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STRUCTURE OF SOFT TISSUES IN CONGENITAL UNILATERAL CLEFT LIP, LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS

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ABSTRACT

Cleft lip (CL), palate (CP), or both (CLP) are one of the most common congenital abnormalities in humans, causing a heavy burden to the affected and their next of kin. We examined biopsy material from CL of seven children: Six 2 to 6 month-old babies and one 4-year-old boy. The samples were taken at the first surgical cleft lip repair. Light microscopy (LM) haematoxylin and eosin stained paraffin sections and toluidine blue stained 0.5–1 µm Durcupan sections from material processed for transmission electron microscopy (TEM), revealed abnormal "ragged" wavy muscle fibres in all seven children. The routine TEM confirmed our results LM; we found no other changes in the soft tissues in TEM; blood vessels of loose connective tissue and nerve fibres were normal. Therefore, we believe that myopathic changes in the cleft lip muscle fibres cannot be of neuronal origin.

Key words: congenital cleft lip; biopsy; light and electron microscopy; myopathy

INTRODUCTION

Cleft lip (CL), *cheiloschisis* may occur either simultaneously, but is often associated with *palatoschisis* – cleft palate (CP), a lesion called *cheilopalatoschisis,* cleft lip and palate (CLP). CLP is a common congenital defect, which occur in one of 300–1400 births in humans depending on the race and region [7, 15, 45, 52, 65, 69].

CL are not as common in animals as in humans. They were described in dogs [41, 46, 51, 56, 60], cats [13, 35], cattle [12, 40, 55, 76,], and rarely in horses [16, 73]. Medial cleft lip is normal in the rabbit, hare, sheep and an incomplete median cleft lip is physiological in the cat family. Llamas and camels have the upper lip split into two parts that they can pull tight to keep out sand.

In the last years, a number of papers on CL and/or CP/CLP have been published, mainly focused on plastic surgery treatment or surgical repair of the CL in humans [49, 57, 65] for the reviews. Some authors used animal model drug induced CL/CP in order to elucidate its pathogenesis [18, 48, 61, 62, 63, 75]. In spite of a great bulk of literature on CL and/or CP or both, the mechanism of pathogenesis of this frequent developmental anomaly remains obscure [9, 33, 58, 71].

We present the results of light (LM) and electron microscopic (EM) observations of tissues in CL biopsies of children treated for plastic reconstructive surgery at UNLP in Košice. The aim of the project was to clarify if and how soft tissue structures are changed in non-syndromic unilateral simple CL, which could help clarify the pathogenesis of lip clefting and provide further background for successful surgical repair.

MATERIALS AND METHODS

We studied samples of seven patients affected by simple non-syndromic unilateral CL, biopsied during the first reparative plastic surgery**.** Biopsy samples were taken from six 2 to 6 month-old babies and one 4-year-old boy**.** Written informed consent has been obtained from the legislative representative(s) of children to publish the data for research purpose.

Light microscopy (LM)

We used (1) haematoxylin and eosin (H&E) stained 5–7 μm paraffin sections from routinely processed material fixed in 4 % neutral buffered formaldehyde and (2) toluidine blue stained 0.5–1 μm plastic Durcupan sections from the material processed for electron microscopy.

Electron microscopy (EM)

We used routine transmission EM. Immediately after biopsy, we fixed material in 3 % glutaraldehyde (Sigma-Aldrich, Bratislava, Slovak Republic) in 0.15 M cacodylate buffer pH 7.2 for 3 hours, rinsed in the same buffer overnight and post-fixed in 1 % OsO_4 (Fluka Chemie AG, Buchs, Switzerland) for 2 hours. Fixed specimens we dehydrated in acetone, and embedded in Durcupan ACM (Sigma-Aldrich Chemie GmbH, Germany). Ultrathin sections we cut on TESLA BS 490 ultramicrotome using glass knives, double stained with uranyl acetate (Sigma-Aldrich, Bratislava, Slovak Republic) subsequently by lead citrate (Merck, Bratislava, Slovak Republic), and photographed in a Tesla BS 500 and/or JEM 1200 EX.

RESULTS

The only significant structural change we found in each of the seven cases of CL were degenerative changes in the skeletal muscle fibres. All other structures such as loose connective tissue well supplied with small blood vessels (Fig. 1 and 2), epidermis and epithelium of oral mucous membrane, hair follicles, sebaceous skin glands (Fig. 3 and 4), blood and nerve supply to the lips were normal, we did not find any pathological changes. TEM confirmed the LM findings. All ectodermal derivatives, e.g. epidermis, hair follicles, skin glands and epithelial cells (Fig. 5) of oral mucous membrane were normal. Lymphocytes (Fig. 5, arrows) were occasionally present in the slightly hypertrophic epithelium of the oral cavity. We did not observe ultrastructural changes of desmosomes (Fig. 5, blue oval) between adjacent cells of the epithelium lining the CL; their normal structure and function are essential for epithelial integrity. The ultrastructure of fibroblasts (Fig. 7 and 8) indicated their normal activity. LM of both routine H&E paraffin and 1 μm plastic sections already revealed changes in the muscle of all CL. Damaged muscle fibres were significantly smaller in diameter, dark, acidophilic in H&E, “ragged” (blue arrows in Figs. 9–11) and always wavy, unlike straight normal muscle fibres. The number of damaged dark wavy “ragged” muscle fibres in individual cases were different, and ranged from about 30 to 60 from case to case. We found no structural evidence of deficient blood and neural supply (Fig. 5) in Simonart´s band of the cleft lip and adjacent tissue. The normal LM structure (Fig. 4, 12) and ultrastructure of small nerve bundles (Fig. 13) and single myelinated (Figs. 14–16) and autonomic nerve fibres gave no evidence of neurogenic muscle atrophy. We did not even detect an increased number of mitochondria in the muscle fibres (Fig. 17). Rarely, just in a few muscle fibres, we observe round nuclei with margination of chromatin (Fig. 18, arrows), a typical sign of apoptosis. We estimated 1–5 % apoptotic fibres depending on the case.

