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PREVENTION OF MYOCARDIAL FIBROSIS BY N-ACETYL-SERYL-ASPARTYL-LYSYL-PROLINE IN DIABETIC RATS

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Short Title: Ac-SDKP and diabetic cardiomyopathy

ABSTRACT

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a physiological tetrapeptide hydrolyzed by angiotensin converting enzyme. In experimental model of hypertension, Ac-SDKP has antifibrotic effects in the heart. The role of Ac-SDKP in diabetic cardiomyopathy is presently unknown.

The aim of the study was to evaluate the effect of Ac-SDKP on cardiac systolic and diastolic function, interstitial and perivascular fibrosis in the heart of diabetic rats.

Diabetes was induced in 55 Sprague Dawley rats by streptozotocin injection. Control rats (n=18) underwent only buffer injection. Out of 55 diabetic rats, 19 were chronically treated with insulin and 13 with the angiotensin converting enzyme inhibitor (ACEi), ramipril (3mg/kg/day). Two months after the onset of diabetes, Ac-SDKP (1mg/kg/day) was administered for 8 weeks to 8 control rats, 13 diabetic rats, 7 diabetic rats treated with ramipril and 9 insulin treated diabetic rats by osmotic minipumps.

Diabetic rats had a significant increase in blood glucose level. Left ventricular interstitial and perivascular fibrosis and TGF β -1 protein levels were increased in diabetic rats, but not in insulin treated diabetic rats and in ramipril treated diabetic rats, as compared with control rats. Ac-SDKP administration significantly reduced left ventricular interstitial and perivascular fibrosis in diabetic rats and in diabetic rats treated with ramipril. This was accompanied by a significant reduction of active TGF β -1 and p-Smad2/3 protein levels in myocardial tissue of diabetic rats. Echocardiography showed that diabetes was associated with increased end systolic diameters, depressed global systolic function and diastolic dysfunction, as assessed by transmitral Doppler velocity profile. These changes were completely reverted by insulin or ramipril treatment. Ac-SDKP treatment partially restored diastolic function in diabetic rats.

In diabetic rats, Ac-SDKP administration reduces left ventricular interstitial and perivascular fibrosis, active TGF β -1 and p-Smad2/3 levels, and improves diastolic function. Altogether these findings suggest that, by inhibiting TGF β /Smad pathway, Ac-SDKP protects against the development of diabetic cardiomyopathy.

Key words: diabetic cardiomyopathy, Ac-SDKP, experimental models, streptozotocin, Smad, ACE-inhibitors, echocardiography, rats.

INTRODUCTION

Diabetes is a major cause of mortality and morbidity [1]. It represents not only a well known risk factor for ischemic heart disease, but it may also affect directly the cardiac structure. In fact, 'diabetic cardiomyopathy' indicates a clinical condition characterized by ventricular dysfunction leading to heart failure in the absence of atherosclerotic coronary heart disease and hypertension [2-4]. Molecular abnormalities behind this condition are complex and caused by the concomitant presence of metabolic alterations such as hyperglycemia, hyperlipidemia and hyperinsulinemia [5]. Finally, the central role of activation of the renin-angiotensin system in the development of diabetic cardiomyopathy has been shown [6].

In line with this concept, angiotensin converting enzyme (ACE) inhibitors are presently among the drugs of choice for the treatment of hypertension, heart failure and renal disease, both in diabetic and non-diabetic patients [7,8]. The effects of ACE inhibitors can not be explained only by their antihypertensive actions [9].

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is an endogenous tetrapeptide, hydrolyzed by ACE. ACE inhibitors increase plasma Ac-SDKP concentration in healthy subjects [10], through the inhibition of its degradation. Ac-SDKP has antifibrotic effects in the left ventricle of angiotensin II treated rats [11] and in aldosterone-salt hypertensive rats [12]. In these experimental models the anti-fibrotic effect of ACE inhibitors is partially mediated by Ac-SDKP [13,14].

The role of Ac-SDKP in diabetic cardiomyopathy is presently still unknown. Of interest, this complication is characterized by an alteration of collagen and extracellular matrix synthesis [15], a possible target of Ac-SDKP, in the absence of hypertension.

The aim of this study was to investigate the effect of Ac-SDKP administration on cardiac function, interstitial and perivascular fibrosis in the heart of diabetic rats, treated or not with insulin. The effect of Ac-SDKP in diabetic rats treated with ACE inhibitors was also evaluated.

METHODS

Animal and experimental design.

Experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experiments were performed in conscious 12 week old male Sprague Dawley rats (body weight 250-275g) (Charles River, Calco, Italy). Animals were individually housed in cages in a temperature-controlled room with a 12:12 light-dark cycle for the whole experimental period and allowed to accustom with the experimental procedures. Rats had free access to a standard rat chow and tap water.

Diabetes was induced in Sprague Dawley rats (n=55) through a single intraperitoneal injection of streptozotocin (75 mg/kg, Sigma) dissolved in 0.1 mol/L citrate buffer. Sham treated rats (n=18) underwent only buffer injection. Two days after streptozotocin administration, blood glucose was measured (Glucometer Bayer) and only rats with blood glucose levels above 13.88 mmol/L entered the experimental protocols. A group of 36 diabetic rats were kept hyperglycemic and received only 2-4 U of insulin two times a week to prevent ketoacidosis. An ACEi, ramipril (3mg/kg/day, in drinking water) was administered to 13 diabetic rats. A group of 19 diabetic rats, two days after the onset of diabetes, received chronic administration of insulin (Limplant, Sustained Release Insulin Implants, 2-4U/24h, Linshin Canada, Canada) to control glycemia. After 2 months, the rats were anaesthetized (sodium pentobarbital, 40 mg/kg i.p.) and osmotic minipumps (Alzet 2004, Palo Alto, CA, USA) were subcutaneously implanted. Osmotic minipumps delivered Ac-SDKP (Primm, Milan, Italy) at the dose of 1mg/kg/day for two months to 8 control rats, 13 diabetic rats, 7 diabetic rats treated with ramipril, and 9 insulin treated diabetic rats. In the corresponding sham treated rats (10 control, 10 diabetic, 6 diabetic rats treated with ramipril, 10 insulin-treated diabetic rats) osmotic minipumps delivered saline solution.

Systolic blood pressure (SBP, mmHg) was measured twice a month. Systolic blood pressure was assessed by the tail cuff method (average of 6 recordings. BP Recorder, Ugo Basile Instruments, Italy) by a single investigator who was unaware of the specific treatment. Body weight (B.W, g.) was measured two times a week and blood glucose level (mmol/L) were measured once a week during the whole experimental period. After 8 weeks, echocardiography was performed as previously described (16,17).

Briefly, the rats were anesthetized with sodium pentobarbital and placed in the prone position and scanned via a 12-MHz transducer connected to an Esaote Sonosite Titan echocardiograph (Bothell, WA, USA); 2D-guided M-mode images of the left ventricle (LV) were obtained in the parasternal short-axis view at the level of the papillary muscles and digitally stored for subsequent analysis. Doppler-derived mitral inflow velocities were obtained in the apical 4-chamber view, at the tip of the valve leaflets. LV end-diastolic and end-systolic wall thicknesses and chamber dimensions were measured by a single operator according to the American Society of Echocardiography recommendations. All echo-derived measures were obtained by averaging the readings of three consecutive beats.

At the end of the experiments the rats were sacrificed. Blood samples were collected to measure Ac-SDKP by immunoassay (Inalco, Milan, Italy).

Hearts were immediately excised and weighted. The apex of the heart was cut and frozen in liquid nitrogen and stored at -80 °C until protein extraction.

