HISTOCHEMICAL, BIOCHEMICAL AND ULTRASTRUCTURAL ASSESSMENT ON MITOCHONDRIAL FUNCTIONS IN PATIENTS WITH NON-SYNDROMIC CLEFT LIP

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by

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LIST OF SYMBOLS

%	Percentage
µl	Microliter
µm	Micrometer
ml	Mililiter
nm	Nanometer
nmol	Nanomole
α	Alpha
β	Beta
g	Gram
g	Gravity

LIST OF ABBREVIATIONS

ATP	Adenosine-5’-triphosphate
ATP-ase	Adenosine triphosphatase
BV	Blood vessels
CL	Cleft lip only
CL/P	Cleft lip and/or palate
COX	Cytochrome c-oxidase
CP	Cleft palate
$CuSO_4H_2O$	Copper sulfate monohydrate
DAB	3,3′-daminobenzidine tetrahydrochloride
df	Degree of freedom
DNA	Deoxyribonucleic acid
EP	Epithelium
et al.	and others
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and eosin
HUSM	Hospital Universiti Sains Malaysia
$MnCl_2$	Manganese bichloride
NADH-TR	Nicotinamide adenine dinucleotide tetrazolium reductase
NaOH	Sodium hydroxide
NSCL/P	Non-syndromic cleft lip and/or palate
O.C.T	Optimal cutting temperature
OD	Optical density
OFCs	Orofacial clefts

PBS	Phosphate-buffered saline
PEH	Pseudoepitheliomatous hyperplasia
pH	Potential hydrogen
SD	Standard deviation
SDH	Succinate dehydrogenase
SG	Stratum granulosum
TEM	Transmission electron microscope
USM	Universiti Sains Malaysia

LIST OF APPENDICES

PENILAIAN HISTOKIMIA, BIOKIMIA DAN ULTRASTRUKTUR KE ATAS FUNGSI MITOKONDRIA BAGI PESAKIT SUMBING BIBIR BUKAN SINDROMIK

ABSTRAK

Sumbing bibir/lelangit bukan sindrom (NSCL/P) adalah salah satu kecacatan kongenital yang menjejaskan bibir atas bayi. Walaupun dengan penyelidikan yang intensif, faktor asas kecacatbentukan kraniofasial ini masih tidak diketahui. Dipercayai bahawa sumbing bibir mempunyai etiologi yang kompleks, termasuk faktor genetik dan persekitaran. Patogenesis sumbing berlaku semasa fasa embrio. Oleh itu, peranan mitokondria dalam mengawal selia sel semasa fasa ini adalah penting. Penemuan yang tidak konsisten telah ditemui dari kajian terdahulu. Oleh itu, hubungkait kecacatan metabolik mitokondria dengan patogenesis sumbing kekal samar. Oleh sebab itu, kami berhasrat untuk merungkai peranan mitokondria dalam tisu sumbing bibir dalam kalangan populasi kita untuk memperolehi penemuan yang lebih mendalam. Objektif dalam kajian ini adalah untuk meneroka perubahan histologi otot orbicularis oris dan peranan mitokondria dari segi struktur dan aktiviti dalam menyebabkan pembentukan sumbing bibir. Tisu sumbing bibir diperoleh daripada persetujuan pesakit sumbing bibir bukan sindrom, manakala tisu kontrol diperoleh daripada persetujuan pesakit yang tidak terjejas oleh sumbing bibir. Sel fibroblas dari gusi manusia normal telah diguna sebagai control untuk ujian adenosina trifosfat (ATP). Empat puluh tisu sumbing bibir dan tujuh kontrol telah diproses mengikut analisis masing-masing: hematosilin dan eosin (H&E), trikrom Gomori yang diubah suai, sitokrom c-oksidase (COX), adenosina trifosfatase (ATP-ase), mikroskop elektron pancaran (TEM), dan ujian ATP. Penemuan histologi telah dianalisis menggunakan mikroskop cahaya dan

perisian pengimejan cellSens, penemuan ultrastruktur pula telah dianalisis menggunakan perisian JEOL JEM 2100F Field Emission TEM, dan analisis statistik ujian ATP telah dianalisis menggunakan ujian-t tidak bersandar. Beberapa keadaan kulit seperti keradangan pada lapisan epidermis dan pembentukan tompok Fordyce telah dijumpai di kawasan mukosa tisu sumbing bibir. Kehadiran ciri khas patologi miopati mitokondria, iaitu gentian merah bergerigis, dan kehadiran gentian COX-negatif novel telah dikesan dalam tisu sumbing bibir. Mikroskop cahaya juga mendedahkan gentian otot yang tidak normal, seperti susunan yang tidak teratur, pembentukan fibrosis, serta peratusan tinggi gentian jenis II. Pengumpulan minima mitokondria dan titisan lipid dalam gentian telah dikesan dengan mikroskop elektron. Melalui penilaian biokimia, kepekatan ATP adalah jauh lebih rendah dalam tisu sumbing berbanding dengan yang control (P = 0.0344). Laporan tentang siri ciri-ciri miopati mitokondria pada pesakit sumbing bibir bukan sindrom ini memberikan lebih banyak bukti dan seterusnya menyokong hipotesis bahawa kecacatan metabolik pada peringkat mitokondria terjadi pada sumbing bibir bukan sindrom.

HISTOCHEMICAL, BIOCHEMICAL AND ULTRASTRUCTURAL ASSESSEMENT ON MITOCHONDRIAL FUNCTIONS IN PATIENTS WITH NON-SYNDROMIC CLEFT LIP

ABSTRACT

Non-syndromic cleft lip/ palate (NSCL/P) is one of the congenital malformations that affects upper lip and/ or palate of an infant. Despite intensive research, the underlying factors of this craniofacial deformity are still vague. It is believed that oral clefts have a complex aetiology including genetic and environmental factors. The pathogenesis of clefts occurs during the embryonic phase, hence, the role of mitochondria in regulating cells during this phase is crucial. Inconsistent findings have been discovered by previous studies, therefore, the association of mitochondrial metabolic defects with cleft pathogenesis remains vague. Therefore, we aim to discover the role of mitochondria in cleft tissues of our population to obtain extensive findings. In this study, the objective is to explore the histological alteration of orbicularis oris muscle and the role of mitochondria in terms of their structure and activity in causing cleft lip formation. Cleft lip tissues were obtained from consented patients with non-syndromic cleft lip, whereas control normal tissues were obtained from consented patients who were unaffected by cleft lip. A normal human primary gingival fibroblast was used as a control for the assay. Forty cleft lip tissues and seven controls were obtained and processed according to the respective analysis: haematoxylin and eosin (H&E), modified Gomori trichrome, cytochrome c-oxidase (COX), adenosine triphosphatase (ATP-ase), transmission electron microscopy (TEM), and adenosine triphosphate (ATP) assay. Histological and histochemical findings were analysed using a light microscope and imaging cellSens software, ultrastructural findings were examined

