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Long-term type 1 diabetes alters the deposition of collagens and proteoglycans in the early pregnant myometrium of mice

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Summary. Introduction: We have previously shown that long-term type 1 diabetes affects the structural organization, contractile apparatus and extracellular matrix (ECM) of the myometrium during early pregnancy in mice. Objective: This study aimed to identify which myometrial ECM components are affected by diabetes, including fibril-forming collagen types I, III and V, as well as proteoglycans, decorin, lumican, fibromodulin and biglycan. Methods: Alloxan-induced type 1 diabetic female mice were divided into subgroups D1 and D2, formed by females that bred 90-100 and 100-110 days after diabetes induction, respectively. The deposition of ECM components in the myometrium was evaluated by immunohisto-chemistry/immunofluorescence. Results: The subgroup D1 showed decreased deposition of collagen types I and III in the external muscle layer (EML) and decreased collagen types III and V in the internal muscle layer (IML). Collagen types I and III were decreased in both muscle layers of the subgroup D2. In addition, increased deposition of collagen types I and III and lumican as well as decreased collagen type V were observed in the connective tissue between muscle layers of D2. Lumican was decreased in the EML of the subgroups D1 and D2. Fibromodulin was repressed in the IML and EML of both D1 and D2. In contrast, decorin deposition diminished only in muscle layers of D2. No changes were noticed for biglycan. Conclusions: Subgroups D1 and D2 showed distinct stages of progression of diabetic complications in the myometrium, characterized by both common and specific sets of changes in the ECM composition.

Key words: Collagens, Extracellular matrix, Myometrium, Proteoglycans, Type 1 diabetes

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Introduction

During pregnancy the myometrium undergoes diverse processes of adaptation to support uterine development and fetal growth, as well as to perform labor successfully. Four major sequential phenotypic phases are recognized in the pregnant myometrium of rats: proliferative, hypertrophic, contractile and labor (Shynlova et al., 2009). Proliferation of smooth muscle cells (SMCs) is coordinated by estrogen, members of the IGF family (Shynlova et al., 2007) and PI3K-Akt-mTOR signaling pathway (Jaffer et al., 2009). The second phase is characterized by hypertrophy of the SMCs and accumulation of extracellular matrix (ECM) components, including collagen types I and III and elastin (Shynlova et al., 2004). SMC hypertrophy is followed by the acquisition of a contractile phenotype and synthesis of basement membrane components (Shynlova et al., 2006). Finally, the labor phase marks the end of pregnancy. Endocrine signals supplied by ovarian steroid hormones combined with mechanical stretch provided by the growing fetus govern the progression through the phenotypic phases of pregnant

myometrium (Shynlova et al., 2004, 2009).

Even though labor-quiescent, contractility of the myometrium is present all through pregnancy, participating for instance, in the regulation of uterine blood flow (Bressan Filho, 2006). The contractile capacity of myometrial SMCs is given by specialized contractile apparatus/cytoskeleton, which interact with the cytoplasmic portion of cell membrane receptors and many regulatory proteins present in adhesion sites (dense plaques), such as integrins. These receptors in turn bind to ECM components through their extracellular domain, anchoring the cells on the ECM (Taggart and Morgan, 2007). Myometrial SMCs express regular levels of alpha-smooth muscle actin throughout pregnancy, whereas gama-smooth muscle actin increases during the second half of pregnancy, accumulating in the periphery of the cytoplasm of these cells by the end of pregnancy (Shynlova et al., 2005). Proteins related to contractile signal integration, such as rho-associated kinases (RKO) alpha and RKO beta, h-caldesmon and myosin light chain (MLC20), are also modulated in the myometrium during pregnancy in mice (Riley et al., 2005).

In rodents, the myometrial ECM is deeply remodeled during the estrous cycle (Salgado et al., 2009) and pregnancy (Morrione and Seifter, 1962; San Martin et al., 2003; Shynlova et al., 2004; Spiess et al., 2007). In addition, the unique physiological conditions of pregnancy stimulate the expression of integrins alpha1, alpha3, and beta1 mRNAs and concentration of these molecules in the cell membrane of myometrial SMCs was detected near term (Williams et al., 2010). Collectively, these data disclose that adjustments in the contractile apparatus of the SMCs together with changes in cell-matrix interactions and ECM composition contribute importantly to the adaptations that take place in the myometrium during pregnancy. Thus, it is expected that abnormal changes in the ECM may negatively affect the contractility and other key cellular processes in the myometrium.

Premature labor represents a major cause of fetal morbidity, as well as mortality (McCormick et al., 2011), and diabetes is among the risk factors related to this condition (Mimouni et al., 1988; Kovilam et al., 2002; Lepercq et al., 2004; Vitoratos et al., 2010). Pregnancies in diabetic women are associated to a higher incidence of both spontaneous and indicated preterm labor, when compared to control population (Mimouni et al., 1988; Kovilam et al., 2002). There is evidence of impairments in the myometrium contractility in diabetic women (Kaya et al., 1999; Al-Qahtani et al., 2012) and animal models of diabetes (McMurtrie et al., 1985; Jawerbaum et al., 1996; Spiegl et al., 2009). A previous study by our group investigated, in mice, the impact of type 1 diabetes on the early pregnant myometrium showing that diabetes-associated complications in this structure occur in a time-sensitive manner. In fact, edema between muscle layers was observed in females bred 90-100 days after diabetes induction, whereas atrophy and fibrosis characterize the myometrium after a longer period of diabetes (101-110 days) (Favaro et al., 2010). In both subgroups, diabetes promoted the narrowing of the myometrial muscle layers, which was associated with a decrease in the proliferation of SMCs. Electron microscope observation and immunohistochemistry showed, respectively, that diabetes alters the organization of the contractile apparatus and the distribution of smooth muscle alpha-actin on the SMCs. Although changes in the organization of collagen fibrils in the myometrial ECM of diabetic females have been identified (Favaro et al., 2010), the contribution of specific collagen types and proteoglycans to these alterations remain to be determined. To expand the knowledge in this field, in the present study we analyze by immunohistochemistry/immunofluorescence, the deposition of fibril-forming collagens (types I, III and V) and collagen-associated proteoglycans (decorin, lumican, biglycan and fibromodulin) during different stages of diabetes-associated complications in the myometrium of mice.