The heart, sectioned into three transverse slices from the apex to the base, was fixed with 10% formalin, embedded in paraffin and used for light microscopic examination and morphometric analysis of interstitial and perivascular collagen.

Histological and morphometric analysis

Changes in cardiac structure were assessed in at least 5 consecutive sections (4 μ m thickness, hematoxylin-eosin stained) of the three transverse slices from each group studied, using a light microscope under normal light.

For all rats a section (4 μ m) of the left ventricle equatorial slice, representative of the whole left ventricle (LV), was chosen for morphometric analysis. The sections were deparaffinized, rehydrated and stained with collagen-specific Sirius Red. The slides were examined with a Leica microscope (Leitz Camera, Wetzlar, Germany) using normal light. A computerized digital camera (Olimpus Camedia 5050, Olympus Inc., Japan) was used to capture 5 Mp (24-bit color depth) images and analyzed for collagen volume fraction in interstitial and perivascular cardiac areas with a computerized imaging software (Metamorph 6.2, UIC, CA, USA).

Image analysis was performed by a pathologist blinded to the experimental source of the samples.

The total collagen volume fraction was measured on pictures of the whole LV equatorial slice (x100 magnification) and was automatically calculated as the ratio between red-stained interstitial area and the total area of the whole heart section.

For analysis of the perivascular collagen volume fraction, all vessels from equatorial slice were traced and semi-automatically measured (x200 magnification).

The collagen immediately surrounding each intramyocardial vessels was considered to represent perivascular collagen deposition and expressed as the ratio between perivascular collagen area and luminal media area.

Collagen analysis by polarized light microscope

For each group, the Sirius Red-stained tissue sections of the left ventricle equatorial slice were also analyzed using a light microscope under polarized light, to evaluated the different type of collagen content occupying the interstitial and perivascular areas. In fact, the analysis using polarized light microscope allows visualization of collagen fibres with different colours (18). Collagen type I presents a yellow/orange, while collagen type III collagen appears green .

For each rat, 5 randomly selected microscopic fields of both interstitial and perivascular cardiac areas were analysed with a light microscope using a X10 objective (Leiz Camera, Wetzlar, Germany). The images were captured by a computerized digital camera (Olimpus Camedia 5050, Olympus Inc., Japan) using SPOT, Diagnostic Instruments (Sterling Heights, MI, USA), and analyzed for different type of collagen content by two pathologists blinded to the experimental source of the samples.

Measurement of TGF β -1 protein

Heart tissue (apical portion) was homogenized in 20mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100 plus protease inhibitors. Samples were centrifuged at 13000 rpm for 30 min and the supernatants were used for the assay. TGF β -1 levels were quantified using a commercially available enzyme-linked immunosorbent assay (Promega, Madison WI, USA), according to the manufacturer's instructions. This assay measures biologically active TGF β -1 in tissue extracts. For each experiment, a TGF β -1 standard curve was constructed using varying concentrations of recombinant human TGF β -1 protein, and a curve-fitting software program (Curve Expert 1.3) was used to quantify TGF β -1 protein concentration in hearts. Data were corrected for heart protein content, measured by Bradford assay (BioRad, Milano, Italy), and expressed in pg/mg proteins.

Western blot analysis

Heart protein samples were diluted 1:2 with a solution containing 60% glycerol, 30% β -mercaptoethanol, and 10% bromophenol blue. 75 μ g of total proteins from each sample were loaded onto 9% SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were blotted onto nitrocellulose membranes (Schleicher and Shull) at 120 mA overnight. The blots were probed with anti-Smad2/3 (Transduction), anti p-Smad2/3 (Santa Cruz) or anti β -catenin (Transduction) as a primary antibodies, followed by peroxidase-conjugated anti-mouse (Smad2/3 and β -catenin) immunoglobulin G (Jackson Laboratories) or biotin-conjugated anti-goat immunoglobulin G (Jackson Laboratories) followed by peroxidase-conjugated streptavidin (p-Smad2/3) as a secondary reagent (Jackson Laboratories). The anti-Smad2/3 antibody detects both the phosphorylated and non-phosphorylated Smad2/3 protein. p-Smad2/3 detects only the phosphorylated protein. The bands were visualized with ECL Western blotting detection system (Perkin-Elmer Life Science) and the signal quantified by densitometry using NIH IMAGE 1.59 software. Protein loading was also confirmed by staining membranes with Ponceau solution (not shown). Data were collected from two different blots. Results are expressed as Smad2-3/ β -catenin and p-Smad2-3/ β -catenin (arbitrary units, a.u).

Statistical Analysis

Data are presented as means \pm SEM. Differences among the groups of rats (control, control+Ac-SDKP, diabetes, diabetes+Ac-SDKP, ramipril treated diabetes, ramipril treated diabetes+Ac-SDKP, insulin treated diabetes, insulin treated diabetes +Ac-SDKP) for body weight, heart weight, systolic blood pressure, echocardiographic parameters, blood glucose levels, Ac-SDKP plasma levels, cardiac TGF β -1 protein, left ventricular interstitial collagen content, perivascular fibrosis and p-Smad2/3 cardiac protein levels were assessed with the use of two-way analysis of variance (ANOVA) followed by the Fisher's protected least-significant test for post-hoc comparisons. Differences between means were considered significant at $p < 0.05$.

RESULTS

At the end of the experimental period, blood glucose levels were significantly increased in diabetic rats and diabetic rats treated with ramipril, while insulin treatment was effective to maintain blood glucose similar to control levels (Table 1). Ac-SDKP did not modify blood glucose levels (Table 1) in control, diabetic, ramipril treated diabetic rats and insulin treated diabetic rats. Neither the development of diabetes nor the administration of Ac-SDKP significantly modified blood pressure (Table 1). In diabetic rats systolic blood pressure was not modified by treatment with ramipril at the dose of 3mg/kg/day (19,20). Ac-SDKP administration through osmotic minipumps was able to significantly increase plasma levels of Ac-SDKP (Table 1). Body weight in diabetic rats and in ramipril treated diabetic rats was lower than in control or insulin treated diabetic groups and was not modified by Ac-SDKP administration (Table 1). No differences were observed in heart weight/body weight ratio among the different groups of rats (Table 1).

Echocardiographic parameters

As shown in Fig. 1, panel A and B, and in Table 2, experimental diabetes was associated with increased end systolic diameters and depressed global systolic function, i.e. by a reduction of endocardial fractional shortening, without any change in wall thicknesses. As to the evaluation of diastolic function, that was performed by analyzing the Doppler transmitral velocity profile, diabetes reduced A wave amplitude, increased the E/A ratio and markedly reduced deceleration time. Two diabetic animals were excluded from the analysis of transmitral flow velocity, since the E and the A waves were fused, probably due to a high heart rate (450 and 447 beat/minute, respectively). ~~In the animal setting and at a comparable heart rate (that was not influenced by diabetes), these findings clearly indicate the presence of diastolic dysfunction.~~ Insulin treatment completely reverted all these changes. ~~indicating that diabetes compensation was associated with preserved systolic and diastolic function in this animal model.~~ The same held true for ACE inhibition, inasmuch as indices of systolic and diastolic function in the treated diabetic animals was comparable to the control rats. Ac-SDKP treatment was associated with a partial correction of transmitral flow velocity profile, whereas end systolic diameters and endocardial shortening fraction were comparable with data obtained from the diabetic rats. The co-administration of both Ac-SDKP and ramipril completely reverted diabetes-associated diastolic as well systolic dysfunction.

Effects of Ac-SDKP on myocardial fibrosis

As shown in Fig. 2, panel A and B, cardiac interstitial collagen content was significantly increased in diabetic rats, whereas in ramipril treated diabetic rats and in insulin treated diabetic rats it was similar to control group. Chronic Ac-SDKP administration in diabetic rats and in ramipril treated diabetic rats significantly reduced cardiac collagen content, despite of the high blood glucose levels. Collagen analysis using polarized light microscope identified the presence of type I collagen fibres (yellow-orange stain) and type III collagen fibres (green stain).

