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Semaphorin 7a regulates inflammatory mediators and permeability in retinal endothelial cells

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Abstract

Research supports a key role for inflammation in damaging the retinal vasculature. Current work is designed to investigate regulation of key inflammatory pathways. In this study, we hypothesized that semaphorin 7a (Sema7a) was involved in the increased inflammatory mediators and permeability changes in retinal endothelial cells (REC) grown in high glucose. For these studies, we used diabetic mouse samples and REC to investigate our hypothesis. Primary retinal endothelial cells were grown in normal (5mM) or high glucose (25mM glucose) for measurements. In a subset of cells grown in high glucose, cells were transfected with Sema7a siRNA or scrambled siRNA. We measured levels of key inflammatory mediators and zonula occludens-1 (ZO-1) and occludin levels by Western blot. Data suggest that high glucose increased inflammatory mediators and reduced the tight junction proteins, which follows what is often observed in cells grown in high glucose. Sema7a siRNA significantly decreased inflammatory proteins and increased levels of ZO-1 and occludin. These data suggest that Sema7a mediates the actions of high glucose in REC. Use of Sema7a siRNA may offer a new avenue for treatment.

Keywords

retinal endothelial cell; inflammation; permeability; semaphorin 7a

Introduction.

Inflammation is a key mediator of retinal damage in diabetes [1, 2]. Current studies are often targeted at reduction of these inflammatory mediators. For the present work, we investigated the role of semaphorin 7a (Sema7a) in the regulation of inflammatory pathways in retinal endothelial cells grown in normal and high glucose.

Contributions: Liu performed the permeability experiments, analyzed the data, and edited the final version; Jiang performed the western blot experiments, edited the final version; Steinle designed the experiments, received the funding, and wrote the text.

Conflicts of Interest: No authors have any conflicts of interest with this work.

Semaphorins are a large family of proteins, including both transmembrane and secreted members. Semaphorins were first found to be axonal guidance proteins, but later shown to have numerous roles in angiogenesis and cytoskeletal rearrangements [3]. Semaphorin 7a (Sema7a) has a GPI-anchored domain and has been reported to play a role in artherosclerosis and tumor growth [3]. Others have shown that Sema7a regulates neuronal guidance, as well as functions in the immune system and morphogenesis [4]. In lung injury, Sema7a has been reported to induce expression of cytokines in endothelial cells, mediating an immune response [5, 6]. Studies designed to explore Sema7a in spinal cord injury showed colocalization of Sema7a in the active astrocytes and in the glial scar at day 7 [7].

Sema7a has also been shown to play a key role in ocular disease. In the cornea, Sema7a has been localized in areas of neovascularization, promoting angiogenesis [8]. Other work in the cornea examined levels of Sema7a in naïve cornea and in cornea following lamellar flap surgery. The authors found that Sema7a was constitutively expressed in the cornea and stimulated nerve regeneration after surgery through activation of the immune response [9]. Others have also reported a role for Sema7a in cornea healing through its axon growth properties [10]. In the retina, exosomes from patients with proliferative vitreoretinopathy showed increased levels of Sema7a vs. samples from patients with retinal detachment [11]. However, less has been done in general on Sema7a in the retina when compared to cornea.

While little has been done on retinal endothelial cells, literature does report a role for Sema7a in endothelial cell actions. Studies on neovascularization of atherosclerotic plaques showed that Sema7a was crucial to promoting VEGF actions and progression of atherosclerosis [3]. Further, studies on carotid ligation models showed that Sema7a is key to endothelial-mesenchymal transition (EMT) in vascular disease [12]. Similar results in lung injury showed that Sema7a mediated inflammation and EMT actions [6].

While there is limited knowledge on Sema7a in diabetic retinopathy models, literature does suggest that Sema7a could play a role. It is well established that tight junctions are substantially altered in the diabetic retina [13, 14]. Additionally, inflammation is key to the changes associated with diabetic retinopathy [15–17]. Since Sema7a mediates changes in permeability and inflammation in other targets, we hypothesized that Sema7a will promote increased levels of inflammatory mediators and decrease levels of ZO-1 and occludin in human retinal endothelial cells.

Methods.

Reagents used for the experiments.