using JEOL JEM2100F Field Emission TEM, and a statistical analysis of ATP assay was analysed using independent samples t-test. A few skin conditions, such as inflammation in the epidermal layer and Fordyce spot formation, were noted in the cleft lip tissues. The presence of the pathological hallmark of mitochondrial myopathy, which is ragged red fibers, and the presence of novel COX-negative fibers were observed in the tissues of cleft lip. Also, light microscopy revealed fibrotic and disorganised fibers, including a high percentage of type II fibers. A minimal accumulation of mitochondria and lipid droplets in the fibers were detected by electron microscopy. By means of biochemical assessment, ATP concentrations were significantly lower in cleft tissues compared to the control ($P = 0.0344$). The report on the series of mitochondrial myopathic features in patients with non-syndromic cleft lip provides more evidence and further supports the hypothesis that a metabolic defect at the mitochondrial level occurs in non-syndromic cleft lip.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Orofacial clefts, also known as OFCs, are a type of congenital abnormality that involve a gap or split in the soft tissues of the lip and/or palate (Chaoui et al., 2015). Cleft anatomy differs from a normal lip due to the splitting of the nasal and oral cavities, affecting the lip, alveolus, hard palate, and soft palate (Neville et al., 2018). Basic facial formation in the human embryo starts at the fourth week until the twelfth week of fertilization (Shkoukani et al., 2013). During this phase, the failure of facial tissue fusion causes separation of the lip and palate, and this deformity can be detected around 16 weeks of gestation (Wattanawong et al., 2016; Smarius et al., 2017). Factors that causing cleft include genetic, environmental, geographic, race, sex, and pregnancy exposure to risk factors such as alcohol consumption, tobacco smoking, poor nutrition, viral infections, and drugs (Kawalec et al., 2015).

Cleft lip and/or palate is one of the most common human embryonic disorder reported in Western countries and the second most common birth deformity among babies (Shaw et al., 1991). It arises about 1 in 700 live births through ethnic and geographic differences (M. J. Dixon et al., 2011). In Malaysia, according to the most recent report, one in every 611 newborn babies has a cleft lip and/or palate in Malaysia (NOHSS, 2007). Most of the orofacial clefts is non-syndromic and considered complex traits. Non-syndromic cleft lip and/or palate (NSCL/P) is a type of cleft abnormality without the presence of any other major abnormalities, representing 70% of cases worldwide. Different from non-syndromic form, syndromic cleft lip and/or palate is a formation of cleft lip and/or palate due to the presence of syndromic disease for example Kallmann syndrome or Van der Woude syndrome, which representing

30% cases worldwide (Stuppia et al., 2011). In this study, we will focus on the non-syndromic form, as our main purpose is to investigate the factors associated with cleft lip deformity only.

The prevalence of clefts differs from one group to another due to factors such as ethnicity, race, and geographical location (Noorollahian et al., 2015). It is heterogeneous with a multifactorial aetiology, as both genetic and environmental factors contribute to the cleft formation (Egbunah, 2022). Cleft deformity affects a child's lifestyle, which also concerns speech, physical health, and stigma among peers (Wydick et al., 2022; Alighieri et al., 2023). As such, further insight into cleft aetiologies should be gained due to the scarce report on NSCL/P malformation in Malaysia. During embryonic development, mitochondria play a critical role in generating adenosine-5'-triphosphate (ATP) to form energy and regulate cell death (Kasahara & Scorrano, 2014). Their role is undoubtedly essential in supporting the cells, critically throughout early embryonic development. The detection of mitochondrial abnormalities in muscle is always related to mitochondrial myopathy. This abnormality will express some pathological hallmarks such as ragged red fiber, oxidative deficiency, and abnormal structures and arrangements of the mitochondria itself.

Orbicularis oris muscle and collagen fibers are the most affected tissues in cleft. Both tissues are constantly reported to be disorganised and disarrayed, depending on the severity of cleft (Khan et al., 2019; Schreurs et al., 2020; Noor, Shah, et al., 2022). We therefore previously discussed the published evidence of the role of mitochondria in cleft pathology in a systematic review, in which the discovery of

mitochondrial abnormalities have been detected in cleft lip and palate specimens, and the association of this abnormality is still inconclusive (Noor, Sulaiman, et al., 2022).

This study is a continuation of our previous preliminary study on NSCL/P carried out on the tissue. We have found abnormal organisation of the collagen and muscle fibers in the cleft lip tissues that was observed on scanning electron microscopy (Shah et al., 2014). This early finding brings up the idea of assessing mitochondrial activity in the cleft lip tissue in order to confirm if the mitochondrial abnormality is related to the disorganisation of fibers, ultimately causing cleft lip formation. Our previous study has focused on qualitative assessment, both histologically and ultrastructurally. This study, however, will have both qualitative analyses through histochemical and ultrastructural assessment, and quantitative analysis through an ELISA assay for biochemical reaction determination for more reliable and significant findings. Histochemical assessment involves the identification of chemical composition in cells and tissues, and it will identify the density of the composition staining in the mitochondria of cleft and normal tissues. Ultrastructural examination will demonstrate a detailed structure (fine structure) of a biological specimen that is magnified at higher magnifications and can be observed only by an electron microscope. Meanwhile, biochemical reactions involve chemical reactions in tissues, which can be measured using an assay, as in this study with the total ATP in the tissue.

1.2 Problem statement

A wide range of studies have been conducted to investigate the aetiology of this anomaly, which is commonly included genetic and environmental factors. On the other hand, the developmental defect of cleft at the mitochondrial level is debatable due to the inconclusive findings and little information regarding the pathological

hallmarks as well as the metabolic deficiency of mitochondria in cleft. As a result, the scarcity of research on the relationship between cleft and mitochondrial abnormality motivates us to extend our investigation to include ultrastructural and biochemical approaches. The discovery of mitochondrial abnormalities in clefts could perhaps broaden the idea and give deeper insights into cleft pathogenesis.

1.3 Significance of the study

Muscle degeneration and disorganisation in cleft patients have been constantly reported in previous studies. This muscle is highly populated by mitochondria, which are known to provide energy for muscle growth and function. This organelle controls, maintains, and regulates cellular metabolism, essentially during critical cellular process such as in early human craniofacial development, whereas it involves the migration of cells and several growth factors. Disturbance during this process could lead to abnormal growth of the facial structure. Hence, the role of mitochondria in controlling cells during embryonic development is essential. Therefore, this study will focus on the evaluation of mitochondria that possibly causing cleft lip formation.