Similar results were obtained for cardiac perivascular fibrosis, as shown in Fig. 3. panel A and B. A significant increase in cardiac perivascular fibrosis, characterized mainly by type I collagen fibers, was observed in diabetic rats. In diabetic rats and in ramipril treated diabetic rats Ac-SDKP administration significantly reduced perivascular fibrosis.

Effects of Ac-SDKP on TGF β /Smad pathway

Diabetic rats showed a significant increase in active TGF β -1 expression in myocardial tissue as compared with control rats (Fig. 4 panel A). In diabetic rats treated with Ac-SDKP the increase in

TGF β -1 levels was not observed, resulting similar to control rats. No differences in cardiac TGF β -1 levels in ramipril treated-diabetic rats, in insulin treated diabetic rats and in the corresponding groups treated with Ac-SDKP were observed.

We examined whether Ac-SDKP antagonizes TGF β signalling leading to fibrosis, through the modulation of Smad pathway. Western blot analysis showed an increase, not statistically significant, of Smad2/3 expression in diabetic rats and ramipril treated diabetic rats. Ac-SDKP reduced Smad2/3 expression only in ramipril treated diabetic rats (Control:235.3 \pm 44.6 a.u; control+Ac-SDKP:263.2 \pm 45.5 a.u; diabetes:347.8 \pm 37.5 a.u; diabetes+Ac-SDKP: 350.0 \pm 124.7 a.u; ramipril treated diabetes:478.6 \pm 171.1 a.u; ramipril treated diabetes+Ac-SDKP: 199.6 \pm 55.8* a.u; insulin treated diabetes:257.1 \pm 56.6 a.u, and insulin treated diabetes+Ac-SDKP: 416.0 \pm 21.0 a.u; * <0.05 vs. ramipril treated diabetes). A significant increase in p-Smad2/3 was present only in diabetic rats as compared to control rats (Fig. 4 panel B). In diabetic rats treated with Ac-SDKP the increase in p-Smad2/3 levels was not observed, resulting similar to control rats. No differences in cardiac p-Smad2/3 levels in ramipril treated diabetic rats, in insulin treated diabetic rats and in the corresponding groups treated with Ac-SDKP were observed (Fig. 4 panel B).

Taken together, these evidences indicate that in diabetic rats Ac-SDKP reduces myocardial fibrosis through the inhibition of TGF β /Smad pathway.

DISCUSSION

Our data demonstrate that Ac-SDKP administration improves indices of diastolic function and reduces left ventricular interstitial and perivascular fibrosis in the heart of diabetic rats. Indeed, the observed changes in myocardial fibrosis were paralleled by a partial correction of diabetes-associated diastolic dysfunction, as assessed by transmitral Doppler flow velocity profile.

In addition, our results show that in myocardial tissue of diabetic rats, Ac-SDKP causes a reduction of TGF β -1 protein expression and p-Smad2/3 levels. These effects occur independently on blood glucose levels or blood pressure modifications.

A reduction of collagen deposition in the heart has been already described in animal experimental models, such as 2-kidney,1-clip hypertension [21] and in rats with heart failure after myocardial infarction [22]. A physiological role of Ac-SDKP in the regulation of collagen content has also been suggested, since decreased endogenous levels of Ac-SDKP promotes organ fibrosis [23]. At present, however, the role of Ac-SDKP in the development of diabetic cardiomyopathy is not known. Myocardial fibrosis is responsible for myocardial dysfunction and heart failure, common features of diabetic cardiomyopathy that occurs independently on other risk factors, such as ischemic heart disease or hypertension [2-4].

In our experimental model, diabetes was associated with reduced A wave amplitude, increased E/A ratio and markedly reduced deceleration time, without any effect on LV wall thickness and heart rate. ~~In the animal setting and at comparable heart rate, these findings suggest the presence of diastolic dysfunction.~~ These changes were completely reverted by insulin or ramipril treatment, whereas Ac-SDKP administration was associated with a partial correction of diastolic function.

Although a much more precise method of assessing diastolic function is represented by the use of *in vivo* pressure-volume relations, the echo-Doppler technique is widely used in the clinical as well as in the experimental setting. At comparable heart rate and loading conditions, an altered transmitral flow velocity profile may suggest the presence of diastolic dysfunction. In the present experiments, heart rate was comparable in the different groups and we did not observe any significant change in left ventricular end diastolic diameter. Therefore, the altered Doppler filling pattern suggest diastolic dysfunction, that was present in diabetic animals, abolished in insulin-treated diabetic rats and partially blunted in Ac-SDKP treated diabetic animals.

Our results demonstrate that chronic Ac-SDKP administration in diabetic rats reduces myocardial fibrosis, evaluated by quantitative morphology, both at interstitial and perivascular levels. No significant differences in myocardial fibrosis were observed in insulin treated diabetic rats, in which myocardial fibrosis was not increased, and in control rats. On the contrary, in ramipril treated diabetic rats, even if myocardial fibrosis was not increased respect to controls, Ac-SDKP administration resulted in a further reduction of fibrosis, strongly suggesting an additive antifibrotic effect of Ac-SDKP in diabetic cardiomyopathy. In the heart of diabetic rats we have measured a significant increase of the main profibrotic cytokine, TGF β -1, in its active form, that, on the contrary, did not increase in diabetic rats treated with Ac-SDKP and in diabetic rats treated with ramipril. The increase in TGF β expression in myocardial tissue of diabetic rats is not surprising. In fact, clinical and experimental evidences demonstrated an activation of cardiac renin-angiotensin system in diabetes [24,25] and angiotensin II is a potent stimulus for TGF β expression. It is known that TGF β /Smad pathway induces the production of extracellular matrix, leading to tissue fibrosis [26]. TGF β -1 was suggested as a major player in cardiac fibrosis by activating the intracellular pathways linking TGF β -1 signals to plasma membrane serine/threonine kinase receptors with the final activation of cytoplasmic effectors, such as Smad proteins. Phosphorylation of Smad2 and its subsequent translocation to the nucleus are the critical steps in cell signalling through this pathway [26,27]. The effects of TGF β -1 induced Smad 2 activation on cell growth depend on the types of

cell involved. In the heart, TGF β -1 stimulates the growth of cardiac fibroblasts, thus increasing fibrosis [28].

In the heart of diabetic rats we have observed a significant increase in p-Smad2/3 levels, indicating an activation of Smad pathway. This could explain why cardiac fibrosis is not increased in diabetic rats treated with Ac-SDKP and is in line with other experimental data demonstrating that Ac-SDKP antagonizes TGF β signalling, through the modulation of Smad pathway [14,27,28].

The pathogenesis of diabetic cardiomyopathy is complex and many factors participate in the development and progression of this condition, such as hyperglycemia, hyperlipidemia, and the activation of the renin-angiotensin system [3,5,6]. In streptozotocin induced diabetic rats, it has been shown that the block of the renin-angiotensin system by ACE-inhibitors is able to reduce cardiac dysfunction [29] and perivascular fibrosis [30]. In addition to the block of the conversion of angiotensin I to angiotensin II and kinin hydrolysis, the block of the hydrolysis of Ac-SDKP has been recently described as a potential mechanism involved in the cardioprotective effects of ACE-inhibitors [13,14].

From a pharmacological point of view, Ac-SDKP, by acting directly on diabetes-specific cardiac damage such as tissue fibrosis, could perspectively represent a therapeutical tool complementary to classic cardioprotective treatment based on antihypertensive drugs.

