P-73 DECREASE OF COLLAGEN TYPE 1 DEPOSITION AND TISSUE-LIKE CONTRACTION IN CLUBFOOT-DERIVED CELLS BY MINOXIDIL ADMINISTRATION *IN VITRO*

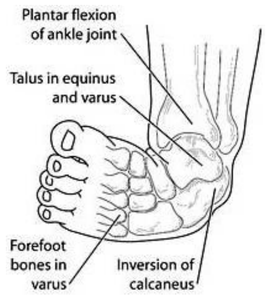
Doubkova M.*[1,2], Knitlova J.*[1], Plencner M.[1], Vondrasek D.[1,3], Eckhardt A.[1], Ostadal M.[4], Musilkova J.[1], Bacakova L.[1] and Novotny T.[2,5]

[1] Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic; [2] Charles University, Second Faculty of Medicine, Prague, Czech Republic; [3] Charles University, Faculty of Physical Education and Sport, Prague, Czech Republic; [4] Charles University and Na Bulovce Hospital, Department of Orthopaedics, Prague, Czech Republic; [5] Masaryk Hospital. Usti nad Labem, Czech Republic

INTRODUCTION

Idiopathic clubfoot (*pes equinovarus congenitus,* PEC) is the second most common congenital orthopaedic deformity, affecting the muscoskeletal system of a lower limb. Stiff, contracted tissue is formed between the *medial malleolus*, *sustentaculum tali* and *os naviculare*, rotating the foot inwards and downwards. The mass of this tissue closely resemble other fibroproliferative disorders, showing signs of fibrosis in histologically stained samples, with high amount of extracellular matrix proteins (collagen I, III, VI) and profibrotic cytokines (TGFβ, PDGF).

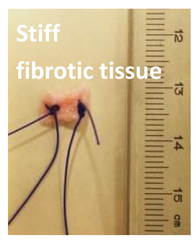
Current treatment consists of physiotherapy, cast fixations and Achilles tenotomy (the Ponseti). However, potential use of antifibrotic agents in treatment of relapsed clubfoot has received little to no attention. Such treatment could potentially reduce tissue contracture in relapsed patients and thus accelerate and/or improve the outcomes of current conservative methods, especially in cases when the relapsed patients are not adequately responsive to Ponseti treatment.

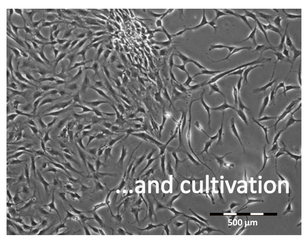


AIMS

Minoxidil (MXD) has an inhibitory effect on enzyme lysyl hydroxylase, which influences the quality of extracellular matrix (ECM) crosslinking – specifically collagen type I. MXD does not degrade collagen fibers already formed in the ECM, instead it alters the type and quantity of crosslinks in newly formed fibers, making ECM more susceptible to degradation. MXD could be used to reduce the stiffness and to improve the flexibility of the tissue.

- We aimed to assess the *in vitro* antifibrotic effects of MXD as a candidate for the development of potential adjuvant treatment for relapsed clubfoot.



