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# Abnormal collagen deposition in synovia after collagen type V immunization in rabbits

**Luciana Tsuzuki Ichicawa Ogido[1], Walcy Rosolia Teodoro[1], Ana Paula Pereira Velosa[1], Cristiane Carla de Oliveira[1], Edwin Roger Parra[2], Vera Luiza Capelozzi[2] and Natalino Hajime Yoshinari[1]**

[1]Discipline of Rheumatology, Faculty of Medicine, University of São Paulo and [2]Department of Pathology, Faculty of Medicine, University of São Paulo, Brazil

**Summary.** Sinovitis in Scleroderma (SSc) is rare, usually aggressive and fully resembles rheumatoid arthritis. Experimental models of SSc have been used in an attempt to understand its pathogenesis. Previous studies done in our laboratory had already revealed the presence of a synovial remodeling process in rabbits immunized with collagen V. To validate the importance of collagen type V and to explore the quantitative relationship between this factor and synovia remodeling as well as the relationship between collagen type V and other collagens, we studied the synovial tissue in immunized rabbits. Rabbits (N=10) were immunized with collagen V plus Freund's adjuvant and compared with animals inoculated with adjuvant only (N=10). Synovial tissues were submitted to histological analysis, immunolocalization to collagen I, III and V and biochemical analysis by eletrophoresis, immunoblot and densitometric method. The synovial tissue presented an intense remodeling process with deposits of collagen types I, III and V after 75 and 120 days of immunization, mainly distributed around the vessels and interstitium of synovial extracellular matrix. Densitometric analysis confirmed the increased synthesis of collagen I, III and V chains (407.69±80.31; 24.46±2.58; 70.51±7.66, respectively) in immunized rabbits when compared with animals from control group (164.91±15.67; 12.89±1.05; 32±3.57) ($p<0.0001$). We conclude that synovial remodeling observed in the experimental model can reflect the articular compromise present in patients with scleroderma. Certainly, this experimental model induced by collagen V immunization will bring new insights in to pathogenic mechanisms and allow the testing of new therapeutic strategies to ameliorate the prognosis for scleroderma patients.

**Key words:** Synovia, Collagen type V, Experimental model, Scleroderma, Fibrosis

*Offprint requests to:* Walcy Rosolia Teodoro, PhD, Discipline of Rheumatology, São Paulo Medical School – University of São Paulo, Av. Dr. Arnaldo, 455, CEP –01246-903, São Paulo, SP, Brazil. e-mail: matrix@lim17.fm.usp.br and vcapelozzi@lim05.fm.usp.br.

## Introduction

Sinovitis in SSc is rare, usually aggressive and fully resembles rheumatoid arthritis. In all other cases sinovial fibrosis is due to time fibrosis and progressive retraction of the fingers. Experimental models of SSc have been used in an attempt to understand its pathogenesis, but most of these models are obtained from genetically modified animals, such as naturally-occurring TSK-1 (Saito et al., 2002), UCD-200 chickens (Sgonc, 1999) or genetically manipulated animals through mutagenesis or genetic modifications (Clark, 2005). Scleroderma-like lesions are also observed following chemical exposure to bleomycin (Yamamoto and Nisshioka, 2005) or transplantation of immune cells originating a graft-versus-host disease (GVHD) (Zhang and Gilliam, 2002). In all of these models the sinovial changes are minimal or absent.

Recently, we discovered a novel experimental model that reproduces most of the histological aspects of scleroderma, induced in healthy female New Zealand rabbits, after animal immunization with human type V collagen (col V) plus Freund's adjuvant. These animals develop progressive fibrosis and vasculitis of all organs normally affected in this disease, including skin, esophagus, heart, synovia, cartilage and kidney (Yoshinari et al., 2002; Teodoro et al., 2003, 2004; Bezerra et al., 2006).

