

# Retinal and Renal Vascular Permeability Changes Caused by Stem Cell Stimulation in Alloxan-induced Diabetic Rats, Measured by Extravasation of Fluorescein

Z. DOCZI-KERESZTESI<sup>1</sup>, J. JUNG<sup>2</sup>, I. KISS<sup>3</sup>, T. MEZEI<sup>2</sup>, L. SZABO<sup>4</sup> and I. EMBER<sup>3</sup>

Departments of <sup>1</sup>Pharmacology and <sup>2</sup>Pathology, University of Medicine and Pharmacy, Targu Mures, Romania;

<sup>3</sup>Department of Public Health, University of Pecs, Hungary;

<sup>4</sup>Crystal Institute KFT, Eger, Hungary

**Abstract.** *Aim: To determine whether treatment with the stem cell stimulator Olimpiq<sup>®</sup> Stem×Cell prevents increase of retinal and renal vascular permeability in alloxan-induced diabetic rats. Materials and Methods: Two groups of Wistar rats were made diabetic by single intraperitoneal injection of Alloxan. The third, the control group, received vehicle alone. One diabetic group received Olimpiq<sup>®</sup> Stem×Cell treatment for 4 weeks. The permeability of the blood–retinal barrier (BRB) and renal vessels were measured by the extravasation of fluorescein–labeled bovine serum albumin. Results: Six weeks subsequently to Alloxan injection, significantly elevated the tissue fluorescence, the renal vascular leakage and BRB breakdown was demonstrated in the diabetic group, compared to the nondiabetic group. Olimpiq<sup>®</sup> Stem×Cell treatment significantly reduced the BRB breakdown, tissue fluorescence, and vascular leakage. Conclusion: Olimpiq<sup>®</sup> Stem×Cell would be a useful choice of treatment for complications associated with increased vascular permeability of diabetes, such as retinopathy or nephropathy.*

Diabetes mellitus is currently a major global epidemiological problem due to the high number of people involved. In 2009 the World Health Organization (WHO) estimated this number at 170 million worldwide, a figure expected to rise to 366 million by 2030 (1). Nevertheless, in August 2011, WHO published a figure of 346 million diagnosed cases. Diabetes kills more than 1 million people annually, out of which almost 80% of cases occur in low- and middle-income countries (1-3).

*Correspondence to:* Zoltan Doczi-Keresztesi, Department of Pharmacology, University of Medicine and Pharmacy, Gheorghe Marinescu str. 38, 540139 Targu Mures, Romania. E-mail: md.doczi@gmail.com

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Diabetes is the most common cause of retinopathy, causing serious visual impairment and blindness. There are a great number of studies confirming that 15 years after the onset of diabetes, 2% of the patients become blind and 10% suffer serious visual impairment (4). About 20-30% of diabetic patients suffer from nephropathy, which can lead to chronic renal failure, eventually requiring dialysis. Diabetes mellitus is the most common cause of kidney failure in adults worldwide in developed countries, causing death in 20% of cases (2, 5).

The purpose of our study was to find new and more efficient means for the treatment of vascular alteration–based complications in diabetes.

The breakdown of the blood–retinal barrier (BRB) is the most characteristic change in diabetic retinopathy, and is responsible for macular edema, the most common cause of visual morbidity in diabetic patients. The breakdown of the BRB is characteristic of early stages of vascular dysfunctions in both human and experimental diabetes (3, 6-8).

The BRB is located at two levels, forming an outer barrier, the retinal pigment epithelium, and an inner barrier, the endothelium of the retinal vessels. Both structures have tight, nonleaky-type junctions, without fenestrations, while the thyroid and renal vessels have very thin endothelial cells with numerous cytoplasmic discontinuities with very thin and irregular basement membrane. The structure of the retinal capillaries is very similar to the one of brain capillaries (9, 10).

In diabetes, the basic morphological change affecting the BRB is related to the incompetent endothelial junctions of retinal vessels. The basal lamina is thickened, pericytes show degenerative changes and the endothelial cytoplasm focally forms fenestrae. The diameter of the fenestrae varies between 50-60 nm, and each is closed by a thin diaphragm. It was also demonstrated that fenestrae with diaphragms can form transendothelial channels. Endothelial fenestration and transendothelial channels are related to high permeability for water and allow passage of electrolytes and large molecules (11).

