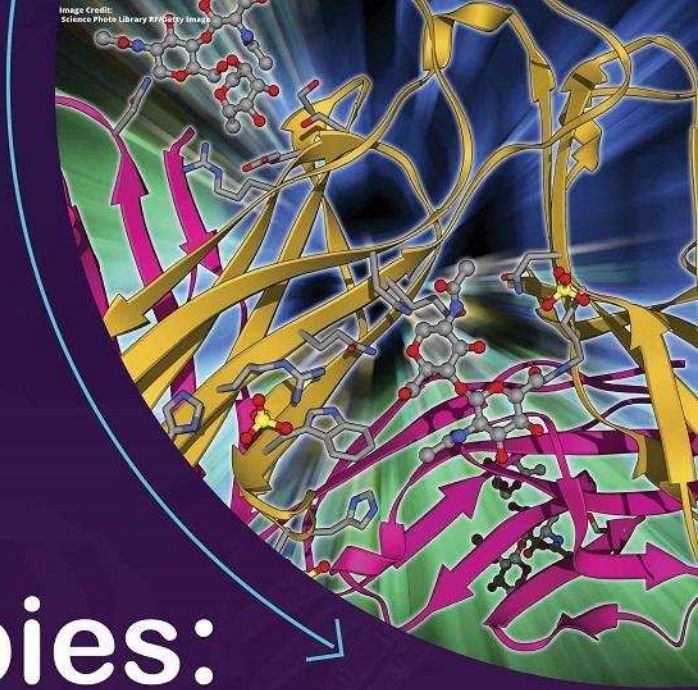


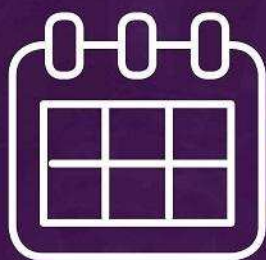
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## Vasculogenesis and Diabetic Erectile Dysfunction: How Relevant Is Glycemic Control?

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### ABSTRACT

Erectile dysfunction (ED) is a complication of diabetes, condition responsible for causing endothelial dysfunction (EDys) and hampering repair mechanisms. However, scarce information is available linking vasculogenesis mediated by Endothelial Progenitor Cells (EPCs) and diabetes-associated ED. Furthermore, it remains to be elucidated if glycemic control plays a role on EPCs functions, EPCs modulators, and penile vascular health. We evaluated the effects of diabetes and insulin therapy on bone marrow (BM) and circulating EPCs, testosterone, and systemic/penile Stromal Derived Factor-1 alpha (SDF-1 $\alpha$ ) expression. Male Wistar rats were divided into groups: age-matched controls, 8-weeks streptozotocin-induced type 1 diabetics, and insulin-treated 8-weeks diabetics. EPCs were identified by flow cytometry for CD34/CD133/VEGFR2/CXCR4 antigens. Systemic SDF-1 $\alpha$  and testosterone levels were evaluated by ELISA. Penile SDF-1 $\alpha$  protein expression was assessed, in experimental and human diabetic cavernosal samples, by immunohistochemical techniques. Diabetic animals presented a reduction of BM-derived EPCs and an increase in putative circulating endothelial cells (CECs) sloughed from vessels wall. These alterations were rescued by insulin therapy. In addition, glycemic control promoted an increase in systemic testosterone and SDF-1 $\alpha$  levels, which were significantly decreased in animals with diabetes. SDF-1 $\alpha$  protein expression was reduced in experimental and human cavernosal diabetic samples, an effect prevented by insulin in treated animals. Insulin administration rescued the effects of diabetes on BM function, CECs levels, testosterone, and plasmatic/penile SDF-1 $\alpha$  protein expression. This emphasizes the importance of glycemic control in the prevention of diabetes-induced systemic and penile EDys, by the amelioration of endothelial damage, and increase in protective pathways. *J. Cell. Biochem.* 118: 82–91, 2017. © 2016 Wiley Periodicals, Inc.

**KEY WORDS:** ERECTILE DYSFUNCTION; DIABETES; ENDOTHELIAL PROGENITOR CELLS; INSULIN; STROMAL DERIVED FACTOR-1 ALPHA

Erectile dysfunction (ED) is a common complication of diabetes, having a profound negative impact on the quality of life of men and their partners [Bortolotti et al., 1997]. In diabetic men, ED occurs at an earlier age than the general population, increasing with

disease duration [Maiorino et al., 2014]. Diabetes-associated ED is a multifactorial disorder, in which hyperglycemia and increased oxidative stress induce changes in central nervous system, peripheral nerve activity, and endothelial cell dysfunction (EDys)

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[Saenz de Tejada et al., 2005; Musicki and Burnett, 2007; Costa, 2012]. EDys comprises a number of functional alterations in the endothelial monolayer, impairing vasorelaxation mechanisms, and blood flow perfusion, leading to ED and systemic vascular disease [Musicki and Burnett, 2007]. Cavernal EDys is recognized as a key factor in diabetic-ED pathogenesis, where significant endothelial injuries may ultimately culminate in apoptosis, disrupting cavernosal tissue homeostasis [Costa and Virag, 2009; Costa et al., 2009; Costa, 2012]. In order to regenerate endothelial lesions, endogenous vascular repair mechanisms of angiogenesis [Folkman and Shing, 1992] and vasculogenesis [Asahara et al., 1999] are usually activated. However, it was reported that besides causing endothelial damage, diabetes may also deleteriously affect re-endothelialization events, exacerbating vasculopathy [Martin et al., 2003; Loomans et al., 2004]. It was demonstrated that due to diabetes, several angiogenic molecules and pathways are altered in penile tissue, which may impair angiogenesis, and endothelial repair [Musicki and Burnett, 2007; Castela et al., 2012]. However, the role of vasculogenesis, mediated by Endothelial Progenitor Cells (EPCs), in the pathophysiology of diabetic-ED remains unknown. EPCs reside within the bone marrow (BM), and are mobilized to the peripheral circulation and recruited to vascular insult sites, where they differentiate into mature ECs, integrating, and repairing damaged vasculature [Asahara et al., 1999]. These processes are regulated by many factors, including Stromal cell-Derived Factor-1 alpha (SDF-1 $\alpha$ ), which by binding CXCR-4 [Tzeng et al., 2011; Suarez-Alvarez et al., 2012], play a relevant role in BM homeostasis, and progenitor cells (PCs) trafficking [Urbich and Dimmeler, 2004] and androgens, which may modulate EPCs functions [Foresta et al., 2008; Castela et al., 2011]. Furthermore, it has been suggested that a good glycemic control, besides improving endothelial function and reducing oxidative markers [Fadini et al., 2011], and may also increase circulating EPCs (cEPCs) [Hortenhuber et al., 2013]. Several reports demonstrated a decrease in cEPCs levels in ED patients with cardiovascular risk factors, including diabetes [Murata et al., 2012; Maiorino et al., 2015]. A recent study, has showed a reduction of cEPCs in type 1 diabetics (T1D) with ED, which correlated with EDys and testosterone levels [Maiorino et al., 2015]. However, these publications have only investigated systemic EPCs levels and their potential association with endothelial, and ED parameters. In addition, the role of glycemic control on EPCs functions and its effects on EPCs modulators has never been assessed in diabetes-associated ED. We aimed to study in an ED-prone experimental model of diabetes [Jin et al., 2009; Castela et al., 2012], the role of insulin therapy on EPCs production/mobilization, on testosterone levels, and its impact in the systemic and penile expression of SDF-1 $\alpha$ .