In our experimental conditions we have observed an increase in left ventricular collagen content and perivascular fibrosis in diabetic rats, that was not present in insulin treated diabetic animals. This is not surprising because, during the whole experimental period, insulin treatment was effective to maintain blood glucose levels near control values, and it is well known that hyperglycemia represents an important stimulus for myocardial fibrosis and for the activation of the renin-angiotensin system [3,5]. Conversely, in clinical practice, an adequate blood glucose control in diabetic patients is still a very difficult target to reach [31], and the uncontrolled increases in glycemia often result in worsening of the fibrotic process linked to diabetic cardiomyopathy. In addition, insulin and in particular hyperinsulinism can promote cardiac hypertrophy and fibrosis [32,33]. However, in our experimental conditions insulin administration was aimed to normalize glucose metabolism and it did not induce hyperinsulinism. This is confirmed by the monitoring of glycemia that in insulin-treated rats showed no hypoglycemic events (and therefore no hyperinsulinemia) during the entire duration of the experimental procedure.

Altogether, these findings strongly support the search for new therapeutic approaches focused specifically on the prevention/remission of diabetic cardiomyopathy. Ac-SDKP in particular appears to be, along with hypoglycemic treatment and ACE inhibition, a powerful tool to counteract the development of fibrosis in diabetic cardiomyopathy.

In summary, our data demonstrate that Ac-SDKP reduces cardiac fibrosis and diastolic dysfunction, as assessed by transmitral flow velocity profile, in diabetic rats, indicating a potential protective role of Ac-SDKP in diabetic cardiomyopathy.

Perspectives

Cardiac fibrosis is an important determinant of the increase in diastolic left ventricular stiffness in diabetic patients with reduced left ventricular ejection fraction [34]. Present therapy is based on strict glycemic control and, when appropriate, on antihypertensive treatment and lipid lowering drugs. These pharmacological approaches ameliorate the prognosis of diabetic cardiomyopathy by reducing risk factors and improving the "environment", but do not affect directly the underlying fibrosis. A direct anti-fibrotic agent is still far from being identified and tested in the clinical arena. In this study we show how early treatment with Ac-SDKP prevents cardiac fibrosis in an experimental model of type 1 diabetes without affecting other clinical parameters.

In perspective, our results are in support of testing Ac-SDKP in clinical trials with the final aim to clarify whether the addition of a direct anti-fibrotic intervention may further strengthen the current therapy for diabetic cardiomyopathy.

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FIGURE LEGENDS

Table 1. Effects of Ac-SDKP administration at the end of the experimental period on blood glucose levels, systolic blood pressure, plasma Ac-SDKP levels, body weight and heart weight in control, diabetic, ramipril treated diabetic and insulin-treated diabetic rats. Control (n=10), control+Ac-SDKP (n=8), diabetes (n=10), diabetes+Ac-SDKP (n=13), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=7), insulin treated diabetes (n=10), and insulin treated diabetes+Ac-SDKP (n=9).

*: $p < 0.01$ vs. Control rats, control+Ac-SDKP, insulin treated diabetes, insulin treated diabetes+Ac-SDKP; §: $p < 0.05$ vs. correspondent sham treated group.

Table 2. Effects of experimental diabetes and of chronic treatment with ramipril, Ac-SDKP, and insulin on end-diastolic (EDD) and end-systolic (ESD) diameters, diastolic interventricular septal (IVSd) and posterior wall (PW) thickness, heart rate (HR), peak early (E) and atrial (A) wave transmitral flow velocity. Control (n=6), control+Ac-SDKP (n=6), diabetes (n=8), diabetes+Ac-SDKP (n=6), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=7), and insulin treated diabetes+Ac-SDKP (n=7).

Data are means \pm SEM. * $p < 0.05$ vs. control; ^ $p < 0.05$ vs. control+Ac-SDKP; † $p < 0.05$ vs. diabetes; ° $p < 0.05$ vs. ramipril treated diabetes; § $p < 0.05$ vs. ramipril treated diabetes+Ac-SDKP; # $p < 0.05$ vs. insulin treated diabetes.

Figure 1. Panel A. Effect of experimental diabetes and of chronic treatment with ramipril, Ac-SDKP, and insulin on left ventricular endocardial fractional shortening (SF%), early-to-atrial (E/A) and deceleration time (Dec Time) of the early transmitral flow velocity. Control (n=6), control+Ac-SDKP (n=6), diabetes (n=8), diabetes+Ac-SDKP (n=7), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=7), and insulin treated diabetes+Ac-SDKP (n=7) rats. Data are means \pm SEM. * $p < 0.05$ vs. control; ^ $p < 0.05$ vs. control+Ac-SDKP; † $p < 0.05$ vs. diabetes; ° $p < 0.05$ vs. ramipril treated diabetes; § $p < 0.05$ vs. ramipril treated diabetes+Ac-SDKP; # $p < 0.05$ vs. insulin treated diabetes; & $p < 0.05$ vs insulin treated diabetes+Ac-SDKP.

Panel B. Representative profiles of echo-Doppler transmitral flow velocity in control, diabetic and Ac-SDKP treated diabetic rat.

Figure 2. Effect of Ac-SDKP administration on left ventricular interstitial fibrosis.

Panel A. Representative photomicrographs of the left ventricular interstitial collagen deposition in control (a.), diabetes (c.), ramipril-treated diabetes (e) and insulin-treated diabetes (g), and in the corresponding Ac-SDKP treated groups (b., d., f., h.). For each group, Sirius Red staining (left) and Sirius Red stained image under polarized light (right), original magnification x100.

Picosirius staining shows an increase of cardiac interstitial collagen content in diabetic rats (c.) versus control (a.), ramipril treated diabetic (e) and insulin treated diabetic (g) rats. Ac-SDKP administration reduced interstitial collagen content in diabetic rats (d.). The polarized light image shows only in diabetic rats (c.) an increase of both types of collagen fibers (type I collagen as yellow/orange and type III collagen as green)

Panel B. Quantification of interstitial fibrosis in the different groups of rats. Control (n=10), control+Ac-SDKP (n=7), diabetes (n=10), diabetes+Ac-SDKP (n=11), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=6), and insulin treated diabetes+Ac-SDKP (n=6). Data are means \pm SEM. * : $p < 0.05$; § : $p < 0.01$.

Figure 3. Effect of Ac-SDKP administration on cardiac perivascular fibrosis.

Panel A. Representative photomicrographs of the left ventricular perivascular fibrosis in control (a.), diabetes (c.), ramipril treated diabetes (e) and insulin treated diabetes (g) rats, and in the corresponding Ac-SDKP treated groups (b., d., f., h.) For each group, Sirius Red staining (left) and Sirius Red stained image under polarized light (right), original magnification x200.

Picrosirius staining shows a significant increase in cardiac perivascular fibrosis in diabetic rats (c.). Ac-SDKP administration in diabetic rats reduces perivascular fibrosis (d.). The polarized light image shows an increase of perivascular fibrosis in diabetic rats (c.) due mainly to type I collagen fibres (orange-yellow)

Panel B. Quantification of cardiac perivascular fibrosis in the different groups of rats. Control (n=10), control+Ac-SDKP (n=7), diabetes (n=10), diabetes+Ac-SDKP (n=11), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=6), and insulin treated diabetes+Ac-SDKP (n=6). Data are means \pm SEM. * : p<0.05; § : p<0.01.

Figure 4. Panel A. Active TGF- β 1 levels in myocardial tissue in the different groups of rats. Control (n=6), control+Ac-SDKP (n=6), diabetes (n=9), diabetes+Ac-SDKP (n=7), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=5), and insulin treated diabetes+Ac-SDKP (n=6). Data are means \pm SEM. * : p<0.05; § : p<0.01.