Multiple interrelated biochemical mechanisms have been proposed to explain the adverse effects of diabetes and hyperglycemia on vascular cells: increased flux of glucose through the polyol pathway, enhanced nonenzymatic glycation, accumulation of advanced glycation end products, activation of protein kinase C and increased formation of reactive oxygen species (ROS), including nitric oxide (NO), superoxide ( $O_2^-$ ) and their product peroxynitrite ( $ONOO^-$ ) (12, 13).

The initial BRB breakdown is associated with increase in expression of both the endothelial and neuronal nitric oxide synthase (eNOS and nNOS), as well as with increase in the vascular endothelial growth factor (VEGF) expression. Increased formation of ROS and lipid peroxides occurs early in diabetic retinopathy (6, 14-16). However, the relationship between morphological and biochemical changes in the structure of the retinal vessels in diabetes is not fully understood.

BRB breakdown has been demonstrated by fluorescein angiography and vitreous fluorophotometry in diabetic humans (17) and rats (18). Vinos *et al.* (19, 20) reported that immunohistochemical staining for albumin was useful in localizing BRB breakdown in retinas from human diabetics, due to the fact that albumin is one of the serum proteins whose extravascular localization signifies BRB breakdown (21, 22).

There are two experimental models which best simulate type I diabetes: in one of the models, the pancreatic beta cells are pharmacologically destroyed with pancreatic beta cell-toxic substances such as streptozotocin (8, 18) or alloxan (23); the other model is the non-obese diabetic (NOD) mouse strain (24). Type II diabetes can be simulated by genetically modified mouse strains with high insulin resistance and also nongenetic models (25).

Recently, promising results have been published on the beneficial effect of cytokine-induced stem cell mobilization in conditions such as kidney failure (26), bone fracture (27), cerebral ischemia (28), and various neurological diseases (29). Granulocyte colony-stimulating factor (G-CSF) or stem cell factor-induced increase in the number of circulating stem cells has been found to be beneficial in myocardial infarction (30). Bone marrow stem cells have been found to differentiate into several cell types such as cardiac muscle cells (31), neurons (32) and liver cells (33). The regeneration and functional improvement of the pancreas and formation of beta cells after local or systemic application of stem cells in beta-cell destruction-based experimental diabetes is also under investigation (33-36). The retinal regenerative effect of stem-cell stimulation has not been reported yet.

The complex mixture Olimpiq Stem×Cell SL<sup>®</sup> (Crystal Institute Ltd., Eger, Hungary), which is a dietary supplement, was found to affect the variation of the number of circulating CD34± cells (37). The mixture contains extracts from

*Ganoderma lucidum* and *Lycium barbarum*, Fucoidan, Black cohosh (*Cimicifuda racemosa*), hemp seed germs, aphanizomenon flos-aquae (AFA) algae, chlorophyll and corn silk (36, 38).

These components are able to increase production of adult stem cells from bone marrow. After leaving the bone marrow, stem cells reach the bloodstream, and are then transported by a specific messenger to the affected organ. After reaching the target organ, stem cells multiply and transform into tissue-specific cells. As a result of this process, the organ will be renewed, regaining health as well as its specific functions (35-40).

Several studies confirm that stem cell mobilization, or stimulation of stem cell production is a veritable possibility to achieve health benefits and can be used with preventive aim in the case of several chronic degenerative diseases (41-43).

Olimpiq Stem×Cell SL<sup>®</sup> is specifically designed for the treatment of diabetes mellitus. Its active components seem to have a role in improving the lives of diabetic patients and in ensuring balanced blood sugar levels, also preventing the occurrence of complications in both types of diabetes and improving the regenerative capacity of the body (34, 36, 38, 40, 42).

## Materials and Methods

All experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments.