## MATERIALS AND METHODS

### EXPERIMENTAL MODEL OF TYPE 1 DIABETES

Procedures described in this study were performed in accordance with the ethical guidelines proposed by the Portuguese General Veterinary Directorate (DGV) in the Directive of 24 November 1986 (86/609/EEC), with the recommendations of 18 June 2007 (2007/526/EC) proposed

by the Council of the European Communities. Male Wistar rats (280–350g; Charles River Laboratories, Barcelona, Spain) were maintained in a 12-h day–light cycle with free access to food, and tap water. A total of 30 rats were randomly assigned to the following groups (n = 10): age-matched healthy controls, 8-weeks diabetics, and 8-weeks diabetics treated daily with insulin. T1D was induced by streptozotocin, as described [Castela et al., 2012]. Immediately after the onset of diabetes, a group of animals initiated a subcutaneous treatment with 2U of insulin (Humulin NPH 100 UI/ml, Lilly), twice a day, for 8 weeks. Glucose levels were monitored before every insulin administration to ensure normalization. To avoid the possible influence of circadian regulation of EPCs production/trafficking, at the 8-week time point all animals were sacrificed at 9am [Thomas et al., 2008]. Femurs and tibias were harvested, and blood samples collected for EPCs characterization. Plasma was stored at –80°C, penises excised and processed for OCT (Optimal Cutting Temperature)-embedding, and immunohistochemistry.

### HUMAN SAMPLES

All patients gave written informed consent for the removal of cavernous tissue, and this study was approved by the local ethics committee according to the World Medical Association Declaration of Helsinki. A total of 22 individuals were enrolled in this study. Eight men without diabetes and without ED [(mean age  $\pm$  standard error (SE); 32.1  $\pm$  3.6)], one had a penile curvature correction, and the remaining had procedures for penile enlargement and/or lengthening. Fourteen patients with diabetes and ED (mean age  $\pm$  SE; 61.5  $\pm$  2.1): 4 with T1D and 10 with T2D, had penile implant surgery. During surgical interventions, cavernosal fragments with  $\approx$ 5–7.5 mm length/2.5 mm width were harvested, and fixed in formaldehyde. Corpus cavernosum (CC) was processed for paraffin inclusion and immunohistochemistry [Costa et al., 2009].

### EPCs EVALUATION BY FLOW CYTOMETRY

BM and peripheral blood (PB)-isolated EPCs were characterized by flow cytometry for the expression of CD34, CD133, VEGFR2, and CXCR4 antigens. These are the most consensual specific surface markers currently used to characterize EPCs, which include antigens that define the hematopoietic lineage, CD34 and CD133, in combination with a marker of endothelial commitment, VEGFR2 [Peichev et al., 2000], and an EPCs mobilization antigen, CXCR4. Combination of these antigens allowed the investigation of several subpopulations displaying vasculogenic properties: CD34<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>CXCR4<sup>+</sup>, and CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup>. Markers of vascular damage were also assessed in PB, through the characterization of putative circulating endothelial cells (CECs) identified by CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>. Briefly, total mononuclear cells (MNCs) were isolated from BM by flushing femurs and tibias with phosphate-buffered saline (PBS). PB MNCs were separated by Histopaque-1077 (Sigma–Aldrich, St. Louis, MO) density gradient centrifugation. Approximately 3–5 millions BM and PB MNCs were stained during 30 min at 4°C with the following rabbit anti-rat antibodies: PE-anti-CD34 (1:50; Antibodies-online Inc., Atlanta, GA), Cy5.5-anti-CD133 (1:10; Bioss Inc., Woburn, MA), Alexa Fluor 488-anti-VEGFR2 (1:10; Antibodies-online Inc.), and PE-Cy7-anti-CXCR4

(1:20; Bioss Inc.). A Fixable Viability Dye eFluor<sup>®</sup> 450 (eBioscience, San Diego, CA) was added to all samples. An isotype matched control was evaluated to achieve the appropriate settings. Results were collected in a FACSanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ) (1–2 million events) and analyzed using FlowJo software (Ashland, OR).

#### SYSTEMIC QUANTIFICATION OF SDF-1 $\alpha$ AND TESTOSTERONE

Plasma SDF-1 $\alpha$  and testosterone levels were measured by ELISA. Systemic SDF-1 $\alpha$  was quantified using the Human CXCL12/SDF-1 $\alpha$  Immunoassay (R&D Systems, Abingdon, UK) and circulatory testosterone with the Testosterone Assay (R&D Systems), according to the manufacturer's protocol. Quantification was performed at 450 and 550 nm using a plate reader (Thermo Electron, Multiskan Ascent).

