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A Systematic Review of the Role of Mitochondria in Cleft Pathology: A Forgotten General?

Rabiatul Adawiyah Mohamad Noor[a], Wan Azman Wan Sulaiman[a], Anani Aila Mat Zin[b], Nurul Syazana Mohamad Shah[a]*

[a]*Reconstructive Sciences Unit, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[b]*Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

*Corresponding author: syazanashah@usm.my

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ABSTRACT

Orofacial clefts (OFC) are one of the most common birth defects that affects the lip, palate, or lip and palate of an infant. The deterioration of clefts is multifactorial involving multiple genes, various interactions from environmental factor and most forgotten, mitochondrial abnormality. The aim of this review is to highlight the importance of mitochondrial activity related to non-syndromic OFC deformity. Despite its important role in cells, the study on mitochondrial activity in cleft pathology was scarce and almost forgotten compared to other genetic investigations. This systematic review was completed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist. The literature search was done via the following databases: Google Scholar, Pubmed and Scopus with a total of nine studies of mitochondrial abnormalities were included. We hypothesise that mitochondria play an important role in early craniofacial development. A decreased in its function or activity may result in cleft lip formation. Hence, we would like to shed light on the remarkable role of mitochondria activity in the pathogenesis of non-syndromic OFC.

Keywords: *Cleft; non-syndromic; mitochondrial DNA; mitochondrial myopathy; orbicularis oris muscle*

INTRODUCTION

Orofacial clefts (OFC) is one of the birth defects that occur in the face area (Pavri & Forrest, 2013; McBride *et al.*, 2016). Cleft occurs in isolation (i.e., non-syndromic) or in combination with other abnormalities (i.e., syndromic). OFC is a result of facial tissues which is not joining properly during early foetal development for an unknown reason (Leslie & Marazita, 2013). The formation of the basic morphology of the face in a human embryo occurs in the fourth week and is completed by the 12th week after fertilisation (Shkoukani *et al.*, 2013). At this stage, the facial tissue has failed to fuse properly which causes separation of the lip and palate known as cleft lip (CL) and cleft palate (CP), respectively (Wattanawong *et al.*, 2016).

OFC can be diagnosed at approximately 16 weeks of gestation (Schoenwolf *et al.*, 2009). The complexity of normal development is controlled by genes at the early embryonic development and any disturbances through these processes

would lead to disruption of normal growth (Sperber, 2002). There has been a lot of studies on the aetiology of OFC, and most scientists are focusing on both genetic and environmental factors (Liu *et al.*, 2015; Xu *et al.*, 2018).

One of the examples of gene-environment interaction in the aetiology of cleft is folic acid metabolism (Bianchi *et al.*, 2000). Folates are involved in the DNA methylation process during the transfer of methyl group to the DNA base, adenine and cytosine (Lucock, 2000; Jones & Takai, 2001). Methyl donor plays an important role and its deficiency will affect the neural crest cells during early embryonic development (Bhaskar *et al.*, 2011). Various studies from Western countries suggested that maternal periconceptional folic acid supplementation has a potential preventive role in preventing OFC (Figueiredo *et al.*, 2015). However, the evidence is inconsistent due to sample selection biases, population as well as different dosages of folic acid supplementation (De-Regil *et al.*, 2015).

Genes expressed in orbicularis oris (OO) muscles are also expected to be involved in the pathogenesis of OFC and this muscle is often damaged in OFC patients, as it is more likely to be involved in the origin of the defect (Masotti *et al.*, 2018). The OO muscle plays different roles in oral functions, from the opening to the movement of the jaw which involve speech, suction, swallowing, mastication and sucking (Regalo *et al.*, 2005; Park *et al.*, 2017). If the OO muscle is not repaired correctly, it may change the shape abnormally and tend to be distorted as the patients grow older (Park & Ha, 1995).

Defects in the mitochondrial respiratory chain in the skeletal muscles may lead to mitochondrial myopathy; weakness and hypotonia of muscles (DiMauro *et al.*, 2014). Mitochondria are essential for eukaryotic life as they are responsible in generating most of the ATP in the cells (Annesley & Fisher, 2019). There are two genomes involve in controlling the mitochondrial functions: nuclear genome (nDNA) and mitochondrial genome (mtDNA) (Taylor & Turnbull, 2005). Mitochondrial myopathies can occur due to pathogenic variants in either of these genomes and it is inherited through maternal lineage (i.e., mtDNA), X-linked, autosomal recessive, or autosomal dominant. The mitochondrial myopathy can be diagnosed based on the following investigations: histological; immunohistochemical and enzymatic techniques; as well as molecular testing (Ahmed *et al.*, 2018). Our previous study observed less dense collagen fibres in the cleft lip and palate (CLP) skin structure compared to the normal ones (Shah *et al.*, 2014). Additionally, we found disoriented and disorganised collagen and muscle fibres in CLP skin using the scanning electron microscope (Shah *et al.*, 2014). Hence, the present review went deeper by studying the role of mitochondria that could be associated with the abnormal regulation of collagen and muscle fibres in non-syndromic CLP.