Panel B. Effect of Ac-SDKP administration on p-Smad2/3 expression in myocardial tissue. Control (n=8), control+Ac-SDKP (n=4), diabetes (n=7), diabetes+Ac-SDKP (n=6), ramipril treated diabetes (n=6), ramipril treated diabetes+Ac-SDKP (n=6), insulin treated diabetes (n=5), and insulin treated diabetes+Ac-SDKP (n=5). *Inset:* example of Western blot showing p-Smad2/3 and β -catenin protein expression in the different groups of rats. Data are means \pm SEM. * : p<0.05.

	Blood glucose (mmol/L)	SBP (mmHg)	Ac-SDKP (nmol/l)	Body Weight (g)	Heart/B.W. (mg/g)
Control	4.89 ± 0.26	117.5 ± 3.9	2.38 ± 0.07	575 ± 20.1	2.45 ± 0.14
Control+Ac-SDKP	5.76 ± 0.49	121.3 ± 8.7	5.64 ± 1.24 §	593 ± 13.9	2.26 ± 0.15
Diabetes	21.52 ± 1.24*	128.2 ± 5.1	1.71 ± 0.14	421 ± 9.3*	2.67 ± 0.09
Diabetes+Ac-SDKP	21.56 ± 1.83*	134.1 ± 5.6	3.94 ± 0.30 §	428 ± 12.5*	2.87 ± 0.12
Diabetes+Ramipril	22.62 ± 1.91*	132.5 ± 4.8	2.98 ± 0.39	416 ± 8.7*	2.99 ± 0.16
Diabetes+Ramipril+Ac-SDKP	21.16 ± 1.87*	134.0 ± 5.1	6.26 ± 1.38 §	430 ± 11.2*	2.87 ± 0.14
Insulin Treated Diabetes	6.82 ± 1.42	127.3 ± 7.6	2.15 ± 0.24	566 ± 21.8	2.44 ± 0.12
Insulin Treated Diabetes+Ac-SDKP	9.26 ± 1.05	130.9 ± 9.9	4.91 ± 1.04 §	537 ± 12.9	2.55 ± 0.11

Table 1

	EDD (mm)	ESD (mm)	IVSd (mm)	PWt (mm)	HR (b/min)	E (cm/s)	A (cm/s)
Control	62.58 ± 2.08	26.18 ± 0.87	20.50 ± 0.22	20.92 ± 0.39	386 ± 13	107 ± 7	54 ± 8
Control+Ac-SDKP	60.83 ± 3.17	28.83 ± 3.17	19.50 ± 0.50	20.50 ± 0.50	328 ± 5	73 ± 1	72 ± 14
Diabetes	59.50 ± 1.35	31.22 ± 1.21*	19.56 ± 0.25	20.17 ± 0.33	374 ± 25	85 ± 10	25 ± 4*^
Diabetes +Ac-SDKP	59.20 ± 1.71	30.13 ± 1.41#§	20.33 ± 0.28	19.87 ± 0.25	358 ± 18	89 ± 13	67 ± 4†
Diabetes+Ramipril	61.17 ± 0.71	25.72 ± 1.09 †	20.44 ± 0.27	20.94 ± 0.36	320 ± 33	82 ± 7	40 ± 7
Diabetes+Ramipril+Ac-SDKP	59.05 ± 0.83	24.43 ± 0.91 †	20.57 ± 0.20	21.00 ± 0.15	327 ± 30	80 ± 2	39 ± 4
Insulin Treated Diabetes	60.00 ± 0.36	24.17 ± 0.80 †	20.12 ± 0.52	21.38 ± 0.24	377 ± 15	94 ± 8	85 ± 13 †°§
InsulinTreated Diabetes+Ac-SDKP	56.44 ± 1.64	25.89 ± 0.87 †	20.33 ± 0.33	21.00 ± 0.19	363 ± 24	82 ± 10	51 ± 8