The Sema7a siRNA (Cat #SR305559 A, B, C) and scrambled siRNA (SR30004) were purchased from Origene (Rockville, MD). Streptozotocin and RNAiMax were purchased from ThermoFisher. The antibodies used in these experiments were from Abcam (Rockville, MD) unless specified. Antibodies were: Sema7a (ab23578), ZO-1 (ab216880), NLRP3 (ab4207), TNFα (ab1973), IL-1β (ab9722), Nek7 (ab166776), P2X7R (ab109054), ASC (ab70627), occludin (Millipore, SAB5700784), and beta-actin-HRP (Santa Cruz, sc-47778).

Mice.

C57BL/6 mice purchased from Jackson Laboratories. Mice were made diabetic using 5 days of injections of streptozotocin (60mg/kg, I.P). Glucose levels were greater than 250mg/dl. At 6 months of diabetes, mice were sacrificed for analyses as we have done in the past [18]. All mouse experiments were approved by the Wayne State University IACUC committee and adhere to the rules provided by the ARVO animal care groups. Glucose levels and body weights for the mice are in Table 1. Six control and diabetic mice were used for these studies.

Retinal Endothelial cells (REC).

Primary human retinal endothelial cells (REC) were purchased from Cell Systems Corporation (CSC, Kirkland, WA) and grown in normal (5mM) or high (25mM) glucose (CSC, Kirkland, WA). Both normal and high glucose medium was supplemented with microvascular growth factors (MVGS), 10ug/mL gentamycin, and 0.25ug/mL amphotericin B (Invitrogen, Carlsbad, CA). Cells were used prior to passage 6. Cells are grown in their respective glucose conditions for 7 days before experiments. Cells are >~95% confluent before experiments are initiated. All cells were grown in medium without MVGS for 24 hours prior to experiments.

Cell Treatments.

Cells were grown in normal glucose and high glucose. Cells in high glucose were transfected with Sema7a siRNA (3 siRNA from Origene combined) or a scrambled siRNA (Origene) using RNAiMax following the manufacturer's protocol. Briefly, cells were reseeded in dishes the day before transfection. Forty-eight hours after transfection, cells were collected, and protein was extracted and stored in −80C for future analyses.

Western blotting.

REC or whole retinal lysates were collected into lysis buffer containing protease and phosphatase inhibitors. Equal protein amounts were loaded and separated onto precast tris-glycine gels (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane. Membranes were blocked in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% BSA, and incubated with primary antibodies to Sema7a, ZO-1, NLRP3, TNFα, HMGB1, IL-1β, occludin, Nek7, P2X7R, ASC or Beta actin. Images were captured on an Azure C500 (Azure Biosystems, Dublin, CA), and blot data measured using Image Studio Lite software, as we have done previously [19]. Five dishes of cells in each group were used for all experiments.

Statistics.

Prism software 9.0 (GraphPad, La Jolla, CA) was used for statistical analyses. A one-way ANOVA with Tukey's post-hoc test was used for analyses with $P<0.05$ taken as significant. A representative blot is provided for Western blot data.

Results.

Sema7a is increased in diabetic mouse retina and in retinal endothelial cells (REC) grown in high glucose.

The initial finding for this work was that Sema7a was significantly increased in whole retinal lysates from diabetic mice when compared to control mice (Figure 1A). Supporting this finding, high glucose culturing conditions significantly increased Sema7a in retinal endothelial cells (REC, Figure 1B).

Inhibition of Sema7a reduced HMGB1 and TNFα levels in REC grown in high glucose.

We have previously reported that high glucose can increase inflammatory mediators in REC grown in high glucose [20-22]. To determine whether Sema7a might mediate some of these changes, we measured TNFα and HMGB1 in REC grown in high glucose. Figure 2 shows that high glucose significantly increased levels of both HMGB1 and TNFα. When REC grown in high glucose were transfected with Sema7a siRNA, levels of both proteins were significantly reduced (Figure 2). Figure 2A is a control to verify successful knockdown of Sema7a.

Reduced Sema7a levels blocked NLRP3 proteins in REC grown in high glucose.