Collagen V is a component of the basement membrane, synovial screen, hyaline articular cartilage, and placenta and surrounds vascular smooth muscular cells (Niyibizi et al., 1984; Linsenmayer et al., 1990; Hay, 1991; Adachi et al., 1997). Some particularities make collagen V different from the other types. The molecule maintains the $NH_2$ and COOH terminal ends making it more immunogenic (Linsenmayer et al., 1990; Hay, 1991). Otherwise, collagen type V is not normally exposed in the tissues, because it is found between collagen types I and III, composing heterotypic fibers, except in the cornea (Niyibizi et al., 1984; Lisenmayer et al., 1990). Type V collagen has also been found to be involved in the pathogenesis of experimental lung allograft rejection (Haque et al., 2002; Yasufuku et al., 2002; Wilkes, 2003). Others authors have prevented bronchiolitis obliterans in rat lung allografts by type V collagen-induced oral tolerance (Yasufuku et al., 2001). Collagen V plays an important role in the proliferation process and tissue remodeling (Leroy et al., 1996; Mckown et al., 2000). Interaction of collagen type V with type I originate heterotypic fibrils, implicated in keeping the integrity of ECM and regulating collagen fibril diameter (Adachi and Hayachi, 1986; Burrows et al., 1996; Michalickova et al., 1998; Birk, 2001). Collagen types I and V heterotypic fibers mask the helical region of type V molecule, becoming epitopes of this region hidden and inaccessible. Otherwise, retention of a large part of the N-propeptide in mature molecules makes it more immunogenic (Linsenmayer et al., 1990; Andrikopoulos et al., 1995).

Previous studies done in our laboratory had already revealed the presence of a synovial remodeling process in rabbits immunized with collagen V (Bezerra et al., 2006). To validate the importance of collagen type V and to explore the quantitative relationship between this factor and synovia remodeling as well as the relationship between collagen type V and other collagens, we studied the synovial tissue in immunized rabbits and herein report our results.

## Materials and methods

### *Experimental protocol*

The experimental protocol was described in previous papers (Teodoro et al., 2003, 2004). Briefly, female New Zealand rabbits (N=10) with similar age and weight (2,500g) were immunized subcutaneously with 1 mg collagen type V isolated from human placenta, extracted by precipitation with sodium chloride, diluted in 1 ml 10 mM acetic acid and added to an equal volume of complete Freund's adjuvant. The antigen specificity was tested by immunoblotting using monoclonal antibodies against collagen types I, III and V.

Four weeks later, rabbits received an identical dose of type V collagen, and two booster immunizations were administered intramuscularly, separated by a 15-day interval (1 mg type V collagen plus 1ml incomplete Freund's adjuvant). Each group of immunized animals was compared to the control group (N=10), inoculated with 10 mM acetic acid in complete or incomplete Freund's adjuvant and the samples were obtained 75 and 120 days after the first immunization.

The synovia was removed under strictly sterile conditions, and samples were immersed in 10% formalin for 24 hours and embedded in paraffin. Two to 3 µm-thick sections were stained by Masson's trichrome and examined under a Nikon light microscope. All slides were coded, randomized, and then examined by three observers (LO, WT and VLC), who did not have access to the code.

### *Collagen immunodetection*

Immunofluorescence of synovia samples was done in paraffin-embedded fragments mounted on slides with aminosylane (Sigma Chemical Co.; St. Louis, Missouri, USA) for collagen characterization. The slides were immersed in xylene and dehydrated in decreasing ethanol concentrations. The immunogenic sites were exposed by enzymatic treatment of the synovia samples with bovine pepsin (10,000 UTD; Sigma Co.; St. Louis, Missouri, USA) in 2 mg/ml acid buffer, pH 2.2, for 30 min at 37°C, followed by incubation in phosphate-buffered 5% milk, pH 7. The slides were incubated overnight at 4°C with polyclonal anti-collagen types I and V antibodies obtained from mice and diluted at 1:25 in phosphate-buffered saline (PBS) (Teodoro et al., 2004), and with monoclonal anti-collagen type III (Oncogen; San Diego, USA) diluted at 1:100. The slides were washed several times in PBS-0.05% Tween 20 and incubated for 90 min with secondary antibody (fluorescein conjugated mouse anti-IgG, Sigma; St. Louis, Missouri, USA) diluted 1:50 in PBS containing 0.005% Evans blue. The slides were again washed several times in PBS-0.05% Tween 20 and mounted with buffered glycerin. The reaction was visualized under a Nikon fluorescence microscope.