Male Wistar rats (250-300 g, supplied by University of Szeged, Faculty of Medicine, Laboratory Animal Facility of Domaszék, 6781 Domaszék, Tanya str. 701, Hungary) were divided into three groups with similar distributions of blood glucose and body weight. Two groups received a single intraperitoneal injection of Alloxan (125 mg/kg – Alloxan monohydrate; Alfa Aesar GmbH & Co KG, in 0.5 mM citrate buffer, pH=4.5) and the remaining control group received vehicle. After 24 hours, the blood sugar was measured and the rats were considered to be diabetic when the fasting blood sugar exceeded 13.7 mmol/l. The blood glucose level was measured weekly using an automated blood-glucometer (Accu-Chek<sup>®</sup> – Roche Diagnostics GmbH Sandhofer Strasse 116, 68305 Mannheim Germany) with blood samples obtained by tail vein puncture. Bodyweight was also measured at the beginning and at the end of the experiment.

Drug treatment was administered *via* food intake and initiated 2 weeks after the Alloxan injection. One group of diabetic rats was treated with Olimpiq Stem×Cell SL<sup>®</sup> (Crystal Institute Kft. 3300 Eger, Faiskola str. 11, Hungary) for 4 weeks (7.14 mg/kg/day dose, calculated for each rat, according to the recommendation of the producer company, administered as a mixture in standard lab chow), whereas the other two groups were fed with standard lab chow.

The blood glucose level (weekly), body weight and fluorescein extravasation (at the end of the experiment, on day forty-two) were measured.

The permeability of the BRB was measured by a partially modified method described by Antonetti *et al.* (21). We compared, for the first time, the permeability of retinal and renal vessels.

After general anesthesia with pentobarbital, 100 mg/kg bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA; Albumin–fluorescein isothiocyanate conjugate, A9771, Sigma-Aldrich Chemie GmbH, Riedstrasse 2, 89555 Steinheim, Germany) was injected *via* the tail vein. Ten minutes after being injected the animals were decapitated, and after eye enucleation ( $n=3 \times 10$ ) and abdominal dissection, retinal and renal tissue sampling was carried out. At the time of death, 3 ml of blood was collected in EDTA tubes. Tissue samples were placed in buffered formalin solution, (4% concentration and  $\text{pH}=7$ ) for further histological processing.

The collected blood samples were centrifuged at  $2000 \times g$  for 10 minutes; the supernatant plasma was assayed with a UV-160°-Shimadzu fluorescence spectrophotometer with excitation at  $433 \pm 2$  nm and emission at  $455 \pm 2$  nm.

Tissue samples were processed as follows: The eyes were sectioned in a horizontal plane to the optic nerve, fixed with agar and embedded in paraffin. Then three, 4-micron-thick sections were made at 80-micron intervals followed by hematoxylin-eosin staining. The kidney was sectioned correspondingly, along a horizontal plane at the surfacing of renal arteries, followed by three sections with the same features described above.

Samples were examined with a fluorescence microscope fitted with a spot camera (Nikon Eclipse E800, DN100 Network Camera). Images were analyzed by using Image J (a public domain Java image processing program, developed by Wayne Rasband, at the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) at 10 different retinal areas ( $200 \mu\text{m}^2$ ) in each section (2 avascular spots, at 5 different retinal layers: external nuclear, external plexiform, internal nuclear, internal plexiform and ganglionar). The renal sample images were analyzed using similar techniques, both in the glomerular (5 areas) and the tubular areas (5 areas). The average retinal and renal fluorescence intensity was calculated and normalized to the fluorescence of the plasma for each animal using the formula:

$$\frac{\text{Tf1}+\text{Tf2}+\dots+\text{Tf10}}{\text{Pf} \times 10} \quad (\text{Tf}=\text{tissue fluorescence, Pf}=\text{plasma fluorescence}).$$

Statistical analysis was carried out with the GraphPad Prism 5.01/2007 (GraphPad Software, Inc.), using one way Anova and Tukey's multiple comparison tests.

## Results

Retinal fluorescence presented differences in the studied groups. Figure 1 captures are taken with the spot camera of the fluorescence microscope. The five retinal layers can be seen on from right to left. The difference in the tissue fluorescence between the three groups can be clearly observed.

Renal tissue fluorescence showed similar differences among the groups. Figure 2 represents images taken with the same camera, in different glomerular and tubular areas.

