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Morphologic, morphometrical and histochemical proprieties of the costal cartilage in children with pectus excavatum

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Abstract

Pectus excavatum (PE) is the most frequent anterior chest deformity occurring in approximately one of 1000 live births. Despite the excellent achievements in the treatment of the disease, the etiology of PE is yet to be clarified. It is believed that the cause for PE is an intrinsic costal cartilage abnormality leading to an overgrowth of the cartilage, which pushes the sternum backward. Several histological studies revealed contradictory results and failed to identify a clear structural abnormality of the costal cartilage responsible for the apparition of PE. In this article, we focused on identifying the microscopic disturbances of the costal cartilage in patients with PE. We obtained cartilage samples from 29 children with PE and 18 control cartilage samples. The samples were subjected to morphologic, morphometrical and histochemical assess. The results indicate a young, immature pattern of the cartilage matrix with a normal cell/matrix ratio. These results sustain the theory that the cause of PE is to be found inside the costal cartilage and the most plausible cause is a global overgrowth of the costal cartilage.

Keywords: pectus excavatum, morphometrical analysis, cartilage, histochemistry, morphology.

Introduction

Pectus excavatum (PE) is the most frequent anterior chest deformity occurring in approximately one in 1000 live births [1]. The deformity consists in the posterior depression of the sternum and the lower costal cartilages [2]. The disease was recognized from the 16th century [3] and the first successful repair was performed by Sauerbruch F in 1913 [4]. Since that more than 50 types of surgical interventions were performed for the correction of PE, Ravitch's technique in the sixties trough eighties and more recently the minimal invasive Nuss's technique, being the most popular ones [5, 6].

Despite the excellent achievements in treating the disease, the etiology of PE is yet to be clarified. Early pathogenic theories included: unbalance between the traction force of the diaphragm and the strength of the rib cage [7, 8], rickets [9], upper airway obstruction [10, 11]. Neither one of these theories were able to fully explain the appearance of PE. Frequent association of PE with several connective tissue diseases led to the supposition that the cause for PE is an intrinsic costal cartilage abnormality. The abnormal costal cartilage overgrows pushing the sternum backward [12]. Several histological studies revealed contradictory results. While some histological studies [13, 14] found no significant differences regarding the number, shape, area of the cell and nucleus between cartilages from PE patients and normal, others found that in PE costal cartilages the number of chondrocytes strongly increases within the singles chondrons [15], greater number of cells and more variable cellular distribution, larger vessel clusters, more frequent myxoid matrix degeneration and focal necrosis [16]. Disturbances of the collagen were found also. Content of collagen is increased by 35–50%, while its capacity to fix water is decreased [17]. Costal cartilage matrix was found to have an increased content of fibronectin, collagen V, procollagen III and IV [18]. More than that, the structural resistance of the cartilage is severely diminished under tensile or compression stress [13]. All this findings strongly support the theory that a costal cartilage disturbance is the origin of PE deformation, but with no direct evidence of it.

In this article, we present the results of our morphologic, morphometrical and histochemical studies of the costal cartilage in children with pectus excavatum.

Materials and Methods

Samples of the deformed costal cartilages from 29 children with PE, age 5 to 18 years, mean 11 years, were obtained during the surgical intervention for the correction of the disease. Costal cartilage specimens were obtained during autopsy from 18 children in whom the cause of death was unlikely to affect the cartilage, age 1 to 19 years, mean 9.3 years. The samples were cut

perpendicular to the long axis of the cartilage and apart from the costo-chondral and the chondro-sternal junction. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Three sections were cut at 3-µm thickness from each cartilage sample. Prior to staining, each slide was dewaxed and rehydrated by standard protocol.

Morphometrical analysis. First of the three sections was stained with Hematoxylin–Eosin (HE). The sections were examined in light microscopy at 100× using a Nikon Eclipse i80 microscope equipped with DS-U2 digital camera and three independent black and white images were obtained for each section. The images were processed and analyzed using NIS elements BR 2.30 imaging software (Figure 1). Density, area and circularity of chondrocytes were assessed.