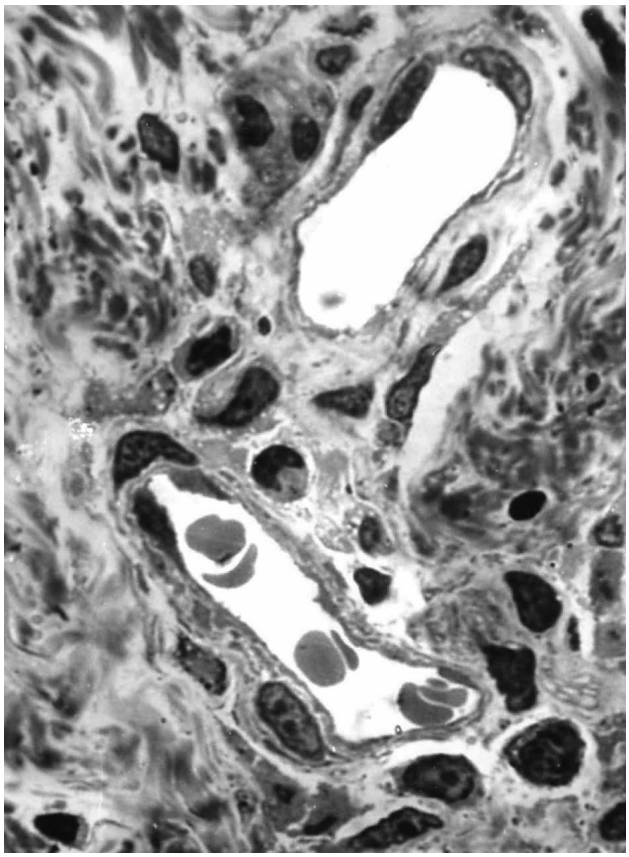

Fig. 1. Vasculature in loose connective tissue.
1 µm Durcupan section, toluidine blue. Magn. x 1000.

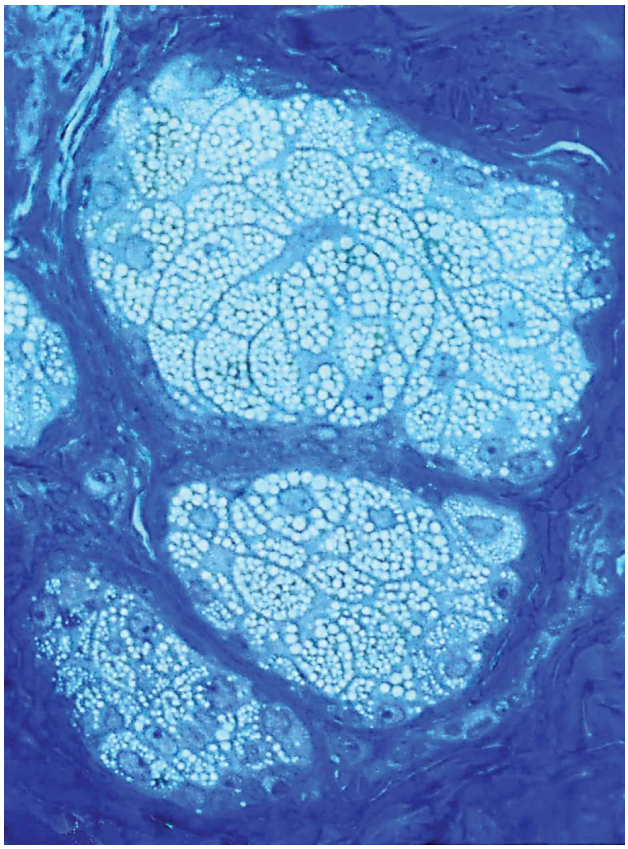

Fig. 3. Sebaceous skin gland, it looks normal.
1 µm Durcupan section, toluidine blue. Magn. x 1000.

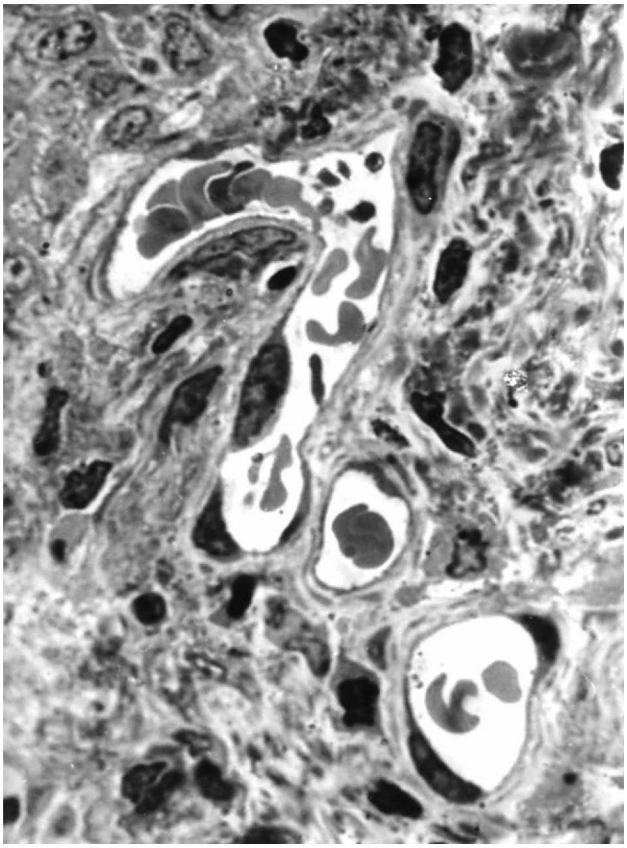

Fig. 2. Rich vasculature in connective tissue.
1 µm Durcupan section, toluidine blue. Magn. x 100x.