This article aims to highlight the deformities in mitochondrial morphology associated with the non-syndromic OFC. This review would deliver deep knowledge regarding the mitochondrial abnormality in OO muscles of non-syndromic OFC and would be helpful for a better diagnosis and prevention. The findings on mitochondrial abnormality in non-syndromic OFC are still unclear since most published studies are focused on the genetic studies. Therefore, the investigation on mitochondrial abnormality will help in providing better prevention, treatment and diagnosis of the defects.

MATERIALS AND METHODS

This review article was done following the PRISMA statement (Moher *et al.*, 2009).

Information Sources

An electronic search was conducted in three different databases: PubMed, Scopus and Google Scholar. The search consisted of the following keywords: non-syndromic,

cleft, OO muscle, mitochondrial myopathy, mitochondrial DNA and aetiology of cleft. There was no limit to publication dates and only studies published in English were chosen. References lists of the eligible articles were manually screened to identify other potential studies. However, we did not contact the authors of the included studies for further information. The final search was completed on 1st October 2020.

Selection Criteria

The following are the inclusion criteria for the studies included in this review: (1) research works focused on groups of patients with non-syndromic OFC only, (2) histological and ultrastructural studies of OO muscles and (3) folate effect on non-syndromic OFC. Commentaries and editorial articles were not included. We also excluded animal-based studies, studies irrelevant to non-syndromic OFC and duplicates within the databases.

Study Selection and Data Analysis

Based on the criteria of inclusion and exclusion, the initial screening was performed on the titles, abstracts and keywords. The next screening was based on the full-text review. The first (RAMN) and fourth (NSMS) authors independently screened the information. The following study characteristics were extracted: (1) the author, (2) the publication date, (3) the population, (4) the source of the sample, (5) the study design and (6) the number of individuals with cleft. In the process of selection and extraction, the final results were discussed by the second (WAWS) and third (AAMZ) authors.

Assessment of Risk of Bias

Data were analysed by summarising the main findings of the studies. The assessment was prepared without any information about the authors of the selected articles, considering only the given data.

RESULTS

The search strategy finally turned out a total of 789 studies; of which, 605 of them were from the PubMed database, 35 from the Scopus database and 149 from the Google Scholar database. A total of 491 items were excluded due to the following reasons: studies that are irrelevant to non-syndromic OFC, duplicates, animal studies and were not written in English. Out of the remaining 298 items, a total of 289 items were dismissed as they did not meet all of the requirements.

In the end, nine studies that were relevant to the mitochondrial abnormality were included in the systematic review as shown in Table 1. A variety of data collection methods were used in these studies. Six studies were conducted in North America (Schendel *et al.*, 1989; 1991; 1994; de Chalain *et al.*, 2001; Franklin *et al.*, 2005; Vieira *et al.*, 2008), two were in Europe (Raposio *et al.*, 1998; Lazzeri *et al.*, 2008), and one was in Asia (Kim *et al.*, 2010). A flow diagram for study selection is presented in Fig. 1, based on PRISMA.

DISCUSSION

Mitochondrial myopathies were introduced in the early 1960s when the studies revealed abnormal-looking accumulations of mitochondria appearing as reddish blotches in the skeletal muscle of patients (Shy & Gonatas, 1964). These mitochondrial abnormalities can be observed using modified Gomori trichrome stain by the presence of "ragged-red fibre" which came to be considered as the pathological indication of traditional histological appearance of mitochondrial myopathy (Engel & Cunningham, 1963). Although it is not used as an indicator of mitochondrial myopathy on muscle biopsy but they are highly suggestive (Franklin *et al.*, 2005).