### *Collagen biochemical characterization*

For extraction of synovia collagen the tissue samples were sliced in EDTA and proteinase inhibitors (50 mM EDTA, 5 mM phenylmethylsulfonyl fluoride and 0.02 mM N-ethylmaleimide) and washed for 48 hours at 4°C. The samples were homogenized with a Teckman polytron and submitted to a 12000 rpm centrifugation for 30 minutes. The residue was treated with pepsin (substrate/enzyme 10:1) in 0.5 M acetic acid at 4°C for 16 h. The reaction mixture was neutralized to pH 8.0 and left overnight at room temperature to inactivate pepsin. Collagen was precipitated by adding NaCl 2 M. The collagen chains were analyzed by SDS-PAGE (7.5%) with reduction by 2-mercaptoethanol according to the methods described by Laemmli (1970) and Sykes et al. (1971). After performing SDS-PAGE of collagen samples, proteins migrated in the gel were electro-

transferred onto a nitrocellulose membrane (Sigma Chemical Co.) The membrane was blocked in 5% skimmed milk/PBS for 1 h at room temperature, and the immunoreaction was performed by incubation with mouse monoclonal anti-collagen type I (Sigma Chemical Co.), mouse monoclonal anti-human type III collagen (Oncogen; San Diego, USA) and rabbit polyclonal anti-human type V collagen according to Teodoro et al. (2004). The collagen chains were evaluated by densitometric method in Image Master VDS (Pharmacia Biotech).

### *Statistical analysis*

To test our hypothesis about the influence of collagen V autoantibody on the pathogenesis of the disease we compared the results obtained from immunized and control animals. We assessed differences and associations in the frequency of densitometric variables by the T-test for independent samples. The statistical procedure was performed with SPSS version 10.0 statistical software (SPSS, 2002).The level of statistical significance was set at $p<0.05$.

## Results

### *Synovial architecture and extracellular matrix Remodeling (ECM) in control and immunized rabbits*

Figure 1 (Panels A to H) shows the morphologic features of synovial tissue obtained from control and immunized animals, stained with H&E and Masson's trichromic. Qualitative analysis of the H&E-stained and Masson's trichromic section of the control groups showed preserved architecture, without inflammatory or repair tissue response in any of the synovial compartments (Fig. 1A-D). In contrast, the subsynovial compartment of immunized animals was characterized by thickening of the connective tissue frame and vessel walls (Fig. 1E,F).