Table 2

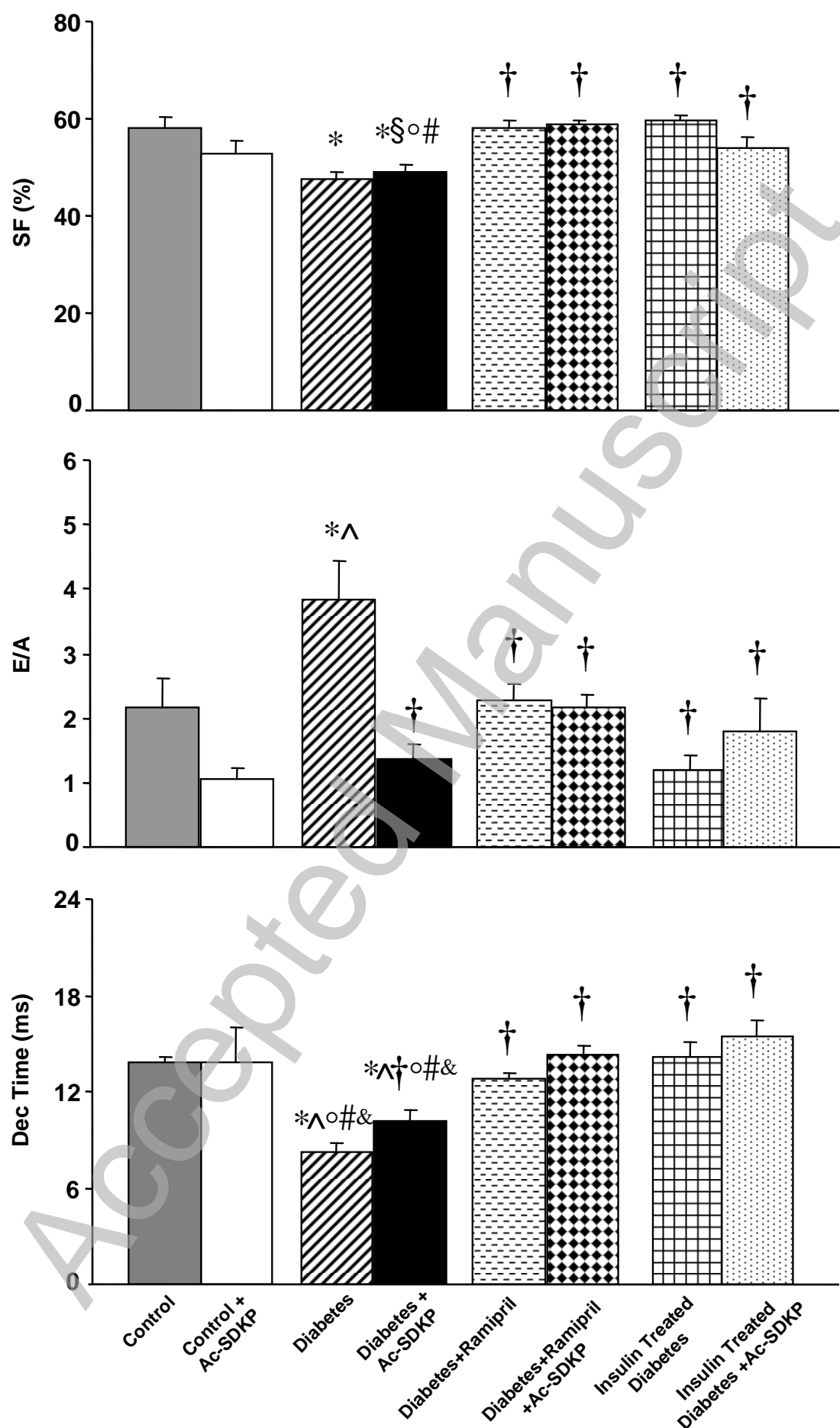


Fig. 1
panel A

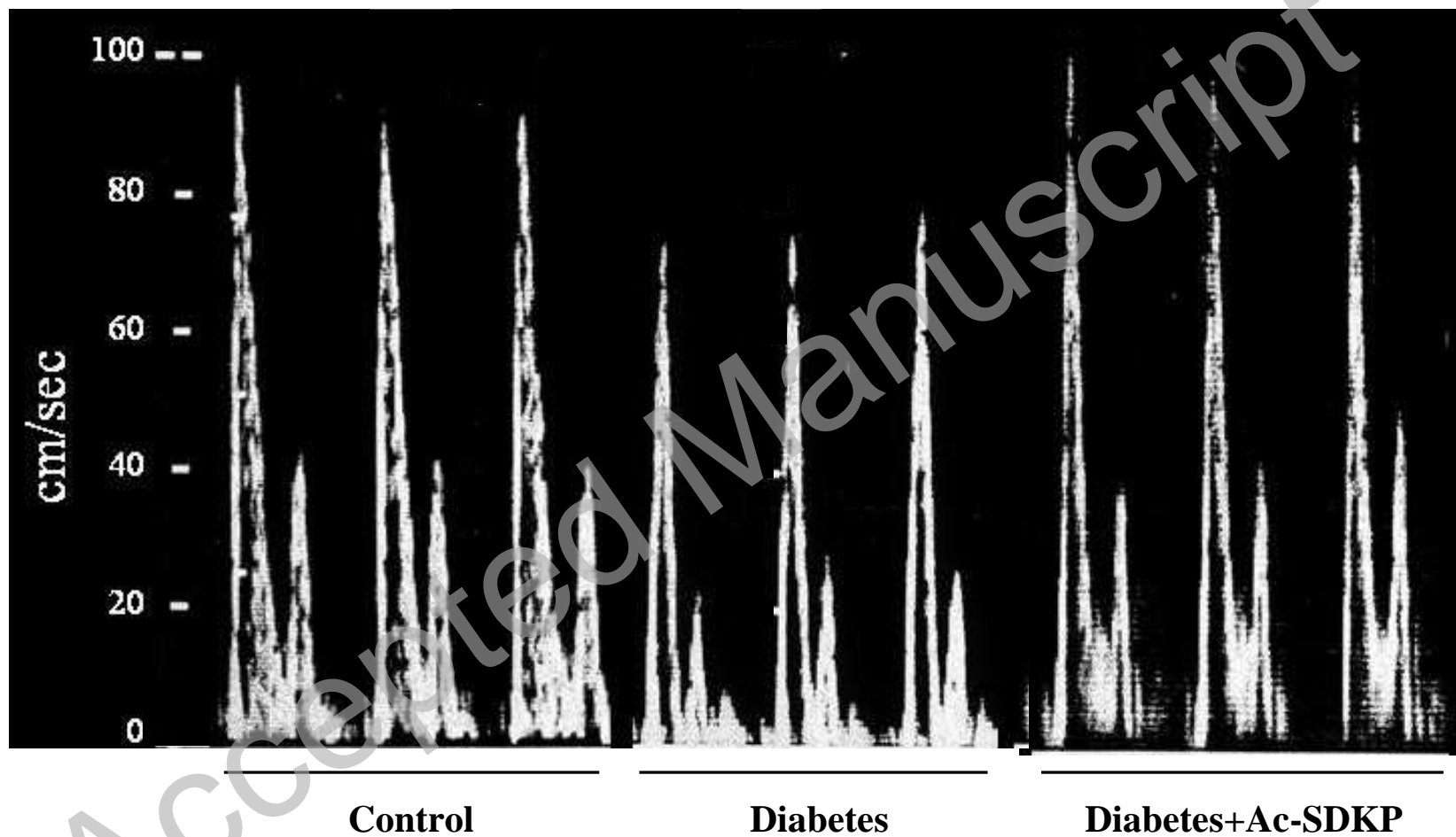


Fig.1, panel B

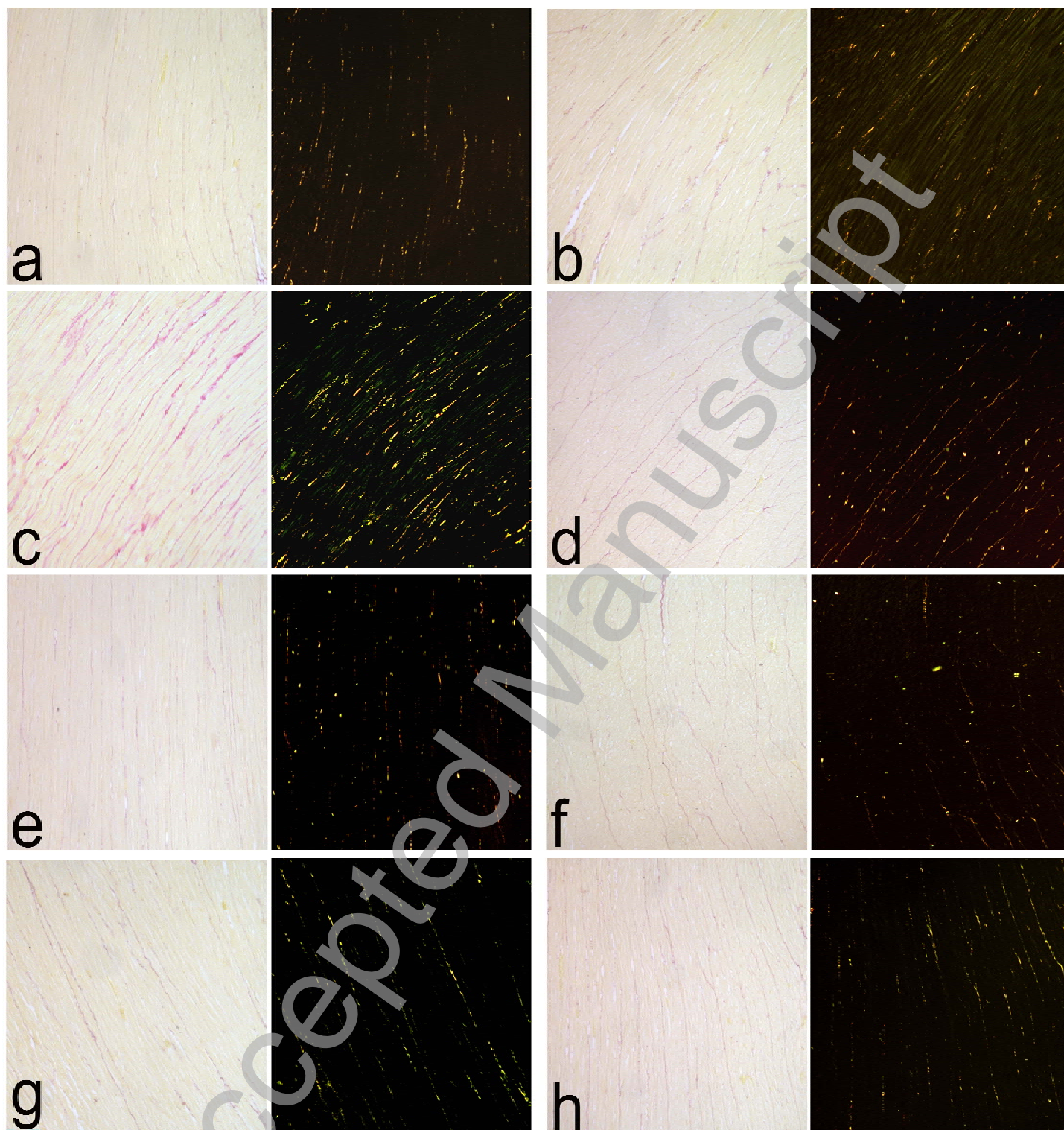


Figure 2, panel A.