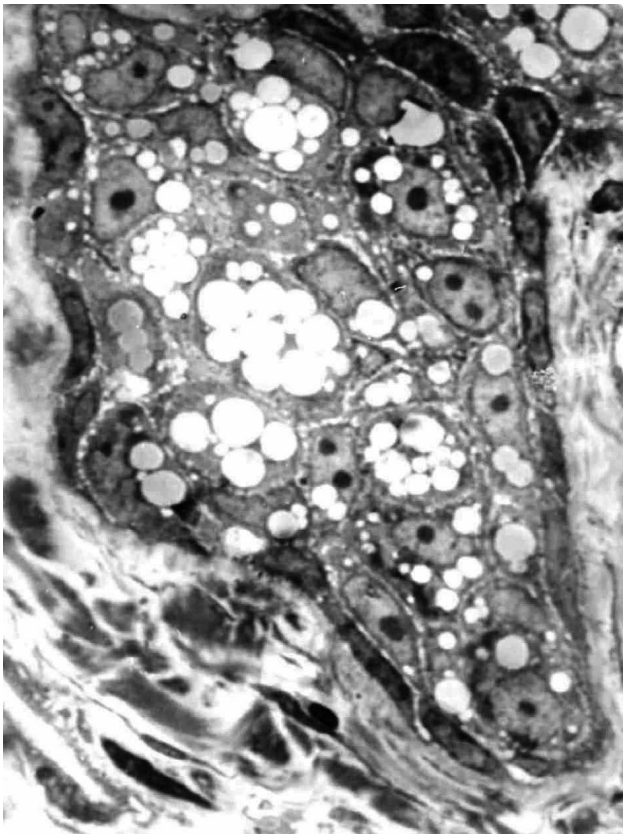

Fig. 4. Sebaceous skin gland from another case.
1 µm Durcupan section, toluidine blue. Magn. x 1000.

