 min), and then observed under Tecnai G^2-20 transmission electron microscope (FEI Company, The Netherlands) at an operative voltage of 200 kV, at the Sophisticated Analytical Instrumentation facility (SAIF)-Electron Microscope Facility, All India Institute of Medical Sciences (AIIMS), New Delhi, India.

Image acquisition and processing

The images were acquired under transmission electron microscope at 60000X using Digital Micrograph (Gatan, Inc.) software. Images were acquired for the medial and lateral sides of each CL specimen (with different levels of the blocks - each section being separated by a distance of 500 nm). The areas and diameter of CFs from each medial and lateral CL tissue specimen were measured using an open-source image processing software, ImageJ (Ver. ImageJ 1.49h, Dresden, Germany). Equating the irregular transverse area (A*i*) of the collagen fibrils to an equivalent circle, led to the determination of edge diameter of CFs ($ED = \sqrt{2\,\mathrm{A}i/\,3.142}$), as previously described (Khan, Nag, Igathinathane, Osuagwu & Rubini, 2015).

To analyse the collagen fibril diameter (CF-ED), our original 8-bit greyscale images were processed to obtain the binary image. Standard commands of ImageJ were used for preprocessing the images (Ferreira & Rasband, 2012). The original greyscale image (Figure 1a) was first segmented using thresholding image with moments scheme and no dark background (Figure 1b). The lower and the upper limit of intensities were then adjusted for clear visibility of CFs of interest, a lower threshold limit of 0 and upper limit of 120-170 for

white background were found to be optimal for images to convert to binary format (Figure 1c). The sequence of assigned operations in ImageJ were followed for a section of input-image to obtain an area desired for each CFs which were color coded using ROI color coder plugin (Ferreira, 2014) for clear visualization of different CFs (Figure 1d-g). The output area of each CF was in pixel unit which made it essential to convert to physical unit by using the line tool of ImageJ. The data were transferred to Excel spreadsheet from ImageJ software for further analysis.

Collagen number density (CF-ND) and fibril-area-fraction (FAF)

Collagen number density is the number of CFs per unit area (μm^2). It was calculated using the formula; $ND = \frac{Total\ CF\ number}{Total\ image\ area\ (\mu m2)}$

We made use of the A*i* of each of the CF included within an image area (A) to calculate the fibril-area-fraction also known as fibril-volume fraction (Starborg et al., 2013) by making use of the formula; $FAF = \sum \frac{Ai}{A}$ (A*i*; transverse area of each fibril and A; is the total area of an image).

Statistical analysis

The distribution of collagen fibrils were checked for normality using Shapiro–Wilk test for small samples in the IBM SPSS Statistics 21. The differences in measurements of CF-ED across the medial and lateral sides of CL was done using Wilcoxon signed rank test. In addition a paired student's *t*-test was used to check for differences in FAF and CF-ND between medial and lateral tissues. Considering the total of four cases included in this study, and assuming an α-error (two-tailed) of 0.05 and a 1-β-error of 0.80, at least 208 CF-ED from each side per sample would be required to detect a change of 20% in CF-ED, FAF or CF-ND, as calculated using G * POWER software 3.1.9.2 version (Faul, Erdfelder, Lang & Buchner, 2007).

Results

The mean CF-ED for the medial and lateral sides of the 4 tissues are presented in Table 1. We found a significantly smaller diameter CFs on the medial side in each of the four cases compared to fibrils on the lateral side. The frequency histogram of CFs on the medial and lateral sides for each of the four cases/tissues are presented in Figure 2. The differences in CFs across the two sides, averaged across the four cases, were significant, with medial side showing smaller CFs (45.69 ± 7.89 nm) than the lateral side (54.18 ± 7.62 nm); $p<0.001$ (Table 1). The transmission electron micrographs of medial and lateral CFs were digitized and color coded to estimate distribution of CF-ED (Figure 3). The pattern of CF-ED distribution is shown in an overall frequency histogram (Figure 4), and percentage of CF-ED falling in different range is presented in Table 2.