B.

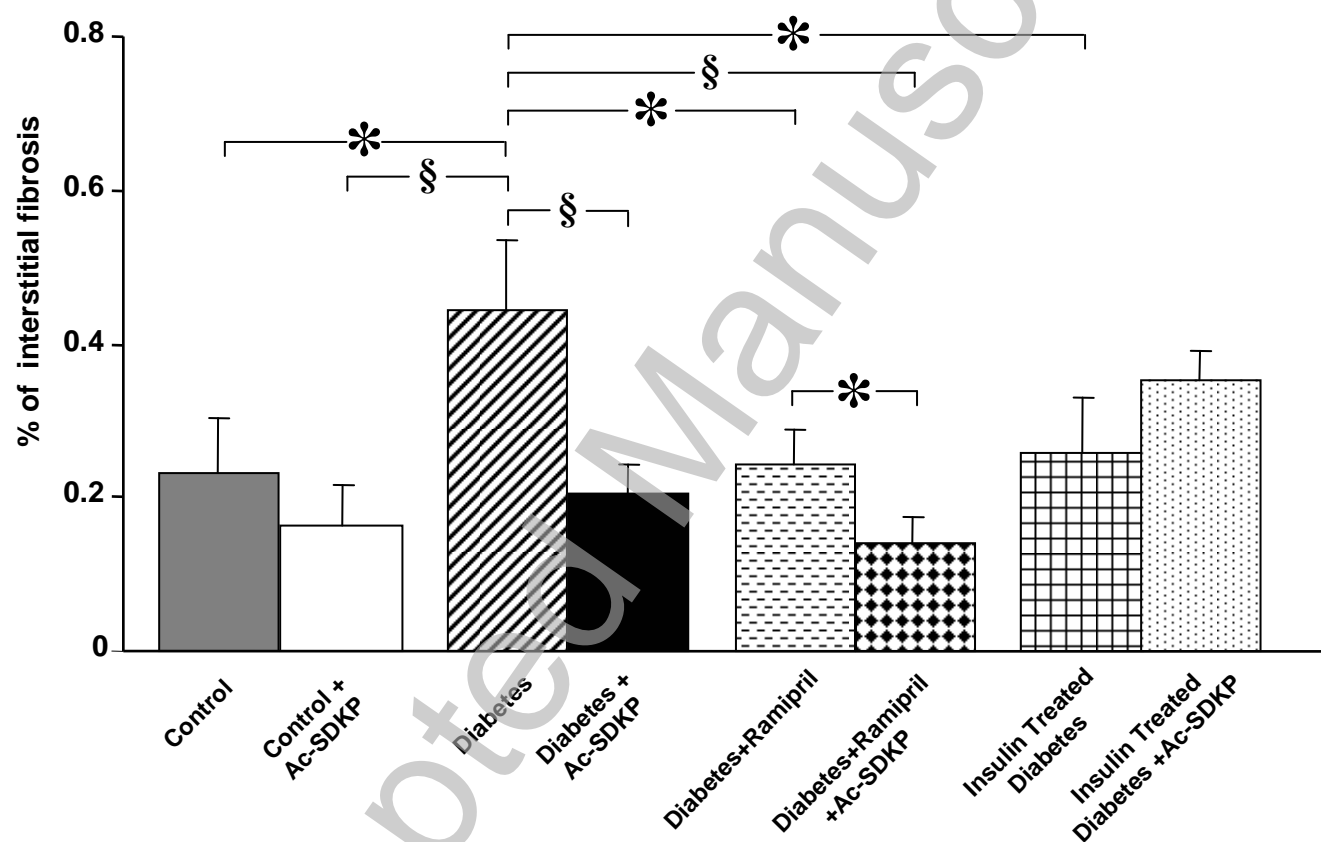


Figure 2, panel B

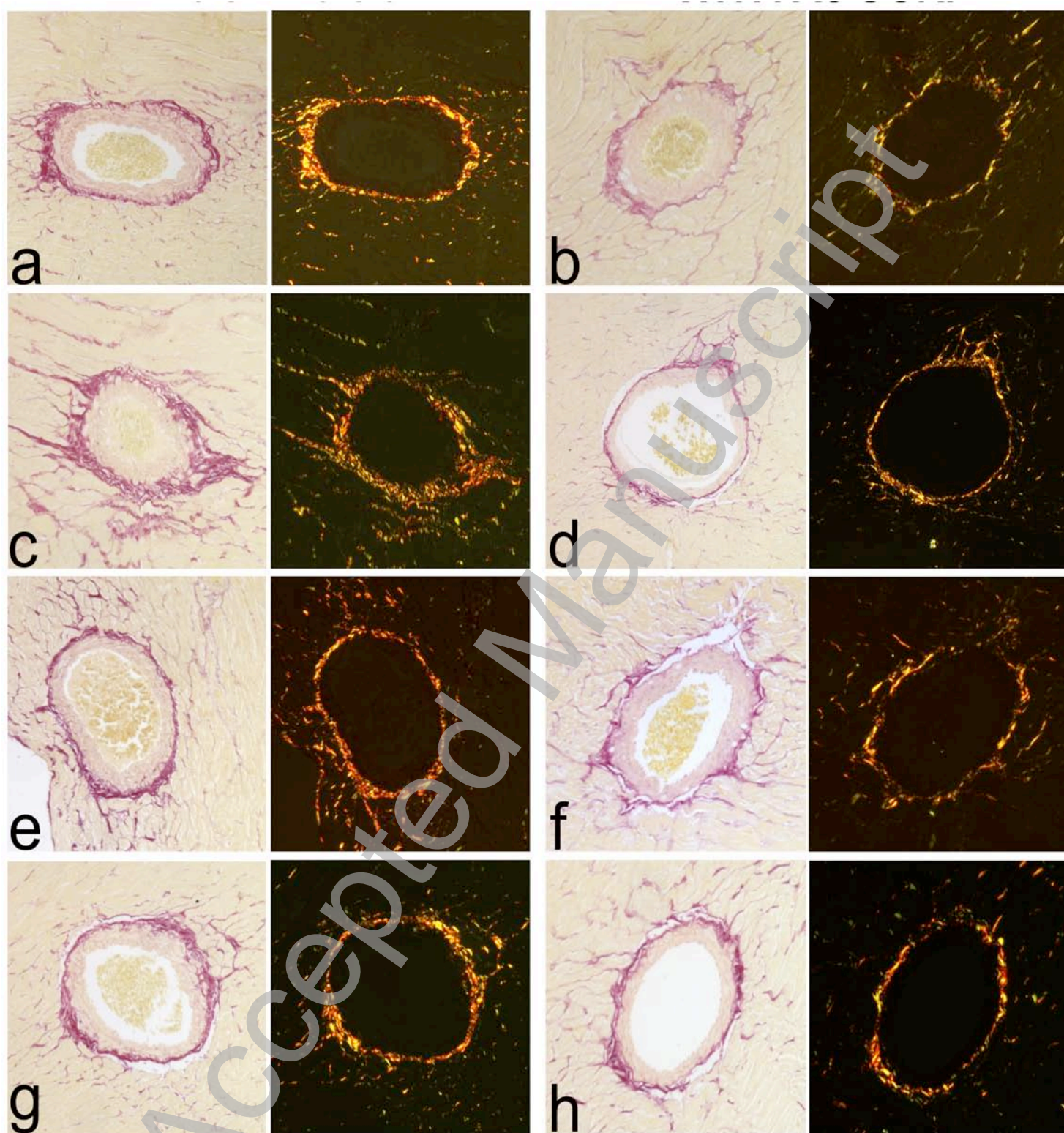


Figure 3, panel A.