The mean tissue fluorescence, assayed with image J, was significantly different between groups. The control, non-diabetic and the treated diabetic group presented significantly lower fluorescence intensity compared to the untreated

diabetic group, with no significant difference between non-diabetic and untreated diabetic groups. Figure 3 A represents the comparative retinal and Figure 3 B the comparative renal fluorescence, normalized to the plasma fluorescence.

The correlation between the retinal and the renal tissue fluorescence was positive and significant (Figure 4).

The control group presented nonsignificant changes in glucose values, whereas both diabetic groups presented decreasing values. Figure 5 represents glycemia changes during the experiment.

Differences between the untreated and the treated group were not statistically significant.

Figure 6 compares the obtained values of glycemia among these groups.

At the end of the experiment, the body weight was increased in the control nondiabetic group, decreased in the diabetic treated group, and slightly decreased in the non-treated diabetic group, compared to the initial weight, without statistically significant differences among the studied groups (Figure 7).

## Discussion

This evaluation mainly focuses on the role of general stem cell stimulation therapy, especially in the management of diabetic retinopathy and nephropathy.

We demonstrate for the first time that stem cell stimulation can reduce BRB breakdown in the retinal tissue of diabetic rats, and compare vascular permeability changes in retinal and renal tissues for the first time. We used an easily reproducible technique that was suitable to demonstrate the effect of different pharmacologically active substances on vascular permeability changes.

There is evidence of morphological (10, 11), and biochemical (12, 14) changes in the structure of the retinal vessels in diabetes, although the relationship between them is not fully understood. The formation of endothelial fenestrations and channels can be determined by the increased formation of ROS, triggered by increased flux of glucose through the polyol pathway, enhanced nonenzymatic glycation, accumulation of advanced glycation endproducts and activation of protein kinase C. These changes appear early in diabetes and are responsible for the increase in vascular permeability. The VEGF increase is probably responsible for later changes in diabetes, such as proliferation and neovascularization, which were not the subject of this study.

Long term Olimpiq® Stem×Cell SL treatment significantly reduced renal and retinal tissue fluorescence, vascular leakage and BRB breakdown, compared to the control and untreated groups. Theoretically, there are two mechanisms that can induce these changes: vascular structural regeneration through stem cell activation and lower glucose values through the