1.4 Objectives of the study

1.4.1 General objective

To explore the role of mitochondrial activity in non-syndromic cleft lip tissue for better understanding of the developmental process in relation to the pathogenesis of cleft lip formation.

1.4.2 Specific objectives

1. To examine the skin structure in cleft lip tissue and normal lip tissue using haematoxylin and eosin staining.

2. To observe mitochondrial abnormality in cleft lip tissue using enzyme histochemical staining and ultrastructural analysis.
3. To compare biochemical modification in cleft lip tissue and normal human gingival primary fibroblast using ATP assay kit.

CHAPTER 2

LITERATURE REVIEW

2.1 Non-syndromic cleft lip and/or palate

Non-syndromic cleft lip with or without cleft palate (NSCL/P) is a craniofacial birth defect that affects 1 in 700 live births globally (M. J. Dixon et al., 2011). In Malaysia, the first prevalence study of orofacial clefts was reported by Stevenson et al. (1966) with an incidence rate of 1.54 per 1000 live births. Then, the subsequent report was carried out by Boo and Arshad (1990), with the incidence was 1 in every 700 newborn babies had a cleft lip and/or palate. The latest report documented that the cleft lip and/or palate incidence was 1 in every 611 babies in Malaysia (NOHSS, 2007).

Craniofacial developmental processes originate in the oropharyngeal membrane, which involves four main stages: cell migration, growth, differentiation, and apoptosis (A. D. Dixon, 2017; Geetha-Loganathan et al., 2022). The formation of the human face occurs with the migration of neural crest cells that merge together with the core mesoderm and pharyngeal ectoderm to form facial primordial and later give rise to form lip and palate structures (Ansari & Bordoni, 2022). This congenital malformation occurs between the fourth to twelfth weeks after fertilization. The lack of the formation of nasal and maxillary processes will result in cleft lip either unilateral or bilateral and cleft palate (Smarius et al., 2017).

One of the affected structures in cleft lip is the orbicularis oris muscle, and the severity varies greatly across the phenotypic spectrum of the cleft defect (Khan et al., 2018). Continuous muscular development is critical throughout this process, and any hindrance or interruption in fusion during upper lip embryonic formation could lead to a orbicularis oris deformity (Deepthi, 2013; Kalantar Motamedi, 2013). Figure 2.1

demonstrates the displacement of the orbicularis oris muscle bundle in the cleft lip; the muscle is redirected towards the alar base on the lateral side and towards the philtrum and columella on the medial side.

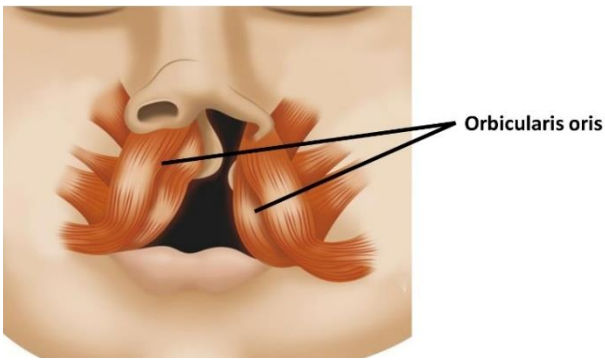

Figure 2.1 Illustration of the orbicularis oris orientation in unilateral cleft lip. From Murthy & Durand (2020).

Clefts can be categorized into non-syndromic and syndromic forms (Hadadi et al., 2017). The affected individuals that have no other physical or developmental abnormalities are known as non-syndromic, whereas syndromic cases have other additional features that can be divided into groups of chromosomal abnormalities, Mendelian disorders, teratogens, and other unidentified syndromes (Leslie & Marazita, 2013). The severity of NSCL/P can be divided into minimal cleft (microform) or incomplete or complete unilateral or bilateral clefts (Carroll & Mossey, 2012). Figure 2.2 below illustrates the three major categories of oral clefts, which are those affecting the lip only (CL), those affecting the lip and palate (CLP), and those affecting the palate alone (CP). These categories are divided into sub-phenotypes such as complete, incomplete, unilateral, and bilateral (Leslie & Marazita, 2013; Swan & Fisher, 2023). An incomplete cleft lip involves 50% of lip height and sometimes has mild nasal deformity. Complete cleft lip affects the lip and nostril parts with a deviation of the columella and the displacement of the alar base (Campbell et al., 2017). Unilateral cleft lip affects only one side of the lip, the separation can be either side of the upper lip that

also involves the nose and alveolus (Vaca & Gosain, 2019). Bilateral cleft lip affects both sides of the lip that involves the columella, nostrils, lower nasal cartilage, and nasal tip, which can be symmetric or asymmetric (Harrison et al., 2021). Sometimes patient with bilateral cleft lip has one complete cleft lip on one side, and incomplete form on the other side (Carroll & Mossey, 2012). Cleft lips can occur unilaterally or bilaterally, with or without a cleft palate, or cleft palate may exist alone. As mentioned by Funato & Nakamura, (2017), the differences among cleft phenotypes may indicate the differences in genetic causes, as genes related to CLP are developmentally and genetically different from incomplete CP, CP only, and submucous CP.

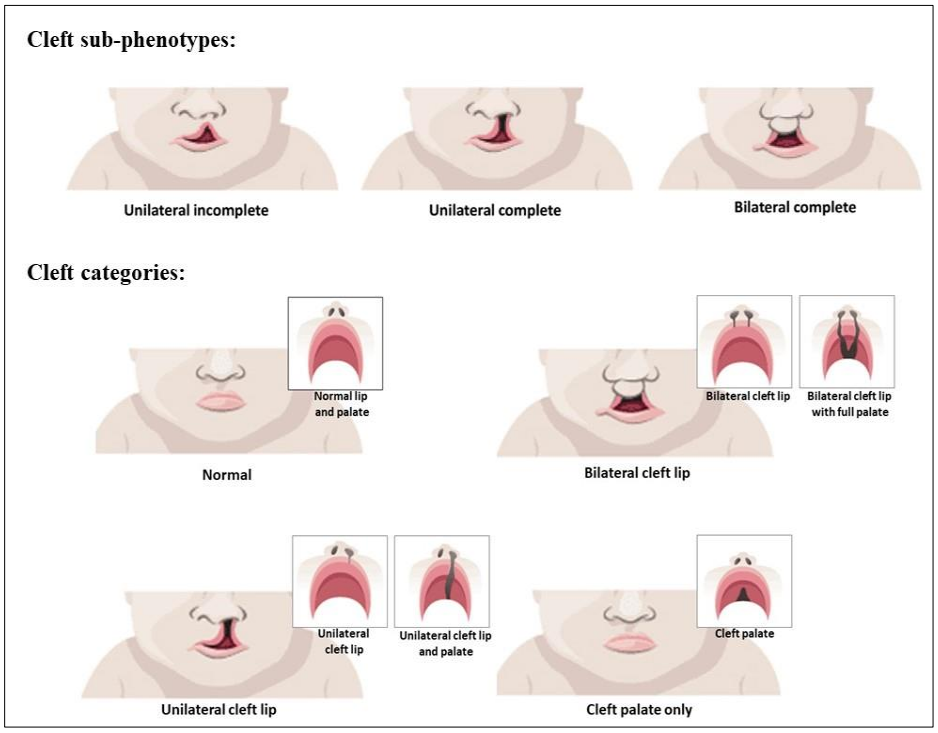

Figure 2.2 Illustration of the normal oral cavity structure and different types of clefts. Adapted from Harun (2020).