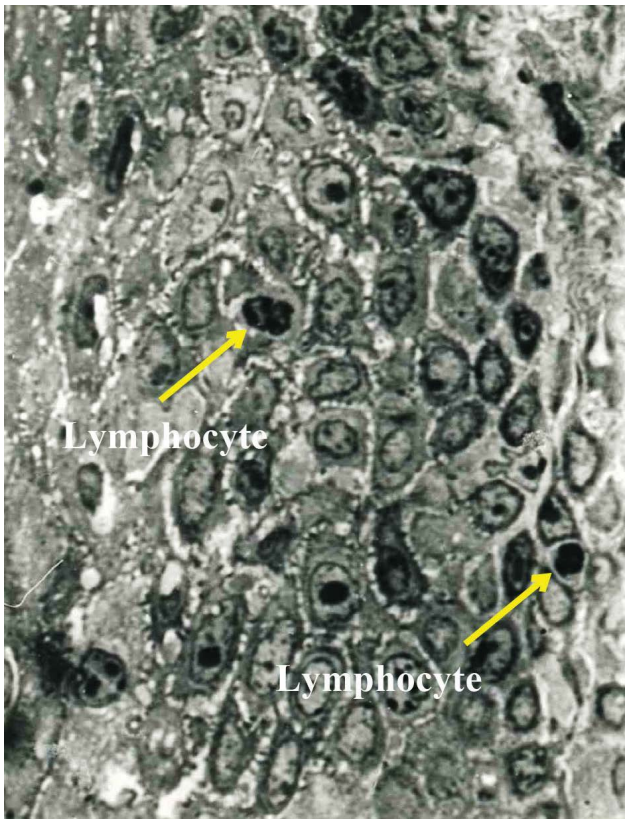

**Fig. 5. Epithelial cells on wet side of mucosa.
LM 1 µ Durcupan section, toluidine blue. Magn. x 1000.**

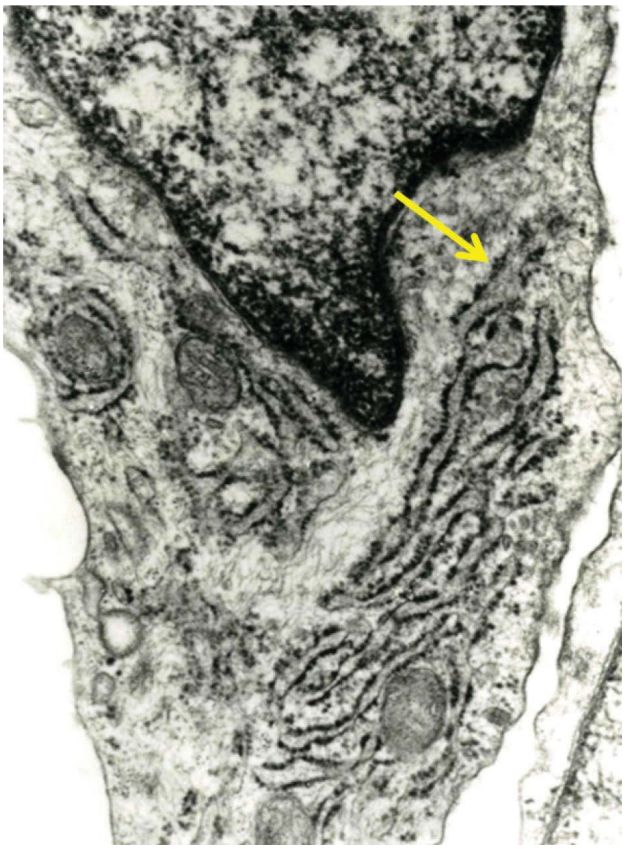

**Fig. 7. Active fibroblast with extensive
endoplasmic reticulum. EM Magn. x 30 000.**

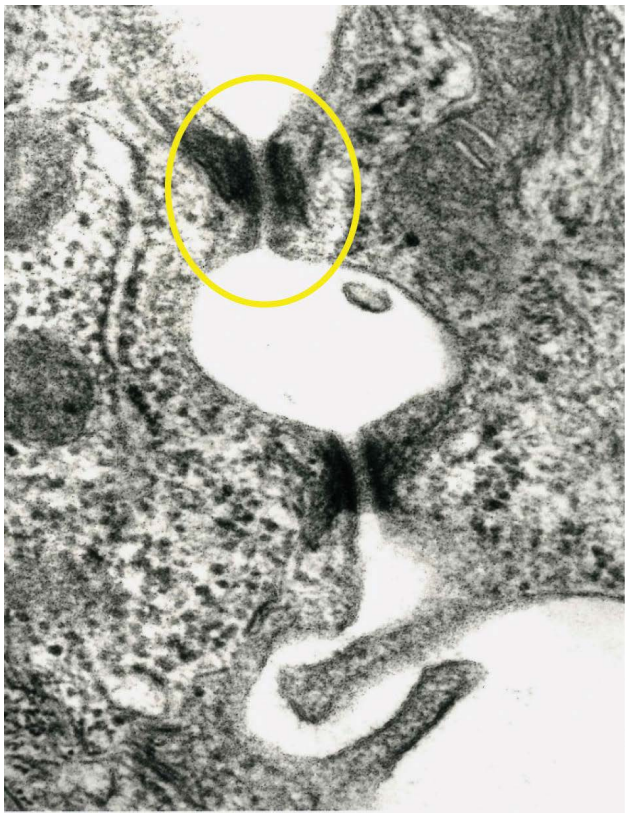

**Fig. 6. Two desmosomes between adjacent
epithelial cells of the epidermis. EM Magn. x 60 000.**

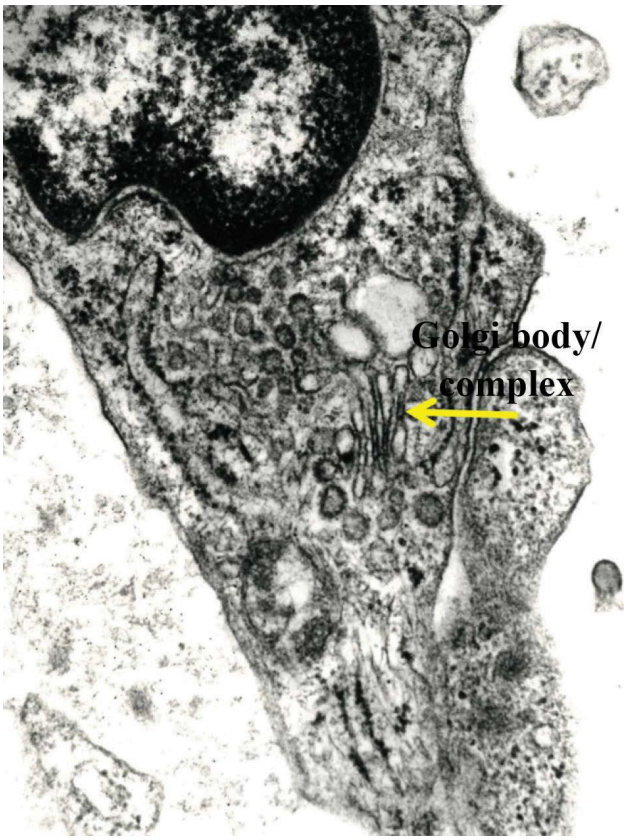

**Fig. 8. Active fibroblast with a Golgi body and
a few cisternae of rough ER. EM Magn. x 36 000.**

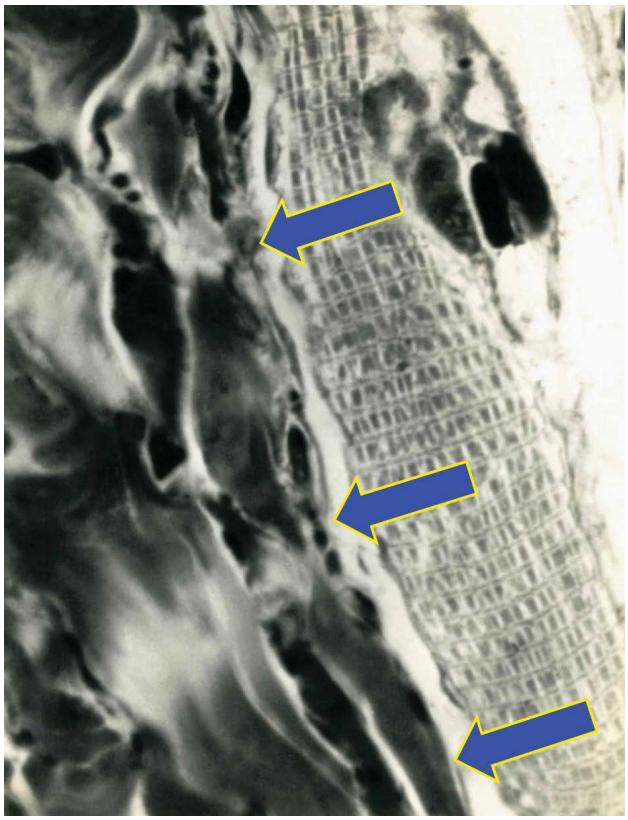

Fig. 9. Dark "ragged" and normal muscle fibres.
LM 1 µ Durcupan section, toluidine blue. Magn. x 1000.

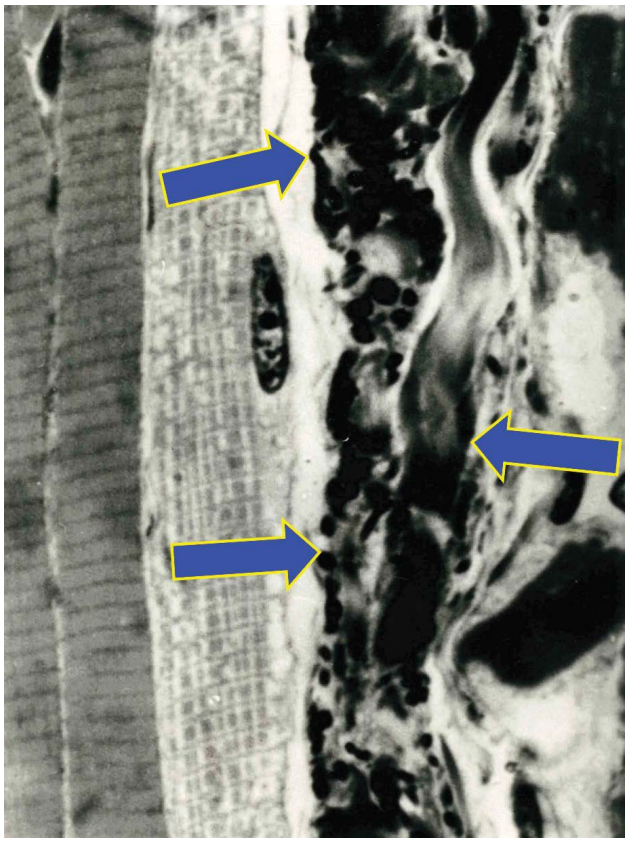

Fig. 10. Dark wavy "ragged" vs. normal muscle fibres;
LM 1 µ section, toluidine blue. Magn. x 1000.

