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ENHANCED ALPHA1(I) mRNA EXPRESSION IN FROZEN SHOULDER AND DUPUYTREN TISSUE

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Abstract
The purpose of this study has been to investigate collagen I and III synthesis during the fibrosing stage of frozen shoulder and Dupuytren samples in comparison to normal capsule tissue.

By using the quantitative PCR significantly increased levels of α_1(I) mRNA transcription in samples of frozen shoulder ($p = 0.016$) and Duypuytren ($p = 0.041$) could be demonstrated, whereas α_2(I) and α_1(III) chains have shown the same mRNA levels as in normal capsule tissue.

Despite an enhancement of α_1(I) mRNA transcription in frozen shoulder and Dupuytren samples the intracellular precursor procollagen I and extracellular mature collagen I was detected immunohistochemically in reduced levels.

The structural alteration of collagen I assembly might be caused by disturbed post-translation from the polypeptide chains into the triple helices procollagen I though α_1(I) mRNA transcription was significantly increased and α_2(I) mRNA transcription was in normal range. Fibroblasts might release high quantities of free α_1(I) polypeptide chains or $[\alpha_1(I)]_3$ homotrimer into the extracellular space during the fibrosing stage of frozen shoulder and Dupuytren disease.

In all samples neither differences of α_1(III) mRNA transcription nor differences of immunohistochemical staining intensity of collagen III could be seen. This might result from apoptosis of myofibroblasts in the final phase of the fibrosing processes.

The stimulating effect of insulin-like growth factor type I (IGF-I) to induce fibrosis in connective tissue such as scarlet is known. In all patients suffering from frozen shoulder and Dupuytren disease the serum IGF-I level was in a normal range and the IGF-I receptor

(IGFR-I) mRNA transcription in the samples was also in the same level compared with normal capsule tissue.

Key words: frozen shoulder, Dupuytren diseases, procollagen, collagen, alpha chains mRNA, collagen synthesis

INTRODUCTION

Fibrosis represents a pathogenic process for almost all forms of chronic connective tissue injuries. Fibrotic processes are caused by vascular dysfunction, cytokines release during inflammation, metabolic disturbance or minor trauma resulting in progressive replacement of the normal connective tissue architecture by fibrotic lesions structure. Fibrosis can appear as severe tissue scarring. Variations of connective tissue are associated with proliferation of resident fibroblast cell types, the increased production and deposition of altered extracellular matrix components or the transition of fibroblasts into cells exhibiting a myofibroblast phenotype [1].

Collagen molecules are considered to be the main extracellular matrix proteins and play the decisive role in the thickening of basement membrane, vascular integrity and in the scar formation processes [2, 3].

Most collagens form polymeric assemblies, such as fibrils, networks and filaments, and can be divided into several subtypes [4].

Frozen shoulder is a disease characterized by fibrosing alteration of collagen structure in the ventral capsule and rotator interval of the joint [5].

Frequently shoulder stiffness is accompanied by Dupuytren disease [6, 7], diabetes mellitus type II [8], hypothyroidism [9] and postmenopausal stage [10].

Structural changes of collagenous tissue revealing thick nodular bands or fleshy mass have been described [7]. Moreover loss of fibril order and twisting of collagen fibrils have been identified [5].

Collagen can be classified in fibrillar such as collagen I, II and III or non-fibrillar organisation such as collagen IV with a amorphous appearance by using of scanning electron microscopy [11].

Morphologically mainly extracellular proteins are differentiated due to the structure of polypeptide α-chains [12].

Collagen type-I represents the major extracellular matrix protein in capsule and tendon tissue.

The synthesis and transcription of α_1(I) und α_2 (I) mRNA followed the translation of amino acid chains to polypeptide by binding on NH_3 and COOH-groups.

Collagen type-I molecules are normally confirmed of two α_1(I) chains and one α_2(I) chain which are transcripted in fibroblasts. Post-translational the three chains are formed intracellularly into the triple helices procollagen I.

The $[\alpha_1(I)]_2\,[\alpha_2(I)]$ heterotrimer procollagen is the predominant molecule which is synthesized and se-

creted by fibroblasts.

Small amounts of stable $[\alpha_1(I)]_3$ or $[\alpha_2(I)]_3$ homotrimers procollagen are detected at variable levels in different tissues [13].