Alcian Blue–Safranin for proteoglycans and glycosaminoglycans. A 3-µm section from each cartilage was stained with Alcian Blue–Safranin at pH 1.42 and analyzed in light microscopy at 100×. Three independent images were obtained from each section. Resulted images were divided in two groups: images that contained both blue (Alcian) and orange-red (Safranin) areas and only blue non orange-red images (Figure 2).

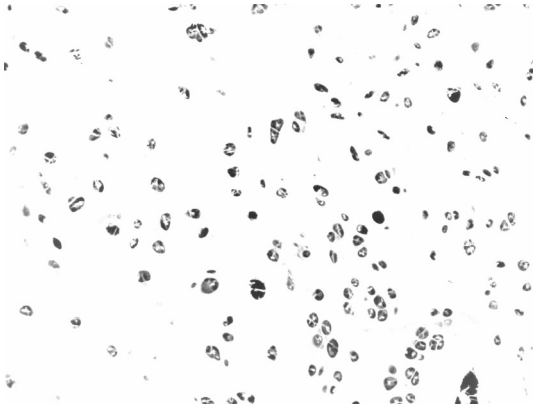
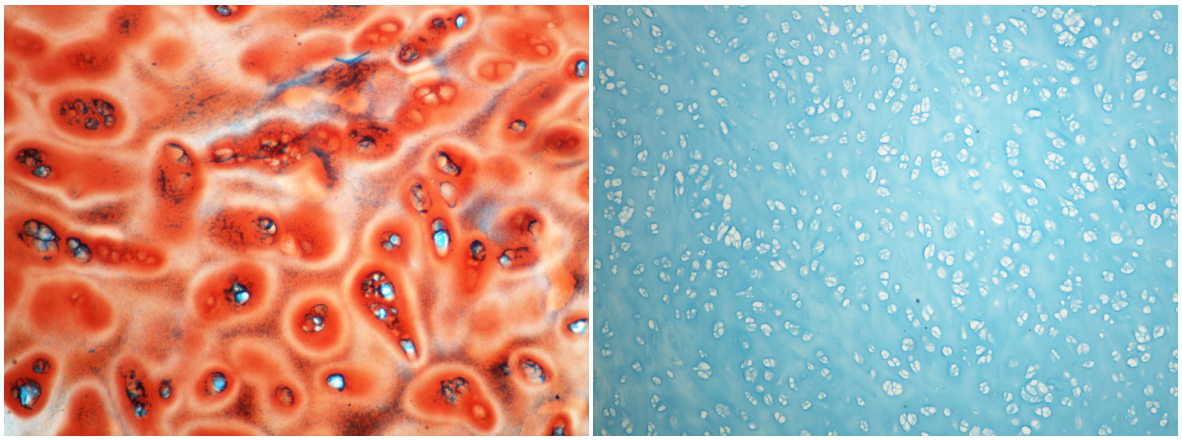


Figure 1 – ***Processed image for morphometric analysis (HE stain, 100×).***



(a) **(b)**

Figure 2 – ***(a) Safraninophilic sample of costal cartilage (Alcian Blue–Safranin stain, 100×); (b) Alcianophilic sample of costal cartilage (Alcian Blue–Safranin stain, 100×).***

The third section from each specimen was stained with Masson trichrome for collagen and examined in light microscopy. We focused on finding the distribution of mature *vs.* immature collagen fibers.

SPSS Statistics 1.7 software was used for statistical analysis. Differences between groups were determined performing the independent samples Student's *t*-test for numeric variables and chi-square test for non-numeric variables; level of significance for *p* was set at 0.05.

Results

By morphometrical analysis we found the density of the chondrocytes raging between 59–2297 cells/µm^2, mean 600 cells/µm^2, for the experimental group and a density of 30–780 cells/µm^2, mean 660 cells/µm^2 for the control group (Table 1).

The area occupied by the cells, calculated in percent of the total area of the image, was between 0.1–9%, mean 2.74% for the experimental group and between 0.3–7.8%, mean 2.37% for the control group (Table 1).

Circularity ranged between 0.62–0.93, mean 0.77, for the experimental group and 0.49–0.95, mean 0.77, for the control group (Table 1).