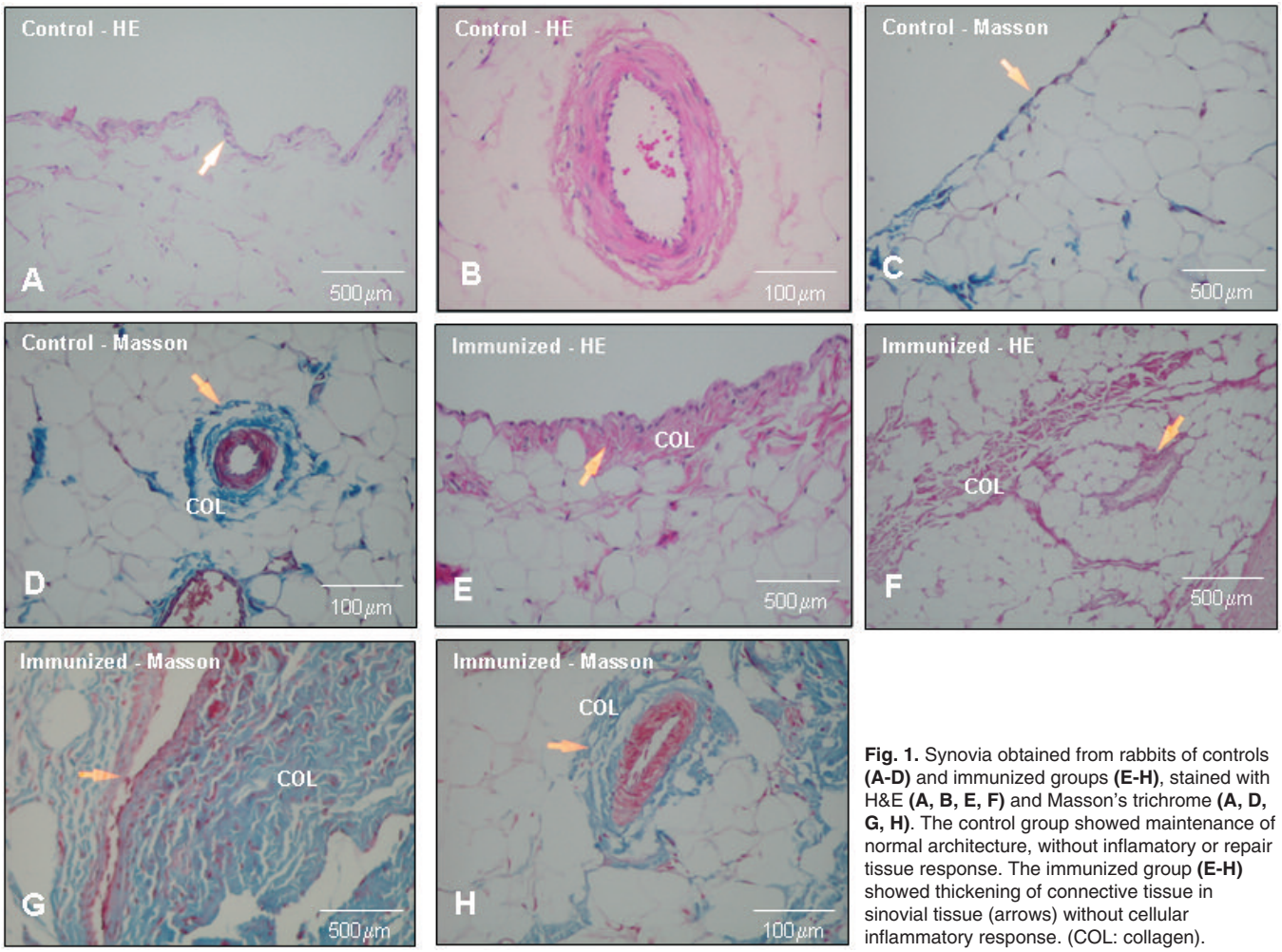


**Fig. 1.** Synovia obtained from rabbits of controls **(A-D)** and immunized groups **(E-H)**, stained with H&E **(A, B, E, F)** and Masson's trichrome **(A, D, G, H)**. The control group showed maintenance of normal architecture, without inflamatory or repair tissue response. The immunized group **(E-H)** showed thickening of connective tissue in sinovial tissue (arrows) without cellular inflammatory response. (COL: collagen).

### *Collagen changes in synovial ECM of control and immunized rabbits*

Figure 2A-H shows the collagen fibers in synovial ECM from control and immunized rabbits, immunostained with types I, III and V collagen and observed using immunofluorescence. Figure 2A,C and E show the synovia from the control rabbits clearly exhibiting the weak fine and fibrilar fluorescent green birefringence for types I, III and V collagen, in synovial (S) and subsynovial (SS) connective frame. The immunofluorescence pattern of types I, III and V collagen is coincident with the preserved architecture observed in normal synovial ECM (Fig. 1A-D). Figures 2B, 2D show the synovia from the immunized rabbits exhibiting strong birefringence for types I and III collagen due to thickening and histoarchitectural distortion, involving the synovial connective frame and ECM of small vessels (Fig. 2B,D). Interesting enough was the high expression of type V collagen, causing diffuse thickening of the synovial connective frame (Fig. 2F) when compared to controls (Fig. 2E). Furthermore, a coexistence of type I and type V collagens was found to determine the thickening of the ECM periadventitial of small vessels (Fig. 2G,H). These alterations were more evident after 120 days of type V sensitization, which was coincident with the modifications discussed at H&E stain (see Fig. 1E-H). To confirm specificity of the staining, some representative control sections were incubated with total serum of rabbits.

### *Biochemical measurement of types I, III and V*

SDS-PAGE profile of pepsin-soluble collagen fractions 2.5 M NaCl-precipitated from synovia of immunized and control rabbits is shown in Figure 3. Types I and III collagen were identified in the presence of ß-mercaptoethanol and urea, presenting bands with