Following the expression of procollagen and the release into the extracellular space the helices are transformed to collagen fibril by proteolysis. The alignment of collagen fibrils is supplied by glycosaminoglycans and proteoglycans.

Collagen-type III, an immature molecule, appeared in embryonic tissue and in early stage of wound healing [14], is derived from procollagen composed of three α_1(III) chains. Predominantly myofibroblasts , a-smooth muscle actin positive cells, synthesize procollagen type-III. The appearance of myofibroblasts in connective tissue of Dupyutren and frozen shoulder samples has been already established [7, 15].

The purpose of this study was to analyze the translation of polypeptide chains and posttranslational modification of collagen I and III in frozen shoulder and Dupuytren samples compared with normal capsule tissue. For measurement of the a-chains mRNA transcription rate the quantitative RT-PCR has been used. For detection of intracellular procollagen I and extracellular collagen I and III immunohistochemical procedures were performed.

Material und Methods

Tissue Sample

Tissue samples were obtained from six patients suffered from frozen shoulder stage II [16] undergoing arthroscopic capsule release. Sample were harvested from the area of rotator interval by incision of the capsule with a 4 mm duck-bill instrument.

Sample were excised from the thickened palmar fascia from 6 patients with Dupuytren disease stage III [17].

In six patients suffering from posttraumatic unidirectional instability after joint dislocation sample were taken in opened operative procedure with duplication of capsule in rotator interval.

For further investigation all samples were divided into two parts for mRNA extraction and for immunohistochemistry.

Tissue for qRT-PCR was snap-frozen in liquid nitrogen and stored at –80 °C until mRNA extraction.

Samples for immunhistochemical staining were fixed in 4% paraformaldehyd.

For the reference of IGFR-I detection intervertebral disc tissues were applied from 6 patients, suffered from prolaps and treated by nucleotomie.

Immunohistochemistry

Immunohistochemistry was performed according to the avidin-biotin-complex. For detection of collagen type-I goat polyclonal antibody L-19 was applied which is raised against a peptide mapping at the amino terminus of mature collagen α_1(I) of human origin was applied.

Procollagen I staining was performed by using goat polyclonal antibody N-17 raised against a peptide mapping at the amino terminus of collagen α_1(I), which reacts exclusive with collagen α_1(I) precursor, while for collagen III goat polyclonal antibody S-17 was used which detects a peptide mapping at the amino terminus of mature collagenα_1(III) were used (Santa Cruz, USA; dilution 1 : 50).

For collagen I and III and procollagen I labelling biotinylated anti-goat secondary antibodies were utilized.

Following application of the ABComplex/HRP (DAKO, Germany) the chromogen Novared (Linaris, Germany) was used for viscualization.

For counterstaining hematoxyline was used.

qRT-PCR

Total RNA was isolated using the Rneasy Mini kit (Qi-

Table 1. Primers used for quantitative RT-PCR.

	Primer	Product length	Accession no.
β_2-MG		165bp	AY187687
Forward	GGTTTCATCCATCCGACATTG	(146-311)	
Reverse	GTTCACACGGCAGGCATACTC		
α_1(I) chain		118bp	NM_000088
Forward	GCAAGGTGTTGTGCGATG	(316-434)	
Reverse	TTGGTCGGTGGGTGACTC		
α_2(I) chain		165bp	NM_000089
Forward	TCCAAAGGAGAGAGCGGTAA	(1194-1359)	
Reverse	GAAGACCACGAGAACCAGGA		
α_1(III) chain		160bp	NM_000090
Forward	ACCTGGAGAGCGAGGATTG	(831-991)	
Reverse	CGCCTGGAAGACCATTTTC		
IGF1R		123bp	NM_000875
Forward	CTACAACTACGCCCTGGTCATC	(381-504)	
Reverse	GGAGAGGTAACAGAGGTCAGCA		