Identification of the aetiology related to NSCL/P may help in the prevention, treatment, and diagnosis of the disease (Ge et al., 2019). Thus, there have been a lot of studies conducted that focus on the aetiology of NSCL/P, especially on both genetic and environmental factors that have been believed to be the cause of the orofacial cleft

development (Murray, 2002; Meng et al., 2009; Y. P. Liu et al., 2015; D. P. Xu et al., 2018). One of the factors that may increase the probability of a baby developing a cleft includes a complex interaction between environmental exposures. For example, maternal lifestyle during pregnancy such as cigarette use and consumption of alcohol (Machado et al., 2016). Specific drug and medicine exposure, chromosomal abnormalities, and genetic factors have also been linked to an increased risk of cleft (M. J. Dixon et al., 2011; Machado et al., 2017). These various etiologic studies on cleft provide additional insights into craniofacial pathogenesis.

2.2 Lip skin histology

Lip skin has hair follicles and a thin, stratified squamous keratinized epithelium covering its exterior (Dimond & Montagna, 1976). The skin transitions to the vermillion zone (red margin), which creates the red area of the lip, and then to the oral mucosa, which lines the inside part of the lip. There are numerous tiny salivary glands (oral glands) beneath the oral mucosa, and the orbicularis oris muscle (skeletal) occupies the centre core of the lip (Arda et al., 2014). Figure 2.3 shows histological staining representing a cross-section of the lip: (a) the skin; (b) the vermilion border; and (c) the mucosa membrane (oral mucosa).

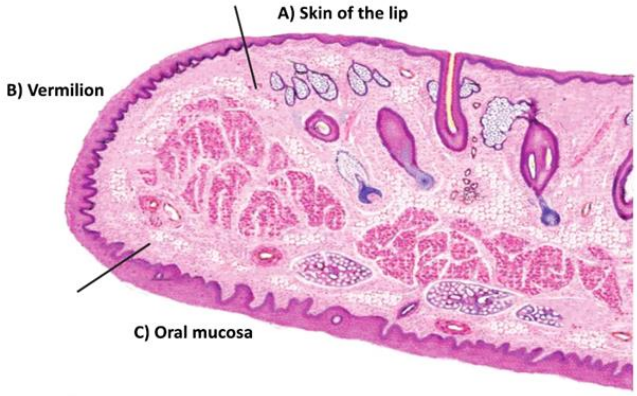

Figure 2.3 Histological representation of the lip section. From Eroschenko (2013).

2.2.1 Skin of the lip

The epithelium of the skin's face is relatively thin and associated with hair follicles and sebaceous glands (Arda et al., 2014). Skin is the outer layer that provides an efficient barrier to the external environment (Jensen & Proksch, 2009). It consists of the superposition of three layers: the epidermis, composed of the keratinized stratified squamous epithelium; the dermis, made of connective tissue; and the subcutaneous tissue, also known as the hypodermis, composed of a layer of loose connective tissue such as adipose tissue (Penna et al., 2009; Yousef et al., 2022). The epidermis of the skin gives rise to hairs and sebaceous glands, and the dermis consists of many blood vessels and sebaceous glands. A histological sagittal section of the lip skin with skin appendages is shown in Figure 2.4A. The circled area of epidermal layer is magnified at higher magnification, as presented in Figure 2.4B.

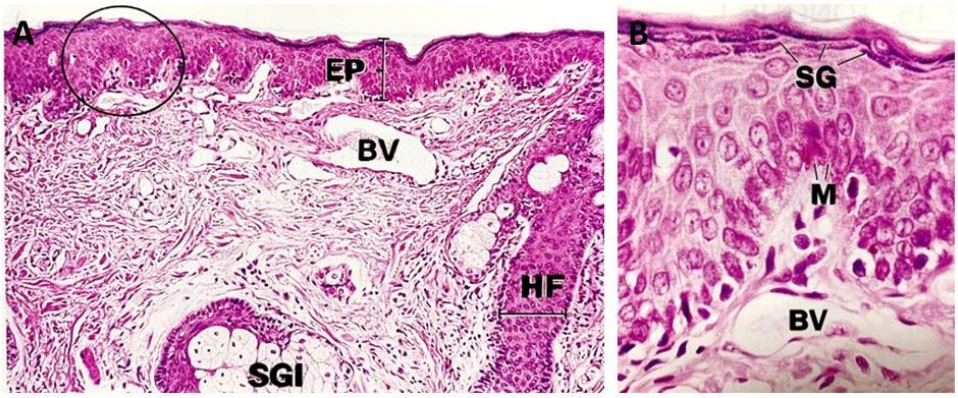

Figure 2.4 Histological stain on skin tissue. A thin epithelial layer associated with blood vessels (BV), hair follicles (HF), and sebaceous glands (SGl) with the presence of stratum granulosum (SG) and melanocytes (M). From Ross et al. (2003).

2.2.2 Vermilion

Vermilion is a rare and limited tissue that has red skin. According to Takashimizu & Yuzuriha (2018), the epithelial layer of the vermilion is less keratinized when compared to the epithelium of the face. The colour of the lip is related to the deep penetration of the heavily packed connective tissue papillae into the epithelial layer. Also, the thinness of the epithelium, along with the extensive venous blood vessels carried close to the surface in these papillae, allows the blood colour to show through in the lips and appear red, as presented in Figure 2.5A. Figure 2.5B shows a higher magnification of the circled layer from epithelium.