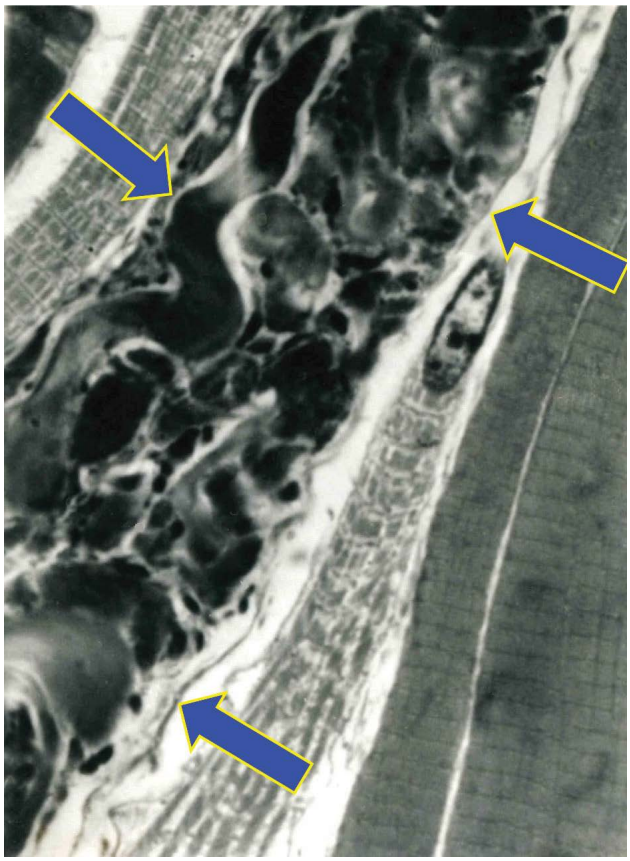

Fig. 11. Dark wavy vs. normal muscle fibres;
LM 1 µ Durcupan section, toluidine blue. Magn. x 1000.

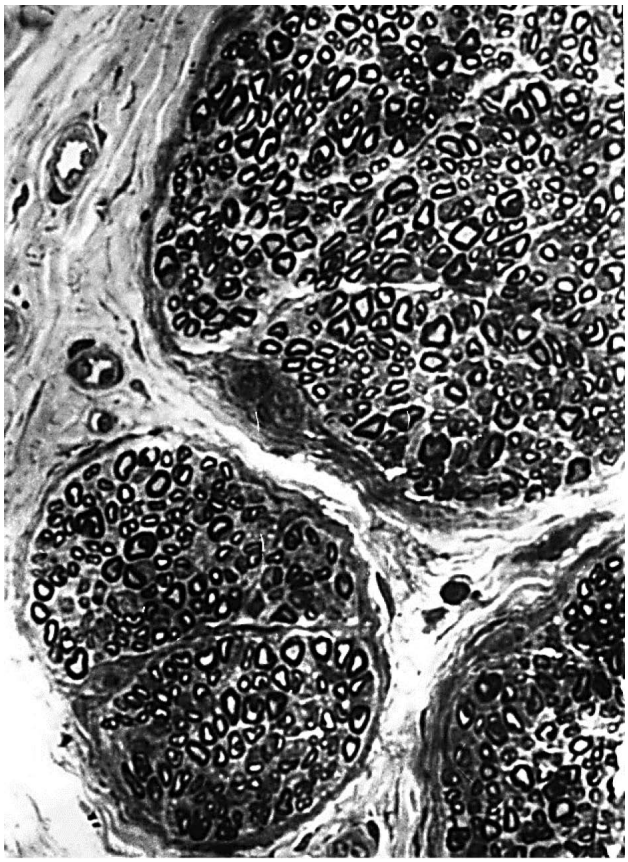

Fig. 12. Parts of three normal nerve bundles.
LM 1 µ Durcupan section, toluidine blue. Magn. x 400.

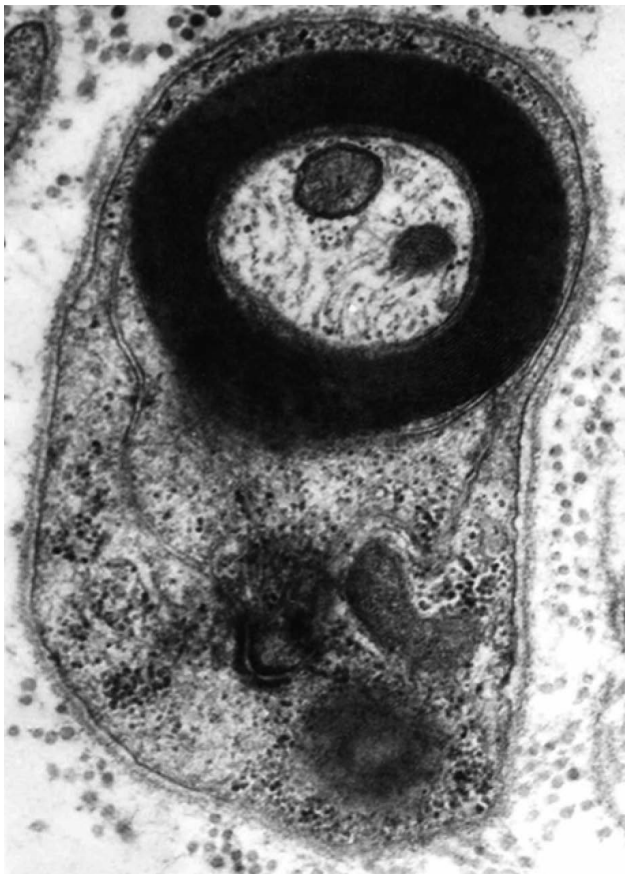

Fig. 13. A small myelinated nerve fibre with normal Schwann cell cytoplasm. EM Magn. x 16 000.