We have also demonstrated that high glucose activated NLRP3 inflammasome proteins [20, 23]. Figure 3 supports our previous work showing increased NLRP3 (A), ASC1(B), and IL-1β (C) levels in REC grown in high glucose. We also showed increased Nek7(D), and P2X7R (E) in the REC. When these cells were transfected with Sema7a siRNA, all 5 proteins were significantly reduced. This strongly suggests that Sema7a regulates the NLRP3 pathway in REC. Combined with the data in Figure 2, data suggest that Sema7a increased inflammatory pathways.

Sema7a regulated ZO-1 and occludin levels in high glucose.

In addition to inflammatory pathways, increased permeability occurred in response to high glucose culturing conditions [24]. To investigate whether Sema7a altered levels of key proteins involved in permeability, we measured ZO-1 and occludin after Sema7a siRNA. Figures 4A and B show that that high glucose decreased protein levels of ZO-1 and occludin, which were significantly increased in REC transfected with Sema7a siRNA, suggesting that Sema7a promotes increased permeability in REC. These data support a role for siRNA as a therapeutic for retinal permeability.

Discussion.

Diabetic retinopathy has been associated with increased inflammation [15]. While less has been done in the retina, Sema7a has been associated with corneal wound healing [10]. We found that Sema7a promoted inflammatory mediators, specifically NLRP3 pathway proteins. We show that that Sema7a induced inflammation, since knockdown resulted in a significant reduction of HMGB1 levels, as well as NLRP3 pathway proteins in the retina. Our findings match those in microglial cells that showing that knockdown of Sema7a reduced apoptosis and inflammation [25]. Others have reported that Sema7a

may play a critical role in metabolic programming in macrophages [4]. Similar to the retina, Sema7a induced inflammation in lung endo- and epithelial cells [6]. Sema7a also caused inflammation in myocardial infarction through activation of the platelet receptor glycoprotein 1b [26]. Thus, our data from retina matches data from lung and heart injury showing that Sema7a induced inflammatory pathways. Our data add to the literature that development of siRNA against Sema7a may offer a new therapeutic target. We also show that the retina findings are similar to other vascular beds.

In addition to inflammatory changes, a study showed that Sema7a caused increased blood brain barrier permeability by the West Nile virus [27]. Others have shown a role for Sema7a in EMT transition, which can be linked to changes in permeability [12]. We found that Sema7a siRNA significantly increased protein levels of ZO-1 and occludin, suggesting that Sema7a induced retinal permeability. Our findings match data in acute lung injury induced by seawater aspiration [28]. Taken together with inflammation, it appears that Sema7a promotes deleterious changes in retinal endothelial cells that are linked to diabetic retinopathy. Further work needs to be done in diabetic animal models to expand these early findings.

The use of high glucose as a stimulus for cells to mimic diabetic-like conditions can be controversial. It is by far the most widely used model for diabetic retinopathy [29-32]. However, some groups feel that activation of REC with inflammatory mediators is more accurate for diabetic retinopathy work [33]. Because our goal was to directly measure inflammatory mediators, we chose to use high glucose as our inflammatory stimuli. To better mimic diabetic retinal disease, we will expand these findings into diabetic mouse models in the future.

This study has specific limitations. We did not perform the in vitro permeability assay, as it is very difficult to perform on cells treated with siRNA, as dextran flux requires 100% confluence, which is counter to the siRNA transfection. We did attempt to treat cells with Sema7a/Fc, but were unable to get a reproducible dose-response curve in the REC. This study also only presents results in from one cell type in culture, which does not represent the entire retina. We will expand into animal models in future work.

In conclusion, data in retinal endothelial cells suggest that Sema7a promotes inflammation and permeability damage in response to high glucose. Based upon this data, development of the Sema7a siRNA into therapeutics offers a new avenue for treatment.

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Highlights

- Semaphorin 7a is increased in the diabetic mouse retina
- siRNA against Sema 7a reduced inflammatory mediators in retinal endothelial cells grown in high glucose
- Sema7a siRNA increased occludin and ZO-1 levels in REC.