Materials and methods

Induction of diabetes

All experiments were approved by the Institute of Biomedical Sciences Animal Ethics Committee (authorization number: 144/2002).

Sixty-day-old Swiss female mice weighing 30-35 g were used in this study. The mice were housed at constant room temperature (21±1°C) and 12 h light /dark cycle with free access to water and standard rodent chow.

Diabetes was induced in female mice by a single injection of alloxan i.v. (40 mg/kg; Sigma, USA), freshly prepared in physiological saline solution (pH 7.0), at least 16 hours after food deprivation. Control mice were injected with physiological saline alone. Seven days after alloxan administration, blood glucose values were evaluated using an Accu-Check blood glucose monitor (Roche Basel, Switzerland). Females with non-fasting glycemia higher than 400 mg/dL (62% of the induced females) were considered diabetic and selected for this study. No insulin was administered to the diabetic females during the experimental period.

Glycemia, insulinemia, glycosuria, ketonuria, body weight, as well as food and water consumption were evaluated every 30 days and at the moment of sacrifice to confirm the maintenance of the diabetic state. The pathophysiological parameters that characterize the present animal model were previously published by Favaro et al. (2010, 2013).

Mating schedule

Mating period occurred from the 90th to the 110th day after alloxan or saline administration. Both control (non-diabetic) and diabetic females were bred with non-diabetic male mice for 3 h. Following this period the

presence of vaginal plug was considered zero hour of pregnancy. Uterine samples were collected at 168 h of pregnancy. This time period matches approximately to day 7-8 of pregnancy, when the vaginal plug is used to define the day 1, and corresponds to the proliferative phase of the pregnant myometrium. To precisely identify the temporal effects of diabetes on the myometrium, the pregnant diabetic mice were divided into two subgroups: D1 (n=5) - females that became pregnant in the first half of the mating period (90-100 days after diabetes induction); D2 (n=4) - females that became pregnant in the second half of the mating period (101-110 days after diabetes induction). Age-matched non-diabetic pregnant females (n=5) were used as controls.

Tissue processing for light microscopy

Prior to sacrifice and collection of samples, the females were deeply anesthetized with an intraperitoneal injection of Avertin® (0.025 ml/g body weight) (Sigma). To avoid undesirable myometrial contractions, a 4% (w/v) papaverin (Sigma) solution in distilled water was dripped onto the uterine horns prior to dissection. Implantation sites were counted and three were randomly isolated from each uterus. Samples were fixed by immersion in Methacarn solution (absolute methanol, chloroform and glacial acetic acid; 6:3:1) for 3 h at 4°C, and processed for paraffin-embedding (Paraplast - Oxford Labware, USA). Sections of 5 μm were cut and adhered onto glass slides using 0.1% poly-L Lysine (Sigma) and subsequently used for immunohistochemistry.

Immunodetection of collagens and SLRPs

Collagens and proteoglycans were localized by immunofluorescence and immunoperoxidase procedures, respectively, following previous protocols established by our group (Spiess et al., 2007; Salgado et al., 2009). Table 1 shows the antibodies and methods of antigen retrieval that were used. All tissue sections were firstly deparaffinized and hydrated. Those used for immunoperoxidase were treated with 3% (v/v) H_2O_2 in phosphate buffered saline (PBS) for 30 min, at room temperature (RT), to block endogenous peroxidase activity. Each of the succeeding steps was followed by a thorough rinse with PBS (except before primary antibody incubation), and performed in a humidified chamber. After antigen retrieval, non-specific staining was blocked with normal goat or rabbit serum diluted 1:1 in PBS-10% bovine serum albumin (w/v), according to the host from which secondary antibodies were produced, for 1 h at RT. Sections were then incubated with primary antibodies diluted in PBS containing 0.3% (v/v) Tween 20, overnight at 4°C. Afterwards, the slides were incubated with FITC-conjugated (Sigma) (immunofluorescence) or biotin-conjugated secondary antibodies (Rockland, USA) (immunoperoxidase) diluted in PBS for 1 h at RT. The immunoperoxidase reaction was followed by incubation with streptavidin-peroxidase complex (Vectastin ABC Kit; Vector Laboratories, USA) for 1h at RT. The peroxidase reaction was visualized using 0.03% (w/v) 3,3'-diaminobenzidine (Sigma) in PBS with 0.03% (v/v) H_2O_2, and the sections were counterstained with Mayer's haematoxilin. In order to achieve standardization of the immunoperoxidase reactions, the slides from all groups were simultaneously incubated with 3,3'-diaminobenzidine and the reaction interrupted with PBS. The immunofluorescence slides were mounted by using ProLong Gold antifade medium (Invitrogen, USA). At least two samples of each animal were independently analyzed by two experienced investigators. Results concerning distribution of

Table 1. Antibodies and enzymes used in immunohistochemistry/immunofluorescence.