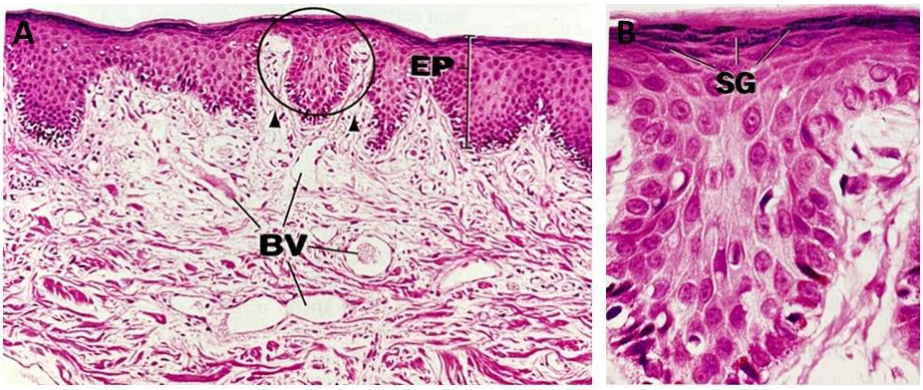

Figure 2.5 Histological stain on vermilion. The epithelium (EP) of vermilion is thicker than the lip skin and noted for its abundance of blood vessels (BV) and stratum

granulosum (SG), with no presence of hair follicles and sebaceous glands. From Ross et al. (2003).

There is no salivary gland in this area, hence, the tongue must constantly moisten the lips to keep them from drying out. The vermilion and intermediate zones represent a transition between a thin keratinized stratified squamous epithelium of the skin and a thick parakeratinized epithelium of the oral mucosa (Barrett et al., 2005). Histologically, epithelial tissue changes subtly from vermilion to oral mucosa, thus making it difficult to see a distinct border between both tissues. Both vermilion and lip mucosa have no epidermal appendages, such as sebaceous glands, hair follicles, or salivary glands, as compared to the lip skin (Tsatsou & Zouboulis, 2014; Kato et al., 2022).

2.2.3 Oral mucosa

The transition from the keratinized red margin of vermilion to the thick stratified squamous parakeratinized epithelium of the oral mucosa, as illustrated in Figure 2.6A. Oral mucosa is a membrane that is made up of two layers: an outer layer of non-keratinized stratified squamous epithelium, and lamina propria which consists of dense connective tissue (Dawson et al., 2013; Brizuela & Winters, 2021). Stratum granulosum cells disappear at the epithelium layer, and nuclei are seen in the superficial cells up to the surface of epithelium, as displayed in Figure 2.6B. The superficial part of the lamina propria is known as the papillary layer, and it is made up of connective tissue papillae that intertwine with the rete ridges of the epithelium (Hamam Dalia & Aly El-Waseef, 2018). Under the lamina propria, there is a loose connective tissue that is a submucosa containing muscle, fats, glands, and numerous superficially located capillaries, which contribute to the red appearance of this structure (Cruchley & Bergmeier, 2018).

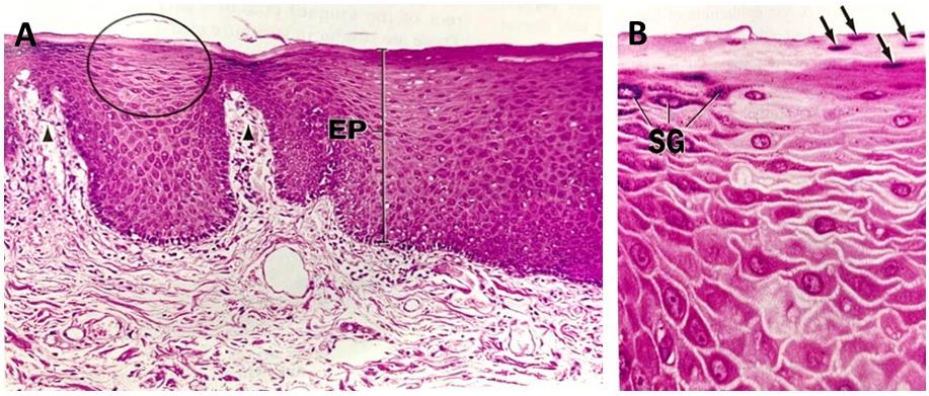

Figure 2.6 Histological stain on oral mucosa. (A) The transition of keratinized vermilion to a thick parakeratinized oral mucosa layer, the disappearance of stratum granulosum (SG) is noted in (B), and nuclei are seen on the superficial layer (arrows). From Ross et al. (2003).

There are three types of oral mucosa: masticatory mucosa (gingivae and hard palate); lining mucosa (lip, cheeks, soft palate, vestibule, alveolar mucosa, and inferior surface of the tongue); and specialised mucosa that mixes of masticatory and lining (dorsum of the tongue). Lining mucosa refers to the inside area of the lip (Ryu et al., 2020). This epithelium layer is similar to skin, which is made up of tightly packed epithelial cells with varying degrees of differentiation, beginning with a basal layer of cells that divide continuously through layers of sup-basal cells undergoing various morphological and biochemical changes dependent on the type of mucosa (Waterhouse, 1984; Dale et al., 1990). The epithelial layer of mucosa is made up of epithelial cells, and just like the epidermis, cells can be found under a light microscope such as melanocytes, Langerhan cells, Merkel cells, and lymphocytes.

The collagen fibers in lamina propria are thin, loosely arranged and have little empty space, whereas the stratified squamous epithelium of oral mucosa exists in non-keratinized state, providing protection against physical, bacterial, and chemical damage as the keratinized layer on the epithelia will decrease the permeability of mucosa (Despotović et al., 2017; Şenel, 2021). A study by Takashimizu and Yuzuriha (2018)

showed the numbers of Melan-A-positive cells in the vermilion were much higher than in the lip mucosa, therefore, the differences in the number of melanocytes and amount of melanin pigment between the vermilion and lip mucosa are responsible for the difference in red colour between the two tissues. The oral mucosa contains orbicularis oris muscle which is positioned in the center of the lip and also tiny salivary glands which is located in the submucosa (J. A. Park et al., 2022; Piccinin & Zito, 2022).

2.3 Orbicularis oris muscle

Musculus orbicularis oris is another name for the orbicularis oris muscle. They are responsible for numerous orofacial movements and facial expression (Sahai & Singh, 2022). During embryonic development, a dense and continuous band of mesenchymal cells reflects the upcoming orbicularis oris muscle formation by 8 weeks of gestation. Later, the visible orbicularis oris muscle fibers appear within 12 weeks, and the architecture is fully completed by 16 weeks (Mooney et al., 1988; Marazita & Mooney, 2004). As illustrated in Figure 2.7, this complex multilayered muscle attaches to the upper and lower lip dermis via a thin, superficial musculoaponeurotic system and functions as an attachment site for numerous other facial muscles in the oral region (Rogers et al., 2005).