#### SDF-1 $\alpha$ QUANTITATIVE IMMUNOHISTOCHEMISTRY

SDF-1 $\alpha$  immunohistochemical detection was performed in experimental and human CC, using the streptavidin-biotin-peroxidase method [Costa et al., 2009]. Briefly, sections were incubated overnight with a cross-reactive rabbit anti-human SDF-1 $\alpha$  (1:1000; Abcam, Cambridge, UK), followed by the biotinylated secondary antibody (Santa Cruz Biotechnology, CA) and the Avidin-Biotin Complex reagents (Vector Laboratories, CA). Omission of the primary antibody was used as negative control. Sections were developed with diaminobenzidine (DAB; Sigma-Aldrich) and counterstained with hematoxylin. Slides were visualized under the Olympus AH3-RFCA microscope and images captured using the Olympus C-35AD-4 camera (Olympus Imaging Europa GmbH, Hamburg, Germany). Signal intensity was quantified using ImageJ color deconvolution (v.1.37a, NIH, MD), as described [Castela et al., 2012]. Briefly, a DAB threshold value of 25 (rat) and 75 (human) was selected for SDF-1 $\alpha$  staining, by measuring the lowest and the highest mean optical density values in 10 random DAB-positive images. For each image, the sum of the intensity values in the regions of interest (ROI), pixels above the brown threshold, was divided by the ROI total area, producing an average unitless intensity value for SDF-1 $\alpha$  staining. All intensity values within the same group were averaged to calculate an overall value, a relative measurement of SDF-1 $\alpha$  levels.

#### DUAL IMMUNODETECTION OF SDF-1 $\alpha$ / $\alpha$ -SMOOTH MUSCLE ACTIN ( $\alpha$ -SMA)

To identify cavernosal cellular components expressing SDF-1 $\alpha$ , a double immunofluorescence for SDF-1 $\alpha$ / $\alpha$ -SMA was performed [Costa et al., 2009]. Briefly, rat and human sections were incubated with the following antibodies: rabbit anti-SDF-1 $\alpha$  (1:1000; Abcam) and mouse anti-human  $\alpha$ -SMA (1:500; Chemicon International, Temecula, UK). Slides were incubated with the respective secondary antibodies: donkey anti-rabbit conjugated with a red fluorochrome (1:1000; Alexa<sup>™</sup> 568, Invitrogen, CA) and donkey anti-mouse conjugated with a green fluorochrome (dilution 1:1000; Alexa<sup>™</sup> 488, Invitrogen). Primary antibodies were omitted in negative controls. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; Invitrogen), slides were observed under the fluorescence microscope (Imager.Z1, Zeiss, Germany) and images captured (Carl Zeiss MicroImaging GmbH, Germany).

#### STATISTICAL ANALYSIS

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software version 5.0, San Diego, CA). Data are presented as mean  $\pm$  SE. In studies with experimental rat model, significant differences between control, diabetes, and diabetes + insulin groups were determined by the One-way Analysis of Variance (ANOVA), followed by the Bonferroni test. Significant alterations in quantitative immunostaining between human non-diabetic, non-ED, and diabetic with ED groups were calculated using Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

#### EVALUATION OF BM-EPCs IN DIABETIC AND INSULIN-TREATED ANIMALS

EPCs subsets were assessed by flow cytometry by the surface expression of CD34/CD133/VEGFR2/CXCR4 antigens. Figure 1A illustrates the flow cytometry gating strategy used to identify EPCs populations. Hematopoietic PCs were selected on the basis of forward and side scatter values excluding the debris, aggregates, and dead cells. The percentages and numbers of the distinct EPCs subtypes were performed, based on the double or triple expression of CD133, VEGFR2, and CXCR4 on CD34<sup>+</sup> gated cells (G4). Quadruple positive cells were quantified by analyzing the CD133 positivity on CD34<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup> cells (G5). Data revealed that hematopoietic stem/progenitor cells characterized by CD34<sup>+</sup> and CD34<sup>+</sup>CD133<sup>+</sup> markers were decreased in diabetics when compared with controls and insulin-treated rats (Fig. 1B and C). Insulin administration significantly prevented diabetes effects on these populations, as displayed in Figure 1B (diabetes:  $0.68 \pm 0.05\%$  vs. diabetes + insulin:  $1.19 \pm 0.15\%$ ;  $P < 0.05$ ) and Figure 1C (diabetes:  $0.24 \pm 0.02\%$  vs. diabetes + insulin:  $0.59 \pm 0.06\%$ ;  $P < 0.01$ ). In diabetic animals, the subpopulation expressing CD34<sup>+</sup>CD133<sup>+</sup> in conjunction with the VEGFR2 was also significantly reduced in comparison to controls (diabetes:  $0.24 \pm 0.02\%$  vs. controls:  $0.54 \pm 0.09\%$ ;  $P < 0.01$ ), and to insulin-administrated animals ( $0.66 \pm 0.07\%$ ;  $P < 0.001$ ) (Fig. 1D). Similar results were obtained for cells co-expressing CD34<sup>+</sup>CD133<sup>+</sup> and CXCR4<sup>+</sup>, the chemokine receptor for SDF-1 $\alpha$  (Fig. 1E). In diabetics the percentage of cells expressing these markers was decreased ( $0.19 \pm 0.02\%$ ), when compared to non-diabetics ( $0.48 \pm 0.14\%$ ) and to insulin-supplemented animals ( $0.51 \pm 0.08\%$ ;  $P < 0.001$ ). The subpopulation quadruple positive for CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup> was significantly decreased in the diabetic BM ( $0.12 \pm 0.04\%$ ) in comparison to control ( $0.43 \pm 0.07\%$ ;  $P < 0.01$ ), and insulin-treated rats ( $0.39 \pm 0.06\%$ ;  $P < 0.001$ ). Importantly, treatment with insulin allowed to maintain all these cell populations to values in the range of controls (Fig. 1F).