agen; Hilden, Germany) from human frozen shoulder, dupuytren, intervertebral disc and normal capsule tissue. Contaminating DNA was destroyed with 1 U DNase/μg total RNA (Gibco-BRL; Karlsruhe, Germany).The RNA was reverse transcribed using Superscript RNase H- Reverse Transcriptase (200 U/onset; Gibco-BRL) for 50 min at 42 °C. Real-time quantitative PCR (qPCR) was done in the I-Cycler (Bio-Rad; Munich, Germany) using QuantiTec SYBR Green PCR kit (Qiagen). Primer sequences, product length and accession numbers are indicated in Table1. The PCR conditions were initial denaturation in one cycle of 15 min at 95 °C followed by 40 cycles of 30 sec at 95 °C, 30 sec at 58.5 °C, and 30 sec at 72 °C. All analyses were done in triplicate. The expression of α_1(I), α_2(I), α_1(III) chains and IGF-I receptor was normalized with β_2-microglobulin (β_2-MG) as a housekeeping gene (Table 1). The relative expression was calculated by comparison of the received CT values. The PCR products were separated by electrophoresis on a 1.5% Tris-acetate-EDTA agarose gel. Sequencing of the PCR products was done by MWG Biotech.

Statistical Analysis

Statistical analysis of data was performed according to Student`s unpaired t test. Probability (p) values <0.05 were considered significant.

Results

Quantitative RT-PCR for Collagen Polypeptide Detection

Using quantitative RT-PCR a significant increase (p<0;05) of α_1(I) mRNA chains in frozen shoulder und Dupuytren samples compared to normal capsule tissue was found.

The quantity of α_2(I) mRNA chains between the three groups have shown no significant differences.

The α_1(III) mRNA transcription rate was similar between frozen shoulder tissue, Dupuytren samples and normal capsule (Fig. 1).

In agarose gel a good quality of mRNA in all samples could be demonstrated by using the housekeeping gene β_2-microglobulin (β_2-MG) (Fig. 2).

Immunohistochemistry

Immunohistochemically abundant intracellular staining of procollagen I (arrows) could be seen in normal capsule tissue (Fig. 3A). Decreased numbers of fibroblast-like cells with intracellular procollagen I staining (arrows) were recognizable in samples of frozen shoulder (Fig. 3B) and in Dupuytren tissue (Fig. 3C).

In comparison to normal capsule tissue (Fig. 4A) weak staining of collagen I was evident in frozen shoulder (Fig. 4B) and Dupuytren tissue (Fig. 4C).

Collagen III staining was revealed a corresponding distribution pattern in all three groups (Fig. 5A-C).

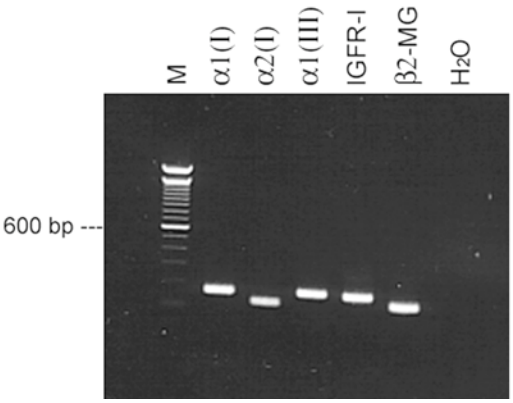

Fig. 1. RT-PCR detection of collagen chains, IGF-I receptor, and RNA integrity control by amplification of the housekeeping gene β_2-MG (165 bp) Lane 1: α_1(I) collagen chains (118 bp); lane 2: α_2(I) collagen chains (165 bp); lane 3: α_1(III) collagen chains (160 bp); lane 4: IGFR-I (123 bp) and lane 5: β_2-MG

Quantitative RT-PCR for IGF-I Receptor Detection

In expectation a significant higher level (p<0.05) on IGF-I receptors mRNA was demonstrated in intervertebral disc samples compared with normal capsule tissue. The IGFR-I mRNA transcription rate could be seen approximate in a same level between frozen shoulder, Dupuytren sample and normal capsule tissue. Differences were not significant (Fig. 6).