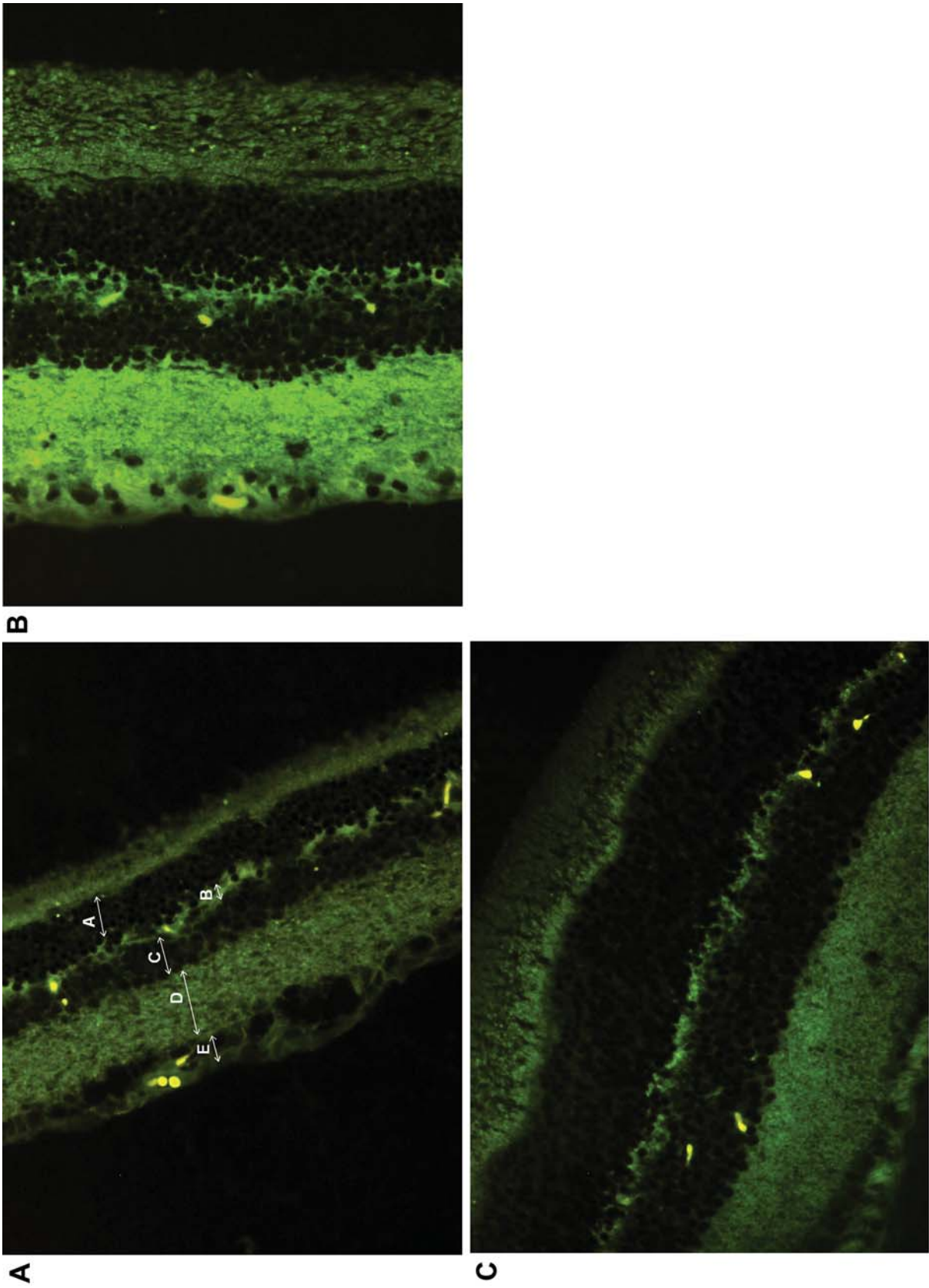


Figure 1. Retinal fluorescence in the nondiabetic (A), untreated diabetic (B) and treated diabetic (C) groups. 1 A, A=external nuclear, B=external plexiform, C=internal nuclear, D=internal plexiform and E=ganglionar (gangl.).