Primary Antibody	Supplier	[ ]	Antigen retrieval	Secondary Antibody	Supplier	[ ]
Rabbit anti-type I collagen	Rockland (USA) 600-401-103	1:50	Pepsin[3] (4 mg/mL) 12 min - 37ºC	Goat anti-rabbit IgG FITC-conjugated	Sigma (USA)	1:150
Rabbit anti-type III collagen	Rockland (USA) 600-401-105	1:50	Pepsin[3] (4 mg/mL) 12 min - 37ºC	Goat anti-rabbit IgG FITC-conjugated	Sigma (USA)	1:150
Rabbit anti- type V collagen	Rockland (USA) 600-401-107	1:50	Pepsin[3] (4 mg/mL) 12 min - 37ºC	Goat anti-rabbit IgG FITC-conjugated	Sigma (USA)	1:150
Rabbit anti-biglycan	LF-113 (NIH-USA)[1]	1:1,000	Chondroitinase ABC[2] (0,2U) 1 h - 37ºC	Goat anti-rabbit IgG Biotin-conjugated	Rockland (USA)	1:2,000
Rabbit anti-decorin	LF-159 (NIH-USA)[1]	1:3,000	Chondroitinase ABC[2] (0,2U) 1 h - 37ºC	Goat anti-rabbit IgG Biotin-conjugated	Rockland (USA)	1:2,000
Rabbit anti-fibromodulin	LF-150 (NIH-USA)[1]	1:400	Chondroitinase ABC[2] (0,2U) 1 h - 37ºC	Goat anti-rabbit IgG Biotin-conjugated	Rockland (USA)	1:2,000
Goat anti-lumican	R&D Systems (USA) AF2745	1:2,000	Chondroitinase ABC[2] (0,2U) 1 h - 37ºC	Rabbit anti-goat IgG Biotin-conjugated	Rockland (USA)	1:2,000

[1]: Prof. Larry Fisher (National Institute of Dental and Craniofacial Research, NIH, USA). [2]: Seikagaku Corp. (Japan). [3]: Sigma (USA).

fluorescence/staining in the myometrial compartments were expressed as increased, decreased or similar comparatively to the control group. Images were recorded using a Nikon Eclipse E600 (Nikon, Japan) microscope, Olympus DP-72 digital camera (Olympus, Japan) and Image Pro Plus software (Media Cybernetics, USA).

Results

Collagen types I, III and V

Immunoreaction for collagen types I, III and V was detected in the myometrium of both control and diabetic groups. The distribution of these molecules, however, differed according to the experimental group, the length of diabetes and the myometrial compartment. Results are summarized in Table 2.

In the control group, collagen types I, III and V were detected (i) in the internal muscle layer (IML); (ii) around bundles of SMCs of the external muscle layer (EML); (iii) in the connective tissue between muscle layers (CT); (iv) in the perimetrium. The distribution of collagen type I in subgroup D1 was similar to the control group. However, a decreased deposition of this molecule was observed in the EML. In subgroup D2, the immunoreactivity for collagen type I was decreased in both IML and EML and increased in CT (Fig. 1).

In subgroup D1, the immunoreaction for collagen type III was decreased in both IML and EML. In subgroup D2, the immunoreaction for this type of collagen increased in the CT (Fig. 2).

Immunoreactivity for collagen type V also decreased in the IML of subgroup D1, as well as in the CT of both diabetic subgroups D1 and D2 (Fig. 3).

Small leucine-rich proteoglycans: decorin, lumican, fibromodulin and biglycan

Decorin, lumican, biglycan and fibromodulin were immunodetected in the myometrium of both control and diabetic subgroups. However, only traces of immunoreaction for fibromodulin were detected in the

Table 2. Summary results of the deposition of collagen types I, III and V in the myometrial compartments of the diabetic subgroups D1 and D2, compared to the control group.

	Collagen type I		Collagen type III		Collagen type V	
	D1	D2	D1	D2	D1	D2
IML	-	↓	↓	↓	↓	-
EML	↓	↓	↓	↓	-	-
CT	↓	↑	↑	↑	↓	↓
PM	↓	↓	↓	↓	↓	↓

D1: diabetic subgroup D1; D2: diabetic subgroup D2; IML: internal muscle layer; EML: external muscle layer; CT: connective tissue between muscle layers; PM: perimetrium. ↑: increased; ↓:decreased; -: no changes.