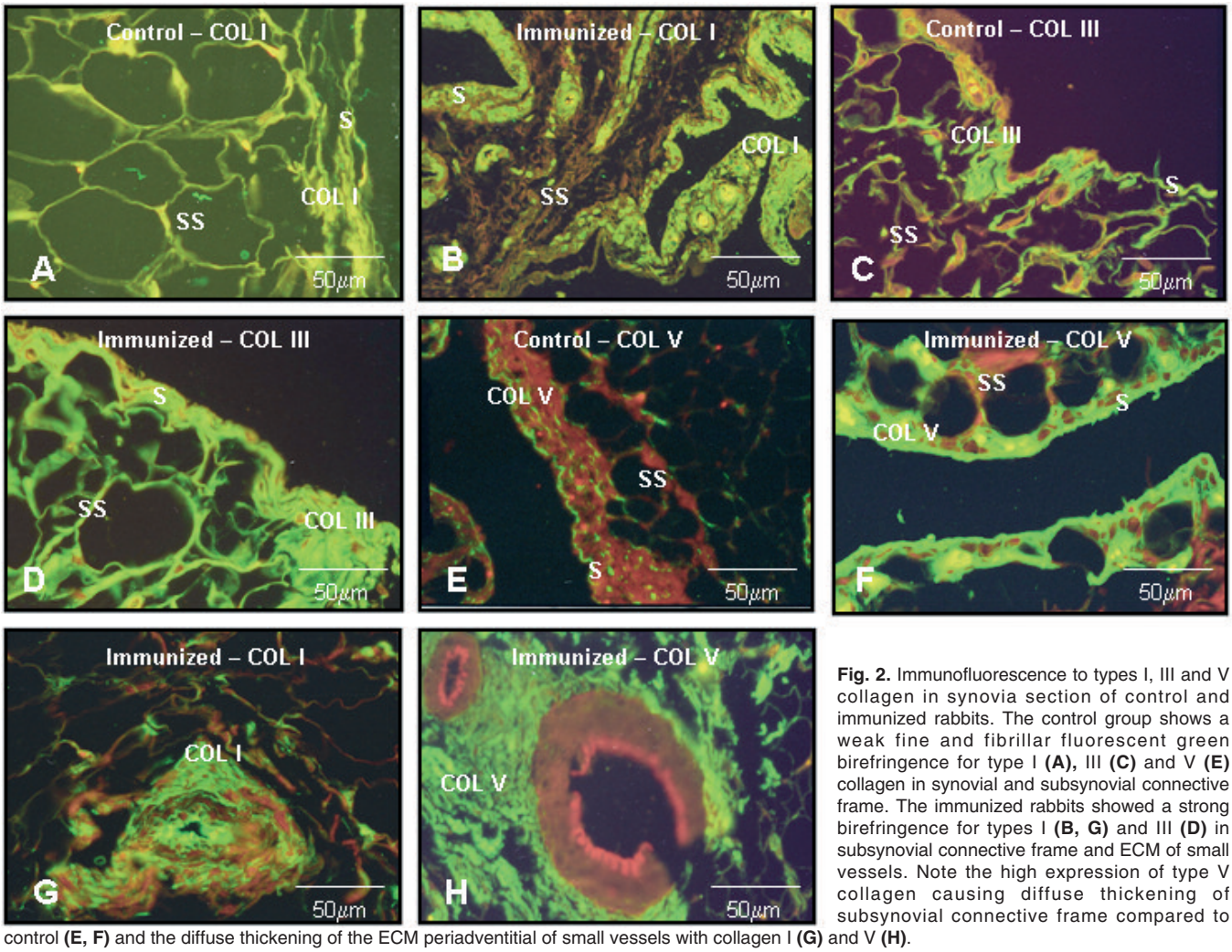


**Fig. 2.** Immunofluorescence to types I, III and V collagen in synovia section of control and immunized rabbits. The control group shows a weak fine and fibrillar fluorescent green birefringence for type I **(A),** III **(C)** and V **(E)** collagen in synovial and subsynovial connective frame. The immunized rabbits showed a strong birefringence for types I **(B, G)** and III **(D)** in subsynovial connective frame and ECM of small vessels. Note the high expression of type V collagen causing diffuse thickening of subsynovial connective frame compared to control **(E, F)** and the diffuse thickening of the ECM periadventitial of small vessels with collagen I **(G)** and V **(H)**.