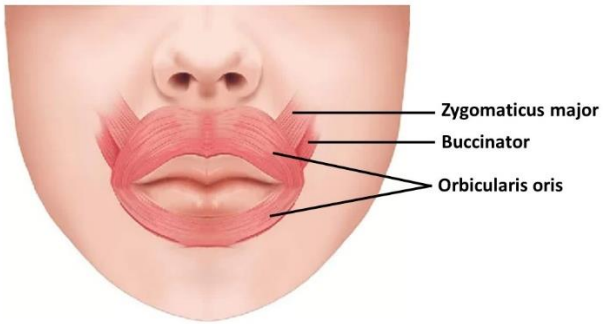

Figure 2.7 Orbicularis oris muscle anatomy. Adapted from Faveret (2015).

Anatomically, this muscle can be considered a single muscle, but it is made of two different components acting independently or together with other facial muscles (Jain & Rathee, 2020). One is a retractor and the other is a constrictor of the lip, and both are known as superficial and deep fiber, respectively (C. G. Park & Ha, 1995). The superficial fiber function exhibits facial expression and lip movement that are required in speech. The role of deep fiber, on the other hand, is associated to keeping food, which involves sphincteric action with other muscular loops of the oropharynx, and is known as the "archaic" part of the muscle (Nicolau, 1983). Thus, the orbicularis oris muscle plays different roles in oral functions from opening to the movement of the jaw such as speech, suction, swallowing, mastication, and sucking (Regalo et al., 2005; J. S. Park et al., 2017).

The connective tissue within skeletal muscle is named according to its association with the muscle fibers as shown in Figure 2.8. Endomysium is a thin connective tissue layer that surrounds individual muscle fibers, perimysium is a denser connective tissue layer that surrounds a group of fibers to form a fascicle bundle, and epimysium is a dense connective tissue sheath that envelops the entire muscle. These connective tissues that surround both individual muscle fibers and a bundle of muscle fibers are essential for force transduction, as they continue as a tendon that attaches to the bone at the end of the muscle (Ross et al., 2003).

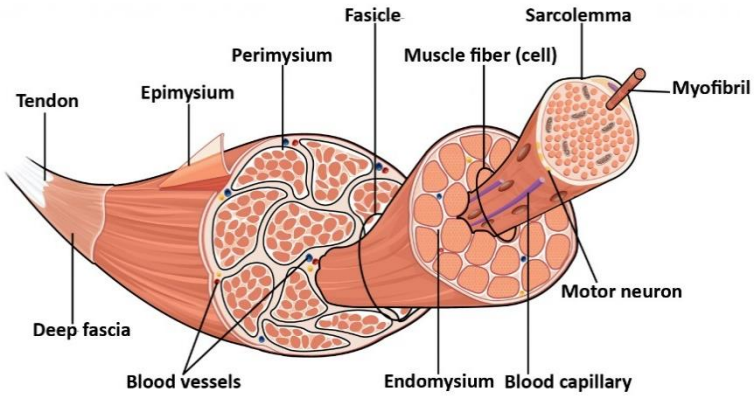

Figure 2.8 Cross-section of a skeletal muscle bundle. From Biga et al. (2019).

Penna et al. (2009) found that the young orbicularis oris muscle has well-defined bundles and fascicles which surrounded by a thin layer of connective tissue. On the other hand, the older orbicularis oris muscle shows signs of atrophy, with smaller bundles and fascicles and a thicker layer of epimysium around them. Muscle fibers are different in diameter, and they can be differentiated using histochemical staining (Talbot & Maves, 2016; Khan et al., 2018). The nuclei, mitochondria, and other organelles of a muscle fiber are located in the cytoplasm beneath the plasma membrane called sarcolemma (Frontera & Ochala, 2015). Muscle fibers represent a collection of myofibrils composed of myofilaments that are made up of functional units called sarcomeres. Myofibrillar proteins myosin (the thick filament) and actin (the thin filament) are found within each sarcomere, as presented in Figure 2.9. The interaction of these two myofibrillar proteins allows muscles to contract (Goldspink & Harridge, 2008). The arrangement of myofibrillar components (actin, myosin, tropomyosin, and troponin) organise into a functional unit named sarcomere. The sarcomere is made up of two interdigitating bands, known as the anisotropic (A) band and the isotropic (I) band that act as contractile properties (Luis & Schnorrer, 2021). The I band which is bisected by the Z line, consists of thin actin filaments that are connected to Z line and

stetch over the I band into the A band. The thick filaments of myosin represent the entire width of the A band, and at the middle of the A band is located M line, which consists of only thick filaments (Valentine, 2017).

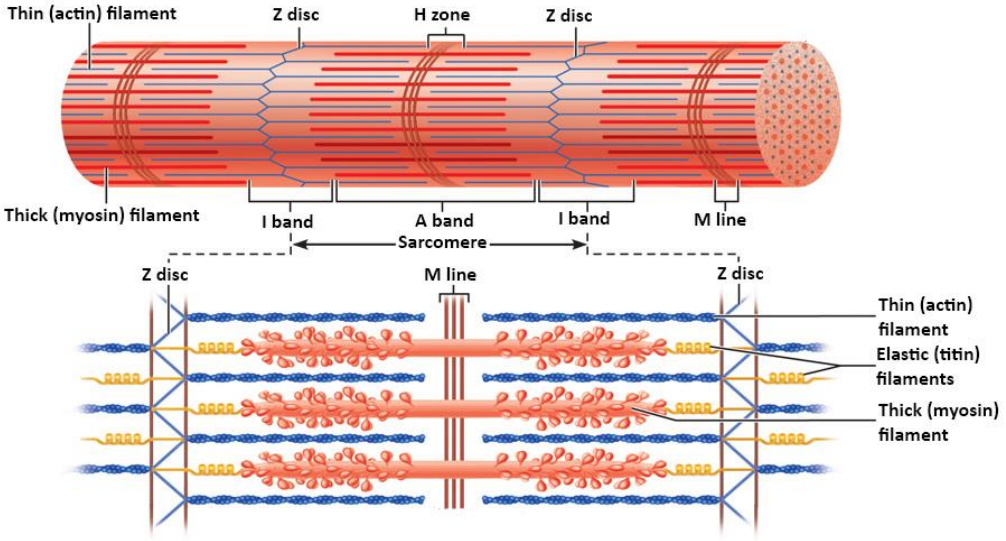

Figure 2.9 Structure of a skeletal muscle fiber. From Marieb & Hoehn (2010).

There are three types of skeletal muscle fibers: 1) red fibers or type I; 2) white fibers or type II; and 3) intermediate fibers (Schiaffino, 2010). Type I fibers are small fibers that contain more blood-carrying myoglobin and a high capillary content, which create darker a colour and contain a large amount of cytochrome complexes and mitochondria. The greater myoglobin and capillary content in red muscles contribute to the greater oxidative capacity of red muscles compared with type II fibers (Klont et al., 1998). They make slow-twitch motor units as slow contraction is needed to maintain an erect posture. This type of muscle can be found in the limb muscles of mammals as well as the breast of migrating birds (Ashmore & Doerr, 1971; Biewener, 2011).