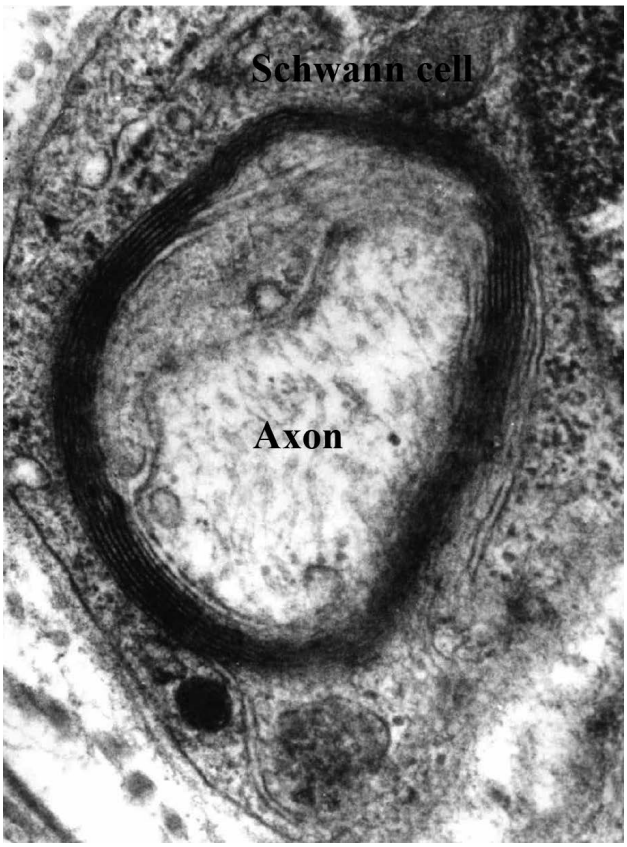

Fig. 15. A medium-sized axon with forming myelin inside the Schwann cell. EM Magn. x 16 000.

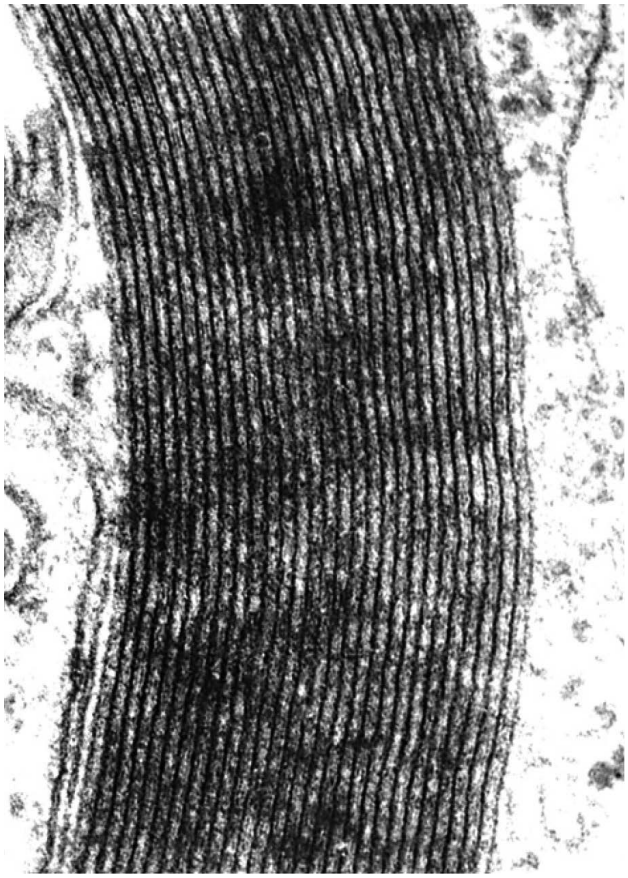

Fig. 14. Myelin with typical alternating dense and pale lines appearing normal. EM Magn. x 100 000.

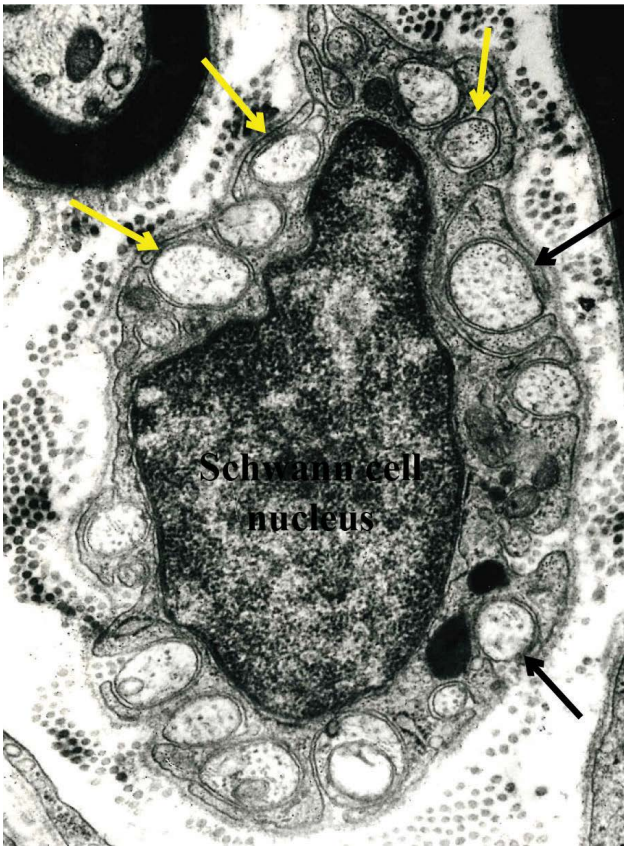

Fig. 16. Several thin unmyelinated nerve fibres in the Schwann cell cytoplasm. EM Magn. x 16 000x.