Schendel *et al.* (1989) proposed in their earlier study of mitochondrial myopathy that a metabolic defect at the mitochondrial

Table 1 History of mitochondrial studies in cleft

Research study	Type of cleft	Type of sample	Histologic and histochemical staining	Other techniques	Outcome
Schendel *et al.* (1989)	Unilateral cleft lip (UCL)	Muscle	Gomori trichrome Adenosine triphosphatase NADH tetrazolium reductase Acetycholinesterase	Electron microscopy	• Presence of large numbers of ragged red fibres • Fibres had star-shaped appearance due to accumulation of mitochondria variable in shape at the central portion of the fibre • Electron microscopy demonstrated large accumulation, variable shapes of mitochondria
Schendel *et al.* (1991)	Secondary revision of UCL	Muscle	Gomori trichrome NADH tetrazolium reductase Acetylcholine esterase Adenosine triphosphatase	Electron microscopy	• No presence of ragged red fibres • Appearance of scattered muscle fibres
Schendel *et al.* (1994)	CLP CP	Muscle	Gomori trichrome NADH tetrazolium reductase Acetylcholine esterase Adenosine triphosphatase	Electron microscopy	• No presence of ragged red fibres • Abnormal accumulation of mitochondria in CLP but not in CP muscles
Raposio *et al.* (1998)	UCL	OO muscle	Gomori trichrome Adenosine triphosphatase Nicotinamide adenine dinucleotide-tetrazolium reductase Cytochrome c-oxidase Succinate dehydrogenase	Transmission electron microscope	• No presence of ragged red fibres • No cytochrome c-oxidase-negative • No succinate dehydrogenase-positive • Electron microscopy showed abnormal sub-sarcolemmal accumulation of glycogen and mitochondria
de Chalain *et al.* (2001)	CL	Muscle	Haematoxylin and eosin Gomori trichrome Adenosine triphosphatase Bielschowsky Masson trichrome Luxol fast blue	Osmium tetroxide Uranyl acetate Light microscopy	• No presence of ragged red fibres • Disorganisation of muscle fibre • Sub-sarcolemmal accumulations of mitochondria in some specimens • The clustering of abnormal-looking mitochondria variations in shape and size were observed
Franklin *et al.* (2005)	CP with other anomalies; hypotonia, rib fusions, ventricular septal dyskinesis	Case study cardiac and skeletal muscle	Gomori trichrome	Multiple clinical investigations Electron microscopy	• Presence of ragged red fibres • Mild hypotonia, cardiomyopathy • Mitochondrial myopathy • Suspected genetic mutations
Lazzeri *et al.* (2008)	CP CLP	Palatal muscle OO muscle	Haematoxylin and eosin Modified Gomori trichrome Adenosine triphosphatase NADH tetrazolium reductase	Light microscopy	• No presence of ragged red fibres • Disorganization of muscle fibres • Increased endomysial fibrosis

(*Continued on next page*)

Table 1 (*Continued*)

Research study	Type of cleft	Type of sample	Histologic and histochemical staining	Other techniques	Outcome
Vieira *et al*. (2008)	Non-syndromic cleft lip with or without cleft palate (NSCL/P)	Blood samples	N/A	Kinetic PCR Molecular beacon assays	• There was an association between RFC1 alleles with CL only among individuals with mtDNA haplotype other than haplotype D • No association between individuals with mtDNA haplotype D with MTHFR alleles
Kim *et al*. (2010)	Unilateral microform CL	Muscle	Haematoxylin and eosin Gomori trichrome NADH tetrazolium reductase	Electron microscopy	• No presence of ragged red fibres • The red granularity were observed within the myofibres indicates the increase of mitochondrial activity • Focal accumulation of sub-sarcolemmal mitochondria • The increased numbers of type 2 fibres were observed

Note: N/A – not applicable.