Discussion

The primary frozen shoulder is characterized by fibrosing alteration of the shoulder capsule accompanied by contracture of the rotator interval and the

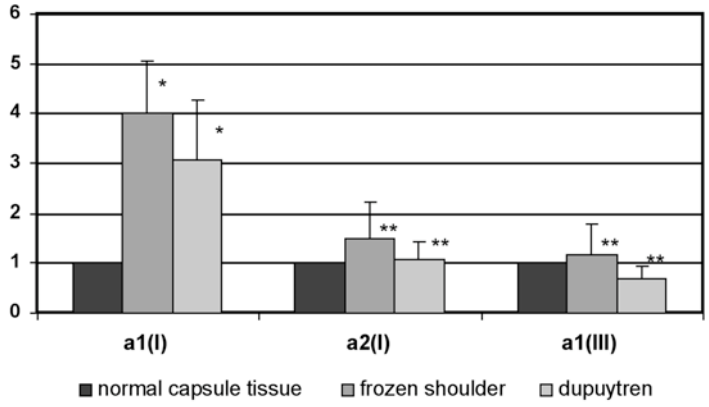

Fig. 2. quantitative RT-PCR of α_1(I), α_2(I) and α_1(III) collagen chains expression in normal capsule tissue, frozen shoulder an dupuytren tissue. *significant to control, **no-significant

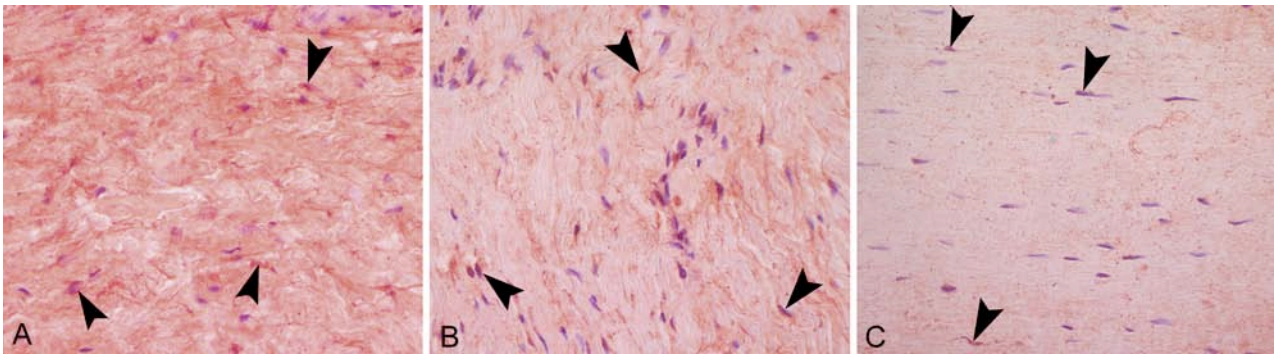

Fig. 3 A - C. Procollagen I immunohistochemistry. Procollagen I is abundant and homogenously distributed throughout the normal capsule tissue (A). Within the tissue of the frozen shoulder (B) moderate staining intensity can be shown, while in samples of Dupuytren (C) weak staining becomes evident. Fibroblasts (black arrows). Magnification x182.

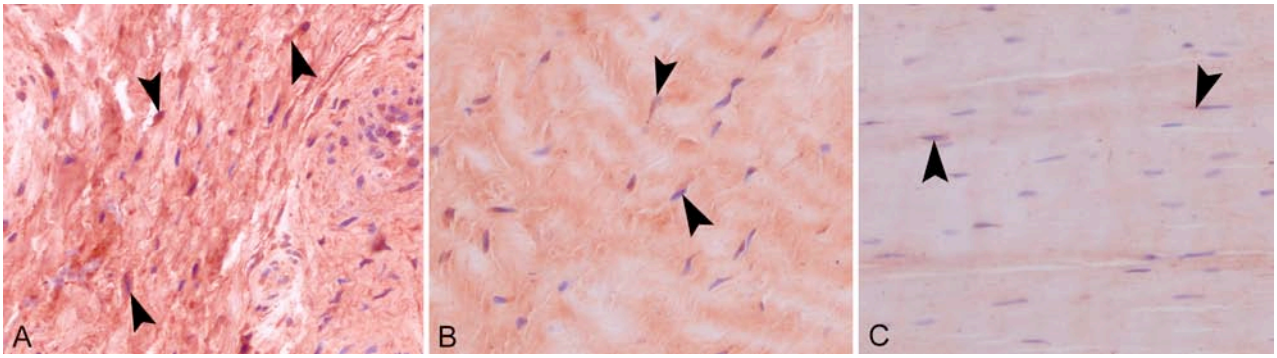

Fig. 4 A - C. Collagen I immunohistochemistry. Intensive immunoreactivity of collagen I can be seen in normal capsule tissue (A), while in samples of the frozen shoulder (B), and especially those of Dupuytren disease (C) the staining intensity decreased. Fibroblasts (black arrows). Magnification x182.