#### cEPCs LEVELS IN ANIMALS WITH DIABETES—EFFECTS OF INSULIN ADMINISTRATION

cEPCs were identified as previously described for BM. Figure 2A shows the gating strategy used to evaluate EPCs in PB. Flow cytometry was used to analyze cEPCs expressing the same

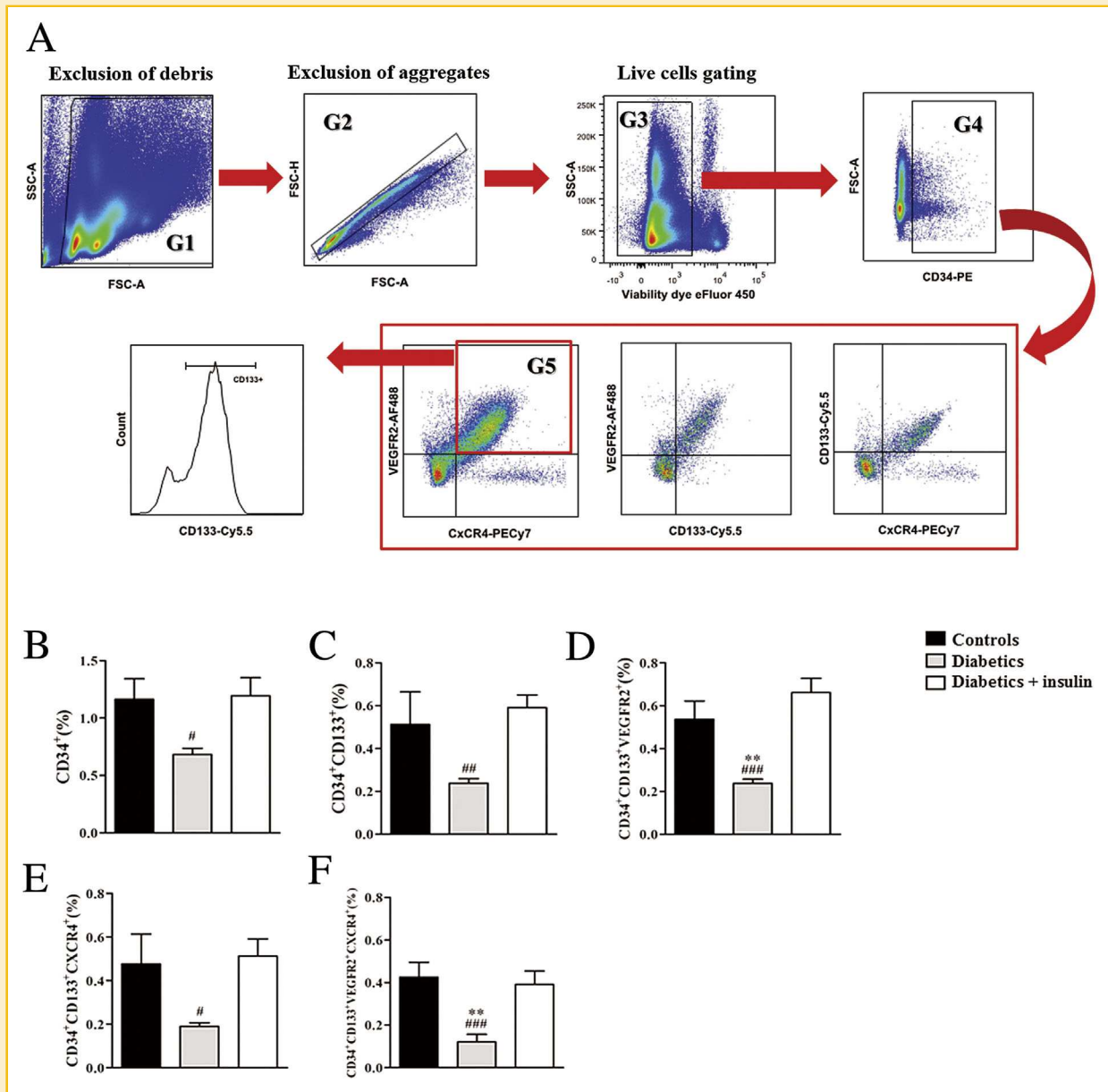
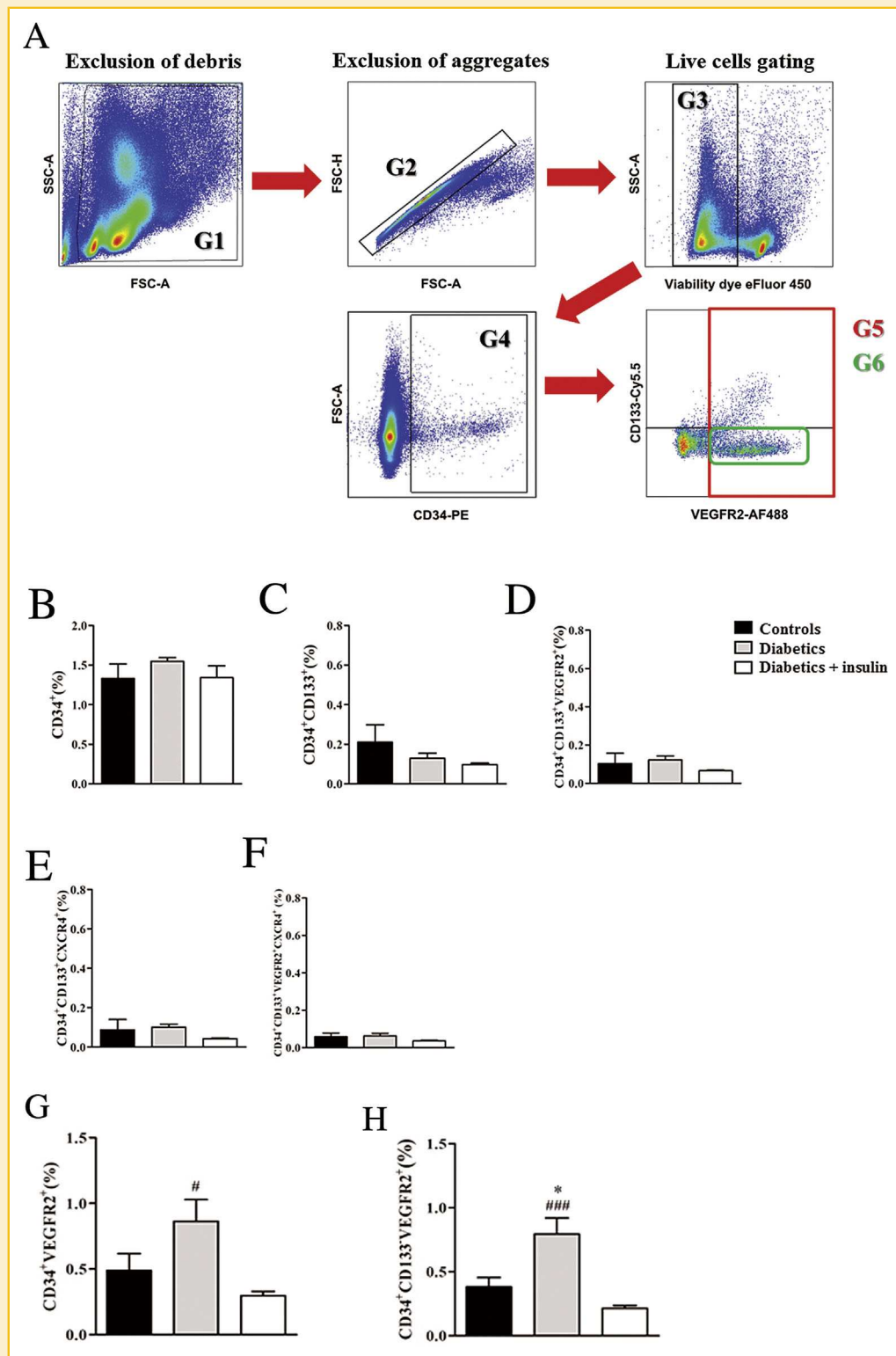