For the medial side, we found that the percentage of CF-ED were almost equally in the 29-44 nm and 45-59 nm ranges. Whereas, the lateral side CF-ED showed a wide-range, with majority (70%) of CF-ED falling within 45-59 nm (Table 2). We further calculated collagen fibril number density (CF-ND) and total cross-sectional area fraction of the CFs as a valuable indicator to determine the packing of the CFs. We found a higher CF-ND for the medial tissue (538) compared to the lateral tissue (376). The calculated averaged FAF showed a higher percentage value of 32.28 ± 8.13% for the medial side and a smaller percentage value of 27.28 ± 3.77% for the lateral side (Table 3, Figure 3).

Discussion

In the present preliminary study we demonstrate for the first time an estimation of CFs in the medial and lateral aspects of upper lip structure obtained from CL/P cases at primary lip surgery. We observed a significant difference in diameter of collagen fibril (CF-ED) between medial and lateral sides of CL. The collagen fibril density (CF-ND) was found to be higher for the medial side. Estimation of collagen fibril-area fraction (FAF) showed a higher percentage value for the medial side compared to lateral side.

We observed a significant difference ($p<0.001$) in CF-ED between medial (45.69 ± 7.89 nm) and lateral (54.18 ± 7.62 nm) CL tissues, with the medial tissue having a smaller CF-ED compared to lateral tissue. This observation of mean difference across the two tissues appears to be compatible with the finding of Moeller et al., (1995) that mechanical overloading on the

connective tissue could eventually cause the thick CFs to become thinner. Based on this finding, we infer that mechanical stimuli may affect CF-ED distribution across the two cleft sides, reflected in the observed differences (Table 2; Figure 4).

Estimation of CF-ND and FAF provided valuable indication on how tightly or loosely packed is the CFs across the two sides of CL. The CF-ND or number of collagen fibrils per micrometer square area was found to be higher for the medial side compared to the lateral side (Table 3). This arrangement may reflect an increased mechanical load distribution across the two sides, with medial side showing higher value for CF-ND. Moreover, our result showed that despite having a smaller CF-ED, increasing CF-ND resulted in an increased FAF (32.28 ± 8.13%) for the medial side compared to lateral side (27.28 ± 3.77%), clearly represented in color coded image (Figure 3e & 3f). This indicates that medial side gains strength and stiffness with increase in FAF, which adds to the mechanical load of the medial tissues.

Studies elsewhere have shown that small and large fibrils have distinct roles in stiff state and only the small fibrils have a role in the disease states and hence lending to changing properties (Goh & Holmes, 2017), which could be the case in our medial tissues. Nonetheless, the possible role of factors apart from CF-ED or a consequence of CF-ED cannot be ruled out for compromised extracellular matrix of the medial tissue, based on previously described ability of metalloproteinases and their inhibitors to participate in matrix remodeling during lip fusion (Smane-Filipova et al., 2016). We postulate that remodeling of the extracellular matrix components (collagen fibers and/or proteoglycans) that forms the migration substrate for the cranial neural crest cells mesenchyme (Henderson & Copp, 1997; Jiang et al., 2006) could be different across the two sides. Some empirical evidence of reduced proliferation and migration of these mesenchymal cells particularly affected on the medial side is shown in a separate animal study (Everson et al., 2017). Moreover, these mesenchymal cells eventually form the continuous bands of the future orbicularis oris muscle (Lazzeri et al., 2008), which is shown to have altered diameter (Khan et al., 2018) and arrangement (Wijayaweera, Amaratunga, & Angunawela, 2000) across the two sides in CL cases. Notably, collagen and muscles share structural functional relationship to ensure proper alignment (Calvi et al., 2012). Therefore, observation of changes in the orbicularis oris muscles across the two sides could be an outcome of changes in distribution of CF-ED, which affects the medial side more than the lateral side in cases with CL.

It is well known that the diameter of the CFs increase with age (Ottani et al., 1998). We consider that our observation of differences in CFs distribution across the two cleft sides is little influenced by ageing as we used tissues derived from the same individual with relatively similar age. However, we realize that inclusion of CL and CLP subtype might have influenced our result but relying on a recent study demonstrating similar rate of development of the two subtypes, the effect might have been neutralized (Sharp et al., 2017).