Table 1 – ***Density, area and circularity of the cells in the costal cartilage***

Group	Density [cells/µm^2]	Area	Circularity
Experimental	599.9±49	2.74±0.1	0.77±0.07
Control	659.4±103	2.37±0.2	0.77±0.09
	t=0.578, p>0.05	*t=1.268, p>0.05*	*t=0.598, p>0.05*

There were no significant differences between the density, area and circularity of the chondrocytes for the two groups ($p>0.05$). In the control group the area and density of the cells decrease with age ($p<0.05$) while the circularity increase ($p<0.05$). In the experimental group there were no significant correlations for all three variables and age of the patients ($p>0.05$).

Alcian Blue–Safranin. A total number of 59 images contained significant proportion of red or orange-red colored areas and were considered as images of cartilage samples with safraninophilic matrix (Figure 2a). The remaining 82 images were stained in blue (Alcian) or had small insignificant orange-red colored spots (Figure 2b) (Table 2).

The number of alcianophilic cartilage samples was significantly higher in the experimental (n=71, 81%) than in the control group (n=11, 20%), ($p<0.05$).

Table 2 – ***Results of Alcian Blue–Safranin staining for proteoglycans and glycosaminoglycans***

Group	Safraninophilic	Alcianophilic	Total
Experimental	16 (19%)	71 (81%)	87
Control	47(80%)	11 (20%)	54
	p<0.05	*p<0.05*	

For Masson trichrome stained samples typical aspects were observed in both groups: nuclei stained dark violet, pale pink cytoplasm and blue stained inter-territorial matrix. Areas of red stained matrix were observed surrounding vascular channels (Figure 3).

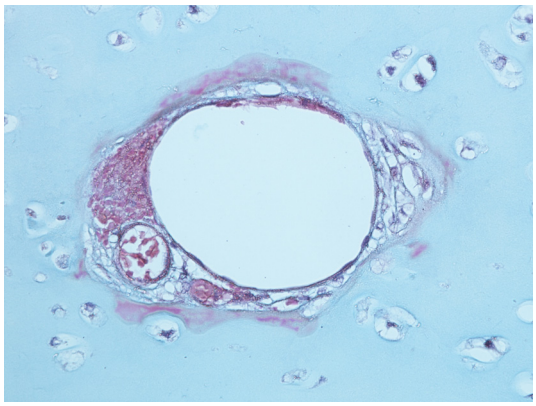


Figure 3 – ***Cartilage channel with surrounding immature collagen (Masson trichrome stain, 100×).***

In two samples from the experimental group spots of immature red stained collagen was observed in the inter-territorial matrix also. Overall, the aspect of the samples was that of a mature hyaline cartilage and with no obvious differences between groups.

Discussion

The most plausible cause for PE is the overgrowth of the costal cartilages caused by an intrinsic disturbance [12]. The exact cause for this overgrowth is until now not known and previous histological studies involving cartilage samples from patients with PE indicated contradictory reports. While some studies revealed hyperplasic findings [15, 16] in the costal cartilages from patients with PE, other revealed no modifications [13, 14]. Neither one of them succeed to find the cause nor to establish with certainty that an overgrowth indeed happen. There reason why we analyzed the shape, density and area of the chondrocytes in cartilages from patients with PE is because a hyperplasic cartilage should theoretically have all this three parameters disturbed. Similar with does found by others [13, 14], our results indicated that the area and shape of the chondrocytes are similar between experimental and control groups. This can be interpreted in two ways. First and most evident one is that the cartilages are not hyperplasic and that the overgrowth of them is not the cause for the disease. The second way is that the overgrowth of the cartilage still exists but has a global pattern with equal involvement of the cells and the matrix.

Shape of the chondrocytes is in concordance with their status. Young chondroprogenitor cells are fusiform, while mature, less active chondrocytes have a much rounder shape [19]. In this study, the circularity index of the chondrocytes in both experimental and control groups are similar. Differences were noted when circularity was linked with the age of the patients. While in control group the circularity increases with age indicating a normal evolving pattern, in experimental group the age of the patients had no influence over the circularity of the cells. In costal cartilages from PE patients, the circularity of the cells does not increase with the age of the patients, indicating that the normal pattern of cell division and maturation is somehow disturbed. This finding, even if is not a direct indication, is supporting the theory of the overgrowth of the coastal cartilages.