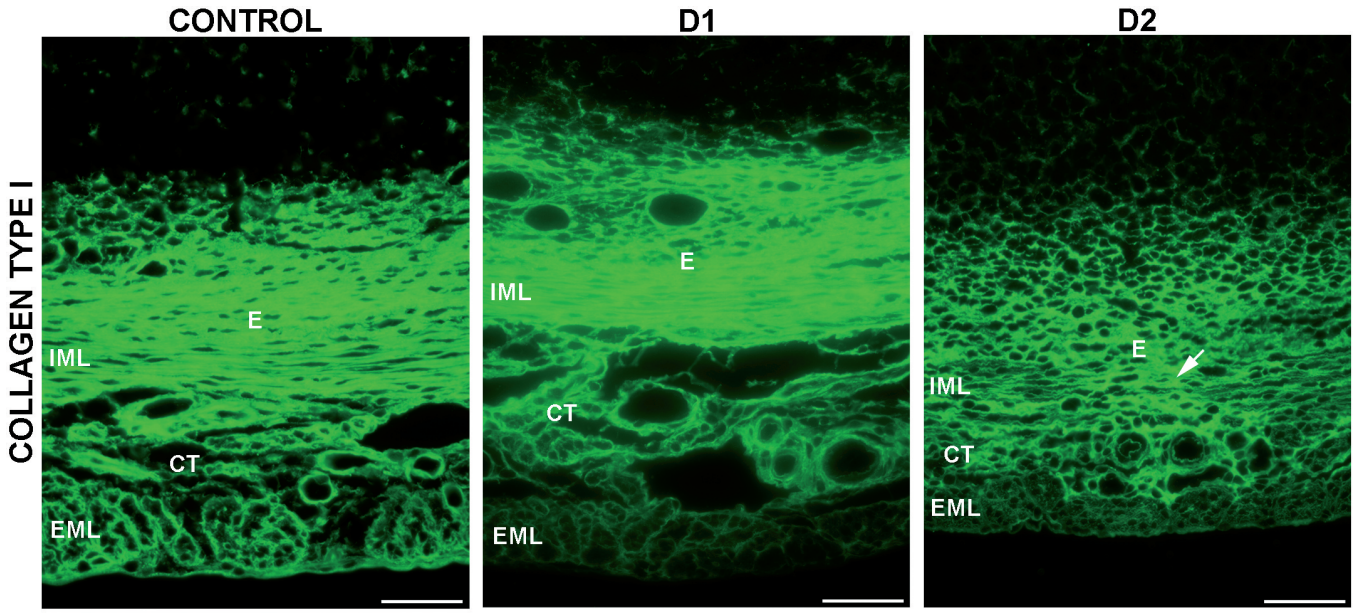

Fig. 1. Immunofluorescence for collagen type I in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control group, collagen type I is present between smooth muscle cells of the internal muscle layer (IML), around smooth muscle cells and muscle bundles of the external muscle layer (EML) and in the connective tissue between muscle layers (CT). Note that collagen type I is decreased in the EML of subgroup D1. In subgroup D2, intense immunoreaction for collagen type I is observed in some areas of the IML (arrow) and in the CT, whereas lower immunoreaction is present in the EML (arrow head). Endometrium (E). Scale bar: 40 μm.

muscle layers of both diabetic subgroups (Table 3).

In the control group, decorin and lumican were immunodetected in the SMCs of the IML and EML, as well as in the CT. The distribution of decorin in subgroup D1 was similar to the control. In contrast, in subgroup D2, no immunoreaction for decorin was detected in the IML and it was very weak in the EML and perimysium (Fig. 4). The immunoreaction for lumican was decreased in the EML and perimysium of subgroups D1 and D2. In subgroup D2, however, intense immunoreactivity for this proteoglycan was detected in the CT (Fig. 5).

In control females, fibromodulin was the sole proteoglycan to be immunodetected in the cytoplasm of SMCs in both IML and EML. A dramatic decrease in the IML and the absence of fibromodulin in the EML were observed in both diabetic subgroups (Fig. 6). Interestingly, some immune cells located in the CT of subgroup D1 showed cytoplasmic and pericellular immunostaining for fibromodulin (insert of Fig. 6).

Biglycan was immunodetected mostly in the IML of both control and diabetic females. A thin network of fibrils was also observed in the CT. Diabetes did not affect either deposition or distribution of this molecule (Fig. 7).

Discussion

In a previous study we showed that type 1 diabetes impairs the structural organization, contractile apparatus and ECM of the early pregnant myometrium in mice (Favaro et al., 2010). The present report adds new information concerning the effects of type 1 diabetes on the myometrial ECM, demonstrating that its composition is disturbed according to the stage of progression of the disease, the myometrial compartment and the class of ECM molecule. Deposition of collagen types I and V, as well as decorin in the muscle layers were differentially modulated during progression of diabetic complications in the myometrium, represented in subgroups D1 and D2. In contrast, other molecules such as collagen type III, lumican and fibromodulin were similarly modulated in the myometrial muscle layers of both diabetic

Table 3. Summary results of the deposition of lumican, decorin, fibromodulin and biglycan in the myometrial compartments of the diabetic subgroups D1 and D2, compared to the control group.

	Lumican		Decorin		Fibromodulin		Biglycan	
	D1	D2	D1	D2	D1	D2	D1	D2
IML	-	-	-	∞	↓	↓	-	-
EML	↓	↓	-	↓	∞	∞	-	-
CT	↓	↑	-	-	-	-	-	-
PM	↓	↓	-	↓	↓	↓	-	-

D1: diabetic subgroup D1; D2: diabetic subgroup D2; IML: internal muscle layer; EML: external muscle layer; CT: connective tissue between muscle layers; PM: perimetrium. ↑: increased; ↓:decreased; ∞: absent -: no changes.