Type II fibers are large fibers with less myoglobin and fewer cytochromes and mitochondria (Berchtold et al., 2000). They make up fast-twitch motor units and fatigue

rapidly, high threshold level that is activated only when the force demands are greater than slow twitch. Intermediate fibers are intermediate size, contained average amount of myoglobin and the number of mitochondria when compared to both red and white fibers (Ogata & Yamasaki, 1985). A few studies with transmission electron microscopy (TEM) have revealed that these different types of fibers vary in mitochondrial structure and the thickness of Z-lines (Ogata & Yamasaki, 1997). Abnormalities in mitochondrial shape and arrangement, such as enlarged and clumping mitochondria, are frequently detected in disease muscle, which indicates mitochondrial dysfunction, also known as mitochondrial myopathy (Milner et al., 2000).

2.4 Mitochondrial myopathy

Muscle fiber has a lot of mitochondria that function in ATP generation via oxidative phosphorylation which provides energy for muscle contraction (Dahl et al., 2015). Figure 2.10 illustrates a cross-sectional muscle fiber with mitochondria located closer to the sarcolemma (subsarcolemmal mitochondria) of the muscle fiber, meant to decrease the gap for oxygen diffusion by the capillary supply. This is particularly useful during aerobic (or endurance) exercise when the demand for oxygen increases. In the area within intermyofibrillar, there is another group of mitochondria known as intermyofibrillar mitochondria (Frontera & Ochala, 2015). The activity of mitochondria is controlled by two genomes: nuclear genome and mitochondrial genome (Ahmed et al., 2018). The mitochondria DNA is strictly maternally inherited and contains in multiple copies ranging from hundreds to thousands within a single cell, distinguishing it from the nuclear DNA (Greaves et al., 2012).

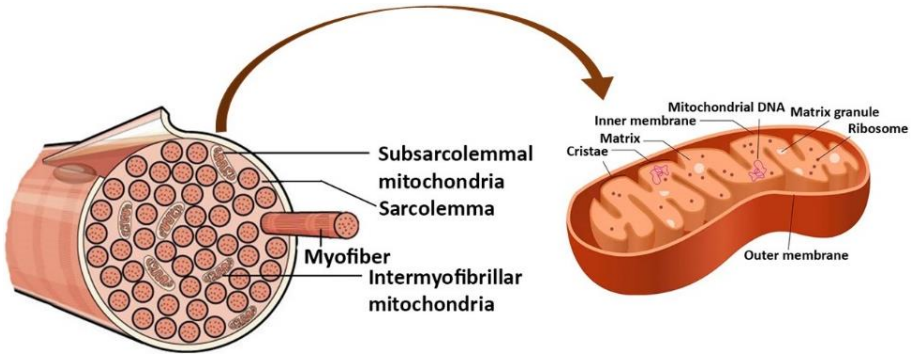

Figure 2.10 Illustration of a structural muscle fiber and a cross section of a mitochondrion. Adapted from Betts et al. (2013) and Kelvinsong (2013).

Mitochondrial myopathies are described, most based on morphological abnormalities of the mitochondria (Dimauro et al., 2014). Histochemical changes in the skeletal muscle indicative of mitochondrial malfunction, despite the fact that in some cases the muscle biopsy can appear normal (Joyce et al., 2012). Several histological and immunological investigations can be used to identify these mitochondrial abnormalities (Ahmed et al., 2018). The formation of ragged red fibers in the fiber is the morphological hallmark of mitochondrial myopathy. This pattern can be detected in skeletal muscle cryosections with the modified Gomori Trichrome stain (Olson et al., 1972; Petty et al., 1986). As stated by Jackson et al. (2015), the disorganisation of muscle fiber observed under light microscopy will be emphasized more in electron microscopy by the arrangement of myofibrillar components. This ultrastructural investigation also provides some insight into mitochondrial myopathic features by detecting mitochondrial morphological abnormalities. A common discovery of electron microscopy in muscle disease is the accumulation of mitochondria that aggregate in the subsarcolemmal and intermyofibrillar regions of a longitudinal section of a muscle fiber, as discovered by Hughes et al. (2019) and J. Q. Lu et al. (2019). Figure 2.11 shows an illustration of a longitudinal section of a muscle fiber and the distribution of

subsarcolemmal mitochondria that located underneath the sarcolemma, and intermyofibrillar mitochondria located within the myofibrils.

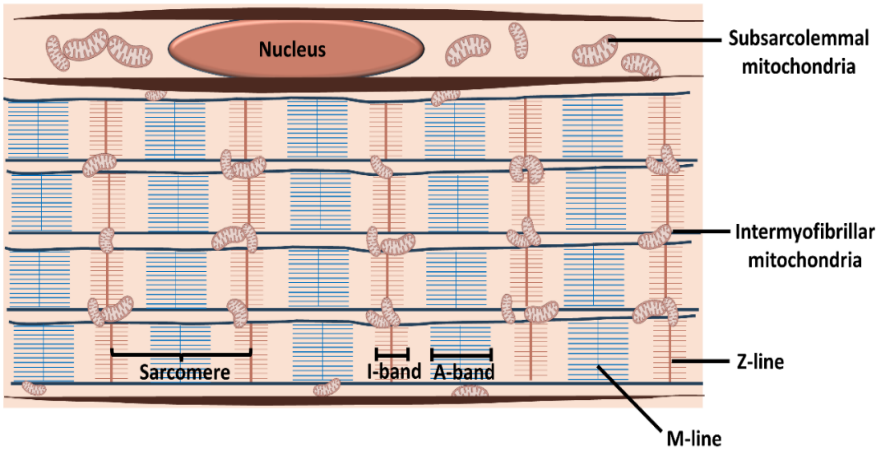

Figure 2.11 An illustration of a longitudinal sectional muscle fiber.

Mitochondrial oxidative phosphorylation is made up of five enzymes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone cytochrome c oxidoreductase (complex III), cytochrome c-oxidase (complex IV), and ATP synthase (complex V) (Lee et al., 2016). Cytochrome c-oxidase is a final electron transport system of the mitochondria (Pitceathly & Taanman, 2018). It plays a vital role for catalysing electron transfer from cytochrome c to molecular oxygen in a process to transport protons (proton pump) for ATP synthesis (Sinkler et al., 2017). These proteins can be found within the inner membrane of mitochondria, as shown in Figure 2.12.