Fig. 1. Flow cytometry quantification of bone marrow (BM) stem and endothelial progenitor cells (EPCs). (A) Representative flow cytometry gating strategy used to analyze BM-derived stem/vascular progenitor cells: exclusion of debris and aggregates, gating of live cells. Subsequently, cells expressing CD34 were gated and among those, subpopulations identified by CD133, CXCR4, and VEGFR2. (B) Diabetic BM presented a decrease in CD34<sup>+</sup> cells and a reduction in subpopulations of EPCs identified by the following immunophenotypes: (C) CD34<sup>+</sup>CD133<sup>+</sup>, (D) CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, (E) CD34<sup>+</sup>CD133<sup>+</sup>CXCR4<sup>+</sup>, (F) CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup>. These alterations were prevented by insulin administration. Data are expressed as Mean  $\pm$  SE. \*\* $P < 0.01$ , diabetic group compared with control group; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ , diabetic group compared with insulin-treated diabetics.

combination of antigens, as for BM. Interestingly, when comparing diabetic animals to controls and insulin-treated, we did not observe significant differences in the percentage of CD34<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>CXCR4<sup>+</sup>, and CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup> cells (Fig. 2B-F). However, we have identified two cell populations, CD34<sup>+</sup>VEGFR2<sup>+</sup> and CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, increased in animals with diabetes (Fig. 2G and H). The percentage of double positive CD34<sup>+</sup>VEGFR2<sup>+</sup>

cells in diabetic rats ( $0.86 \pm 0.47\%$ ), was augmented as compared to the non-diabetes group ( $0.57 \pm 0.29\%$ ) and with insulin-administered animals ( $0.28 \pm 0.09\%$ ;  $P < 0.05$ ) (Fig. 2G). CD34 and VEGFR2 are surface markers present in both EPCs and in mature ECs, yet, the latter is negative for CD133. Our results demonstrated an increase in CD34<sup>+</sup>CD133<sup>-</sup>VEGFR2<sup>+</sup> cells in diabetics ( $0.79 \pm 0.13\%$ ), in comparison to controls ( $0.39 \pm 0.07\%$ ;  $P < 0.05$ ) and to insulin-treated rats ( $0.22 \pm 0.06\%$ ;  $P < 0.001$ ) (Fig. 2H).



**Fig. 2.** Flow cytometry quantification of peripheral blood cells. (A) Representative flow cytometry gating strategy of putative circulating endothelial cells (CECs): exclusion of debris and aggregates, gating of live cells. Subsequently, cells expressing CD34 were gated and among those, subpopulations identified by VEGFR2 positivity and CD133<sup>-</sup>. (B–F) Percentage of CD34<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>CXCR4<sup>+</sup>, and CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup> cells, no significant difference was observed when comparing diabetic animals to controls and insulin-treated diabetics. (G and H) In the diabetic group a significant increase was observed in subsets of cells characterized by (G) CD34<sup>+</sup>VEGFR2<sup>+</sup>, (H) CD34<sup>+</sup>VEGFR2<sup>+</sup>CD133<sup>-</sup>, which may identify matured ECs sloughed from vascular walls. This effect was rescued to control levels, by insulin therapy. Data presented as Mean ± SE. \**P* < 0.05, diabetic group compared with control group; <sup>#</sup>*P* < 0.05, <sup>###</sup>*P* < 0.001; diabetic group compared with insulin-treated diabetics.

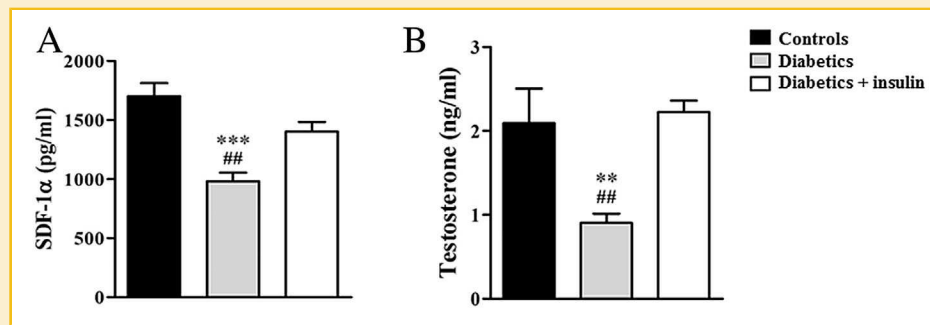


Fig. 3. Plasmatic quantification of SDF-1 $\alpha$  and testosterone. (A) Diabetic animals presented a significant reduction in systemic SDF-1 $\alpha$  and (B) testosterone. Insulin therapy prevented the effects of diabetes, increasing SDF-1 $\alpha$  and testosterone levels. Data presented as Mean  $\pm$  SE. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  diabetics compared to control group; ##  $P < 0.01$  diabetic group compared to insulin-treated diabetics.

### SDF-1 $\alpha$ AND TESTOSTERONE LEVELS

Systemic SDF-1 $\alpha$  and testosterone were evaluated by ELISA. As shown on Figure 3A, animals with diabetes had a significant decrease in SDF-1 $\alpha$  (982.60  $\pm$  72.46 pg/ml), as compared to controls (1704.00  $\pm$  180.30 pg/ml;  $P < 0.001$ ) and to insulin-treated rats (1402.00  $\pm$  82.26 pg/ml;  $P < 0.01$ ). Similarly, plasma testosterone was reduced in diabetics (0.90  $\pm$  0.11 ng/ml), and rescued to control values (2.09  $\pm$  0.41 ng/ml) by insulin supplementation (2.23  $\pm$  0.1 ng/ml;  $P < 0.01$ ) (Fig. 3B).