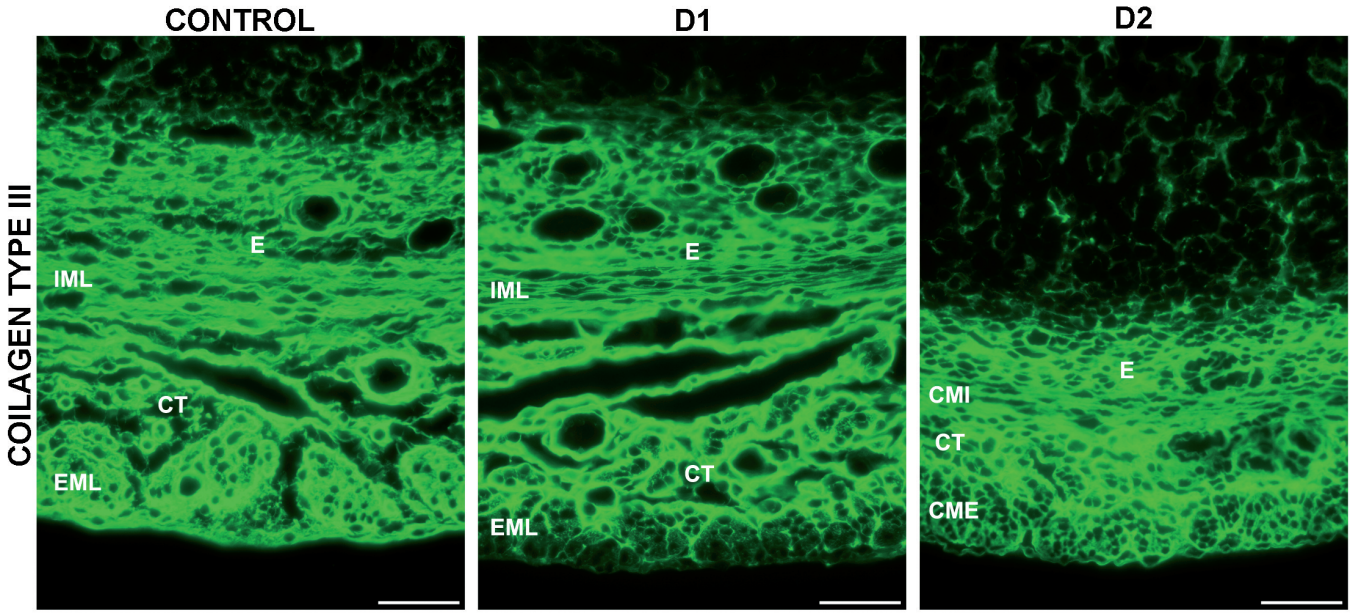

Fig. 2. Immunofluorescence for collagen type III in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control group, collagen type III is present between smooth muscle cells of the internal muscle layer (IML), around smooth muscle cells and muscle bundles of the external muscle layer (EML) and in the connective tissue between muscle layers (CT). Note that fluorescence is weak in the IML and EML of the diabetic subgroup D1. In subgroup D2, in addition to the findings described in D1, there is accumulation of collagen type III in the connective tissue between muscle layers. Endometrium (E). Scale bar: 40 μm.

subgroups. Besides, subgroup D1 was distinguished by decreased deposition of ECM molecules in the CT, whereas increased deposition typified subgroup D2.

In addition to the important structural roles of the fibril-forming collagens, these molecules also signal to cells through cell membrane receptors (integrins), participating in a variety of cellular functions (Leitinger and Hohenester, 2007). Collagen types I, III and V were previously immunolocalized in both myometrial muscle layers from days 5 to 8 of pregnancy (Spiess et al.,

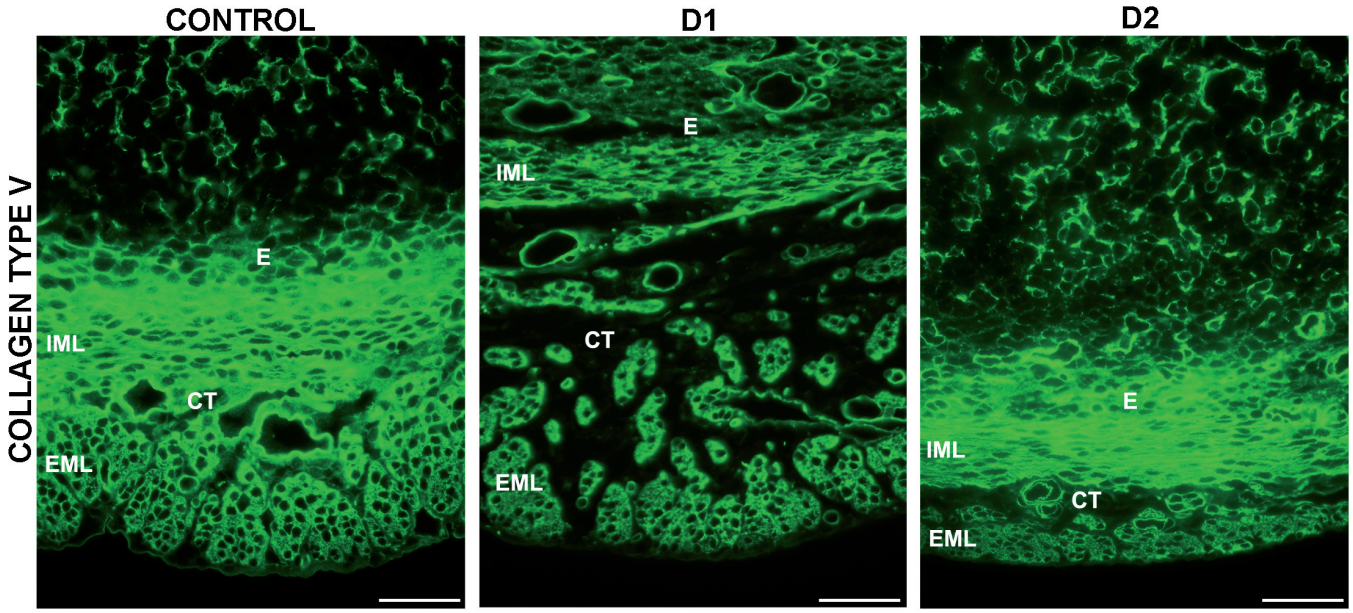

Fig. 3. Immunofluorescence for collagen type V in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control, collagen type V is present between smooth muscle cells of the internal muscle layer (IML), around the smooth muscle cells of the external muscle layer (EML) and in the connective tissue between muscle layers (CT). Note that the immunofluorescence for this collagen is decreased in the IML of subgroup D1 and in the CT of both D1 and D2. Endometrium (E). Scale bar: 40 μm.