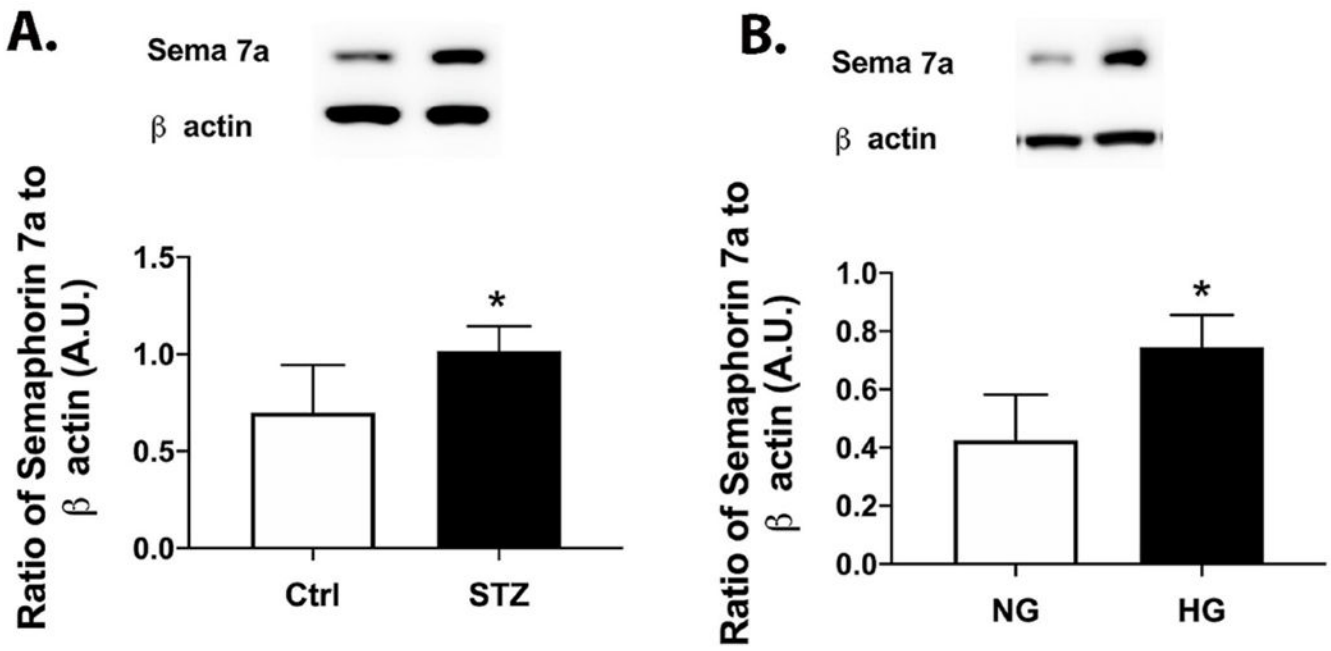

Figure 1.

Sema7a is increased in diabetic mice and retinal endothelial cells grown in high glucose. Western blot results for Sema7a from control (Ctrl) and diabetic (STZ) mice (Panel A) and from retinal endothelial cells (REC) grown in normal (NG) or high glucose (HG). *P<0.05 vs. NG or Ctrl. N=6 for mice and N=5 for cells.