### SDF-1 $\alpha$ EXPRESSION IN RAT PENILE TISSUE

We evaluated if diabetes and insulin treatment had a role in cavernosal SDF-1 $\alpha$  expression, as quantified in DAB-labeled sections [Castela et al., 2012]. Additionally, we identified CC components expressing SDF-1 $\alpha$ , by dual immunofluorescence for SDF-1 $\alpha$ / $\alpha$ -SMA. We observed that SDF-1 $\alpha$  (Fig. 4A) was significantly decreased in diabetic CC (62.34  $\pm$  3.23;  $P < 0.05$ ) in comparison with controls (75.35  $\pm$  2.60), an effect prevented by insulin administration (74.92  $\pm$  2.22) (Fig. 4A). Dual immunofluorescence revealed that SDF-1 $\alpha$  was predominantly detected in perivascular smooth muscle cells (SMCs) (Fig. 4C), with some expression also perceived in endothelium of control and insulin-administered animals (Fig. 4C, arrow head).

### SDF-1 $\alpha$ IMMUNOLABELING IN HUMAN CC

To verify if alterations in diabetic cavernosal SDF-1 $\alpha$  expression were not specific of rat, we performed analogous experiments in human non-diabetes, non-ED, and in diabetes with ED samples. Consistent with the results obtained in experimental CC, human diabetic-ED penis displayed a significant decrease in SDF-1 $\alpha$  protein (mean intensity values 105.40  $\pm$  3.22 in non-diabetes, non-ED vs. 96.01  $\pm$  0.82 in diabetes with ED samples;  $P < 0.01$ ) as quantified in SDF-1 $\alpha$ /DAB-stained slides (Fig. 5A and B). SDF-1 $\alpha$ / $\alpha$ -SMA immuno-colocalization revealed that SDF-1 $\alpha$  was predominantly present in SMCs of both human non-diabetic and diabetic fragments (Fig. 5C).

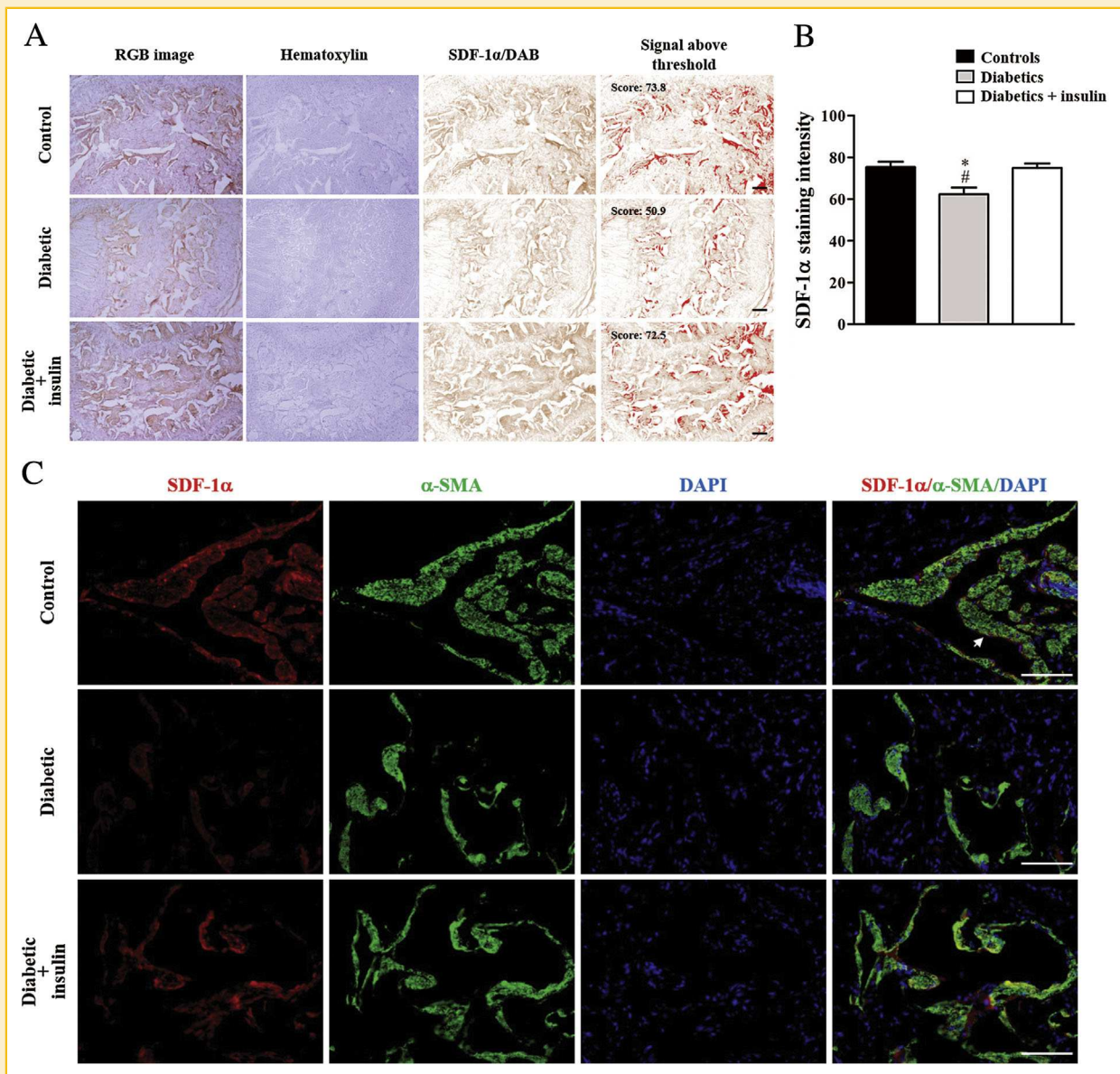
## DISCUSSION

In this study, we evaluated in a T1D experimental model the effects of insulin treatment in EPCs functions, on testosterone levels, and in

the systemic and penile expression of SDF-1 $\alpha$ . The main findings suggested that insulin administration rescued diabetes effects in endothelial damage and improved systemic, and cavernosal protective proteins/pathways involved in EPCs function/modulation, highlighting relevance of glycemic control in preventing endothelial dysfunction associated to diabetes.