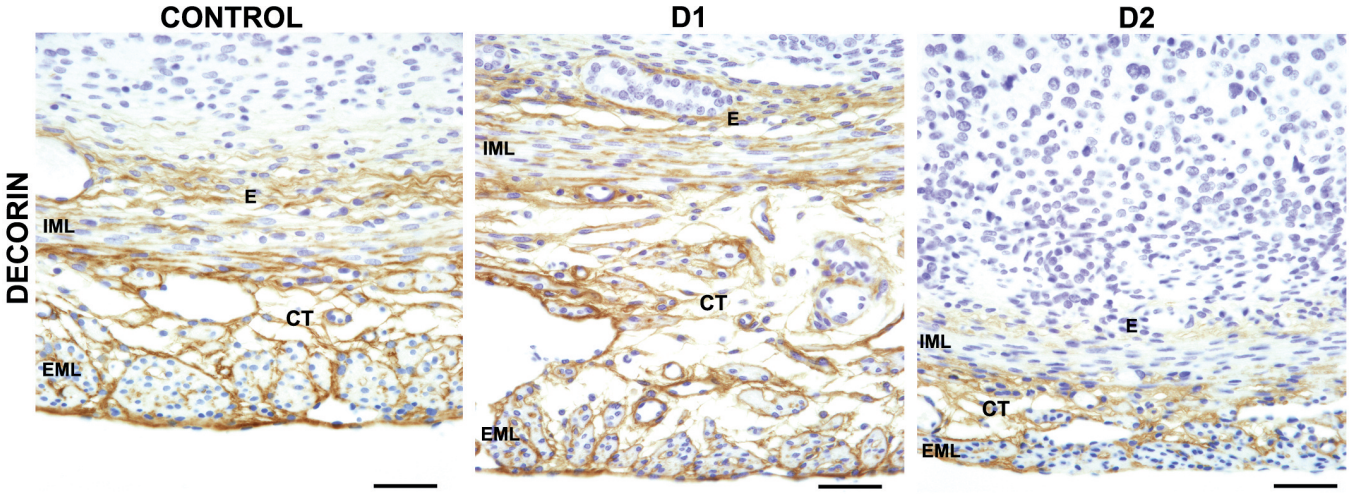

Fig. 4. Immunoperoxidase for decorin in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control group, immunoreaction for decorin is present between smooth muscle cells of the internal muscle layer (IML) and around muscle bundles of the external muscle layer (EML). Similar distribution is present in subgroup D1. In contrast, no immunoreaction for decorin is observed in the IML in subgroup D2 and it is decreased in the EML. CT: connective tissue between muscle layers. Endometrium (E). Scale bar: 40 μm.

2007). Formation and organization of collagen fibrils in the ECM comprise intricate biological processes that requires the interaction of different types and amounts of collagen, as well as collagen-associated proteoglycans (Banos et al., 2008). As previously shown by us in an ultra-structural study, collagen fibrils in subgroup D1 were scarcer and disperse in the extracellular space, whereas in the atrophic myometrium of subgroup D2, collagen fibrils were numerous and spatially disoriented (Favaro et al., 2010). In the decidualized endometrium of diabetic mice, changes in the ratio of collagen types (I, III and V) and proteoglycans (biglycan and lumican) were associated with impaired collagen fibrillogenesis, revealed by transmission electron microscopy (Favaro et al., 2010). In conjunction, these data emphasize the relevance of combining different approaches, such as