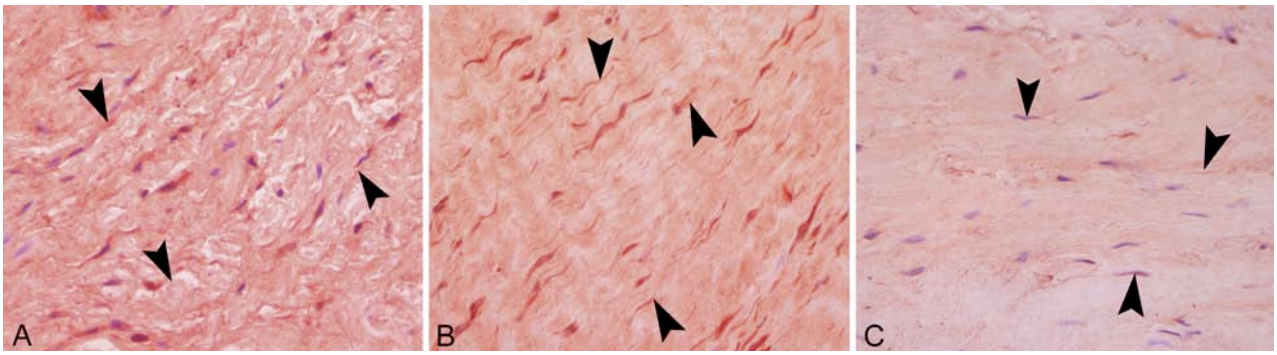

Fig. 5 A - C. Collagen III immunohistochemistry. Corresponding staining intensity and distribution pattern of collagen III becomes obvious in normal capsule tissue (A), and samples of the frozen shoulder (B), whereas in Morbus Dupuytren (C) only weak collagen III staining can be detected. Fibroblasts (black arrows). Magnification x182.

coracohumeral ligament.

The prolonged clinical history characterized by initial inflammation and the acute painful freezing stage is followed by the frozen phase with the typical painless stiffness of the shoulder.

Arthroscopic finding in the freezing phase is primary an acute synovitis, whereas in the frozen stage a thickened capsule and a decreased joint space are seen. Similar fibrosing alterations occur in the course of Dupuytren disease. The frozen shoulder is also called "Dupuytren-like disease" [6, 7, 18].

It has been assumed that initial synovitis in frozen shoulder is induced by the release of cytokines, growth factors and matrix metalloproteinase expressed lymphoid cells, platelets epithelial cells, mesangial cells and fibroblasts [19].

In previous studies the presence of a smooth muscle actin (ASMA) expressing cells was described in frozen shoulder tissue. Fibroblasts induce differentiation of ASMA cells, which express collagen III by the

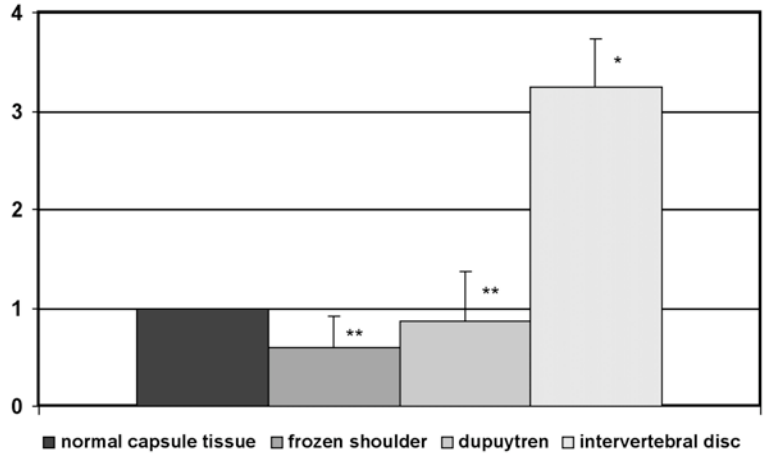

Fig. 6. quantitative RT-PCR of IGFR-I expression in normal capsule tissue, frozen shoulder, dupuytren and intervertebral disc. *significant to control, **no-significant

effect of transforming growth factor-beta (TGF-β).