We started by evaluating BM and peripheral EPCs by flow cytometry, analyzing five subpopulations identified by the following immunophenotypes: CD34<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>, CD34<sup>+</sup>CD133<sup>+</sup>CXCR4<sup>+</sup>, and CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup>CXCR4<sup>+</sup>. All subsets may comprise cells with vasculogenic capabilities, although triple and quadruple antigen positive subpopulations, are considered as bonafide EPCs [Peichev et al., 2000]. We observed a decrease in all immunophenotypes in diabetic BM, as compared to control and insulin-treated. In agreement, previous studies also demonstrated a reduction in BM-derived EPCs in experimental models of diabetes [Mohler 3rd et al., 2009; Saito et al., 2012]. It was also reported that diabetes may be responsible for BM microenvironment alterations [Oikawa et al., 2010; Hazra et al., 2013; Westerweel et al., 2013] impairing stem, and PCs production. Interestingly, this effect was prevented by insulin, which seemed to restore BM functionality. These results, corroborated data from a single publication demonstrating in a mouse model of diabetes the beneficial effects of insulin administration on BM cell production [Saito et al., 2012].

Next, we investigated if diabetes and insulin therapy played a role in cell trafficking to PB. We did not observe significant alterations, between the three groups, in any of the analyzed EPCs subpopulations. In fact, contradictory findings have been reported in what regards cEPCs in diabetes. Several studies have presented a decrease in vascular progenitors in diabetic PB [Murata et al., 2012; Saito et al., 2012; Westerweel et al., 2013; Maiorino et al., 2015], whereas, others have shown an increase in these cell populations [Fadini et al., 2006; Liu et al., 2010; Flammer et al., 2012]. This relates to the so-called diabetic angiogenic paradox [Costa, 2011]. Decreased cEPCs were reported in diabetes challenged with: ischemia, wound healing, coronary, and peripheral artery disease [Fadini et al., 2005; Gallagher et al., 2007; De Falco et al., 2009; Antonio et al., 2014]. Whereas, EPCs increase was associated to diabetic retinopathy (DR), characterized by excessive neovascularization [Fadini et al., 2006; Liu et al., 2010]. However, deficient vascular formation may co-exist



**Fig. 4.** Quantitative immunohistochemistry of SDF-1 $\alpha$  experimental penile tissue. (A, B) SDF-1 $\alpha$  protein expression levels as quantified in SDF-1 $\alpha$ /DAB stained slides by ImageJ color deconvolution, revealed a significant decrease in SDF-1 $\alpha$  in diabetic corpus cavernosum (CC), when compared with age-matched controls. Insulin therapy increased SDF-1 $\alpha$  protein to values in the range of controls. Data presented as Mean  $\pm$  SE. \* $P < 0.05$ , diabetics compared to control group; # $P < 0.05$  diabetic group compared to insulin-treated diabetics. RGB, Red Green Blue; DAB, diaminobenzidine. (C) Co-localization of SDF-1 $\alpha$  and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) by dual immunolabeling demonstrated that SDF-1 $\alpha$  protein was predominantly present in perivascular smooth muscle cells (SMCs) in all groups analyzed. In certain vascular spaces SDF-1 $\alpha$  was clearly identified lining the sinusoids (upper panel; white arrow). DAPI, 4',6'-diamidino-2-phenylindole (DNA intercalator). Bars, 100  $\mu$ m.

with DR in the same subject, making it a paradigm. Our 8-week T1D model, does not develop DR, and was not challenged with any vascular trauma, which can explain the lack of difference in cEPCs between groups. Additionally, to avoid the possible influence of circadian regulation, we isolated MNCs in all groups at the same hour of the day [Thomas et al., 2008]. Nonetheless, our results do not corroborate recent data in T1D ED patients, where a decrease in peripheral EPCs was observed [Maiorino et al., 2015]. This study enrolled long-term men with diabetes, and EPCs reduction could be attributed to other vascular complications associated to the disease.

Our results, have however, shown that a CD34<sup>+</sup>CD133<sup>+</sup>VEGFR2<sup>+</sup> subpopulation was increased in diabetic PB. These may identify putative CECs that have sloughed from vessels walls. In fact, augmented CECs have been considered surrogate markers of severe endothelium injury [Blann and Pretorius, 2006] and related to systemic vascular disease associated to diabetes [Abraham et al., 2004; Ascioglu et al., 2010]. This unbalance towards vascular damage was successfully prevented by insulin therapy, suggesting a protective role in ameliorating diabetic-related vascular complications. In support, one publication has showed that optimized