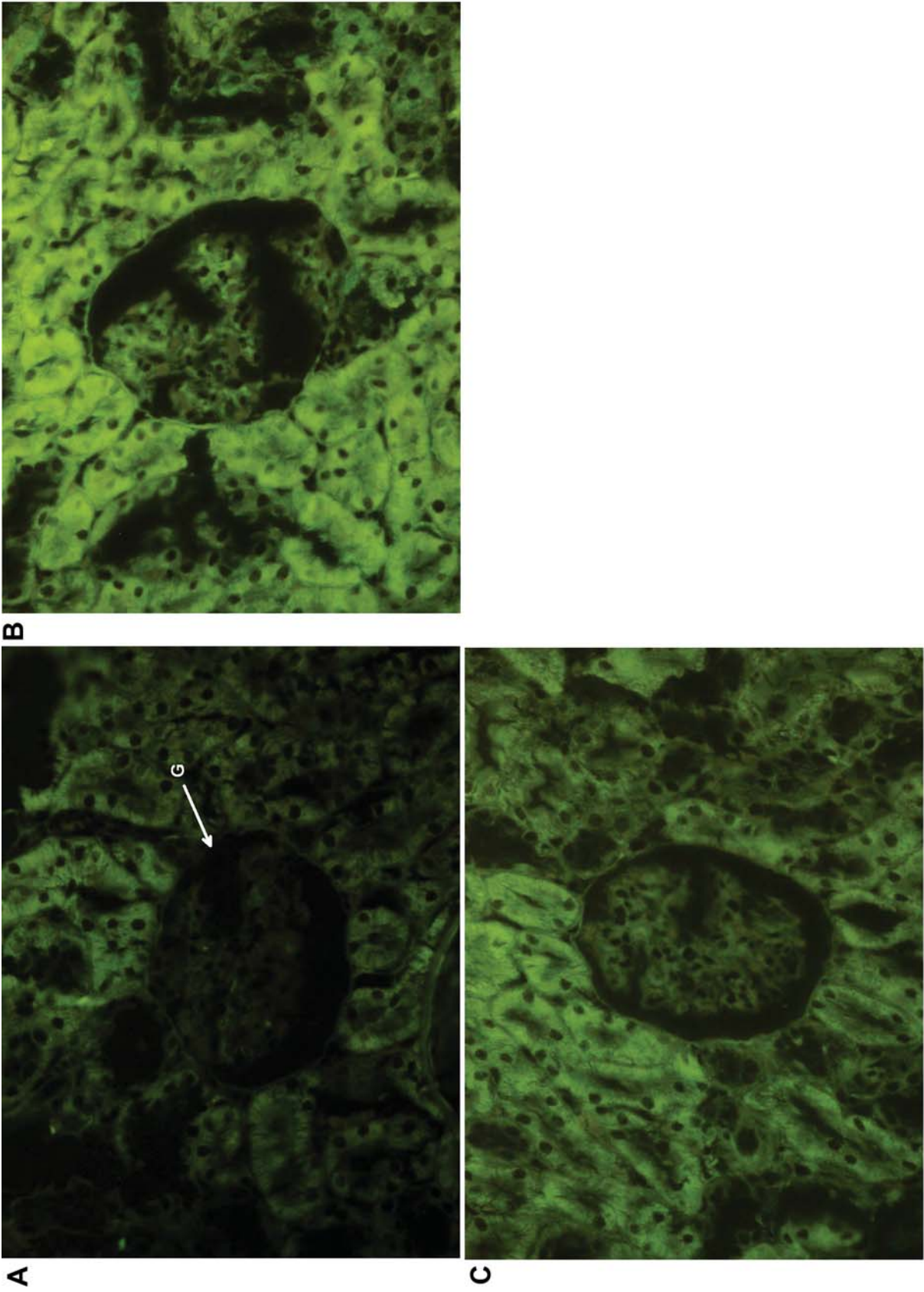
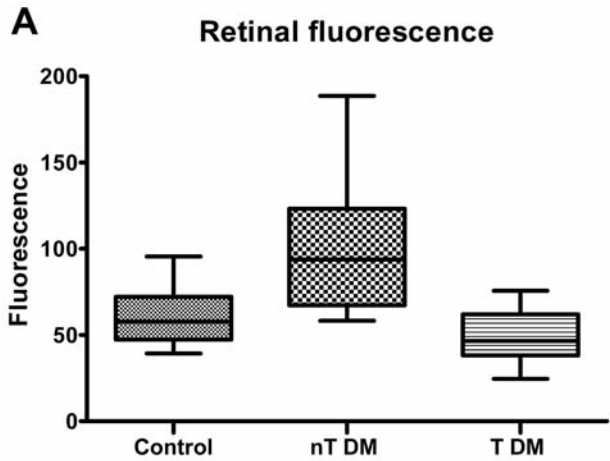
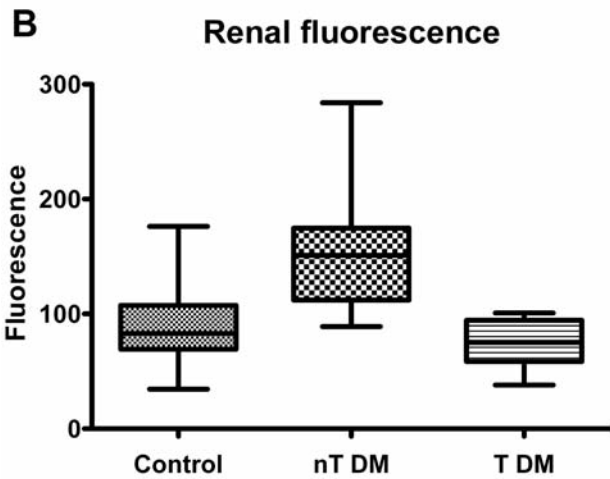


Figure 2. Renal fluorescence in the nondiabetic (A), untreated diabetic (B) and treated diabetic (C) groups. G=glomerulus.