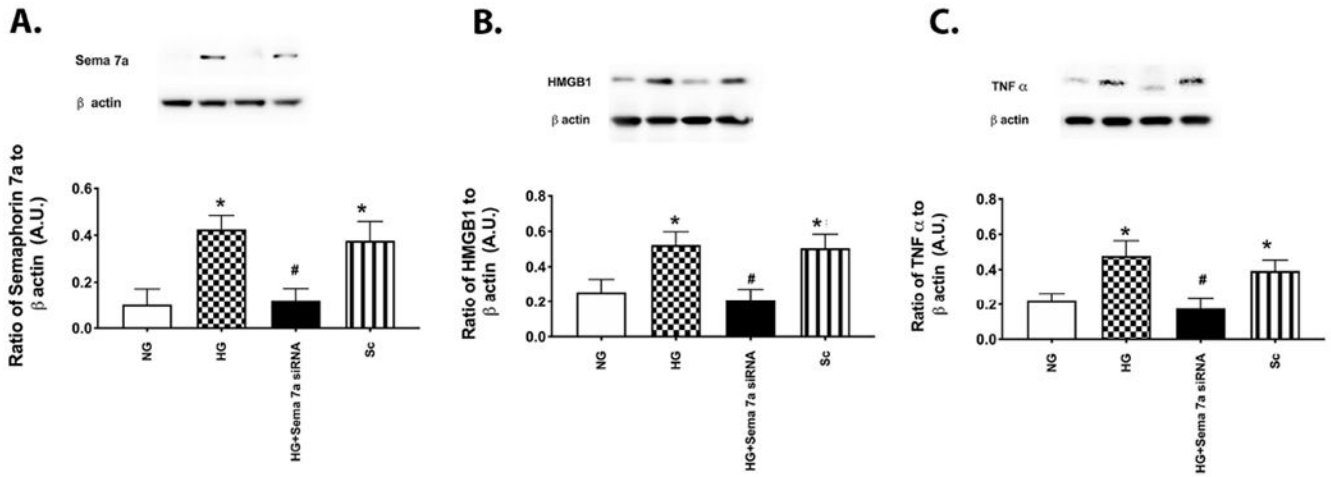

Figure 2.

Sema7a siRNA reduced inflammatory mediators. Western blot results for Sema7a (A), HMGB1 (B), and TNFα (C) in retinal endothelial cells grown in normal glucose (NG), high glucose (HG) or high glucose and transfected with Sema7a siRNA (HG+Sema7a siRNA) or scrambled siRNA (Sc). *P<0.05 vs. NG, #P<0.05 vs HG. Data are mean ±SEM. N=5.

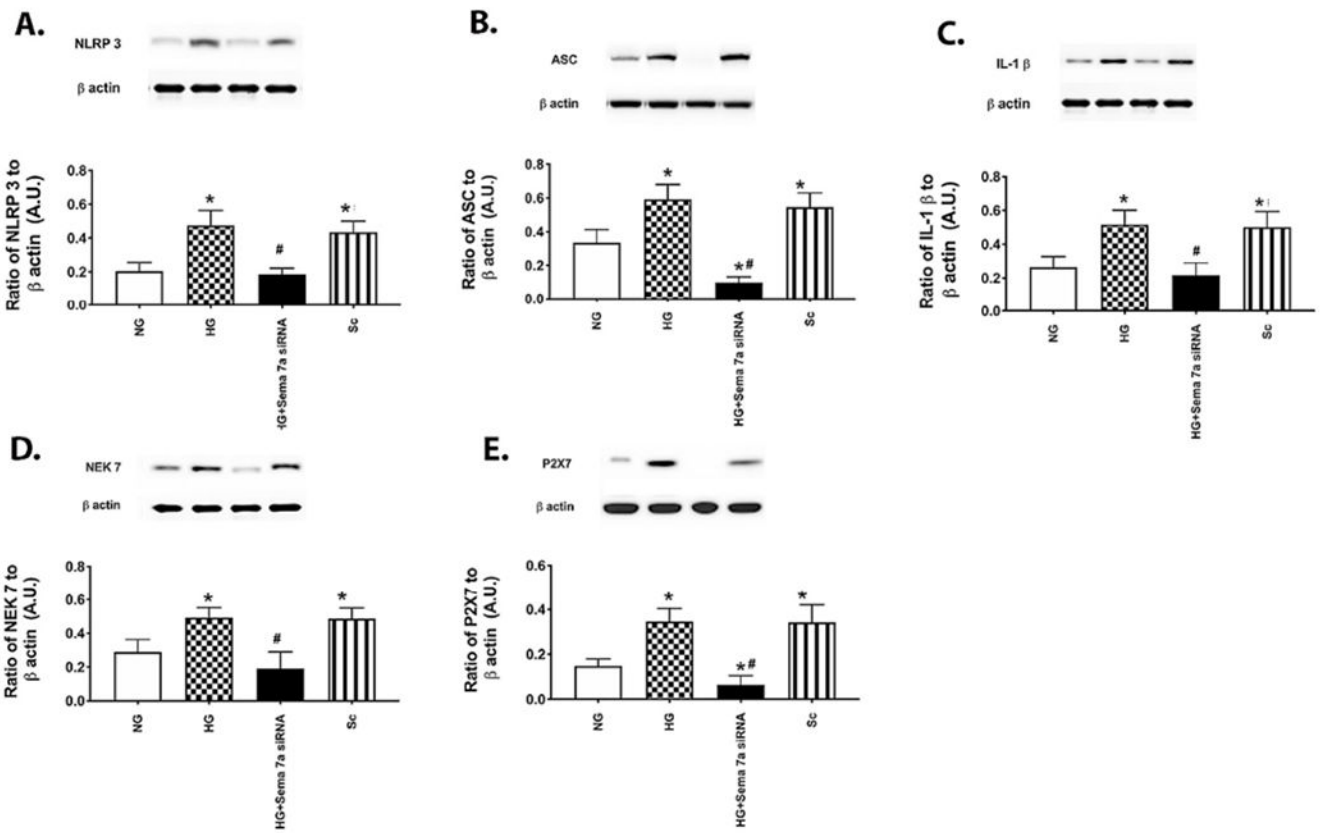

Figure 3.