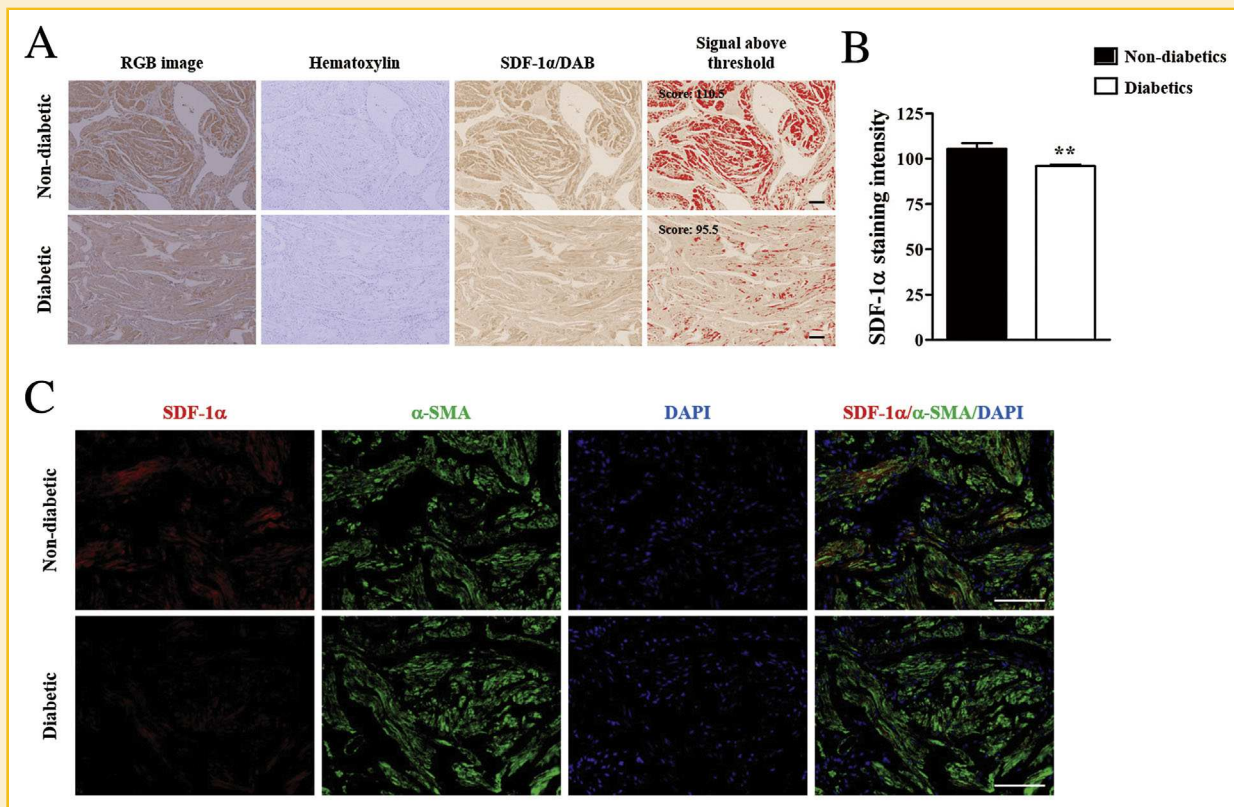


Fig. 5. SDF-1 $\alpha$  protein evaluation in human non-diabetic and diabetic cavernosal samples. (A, B) SDF-1 $\alpha$  protein levels were quantified in SDF-1 $\alpha$ /DAB stained corporal tissues using ImageJ color deconvolution. Quantitative immunohistochemistry showed a significant reduction in SDF-1 $\alpha$  protein expression in human diabetic erectile. (C) Double immunofluorescence for SDF-1 $\alpha$ / $\alpha$ -SMA demonstrated positive labeling in cavernosal SMCs. Data presented as Mean  $\pm$  SE. \*\* $P$  < 0.01. RGB, Red Green Blue. DAB, diaminobenzidine. DAPI, 4',6-diamidino-2-phenylindole (DNA intercalator). Bars, 100  $\mu$ m.

glycemic control in patients with diabetes improved indexes of endothelial damage, as assessed by CECs evaluation [Fadini et al., 2011].

Next, we investigated the effect of insulin on EPCs modulators SDF-1 $\alpha$  and testosterone. We observed that insulin administration significantly increased systemic SDF-1 $\alpha$ , which was reduced in diabetics. To our knowledge, SDF-1 $\alpha$  plasma levels have not been investigated in the same diabetic conditions as ours. A single report has assessed plasmatic SDF-1 $\alpha$  in STZ-induced animals after being challenged with acute hind limb ischemia [De Falco et al., 2009]. Another showed that circulating SDF-1 $\alpha$  was reduced in transgenic db/db mice [Chen et al., 2012]. To date there are no studies referring the effects of insulin in SDF-1 $\alpha$  levels altered by diabetes. We hypothesized that normalized glucose prevented diabetes metabolic alterations, rescuing SDF-1 $\alpha$ . Additionally, putative CECs decrease in treated-insulin rats may also be related to SDF-1 $\alpha$  increase. Mature ECs also express CXCR4 and SDF-1 $\alpha$  is thought to exert a direct action on endothelial functions [Carr et al., 2006]. Further, it is known that testosterone deficiency is a prevalent disorder associated to diabetes [Tesone et al., 1980; Xu et al., 2014]. Concordantly, we observed a reduction in testosterone levels in animals with diabetes, which was rescued to control values by insulin. These

data are corroborated by previous research demonstrating a reduction on plasma testosterone in diabetic models, which was also restored to normal levels by insulin replacement therapy [Tesone et al., 1980; Xu et al., 2014]. It was also shown that androgens may play a role in EPCs modulation and EC function [Foresta et al., 2008; Castela et al., 2011]. Although no alterations in PB EPCs were observed after insulin treatment, the decrease in endothelial damage may also be related to a testosterone protective effect in vascular endothelium.

In addition to the regulatory role of systemic SDF-1 $\alpha$ , we also evaluated if insulin modulated this chemokine in CC. To the best of our knowledge, this is the first study evaluating SDF-1 $\alpha$  in diabetic cavernosal tissue. We observed a decrease in SDF-1 $\alpha$  protein in both diabetes experimental penile tissue and in human samples harvested from men with diabetes [Costa et al., 2009; Angulo et al., 2010]. This alteration was prevented in rat CC when treated with insulin. This observation suggests a possible role for SDF-1 $\alpha$  in penile vascular homeostasis. Given more consistency to this result is the fact that insulin administration prevented the effect of diabetes in SDF-1 $\alpha$  expression. We thus, hypothesized that SDF-1 $\alpha$  may mediate cavernosal endothelial homeostasis by binding to CXCR4 in the endothelium [Carr et al., 2006]. The axis SDF-1 $\alpha$ /CXCR4 may be relevant for the maintenance of penile vascular function, most

likely mediating locally re-endothelialization mechanisms. In sum, we demonstrated the beneficial effects of insulin therapy in preventing the noxious effects of diabetes in vascular function associated with diabetic-ED. Glycemic control was able to promote BM function, reducing levels of CECs, modulating systemic SDF-1 $\alpha$  and testosterone, and regulating penile SDF-1 $\alpha$  protein expression.

We provided evidence of the relevance of early glycemic control in preventing vascular-related alterations in diabetes. The improvement in systemic and cavernosal endothelial health was attained by the amelioration of vascular damage markers, and increase in protective proteins and pathways. These findings highlight the importance of insulin-induced normoglycemia in modulating endothelial mediators and preventing EDys. Ongoing research will unveil if EPCs have the ability to be recruited to injured diabetic penile vasculature and functionally integrate cavernosal endothelium, contributing to endogenous repair.

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