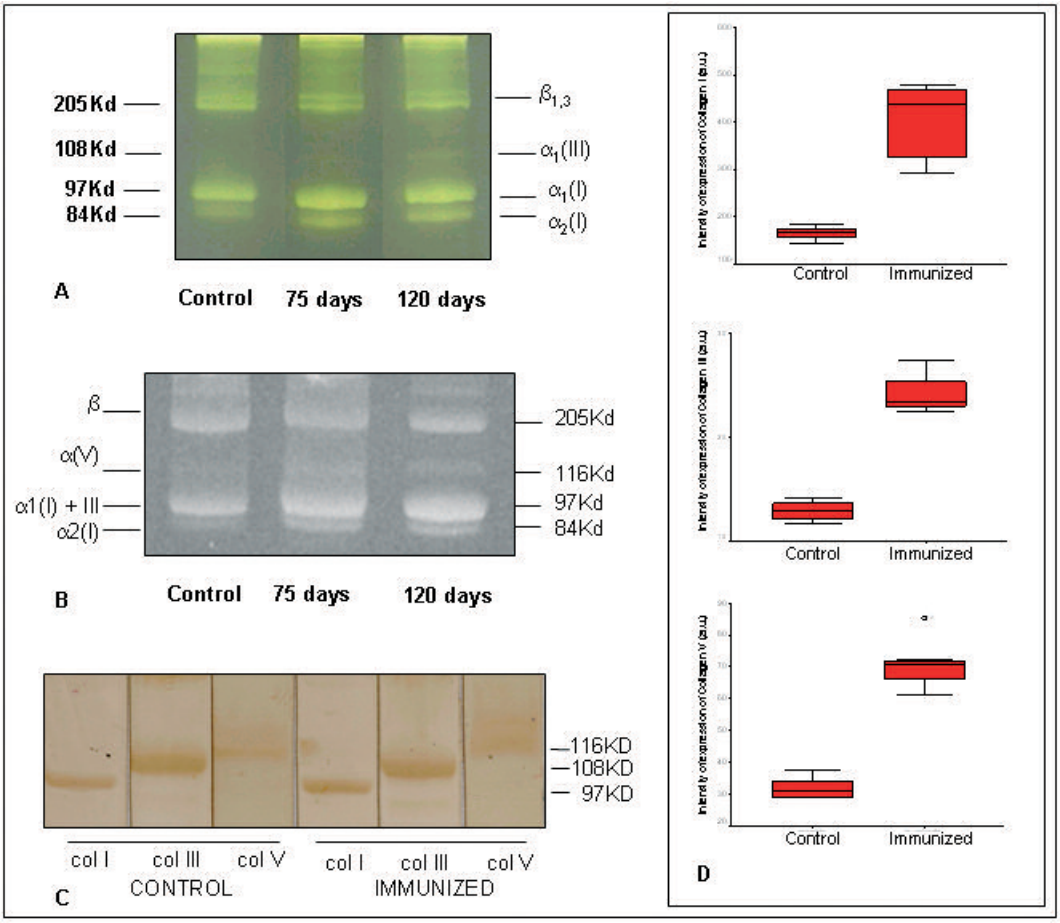


**Fig. 3.** Collagen types I and III fractions in SDS-PAGE profile of immunized and control rabbits identified in the presence of b-mercaptoethanol and urea **(A)** and collagen type V fractions identified in the presence of b-mercaptoethanol without urea **(B)**. Immunoblotting shows types I, III and V collagen in control and immunized animals **(C)**. Graph obtained after densitometric quantification of types I, III and V collagen shows increased amount of collagen types I, III and V in immunized animals compared with control group **(D)**.

molecular masses of 108, 97 and 84 Kd, corresponding respectively to α1(III), α1(I) and α2(I) chains, in synovia from control and immunized rabbits (Fig. 3A). Type V collagen α [α1(V), α2(V) and α3(V)] chains were observed around 116Kd by SDS-PAGE electrophoresis without urea (Fig. 3B). Immunoblotting analysis using specific antibodies confirmed the electrophoretic motility of types I, III and V collagen chains in synovia samples from immunized and control rabbits (Fig. 3C).

The amount of types I, III and V collagen α-chains isolated from synovia of experimental groups and controls was determined by densitometry. The statistical analysis of the densitometric results revealed increased amounts of collagen types I, III and V (407.69±80.31; 24.46±2.58; 70.51±7.66) when compared with the control group (164.91±15.67; 12.89±1.05; 32±3.57) ($p<0.0001$) (Fig. 3D).