Proteoglycans and glycosaminoglycans are a major component of the cartilage matrix forming together with the collagen a complex architecture around the cell [20]. They are involved in modulation of cell proliferation and differentiation, response to growth factors, and signal transduction pathways [20]. The proportion and content of proteoglycans and glycosaminoglycans in the hyaline cartilage is changing with age and different pathological conditions [21]. Maturation of the cartilage is taking place up to approximately 20-year-old and during this period, the chondrocyte is most actively synthesizing glycosaminoglycans accompanied by major changes in sulfation pattern of glycosaminoglycans [22]. With increasing age, the sulfation of condroitin sulfate chains decreases linearly [23]. On the other hand, with increasing age the content of highly sulfated keratan sulfate increases rising to a plateau between 20 and 30-year-old [21, 24, 25]. This means that during the normal process of human growing and maturation the total content of sulfur and sulfated polysaccharides of the human hyaline cartilage increase also [25, 26]. Alcian Blue–Safranin reaction distinguishes between weakly sulfated and strongly sulfated mucopoly-saccharides by a shift from Alcian Blue to Safranin staining [27]. We found that when stained with Alcian Blue–Safranin most of the samples from the normal costal hyaline cartilage are safraninophilic (Figure 2a). In contrast cartilage, the majority (82%) of samples from children with PE are almost exclusively alciano-philic (Figure 2b). This is strongly suggesting a there is a significant lower percent of mature proteoglycans and glycosaminoglycans in the matrix of costal cartilages from children with PE. By similarity with the osteo-arthritic articular cartilage in which the affected cartilage has an increased content of immature glycos-aminoglycans [28], it can be speculated on this basis that the costal cartilage from children with PE has a chondroblastic phenotype.

On the other hand, this alteration in the sulfation pattern of proteoglycans found by use may be interpreted otherwise. It is a known thing that the ionic interaction between aggrecan and hialuronic acid is the main factor responsible for the water content of the cartilages and that the water and electrolytes content are a major factor contributing to the physical strength of the hyaline cartilage [29, 30]. Indeed, the strength of cartilages from children with PE in terms of tension, compression and flexure are less than does in the normal cartilage [13]. Moreover, in these cartilages,

there is a diminished content of zinc and the cartilages have a low capacity to bind water molecules [17, 18]. These findings are leading to another possible cause for PE: a diminished strength of the costal cartilage that fails to maintain the normal position of the sternum during the respiratory movements of the thorax [14]. Further investigations are necessary in order to get enough evidence to support this theory.

In this study, we tried to find an alteration of the normal distribution of the immature/mature collagen fibers. We presumed that if the costal cartilages from patients with PE are overgrowing there should be an increase content of immature collagen fibers. Previous studies revealed several disturbances of the collagen in costal cartilages from patients with PE. Increased total content of collagen and increased content of fibronectin, collagen V, procollagen III and IV was found [17, 18]. Feng J *et al.* [13] found that the collagen in the cartilages from patients with PE is disorderly arranged and distributed in the deeper zones of the cartilage and there for the structural resistance of the cartilage is severely diminished under tensile or compression stress [13]. Our findings did not reveal any disturbances of the collagen in the costal cartilages from children with PE, but because our examination implied only light microscopy studies, which does not give us a detailed image of the collagen network, we cannot conclude that the collagen content and distribution is normal.

Overall, it is obvious that the cartilages from children with PE are different than normal, abnormalities being found especially in the non-collagenous content of the matrix. This is sustaining the theory that the cause of PE is to be found somewhere inside the costal cartilage.

Conclusions

The number, area and shape of the chondrocytes are similar in PE cartilage and normal cartilages. We found a disturbance of the sulfation patter of the proteoglycans in the matrix of cartilage from children with PE. These cartilages have a lower content of strongly sulfated mucopolysaccharides indicating an immature pattern that may influence the physical strength of the cartilage. Light microscopy study of the collagen revealed no abnormalities but did not offer direct evidence that collagen has a normal structure. The modifications that we found sustain the theory that the cause of PE is to be found inside the costal cartilage and the most plausible cause is a global overgrowth of the costal cartilage.

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