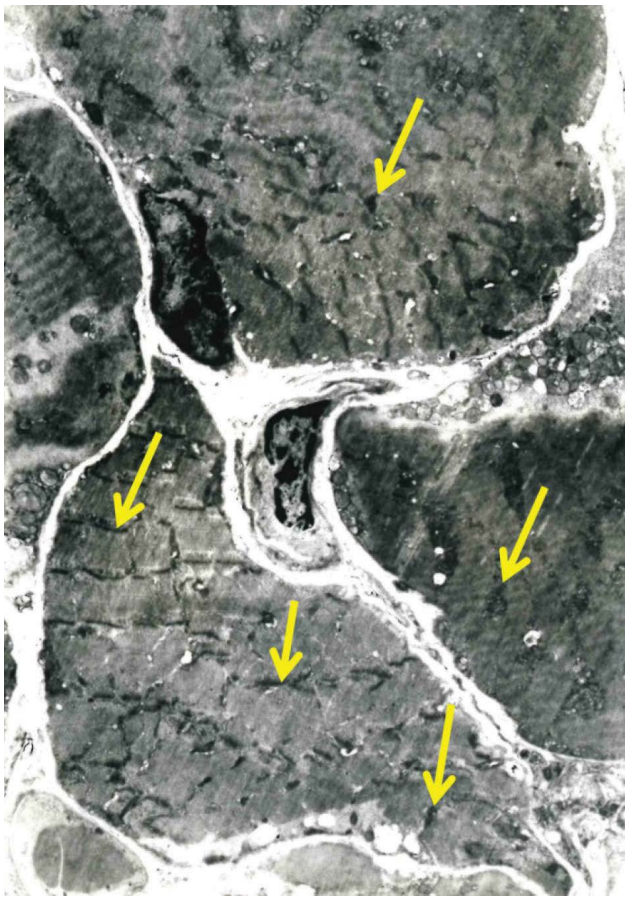

Fig. 17. Parts of four myopathic muscle fibres in cross section, none of them shows typical myofibrils; mitochondria (arrows); EM Magn. x 6 000.

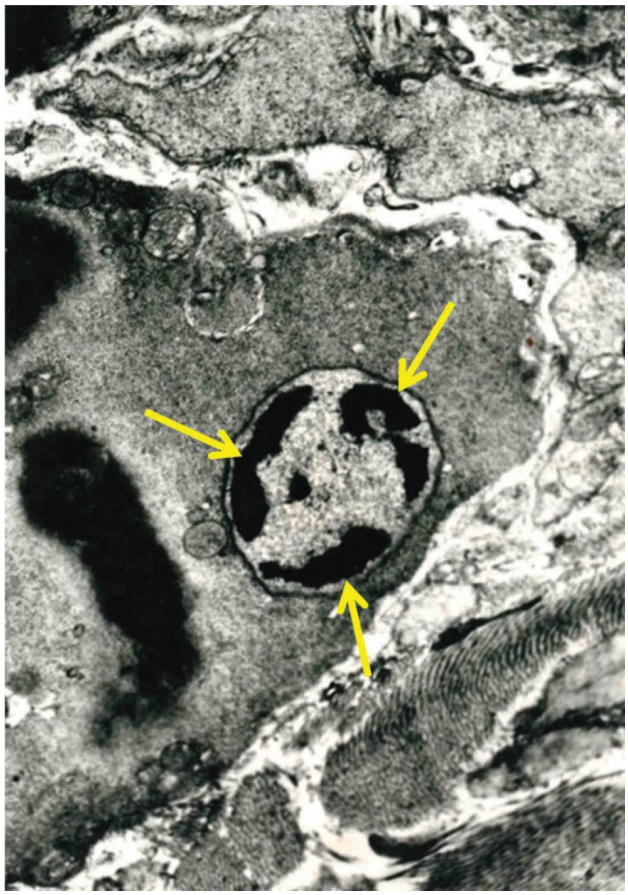

Fig. 18. One apoptotic muscle fibre in cross section, its nucleus has a chromatin margination (arrows) typical for apoptosis. EM Magn. x 6 000.

DISCUSSION

The simple cleft lip (CL) with or without associated cleft palate (CP), is a common human congenital defect, one of the most frequent human malformations [30, 69, 71]. Pathogenesis of CL/CP/CLP is still not fully understood [11, 71] and remains a matter of controversy [72]. The aetiology of the non-syndromic form is multifactorial [5, 39, 65], only some CL/CP are caused by mutations in single genes [5, 27, 29]. In most cases, it is caused by environmental and/or genetic factors and/or their combination [11, 16, 33, 37]. Most CL/CP/CLP have a multifactorial aetiology comprising both genetic and environmental factors [17, 27]. Orofacial clefting is genetically complex, no single gene being responsible for all forms [8]. However, some human cleft lips may result from the action of a single major gene, e.g. coding TGF-alpha variants [37] or mutations of a single HYAL2 gene for hyaluronidase 2 [42]. CL can be a simple (non-syndromic) as an isolated phenotypic effect [5, 7, 43, 44] or it can be a part of multiple developmental anomalies [15] as a result from a single gene defect [42], either as part of a syndrome, e.g. van der Woude syndrome, Treacher-Collins syndrome and other [8, 43]. In animal models, CL can be caused by a lack of mesencephalic neural crest cells in chick [70], insufficient cell migration and proliferation in the embryo due to abnormal mitochondria [1, 53, 58], or genetic involvement of loci on chromosome 17, including retinoic acid receptor alpha (RARA) in non-syndromic oral cleft gene mutations [4, 38, 50]. Several genes causing syndromic CLP have been discovered [27, 24, 42]. Recently L a n s d o n et al. [29] identified COBLL1, RIC1, and ARHGEF38 as further clefting genes.