## Discussion

In this study, histoarchitectural changes of interstitial and vascular extracellular matrix characterized the remodeling process of the synovia in the presence of a poor inflammatory response after 120 days of rabbit immunization with human collagen type V (col V). Immunofluorescence and biochemical analysis confirmed collagen types I, III and V deposits at synovial extracellular matrix. These findings are very similar to histological changes found in human synovia of patients with scleroderma. In both cases, the presence of important remodeling and scarce inflammatory process are common features (Bezerra et al., 2006). Interestingly, the immunodetection of collagen type V at the synovial tissue showed the same abnormal expression of this molecule, similarly to those previously observed in lung and skin of animals and patients with systemic sclerosis (Teodoro et al., 2004; Bezerra et al., 2006). Specifically, increased quantities of structurally different collagen V were found distributed in the subsynovial connective tissue and vessels, forming thick fibers with a network aspect, causing histoarchitectural distortion of the extracellular matrix. In general, this molecule displays discrete labelling, forming long and slim fibrils, composing heterotypic fibers with collagen types I and III (Adachi and Hayashi, 1986).

The above results confirm the relevance of this novel experimental model, induced in healthy animals,

following col V immunization, since it reproduces similar histological abnormalities seen in scleroderma in all organs studied until now, including sinovial tissue. Also, the overexpression of abnormal collagen type V was a common feature observed in animals and patients, suggesting the existence of a final common pathogenic pathway between the experimental model and patients with scleroderma (Bezerra et al., 2006).

We speculate the existence of at least two complementary pathways, responsible for inflammatory and remodeling processes, viewed in rabbits immunized with col V. Probably, the mechanism is related to direct binding of collagen V epitopes on endothelial cells, enhancing synthesis of collagen by fibroblasts or myofibroblasts. Prolonged attack can provoke basement membrane injury and exposition of hidden collagen V epitopes, originating neoantigens, which will trigger an auto-immune disease. The presence of this pathway may be corroborated by the presence of persistent circulating immunocomplexes and the appearance of antibodies to collagen types IV and continuous presence of antibodies to collagen V in the sera of immunized rabbits.

Our results are supported by previous studies in atherosclerosis. Underwood et al. (1998), demonstrated in an experimental study that collagen type V secreted into the ECM by human intimal vascular smooth muscle cells in culture, is inhibitory for adhesion and proliferation of endothelial cells isolated from the umbilical artery. Moreover, previous studies had demonstrated the cessation of endothelium recovery, following endothelia cell damage, observed during any surgical procedure in an attempt to alleviate arterial stenosis (Shirotani et al., 1993; Rogers and Edelman, 1995). The reason for such inhibition was suggested by the formation of a neointima secreted by smooth muscle, which was rich in inhibitory factors, including collagen type V (Ziats and Anderson, 1993). These studies suggested an important role for col V in the inhibition of endothelium recovery after vascular injury. We postulate that in our experimental model, similar pathogenic mechanisms are present.

Synovia remodeling present in this experimental model probably mimicks the articular compromise observed in patients with SSc. Previous studies done in our laboratory had already revealed the presence of a synovial remodeling process in rabbits immunized with collagen V (Teodoro et al., 2003). Studies on synovial tissue are of great interest in rheumatology, since joint symptoms are frequent and in general, each rheumatic disease presents particular clinical and histological aspects, helping to discriminate a given disease. There are few studies on synovia tissue in scleroderma and some questions have intrigued researchers. For example, how is it possible for the same pathogenic mechanism responsible for disease setting to promote distinct histological presentations in different organs? Lung involvement in the active stage of scleroderma patients is characterized by the presence of a remarkable inflammatory process, while the remodeling process with discreet cell infiltration, is observed in synovial tissue.

The next question is to understand the remodeling process in the presence of a poor inflammatory response observed in synovia of the immunized rabbits. It is possible that collagen V isoform found in synovia could be different from that found for example in lung, or the quantitative expression of collagen V is minor in synovia, when compared with those seen in other organs. It is known that synovial cells are not supported by basement membrane, which is an important source of collagen V molecules.

We conclude that synovial remodeling observed in the experimental model can reflect the articular compromise present in patients with scleroderma, overexpression of abnormal collagen type V being a common feature in both conditions. Certainly, this experimental model induced by collagen V immunization will bring new insights in to pathogenic mechanisms and allow the testing of new therapeutic strategies to ameliorate the prognosis for scleroderma patients.

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