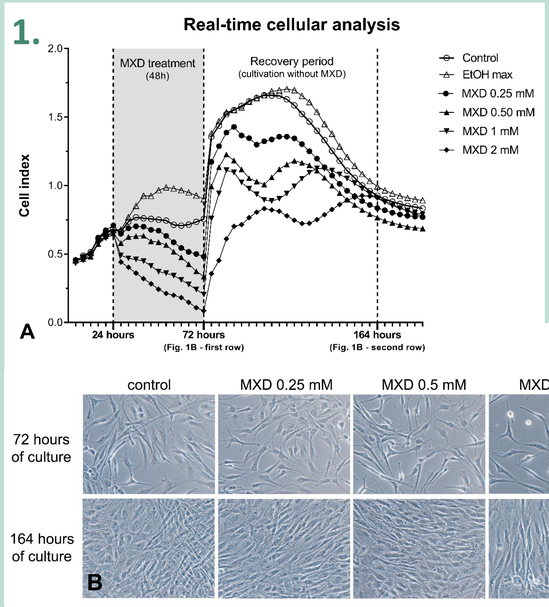


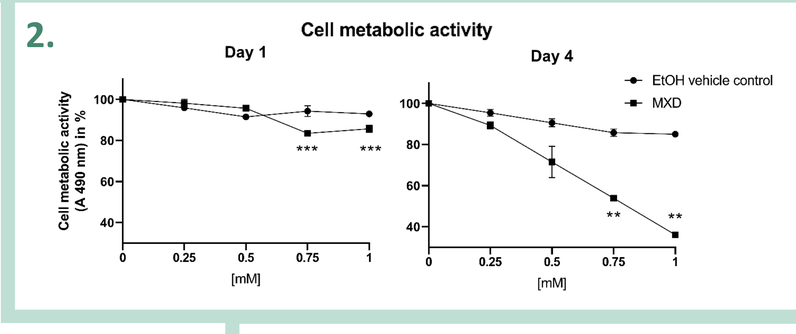
CLUBFOOT PRIMARY CELL CULTURE

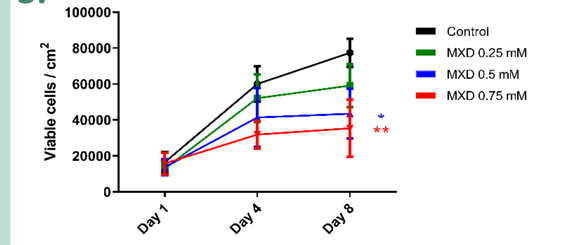
Cell type	Analyzed markers								% in culture
	CD34	CD45	CD90	Col1	αSMA	SMM MYH11	vimentin	desmin	
Fibroblasts	-	-	+	+	-	-	+	-	96%
Myofibroblasts	-	-	+	+	+	-	+	-	< 3 %
Fibrocytes	+	+	+	+	-	-	+	-	
Vascular smooth muscle cells	NT	NT	NT	dim+	+	+	-	+	< 0.1 %

↑ Cells derived from the relapsed clubfoot were characterized with emphasis on the presence of cell types associated with fibrosis. Pure fibroblast culture was obtained in the 4rd passage → We use only 1st to 3rd passage for the experiments. +/- sign = positivity/negativity of markers for the cell to be identified as a particular type. NT = marker not tested. dim+ = marker is at least dim positive

CYTOTOXICITY ASSESSMENT



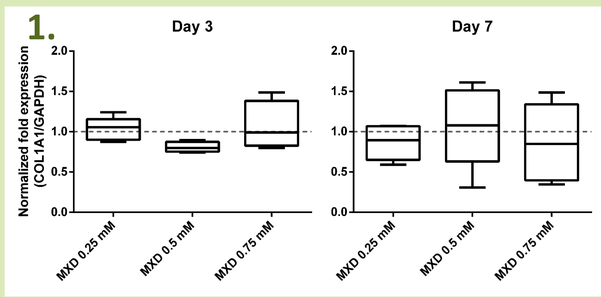


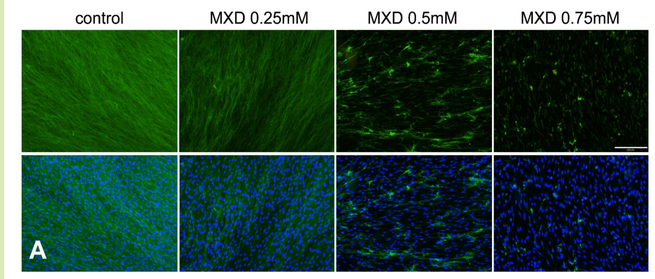


↑ Fig. 1., 2., 3.: Concentrations of 0.25, 0.5 and 0.75 mM MXD inhibited proliferation and metabolic activity of clubfoot-derived cells in a concentration-dependent manner without causing direct cytotoxic effect. Viability of the cells during this treatment was always above 98%. Concentrations of 1 and 2 mM MXD caused cessation of cell growth, changes in cell morphology, lowered viability and therefore was excluded from further experiments. Bar=50µm.

EFFECT OF MXD ON COLLAGEN TYPE I DEPOSITION

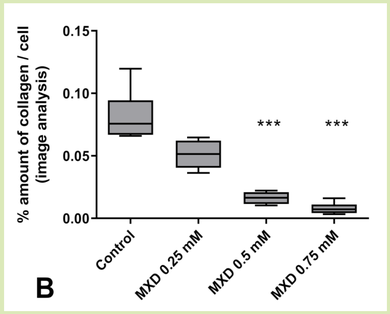
→ Collagen I gene expression. No concentration-dependent effect of MXD on collagen type I alpha chain mRNA expression was found after 3 and 7 days in culture. Therefore it is safe to assume that the inhibitory effect of MXD on collagen is realized downstream from the COL1A1 expression.

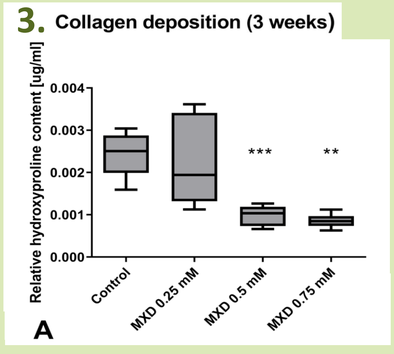




← A. Short-term collagen deposition is lower in the MXD-treated samples. The amount of collagen (green signal) after 8-day treatment with increasing MXD concentrations decreased gradually, but not in proportion to the lower number of cells in culture (blue signal, merge). Bar=200µm.

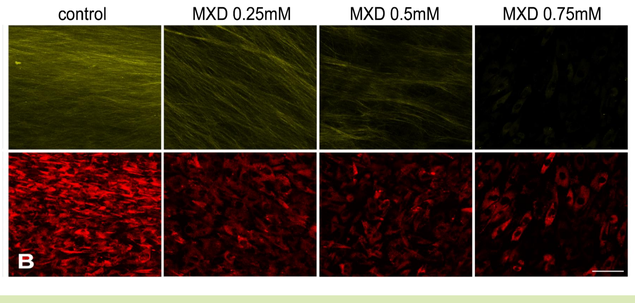
→ B. Percentage of the fluorescently stained collagen area expressed per single cell.