Rodeo et al. [20] have shown an elevated level of TGF-β in frozen shoulder by immunocytochemistry, whereas Bunker et al. [19] could not demonstrated increased levels of TGF-β mRNA in frozen shoulder compared with normal capsule tissue.

Immunohistochemical investigation in order to study the effects of cytokines and growth factors on cells and tissues bear the problem of inadequate availability of appropriate human tissue , because only in the final stage of both frozen shoulder and Dupuytren an operative procedure with arthroscopic adhesiolyse and capsulotomie of the shoulder or resection of palmar fascia acquisition of samples is possible.

In contrast human tissue sample can not be taken during the initial inflammatory phase because of the operative counter indication. Therefore, only reparative and remodelling processes could be seen in the frozen shoulder stage II and Dupuytren disease phase III.

In the present study we have investigated the collagen I and III synthesis in frozen shoulder and dupuytren disease and a significant enhancement of the α_1(I) mRNA transcription rate together with a decreased expression rate of intracellular precursor procollagen I and extracellular mature collagen I in comparison to normal capsule tissue could be detected.

The α_1- and α_2-chains consist distinct terminal amino- and carboxyl propeptides [21].

The assembly of the procollagen I heterotrimer is induced by the interaction of the carboxyl-terminal peptide of the a-chains, which stabilize the propeptide by the formation of interchain disulfide bonds [22]. The triple helix folding is initiated by associated carboxyl terminal peptide to the amino terminal peptide [21].

The intracellular procollagen I staining by using the polyclonal N-17 antibody appears in decreased levels of the fibrotic altered tissue. The enhanced α_1(I) mRNA transcription rate and the reduced intracellular procollagen I staining indicate the disturbed posttranslation rate.

In results of the posttranslational modification an enhancement of free α_1(I) polypeptide chains expression may be possible.

The applied antibodies bind to the terminal amino groups of the α_1(I) polypeptide chains.

The reduced extracellular level of mature collagen I is explained by the decreased expression of procollagen I.

It is known that no biological activities in the extracellular matrix are induced by arranged polypeptide chains. The function of the matrix depends on the conformation of the polypeptide chains to three-dimensional configuration [4].

The enhanced levels of mRNA encoding α_1(I) chains taken together with the decreased

procollagen I staining found in immunohistochemistry indicates either that the message was not translated or that the chains were secreted rapidly into the extracellular space .

In this study it has been shown that of α_1(III) chains mRNA and the extracellular collagen III levels are expressed in similar amounts in frozen shoulder, Dupuytren samples and normal capsule.

α-smooth muscle actin positive myofibroblasts, expressing EDB-fibronectin and collagen III, are numerous in frozen shoulder and Dupuytren tissues. In contrast, the presently observed lack of enhanced α_1(III) chains transcription for collagen III synthesis points towards the immunohistochemical absence of myofibroblasts (data not shown). This lack of expression in final stage of frozen shoulder and dupuytren disease could be caused by apoptosis of myofibroblasts.

It seems likely that diabetes mellitus, hypothyroidism and postmenopausal age represent a predisposition to frozen shoulder disease.

A stimulating effect of cytokine IGF-I on fibroblasts collagen expression is described in previous studies and is dependent on fibroblastic IGF-I receptors [23-25].

Diabetes mellitus type II, hypothyroidism and postmenopausal age are associated with increased IGF-I serum level [8-10]. In fibroblast extracellular matrix IGF-I cytokines equilibrate with IGF-I receptors dependent on presence of insulin-like growth factor-binding proteins [24].

Hyperstimulation of the insulin-like growth factor-I receptor (IGFR-I) is thought to be closely linked to abnormal fibroblast proliferation and differentiation in connective tissue diseases such as keloid [26, 27].

In our study an enhancement of IGFR-I mRNA could not be seen in comparison to normal capsule tissue by using the polymerase chain reaction. Addi-

tionally all patients revealed normal IGF-I serum levels was also in a normal range (data not shown).

In summary we have shown increased levels of α_1(I) polypeptide chains and reduced levels of intracellular procollagen I and extracellular mature collagen I in frozen shoulder and Dupuytren samples.

Free α_1(I) polypeptide chains might induce the alteration of collagen structure demonstrated by means of transmission electron microscopy in a previous study [5].

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