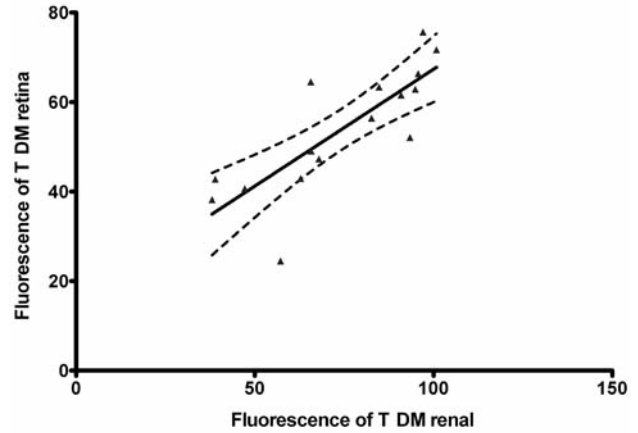
Tukey's Multiple Comparison Test	Mean Diff.	q	p<0.005
Control vs. nT DM	-41.44	8.501	Yes
Control vs. T DM	10.58	2.171	No
nT DM vs. T DM	52.03	10.67	Yes



Tukey's Multiple Comparison Test	Mean Diff.	q	p<0.005
Control vs. nT DM	-65.78	8.278	Yes
Control vs. T DM	14.91	1.877	No
nT DM vs. T DM	80.69	10.15	Yes

Figure 3. Comparison of retinal (A) and renal (B) fluorescence among groups. nT DM, untreated diabetic; T DM, treated diabetic.

regeneration of insulin-secreting cells. Gradually decreasing glycemia was demonstrated in all groups, with no statistically significant differences between them, therefore the regenerative mechanism of insulin-secreting cells was not demonstrated in this experiment. However, there is evidence of a glycemia-normalizing effect of stem cell stimulating therapy in experimental diabetes (36).



Pearson r	0.8035
95% confidence interval	0.5114 to 0.9291
p-value (two-tailed)	0.0002
p-value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.6456

Figure 4. Correlation between renal and retinal tissue fluorescence in the treated diabetic group.

The mechanism for permeability decrease by stem cell stimulation seems to be the structural recovery of the vascular endothelium, both in retinal and renal tissues.

### Conclusion

The results of this study suggest that Olimpiq® Stem×Cell SL is useful for the treatment of diabetic complications such as nephropathy and retinopathy, associated with BRB leakage, such as diabetic macular edema, exudative retinopathy and hemorrhage. Fluorescein extravasation in different tissues is an appropriate technique to demonstrate the effect of different pharmacologically active substances on vascular permeability changes which appear in early stages of diabetes.

### Conflicts of Interest

The Authors declare no potential conflicts of interest.