CL, CP, and CLP were also induced in animal models mainly mouse [14, 18] by some drugs e.g. triamcinolone (61), 5-fluorouracil [64], bromodeoxyuridine [62], hadacidin [63], cyclophosphamide [75], phenytoin [2] and others [48, 67]. The appropriateness of animal models should always be questioned because “human and animal/mouse facial traits are utterly dissimilar” [14].

Despite great progress in identifying and understanding the genetic and environmental triggers of CP and CLP syndrome, the aetiology of the most common non-syndromic (isolated) forms remains poorly characterized [11] and "the nature and extent of pathophysiologic changes in CL muscle are controversial" [9]. Some authors [19, 31, 32] conclude that epithelial integrity and appropriate epithelial-mesenchymal interaction are necessary for normal facial development. Others found large variation in the size of muscle fibres, their asymmetric distribution and their anomalous insertion [53, 54]. They concluded that structural anomalies in the examined tissues could not explain the malformation, but might be a consequence of the surgical repair of CL. De Chalain et al. [9] as well as we found characteristic myopathic changes in CL. Similar to some authors [9, 26, 58], we believe that there is no evidence of deficient nerve supply in the CL and "no evidence of neurogenic muscle atrophy" [9] in CL.

Senders et al. [59] reanalysed Georg L. Streeter's 1920 dynamic fusion "merging theory" of lip development in primates and concluded that essential is the interaction between epithelial layers, "epithelial layers must fuse properly to avoid CL deformities" [59]. We observed a normal structure of epidermal cells and epidermal derivatives in all our samples. We did not find deviant epithelial structure in LM/EM or any direct evidence of lack of epithelial fusion in CL. Besides that, merging theory cannot explain degenerative changes in CL muscle. According to Noor et al [47] and Schendel et al. [58], a metabolic defect in the mitochondrial function could cause a deficiency in proper cell migration and proliferation resulting in the malformation. Similar to Rajabian et al. [53], we did not find abnormal mitochondria or changes in their volume and number in CL. We also did not observe the larger number of lysosomes (and their increased activity) reported by [61, 62, 64] in drug induced CP in hamsters. Therefore, we suppose that mitochondrial and lysosomal pathology are not involved in the pathogenesis of CL. Other authors [9, 25, 26] found increased amount of connective tissue in cleft specimens with size variation of muscle fibres, hypoplasia, myopathic changes and non-neurogenic atrophy of muscle fibres [9, 22, 26, 46, 58, 77]. Khan et al. [22] by image processing determined the muscle fibre diameter of 18 infants with CLCP, with most fibres between 6 to 11 μm in diameter. We found similar values, but we estimated the thickness of some muscle fibres is more than double. Khan et al. [23] found that even diameter of collagenous fibres is significantly smaller on the medial side than on the lateral one but their density was higher there than that on the lateral side. All this provides a new insight into the biomechanics of underlying basic tissue. However, the authors do not state the age of the patients, so it is not possible to assume whether the differences are related to the cause or the result of CL. Kohn at al. [28] in their LM, EM and immunohistochemical study found that the myogenic structures surrounding cleft margins are an inferior tissue incapable of regeneration or normal function. They concluded for clinical practice that "the tissue in the vicinity of the clefts should be generously excised when doing cheiloplasty" [28].

Regarding muscle, all authors agree that LM/EM muscle structure in CL is significantly different from control [9, 26, 23, 54, 58, 77]. Identical changes of muscle fibres as we found around the cleft lip, previously described [23, 25]. Similar to de Chalain et al. [9], we also found no evidence of lack of nerve supply in tissue surrounding CL. We assume that the muscle changes in CL are not the result of nerve injury or muscle denervation during surgery, because we found myopathy in all CL biopsies taken during the first reconstructive surgery. This excludes that they are its consequence. Based on the above findings, we are of the same opinion as some authors [9, 25, 26, 58] that in CL there is non-neurogenic muscle atrophy without mitochondrial myopathy in the orbicularis oris muscle. It is worth noting that human Wnt7a protein induces vigorous regeneration of the orbicularis oris muscle [34], which unlike typical somite-derived skeletal cross striated muscles, originates from the second branchial arch [10]. Some authors identified altered expression of genes previously implicated in CL/CP/CLP [31, 32]. Findings provide the foundation for detailed investigations using animal models to examine pathways for normal orofacial development, the failure of which leads to orofacial clefts in humans. Several authors developed an animal model of foetal CL repair mainly in lambs [21, 36, 66, 74] with the aim of developing a method for surgical repair of CL in humans, which would prevent or minimize the formation of scars after the treatment. Kaban et al., [20] surgically repaired CL in rabbit foetuses, which resulted in healing without scar formation. On the contrary, Wenghoefer et al. [74] reported, that their experiments in foetal lambs did not bring the expected results. However disputed, the

fact remains that foetal wounds heal without scarring [3, 6, 68]. This phenomenon may be used for surgical treatment of CL/CP/ in humans in the future, but extensive experimental work must first be carried out to prove the benefits of in utero repair.

Cleft lip is a great trauma for the sufferer and the next of kin. Therefore, in conclusion, we quote the realistic vision of A l m a s r i, the editor of the monograph of paper [41]: "I am confident that one day the surgical interventions that bombard the patients from the day of newborn delivery and throughout the years of youth should be significantly decreased based on the genetic prophylactic intervention."

CONCLUSIONS

Both light (LM) and routine transmission electron microscopy (TEM) revealed: 1. variations in the diameter of skeletal muscle fibres. 2. Degeneration of skeletal muscle fibres in *m. orbicularis oris*; its muscle fibres were dense, wavy "ragged" in LM. 3. LM revealed well-preserved nerve bundles, EM of all individual nerve fibres was normal. 4. Both LM and TEM of epidermis, hair, hair follicles, skin glands and blood vessels in surrounding connective tissue did not reveal their abnormal structure. Both the blood and nerve supplies to lip muscle in CL were normal. We found no structural signs of neurogenic muscle pathology and/or its atrophy. Consequently, we believe that degenerative changes in CL muscle fibres must be of other than neurogenic origin.

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