Sema7a regulated NLRP3 proteins. Retinal endothelial cells were grown in normal glucose (NG), high glucose (HG) or high glucose and transfected with Sema7a siRNA (HG+Sema7a siRNA) or scrambled siRNA (Sc). Western blot results for NLRP3 (A), ASC (B), IL-1β (C), Nek7 (D) and P2X7R (E). *P<0.05 vs. NG, #P<0.05 vs HG. Data are mean ±SEM. N=5.

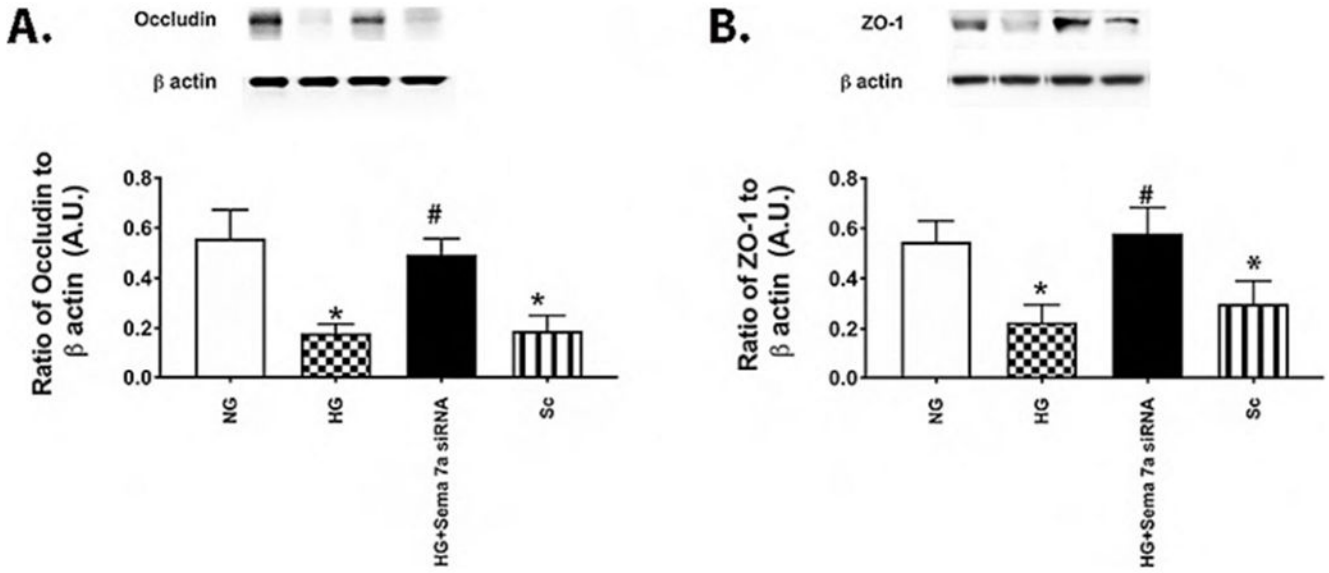

Figure 4.

Sema7a regulated permeability proteins. Retinal endothelial cells were grown in normal glucose (NG), high glucose (HG) or high glucose and transfected with Sema7a siRNA (HG+Sema7a siRNA) or scrambled siRNA (Sc). Western blot was done for occludin (A) and ZO-1 (B). *P<0.05 vs. NG, # P<0.05 vs. HG. Data are mean ±SEM. N=5.

Table 1.

Data represent mean ± SD;

	n	body weight (g)	Blood Glucose (mg/dL)
Ctl 6 mon	5	29±1.3	129±6
STZ 6 mon	5	19.5± 2.5*	509±49*

* $p < 0.05$ v.s control