Moreover, our study has potential limitations with respect to small sample size with low statistical power owing to difficulties in collecting tissues (Stock et al., 2016), and therefore we were unable adequately to assess inter-individual variability. We also note that lack of clarity or darker patches in our original image could have interfered in the binary image creation, leaving holes that might have effect on the measured parameters. Therefore, the differences in CFs across the two sides should be interpreted with caution until replication data become available; these would be based on a larger sample size and good quality input images for better segmentation of CFs while processing to binary images. Another limitation is the lack of data on lip tissues from normal babies to determine whether the difference between the sides is present in general population or is specific to infants with clefts. Nevertheless, investigation of differences using tissues from CL/P cases is a promising approach in investigating the etiopathology of CL/P.

In conclusion, the result of this study suggest of differences in CF-ED across the two cleft side, with medial side having smaller diameter and higher stiffness that might play a role in migration of mesenchymal cells in these tissues, causing a tissue deficiency that prevents contact with opposing lateral tissues, resulting in cleft lip. Additionally, our study could provide knowledge towards underlying tissue biomechanics that may help reconstruction of perioral tissue scaffolds, ultimately resulting in better treatment of patients with oral clefts.

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Author contributions

MFJK designed the study, carried out experiment and drafted the manuscript. MR, LA and MCM managed recruitment of PENTACLEFT lip tissue samples. The data was analyzed by MFJK together with MR. MR, JL, PM, TCN and AM checked and revised the manuscript. MFJK and AS captured images on TEM. All authors read and approved the final manuscript.

Conflicts of interest

None to declare.

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Table 1. Mean ± Standard deviation (SD) values of collagen fibril edge diameter (CF-ED in nm) on the medial and lateral sides of four N1-N4 (number of measured CFs) infants with cleft lip.

Cleft lip tissues	**N1 (**532**)**	**N2 (**289**)**	**N3 (**509**)**	**N4 (**511**)**	**Mean N1-N4 (**1841**)**
Medial	36.32 ± 4.21	47.99 ± 6.03	46.85 ± 4.31	52.98 ± 4.21	45.69 ± 7.89
Lateral	51.74 ± 7.04	49.63 ± 5.36	58.73 ± 7.82	54.76 ± 6.57	54.18 ± 7.62
	Z= -19.637 p<0.001	Z= -2.774 p=0.006	Z= -18.568 p<0.001	Z= -5.792 p<0.001	Z= -28.387 p<0.001

Table 2. Percentages of collagen fibril-edge diameters (CF-ED) in different ranges on the medial and lateral sides of the cleft lip in four affected infants.

Cleft lip tissues	**15-29 nm**	**30-44 nm**	**45-59 nm**	**60+ nm**
Medial	2	40	57	1
Lateral	<1	10	70	19

Table 3. Collagen number density (CF-ND) and fibril area fraction (FAF) on the medial and lateral sides of the cleft lip in four affected infants.

Cleft lip tissues	**Density of collagen fibrils** (μ^2)	**Area fraction of collagen fibrils** (%)
Medial	538	32.28 ± 8.13
Lateral	376	27.28 ± 3.77
	p=0.477	p=0.415

Figure 1. **Preprocessing of micrographs using ImageJ standard commands to obtain digital color-coded image.** (a) original 8-bit grey-scale image; (b) thresholding image with "moments" scheme and "no dark background" to convert image to binary format; (c) mask prepared by analyzing the particles with "no exclude edge" and "fill holes" options; (d) inverted mask giving black background using "invert"; and (e) "watershed" segmented image; (f) labelled collagen bundles; and (g) area based color-coded collagen bundles for better visualization of sizes.

Figure 2. **Frequency histogram of collagen fibril diameter (CF-ED) for medial and lateral cleft sides of each of the four cases (**N1, N2, N3 & N4**).**

Figure 3. **Electron micrograph, binary images and digital images of collagen fibrils of the medial and lateral sides of CL.** (a) original grey-scale image of medial side with smaller CF-ED; (b) original grey-scale image of the lateral side larger CF-ED; (c) binary image of medial and (d) lateral side; (e) digital colour coded image of medial side with higher CF-ND and FAF; (f) lateral side showing smaller CF-ND and smaller FAF.

Figure 4. **Overall (**N1-N4**) frequency histogram of collagen fibril diameter (CF-ED) for medial and lateral cleft sides.**