← A. Long-term collagen deposition after 3 weeks is lower in the MXD-treated samples than in the untreated control, as represented by a lower relative hydroxyproline content.

→ B. SHG signal decrease after 3 weeks of treatment demonstrates the effect of MXD on correct structural assembly of collagen (green signal). The persistent concentration-dependent suppression of cell by MXD is still apparent by lower cell number (red), but viability was good.

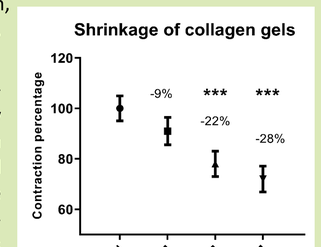


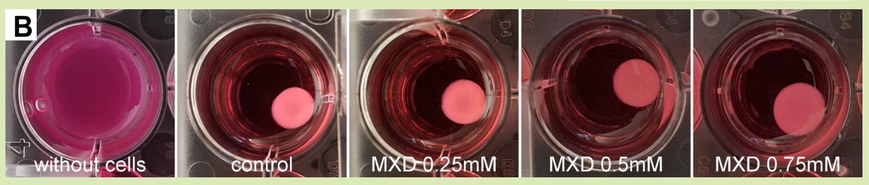
EFFECT OF MXD ON TISSUE-LIKE CONTRACTION

...in 3D model of the cell-populated collagen gel lattices after 2 days of treatment.

The contraction of gel occurs due to (a) changes in cell morphology – the cells elongate, which generates forces that translocate collagen fibrils in the lattice. MXD can also (b) inhibit TGFβ/Smad3 signallization, which reduces collagen formation, or (c) decrease fibroblast secretion of glycosaminoglycans, an ECM component in direct interaction with collagen.

→↓ MXD significantly inhibited the contraction of cell-populated collagen lattices (0.5 mM by 22%; 0.75 mM by 28%). No shrinkage occurred in gels without cells when treated with MXD. The cell-populated MXD-untreated Control gels were the most contracted, and were therefore regarded as a 100% value of the area shrinkage. The cell-mediated gel contraction after 2 days of MXD treatment is presented as a % of decrease in the gel contraction in comparison with the untreated Control gel.





MATERIALS

- **Primary cell culture** was isolated from the tissue samples of 11 patients (surgery of ponseti-resistant relapsed clubfoot; mean age = 5 yrs) from the area between the medial *malleolus*, *sustentaculum tali* and *os naviculare*.
- **Minoxidil** solution: MXD powder (M4145, Sigma-Aldrich) dissolved in 96% EtOH to 0.25, 0.5, 0.75, 1 and 2 mM.

METHODS

- **Cultvation in presence of MXD:** 10 000 or 16 000 cells/cm^2 (depending on the experiment) in DMEM with 10% FBS + Minoxidil (0-2 mM) in PS 24-wp culture plates. **Cultivation of controls:** Vehicle control (sample EtOH max; DMEM with 10% FBS + EtOH; no MXD) and Control sample (DMEM with 10% FBS)
- The primary cell culture was characterized by **flow cytometry.**
- Cell viability and proliferation were quantified by **xCELLigence, MTS metabolic assay, Live/Dead assay.**
- Amount of collagen type I deposited into the extracellular matrix was quantified by **immunofluorescence** with subsequent **image segmentation analysis, hydroxyproline assay, Second Harmonic Generation imaging (SHG).**
- Effect of minoxidil on gene expression of collagen I (COL1A1) was determined by **RT q-PCR** (HG:GAPDH; $2^{-\Delta\Delta Ct}$ method, data normalized according to the gene expression in the Control sample).
- Extracellular matrix contraction was studied in a **3D model of cell-populated collagen gel lattices.**

CONCLUSIONS

This study provides important preliminary data demonstrating the potential relevance of MXD (or a substance with similar effects) for adjuvant pharmacological therapy in standard treatment of relapsed clubfoot, which could potentially decrease the contracture and facilitate the casting therapy. MXD exerts an in vitro inhibitory effect on the cell proliferation, collagen accumulation and extracellular matrix contraction processes that are associated with fibrosis in clubfoot:

- Dose-dependent inhibition of cell proliferation (desirable in tissue fibrosis and hyperproliferating fibroblasts).
- Lower deposition and structural maturation of collagen type I.
  - Exposure to ≥ 0.5 mM MXD resulted in a decrease in collagen I accumulation in extracellular matrix after 8 and 21 days in culture.
  - Changes in collagen fiber assembly were observed after immunofluorescence staining and Second Harmonic Generation signal imaging.
- No effect on expression of COL1A1 gene.
- Decrease of the cell-mediated contraction in cell-populated collagen gel lattices.

NEXT STEP: To investigate the mechanism of action of MXD on clubfoot-derived cells in more detail.

Martina.Doubkova@fgu.cas.cz

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* M.D. and J.K. contributed equally to this work.