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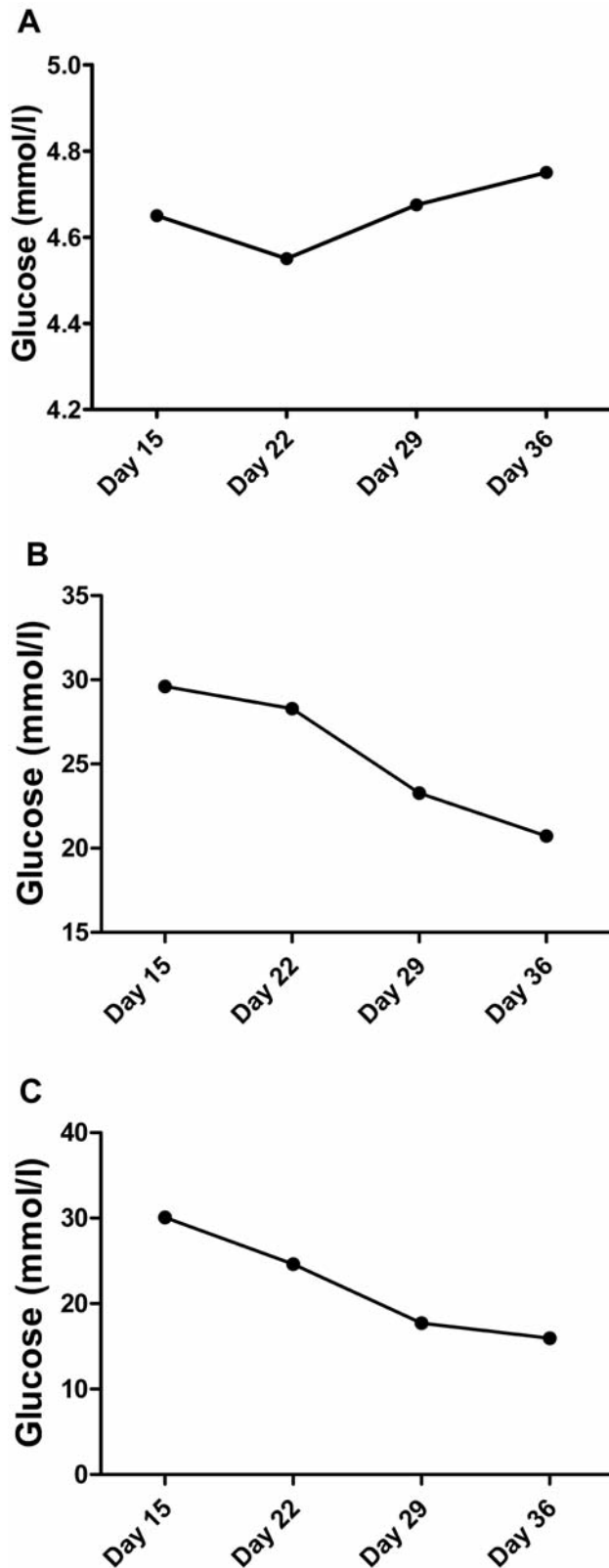
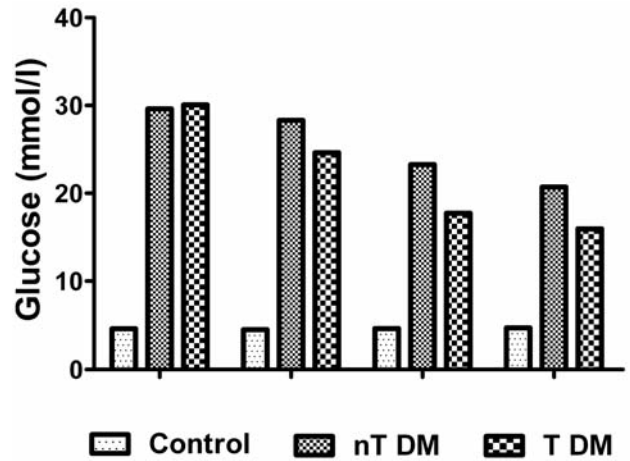
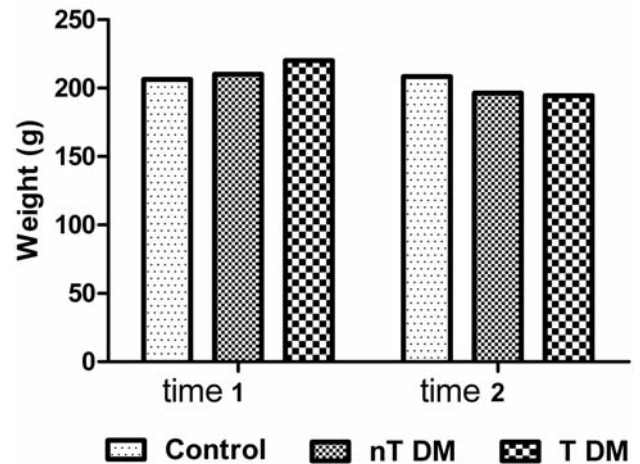


Figure 5. Average glucose values in the nondiabetic (A), untreated diabetic (B) and treated diabetic (C) groups.



Tukey's Multiple Comparison Test	Mean Diff.	q	p<0.005
Control vs. nT DM	-20.819	12.351	Yes
Control vs. T DM	-17.438	10.345	Yes
nT DM vs. T DM	3.3813	2.006	No

Figure 6. Comparison of obtained glycemia values among groups.



Tukey's Multiple Comparison Test	Mean Diff.	q	p<0.005
Control vs. nT DM	4.250	0.6102	No
Control vs. T DM	0.125	0.01795	No
nT DM vs. T DM	-4.125	0.5923	No

Figure 7. Comparison of body weight alterations.

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