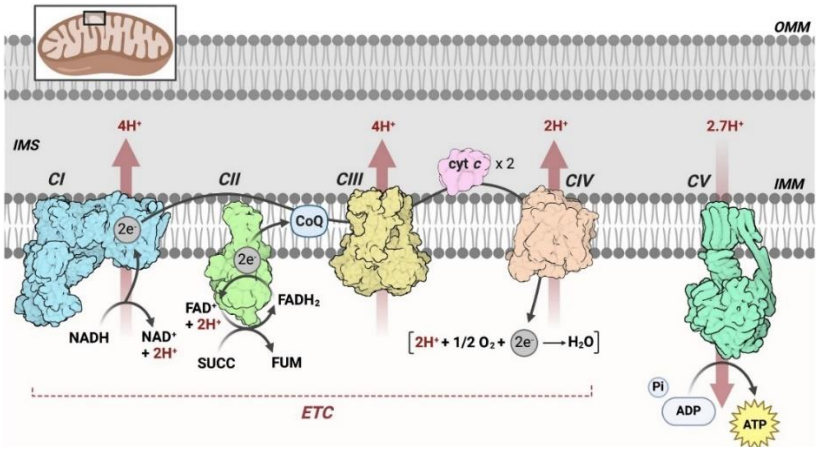

Figure 2.12 Mitochondrial oxidative phosphorylation pathway. From Brischigliaro & Zeviani (2021).

Defects in function and formation of oxidative phosphorylation complexes associated with nuclear or mitochondrial DNA can induce mitochondrial myopathy (Bergman & Ben-Shachar, 2016). Mitochondrial defects can be detected using cytochrome c-oxidase (COX) histochemical staining, which identifies the deficiency of COX activity in complex IV of mitochondria. White COX-negative fibers and brown COX-positive fibers form a mosaic pattern with the muscle fibers (Orsucci et al., 2021). Another histochemical stain involved in the oxidative activity of mitochondria is succinate dehydrogenase (SDH, complex II), which has the ability to identify mitochondrial aggregation in the subsarcolemmal area of the fibers, which is another diagnostic hallmark of mitochondrial myopathy (Moraes et al., 1992).

Energy production from glycolytic pathways acts as an precursor sources for biochemical reactions to the Krebs cycle and oxidative phosphorylation process (Kanungo et al., 2018). A defect in mitochondrial function may attenuate ATP production; thus, these three pathways must be accounted for to quantify the total ATP of the cells (Desousa et al., 2022). Quantification of ATP levels has been widely used to observe metabolic activity in cells and tissues, its depletion becomes an indicator of

impaired mitochondrial function. However, genetic and biochemical defects of mitochondrial function are the main cause of human illness, but their connection to mitochondrial morphology is unclear (Vincent et al., 2019).

Eight cleft studies have been conducted for mitochondrial myopathy investigation by using histological and histochemical staining, and also ultrastructural analysis (Schendel et al., 1989, 1991, 1994; Raposio et al., 1998; De Chalain et al., 2001; Franklin et al., 2005; Lazzeri et al., 2008; E. K. Kim et al., 2010). The most significant findings were the detection of ragged red fibers through histochemical staining in patients with non-syndromic cleft lip by Schendel et al. (1989) and in a patient with cleft palate diagnosed with multiple diseases, as reported by Franklin et al. (2005). This pathological hallmark, however, was never identified in another six studies. Instead, electron microscopy revealed that these cleft lip muscle fibers were frequently shown to have large size of mitochondria accumulated within sarcolemmal regions, causing irregular striations of the myofibrils, as reported in the studies conducted in North America by Schendel et al. (1994) and De Chalain et al. (2001), in Europe by Raposio et al. (1998), and in Asia by E. K. Kim et al. (2010). All these studies, however, were more than 20 years old, so the methods used in the past differ from now as they have evolved throughout time. So, it is understandable that the complexity of cleft pathology and insufficient investigations have not provided any substantial findings supporting the presence of myopathy in cleft.

CHAPTER 3

MATERIALS AND METHOD

3.1 Sample collection for cleft lip tissues

Lip skin tissues were taken from patients from January 2020 until June 2022, at Hospital Universiti Sains Malaysia (HUSM), Kelantan. All cleft lip tissues were obtained from patients with NSCL/P who underwent cleft lip repair operation at the upper lip skin area conducted by the plastic surgeons of the Reconstructive Sciences Unit at HUSM under general anaesthesia. The age of NSCL/P patients was between 3 months to 7 years old. Forty cleft lip tissues were obtained and processed based on the respective analysis: eight tissues were fixed in formalin for haematoxylin and eosin (H&E) staining and modified Gomori trichrome staining; five tissues were frozen in the optimal cutting temperature compound (O.C.T.) for cytochrome c-oxidase (COX) staining and adenosine triphosphatase (ATP-ase) staining; four tissues were fixed in glutaraldehyde for electron microscopy; and 23 tissues were cryopreserved for assay.

3.2 Sample collection for controls

Control tissues were collected from January 2020 until June 2022, at HUSM, Kelantan. Normal patients were aged between 1 and 14 years old. Seven controls were used in this study. For objective 1, two control lip tissues were obtained from consented normal individuals who underwent lip excision for trauma injuries due to road traffic accidents. Meanwhile, for objective 2, two muscle tissues from lower limbs were obtained from two different individuals who underwent muscle biopsies. A normal human primary gingival fibroblast (ATCC PCS-201-018) was purchased and cultured for three passages as controls for objective 3.

3.3 Selection criteria

3.3.1 Inclusion criteria

3.3.1(a) Cleft lip patients

Patients were screened by specialists of plastic surgery. Non-syndromic unilateral or bilateral cleft lip patients that age between 3 months – 7 years old were included. Non-syndromic cleft lip patients with one minor anomaly identified such as low-set ears, hypertelorism, clinodactyly and single palmar crease were included as these anomalies will not interfere with cleft abnormalities.

3.3.1(b) Normal patients

Individuals who are normal, aged between 1 and 14 years old, were included. Individuals who underwent lip injury excision and maintained their epithelial and dermal structures were chosen for objective 1. Normal lip tissue that maintained orbicularis oris muscle structure or skeletal muscle tissue from any part of the body of normal individuals were selected for objective 2. Fresh normal lip or oral tissues from individuals who got into traffic accidents were selected for objective 3.

3.3.2 Exclusion criteria

3.3.2(a) Cleft lip patients

Syndromic cleft lip and/or palate patients, facial clefts and non-syndromic cleft lip and/or palate patients with any major abnormalities such as heart problem, brain defect detected during the screening were excluded.

3.3.2(b) Normal patients

Normal control individuals who displayed non-syndromic or syndromic cleft lip and/or palate formation, or other syndromic diseases, or had a family history of cleft were excluded from this study. Individuals with brain defects, heart problems, or any