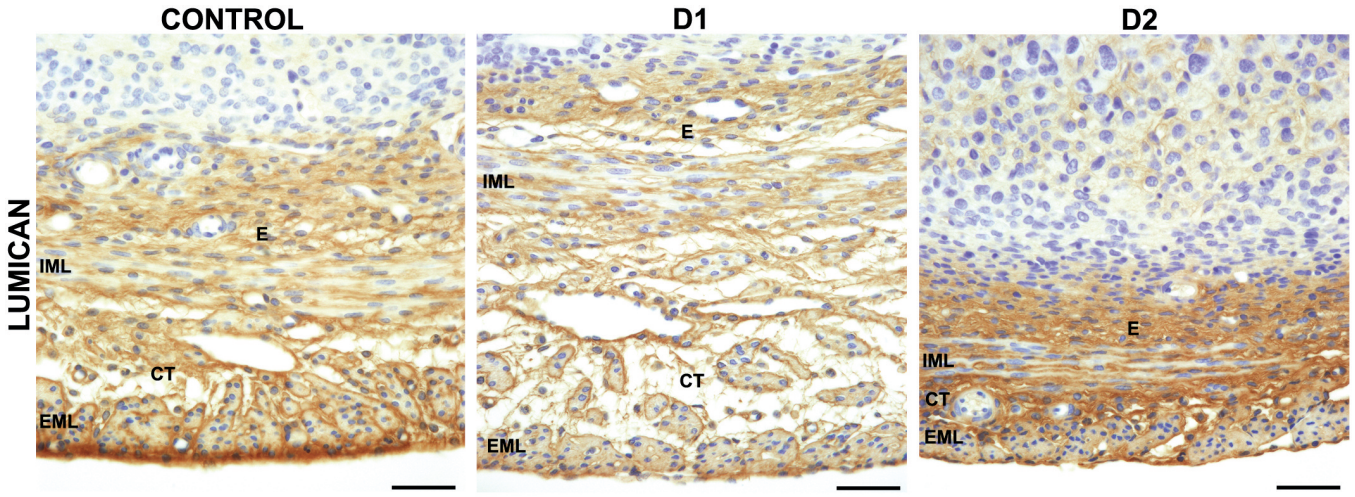

Fig. 5. Immunoperoxidase for lumican in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control group, immunoreaction for lumican is observed between smooth muscle cells of the internal muscle layer (IML), around muscle bundles of the external muscle layer (EML) and in the connective tissue between muscle layers (CT). Observe that this proteoglycan is decreased in the CT and EML of subgroup D1. In D2, the immunostaining in the EML is also diminished, whereas accumulation of lumican is detected in the CT. Endometrium (E). Scale bar: 40 μm.

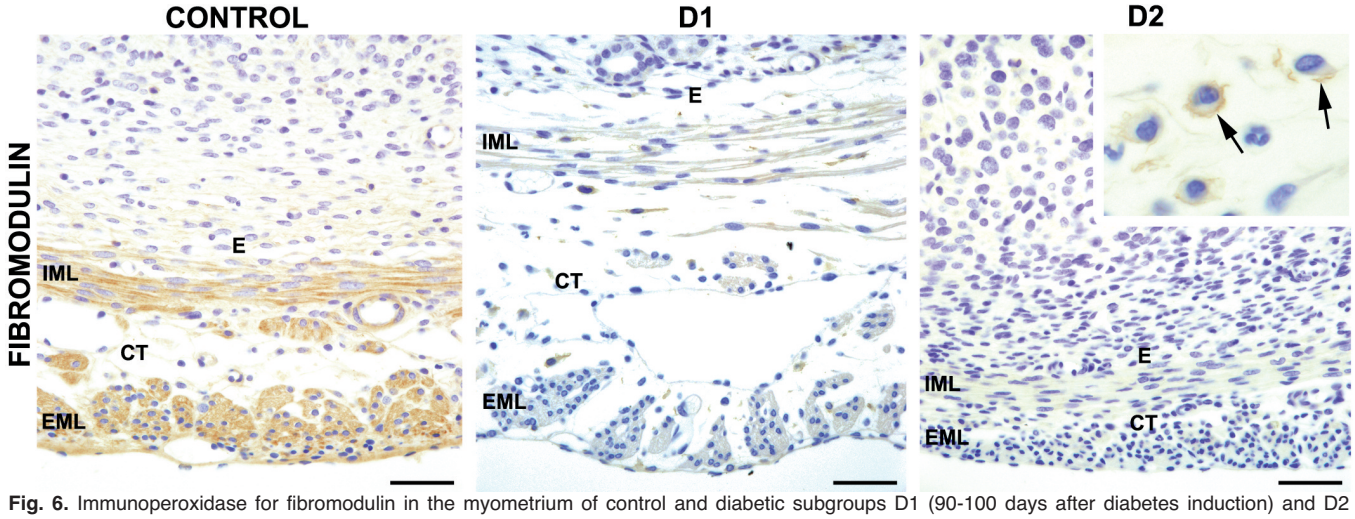

Fig. 6. Immunoperoxidase for fibromodulin in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). In the control group, fibromodulin is present in the cytoplasm of smooth muscle cells of both internal (IML) and external muscle layers (EML). The immunoreaction for this proteoglycan is dramatically reduced or even absent in the myometrium of diabetic subgroups D1 and D2. The insert shows pericellular reaction (arrows) in immune cells localized in the connective tissue between layers (CT) of diabetic subgroup D1. Endometrium (E). Scale bar: 40 μm.

light and electron microcopy, as well as immunohistochemistry to better understand complex biological phenomena, including the relationship between structure and molecular composition of the ECM.

Taking into account the role of the ECM in cell proliferation (Moreno-Layseca and Streuli, 2014) and SMC contractility (Gunst and Zhang, 2008) it is expected that disturbed deposition of collagens and proteoglycans in the myometrium of diabetic mice, as observed in the present study, may affect those cellular processes. In fact, it has been reported that diabetes is associated with impairments of uterine contractility (Jawerbaum et al., 1996; Kaya et al., 1999; Spiegl et al., 2009; Al-Qahtani et al., 2012) and proliferation of the SMCs (Favaro et al., 2010). Similarly to reported by our group in the myometrium of type 1 diabetic mice (Favaro et al., 2010), Al-Qahtani and cols. (2012) described diminished muscle content in the myometrium of pregnant women carrying gestational and type 1 diabetes. However, in contrast to our current results, those authors did not find significant changes in the myometrial collagen deposition. This divergent outcome may result from limitations of the technical approaches used in that study in women. Although Masson's Trichrome staining is able to identify collagen deposition, this method is unable to distinguish the different collagen types in the tissues.

The SLRPs participate in collagen fibrillogenesis and interact with growth factors, tirosine kinase receptors and toll-like receptors, playing a role in cell growth, morphogenesis and immunity (Iozzo and Schaefer, 2010). Fibromodulin and lumican show a dynamic process of synthesis, deposition and degradation during the estrous cycle of mice (Salgado et al., 2009). In contrast to decorin and lumican, present in the myometrium from day 1 to 7 of pregnancy, biglycan and fibromodulin are expressed only after day 5 of pregnancy period in which the endometrial decidualization starts and SMCs proliferation occurs (San Martin et al., 2003; Favaro et al., 2014). Taking together, these data indicate a link between the proliferative phenotype of the myometrium and adjustments in the composition of the ECM.