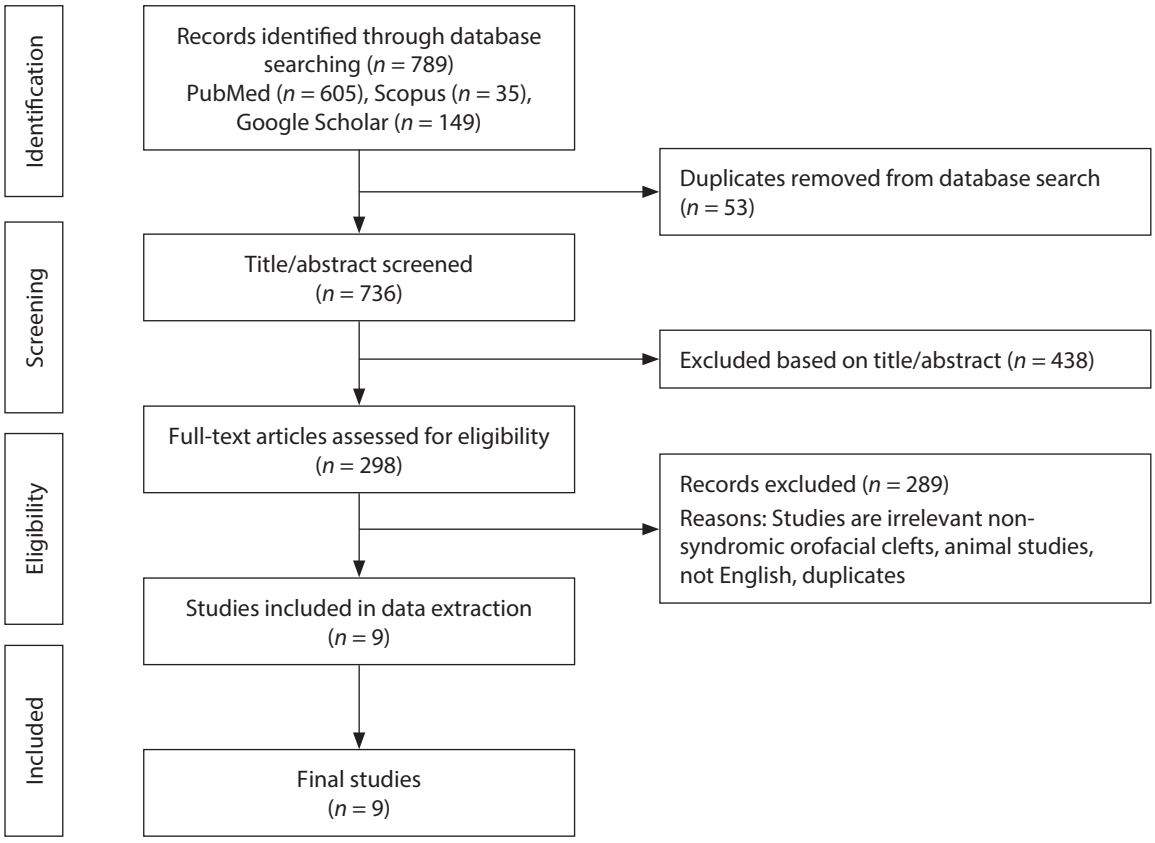

Fig. 1 PRISMA flow diagram of search results, study selection and inclusion process (Moher *et al.*, 2009).

level was expected to be involved in the CL aetiology. Sixty-six specimens from primary CL repair were obtained and submitted to histological stains, enzyme histochemical stain and electron microscopy. Modified Gomori trichrome stain and electron microscopy demonstrated the presence of ragged red fibres and accumulation of mitochondria in all muscle biopsies of CL. They postulated that a metabolic myopathy in the mitochondria on the CL was probably due to the presence of ragged red fibres and the deformities of mitochondrial size, structure, and grouping. Therefore, they suggested myopathy in the facial mesenchymal was due to the insufficiency of energy production by the mitochondria, which led to the failure of mesenchymal reinforcement of the facial process in CL (Schendel *et al.*, 1989).

In their next study, however, Schendel *et al.* (1991) were unable to demonstrate the appearance of ragged red fibres in all muscle biopsies obtained from CL during secondary lip revision. For that reason, they postulated that the "pattern of disappearance" of abnormal mitochondria could be because of the early ages of the patients when the primary CL is repaired. Therefore, the aetiology of this condition remains unknown. Mitochondrial myopathy studies then proceeded with 30 specimens from 16 patients that had CLP and 7 had isolated CP (Schendel *et al.*, 1994). Gomori trichrome and NADH stains revealed the presence of abnormal mitochondrial accumulations in palatal muscle in CLP specimens but not in isolated CP muscle specimens; this appearance was not as intense as those in the primary lip defects. They postulated that the appearance of this central mitochondrial accumulation might be functionally induced or due to secondary origin. However, there was no sign of mitochondrial myopathy found in the CP samples (Schendel *et al.*, 1994).

On the other hand, Raposio *et al.* (1998) observed the increased mitochondrial activity under electron microscopy, differentiated by red granularity and sub-sarcolemmal accumulation of mitochondria in certain parts of muscle specimens that were obtained from 10 (unilateral) CL infants. However, with histological stain, they failed to demonstrate ragged red fibres or a metabolic myopathy that would support the theory of mitochondrial myopathy. Additionally, de Chalain *et al.* (2001) investigated about 40 fresh muscle biopsies from CL patients and 8 control muscle tissues from patients who underwent lip laceration. Histological examination revealed increased sarcolemmal accumulation of mitochondria in some CL specimens with identification of muscle fibre size variations. Also, the ultrastructural findings observed abnormal clustering of abnormal-looking mitochondria in muscle specimens, but no ragged red fibres was observed.