B.

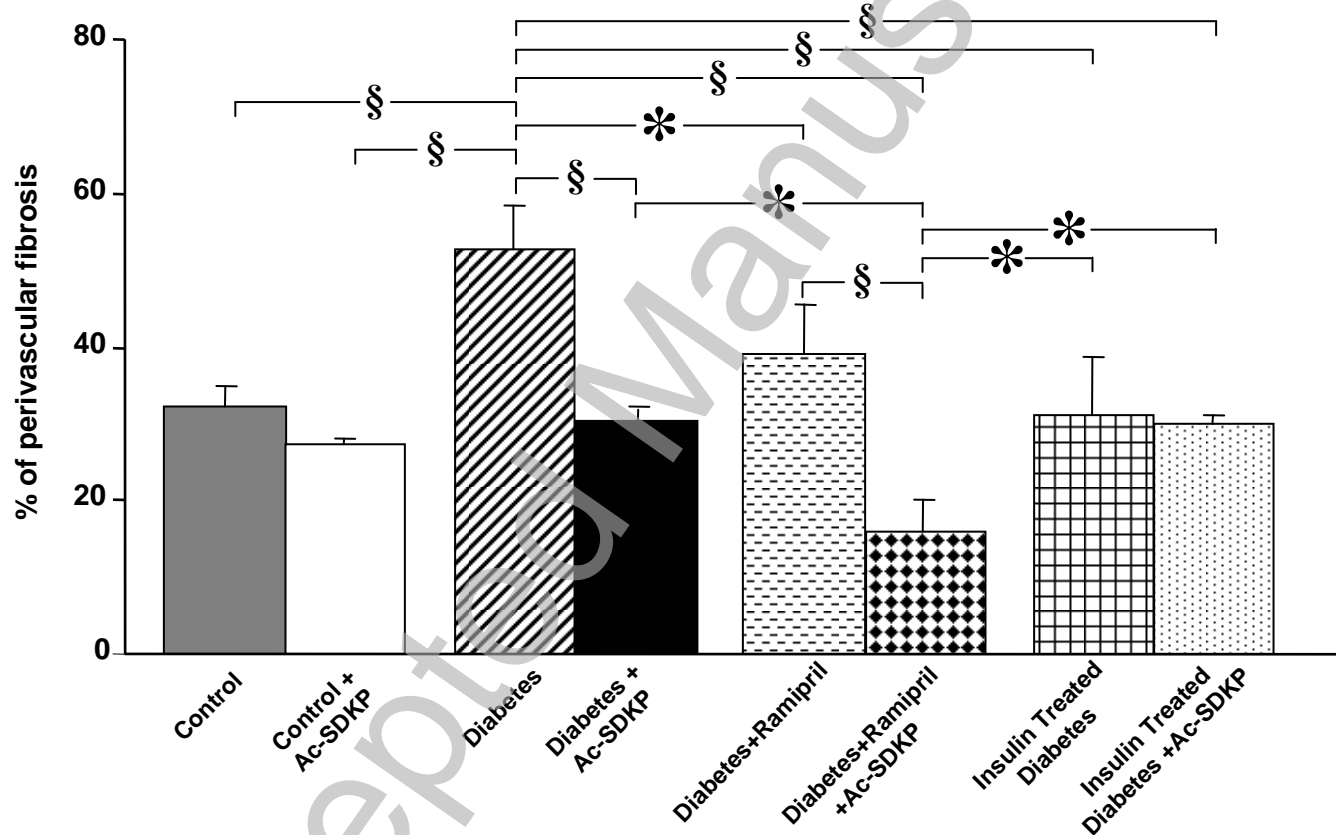


Figure 3, panel B

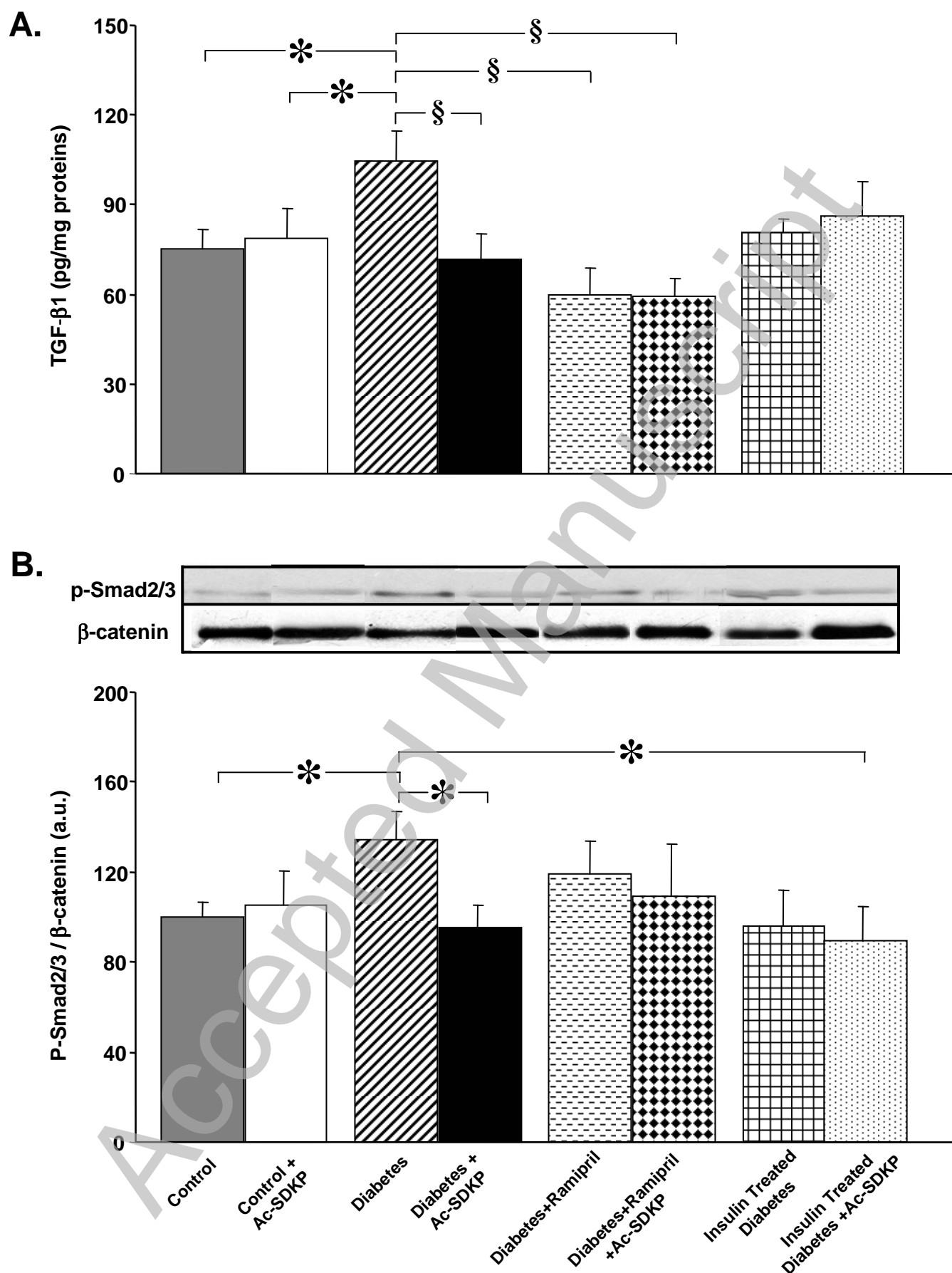


Figure 4, panel A, B