Decorin is known to bind to cell surface receptors and also harbor growth factors, controlling their availability within the cellular microenvironment (Neill et al., 2012). These properties were associated with the ability of decorin to prevent cell proliferation (Yamaguchi and Ruoslahti, 1988; Xu et al., 2002). In this context, we argue that the decrease in decorin deposition in the muscle layers of D2 diabetic group may be a mechanism to sustain cell proliferation, severely depressed by diabetes (Favaro et al. 2010).

Results from our group demonstrate that expression and deposition of SLRPs in the endometrium and myometrium of ovariectomized mice are modulated by ovarian hormones (Salgado et al., 2011). Moreover, variations in the levels of circulating progesterone and intensification of mechanical tension exerted by the growing fetus were proposed as key mechanisms controlling myometrial ECM remodeling during pregnancy (Shynlova et al., 2009).

Diabetes and estrogen have a relevant and complex relationship (Dantas et al., 2012). Studies in rats showed that diabetes interferes with estrogen signaling in the aorta and uterus (Bolego et al., 1999; Manabe et al., 2013). In this context, alterations in the deposition of ECM molecules in the myometrium of diabetic females may be associated with disturbed levels and/or impaired action of ovarian hormones promoted by the disease

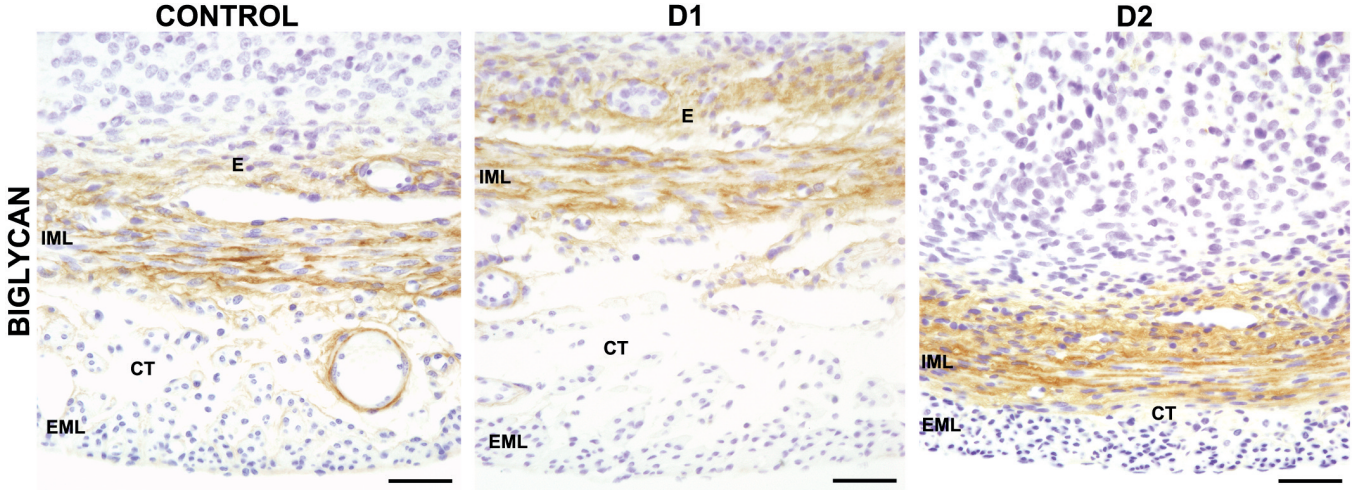

Fig. 7. Immunoperoxidase for biglycan in the myometrium of control and diabetic subgroups D1 (90-100 days after diabetes induction) and D2 (100-110 days after diabetes induction). Biglycan is imunodetected exclusively between smooth muscle cells of the internal muscle layer (IML) of both control and diabetic groups, and no differences are noted between them. CT: connective tissue between muscle layers; EML: external muscle layer. Endometrium (E). Scale bar: 40 μm.

(Garris, 1988; Zakaria et al., 2000; Albaghdadi and Kan, 2012; Manabe et al., 2013).

Diabetes promotes the development of complications in different organs of the genitourinary system. Studies performed in vagina of diabetic rats demonstrated the narrowing of the epithelium and muscle layer, a reduction of the vasculature and expansion of the connective tissue (Park et al., 2001; Kim et al., 2006). This fibrotic response was associated with high levels of TGF-beta (Park et al., 2001). On the bladder of diabetic rats, whereas the area occupied by smooth muscle increases, collagen content decreases (Pitre et al., 2002). A further analysis of the diabetic bladder by 2D-gel electrophoresis followed by mass spectrometry (MALDI-TOF) showed that proteins related to cell cycle and inflammation were enriched, whereas proteins related to cell adhesion, cytoskeleton and ECM were reduced, such as collagen type 1 and lumican (Yohannes et al., 2008). In our study, a similar trend was observed for these ECM molecules in the myometrium of the diabetic females. Furthermore, the development of diabetes-associated complications in the bladder (Pitre et al., 2002; Yohannes et al., 2008) and myometrium (Favaro et al., 2010) is correlated with the length of the disease.

In summary, the present data demonstrate common and specific sets of changes occur on the ECM of the myometrium, according to the progression of diabetic complications in this uterine compartment, enlarging the understanding of the effects of diabetes on the pregnant myometrium of mice.

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