Lazzeri *et al.*, (2008) analysed histological and histochemical characteristics of palatal muscles and OO muscles in CP and CLP patients. The histological stains showed increased endomysial fibrosis, disarrayed arrangements and size variability of OO muscles fibres. In contrast to Schendel *et al.*, (1989), they could not find any signs of mitochondrial myopathy in the muscle of patients. The studies by Raposio *et al.* (1998) and de Chalain *et al.* (2001) were supported by Kim *et al.* (2010) in their study on OO muscles from 11 CL muscle specimens. The results revealed scarce and hypoplastic with variation in size, with the focal sub-scarcolemmal accumulation of mitochondria in the specimens. Kim *et al.* (2010) suggested the presence of red granularity had indicated the increase of mitochondrial activity within myofibres, and again, there were no signs of mitochondrial myopathy such as ragged red fibres were detected as mentioned by Schendel *et al.* (1989).

Although the mitochondrial myopathy has not been consistently identified in muscle specimens from cleft repairs, histological studies by Franklin *et al.* (2005) however, demonstrated the appearance of ragged red fibres on muscle biopsy suggesting mitochondrial myopathy. However, the muscle specimens were obtained from an infant diagnosed with a CP accompanied by other deformities; hypotonia, rib fusions and ventricular septal dyskinesia. The association between clefting and mitochondrial myopathy has not been established, hence, Franklin *et al.* (2005) suggested further research on similar patients is needed to reveal more links between these two diagnoses.

To date, the association of mitochondrial myopathy with OFC has not been established. Therefore, the researchers postulated that the evidence of mitochondrial myopathy could not be demonstrated in other studies due to the pathologic changes in mitochondria affecting certain areas of tissue populated by normal mitochondria, or the difference in terms of aetiologic mechanism (de Chalain *et al.*, 2001). Studies on mitochondrial ultrastructure from cleft tissues have been previously reported (Kim *et al.*, 2010), and researchers nowadays are currently exploring on mitochondrial myopathy and its possible role in the pathogenesis of non-syndromic OFC using several methods including histological and histochemical staining, biochemical reaction and ultrastructural analysis.

Maternal folate plays a crucial role in development in the early life, where its deficiency would disturb embryo and foetal cells proliferation such as neural crest cells which consequently involve in the maxillofacial bone and cartilage development (Jones & Takai, 2001; Bhaskar *et al.*, 2011). The mtDNA is used to trace the maternal ancestry of individuals, and because of its heritability, it is also subjected to observe mutational changes in its sequence (Hájek *et al.*, 2008). The previous study by Vieira *et al.* (2002) suggested that mtDNA variant known as mitochondrial haplotype D was commonly discovered among CL/P cases born in South America. However, their

next study on the folate effects on OFC demonstrated the association between CL and the reduced folate carrier 1 (RFC1) with the individuals who have mtDNA other than haplotype D in the same population of South America (Vieira *et al.*, 2008). Therefore, they suggested that the mutations in mitochondrial genes that took part in the folate pathway might contribute to CL only, dependent on ethnic group and specific genetic background such as maternal mitochondrial origin.

Lack of findings on the association between CL and variation in RFC1 has been consistently reported; hence, further investigations should be considered to discover the differences in the clinical expression of OFC. Although there is insufficient evidence for folate effects on OFC, along with its effects for neural tube defects, a previous study had suggested that genetic mutations existed in the folate pathway (Vieira *et al.*, 2008). Thus, the folate effect on OFC is still an issue that needs to be studied further (Millacura *et al.*, 2017).

CONCLUSION

This review provides an overview of the inconsistency of the previous studies on mitochondrial abnormalities in non-syndromic OFC. Due to the lack of investigations and knowledge, there was no reported significant role of mitochondrial abnormality in cleft pathology. It seemed that the pathological study of mitochondria on cleft is not of interest to the researchers. It has been almost forgotten, hence the lack of information on this subject matter. However, with the advancement of genetic technology, studies on mtDNA had shown a significant potential in determining the important role of mitochondria in the orofacial development. Therefore, this article will provide more information regarding the association of mitochondrial abnormality in non-syndromic OFC and would offer more understanding of the pathogenesis of this malformation.

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