

# Vascular Permeability Changes in Experimental Diabetic Retinopathy, Under the Effect of Stem Cell Stimulation, in Rats

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**Objective:** The purpose of this experiment was to determine the long-term effect of stem cell stimulator Olimpiq® StemXCell treatment on retinal vascular permeability and breakdown of the blood-retinal barrier (BRB) in alloxan-induced diabetic rats.

**Methods:** Male Whistar rats were divided into three groups. Two groups received a single intraperitoneal injection of Alloxan (125 mg/kg) – a specifically pancreatic beta cell-toxic substance, and the other control group received vehicle. The Alloxan-induced diabetic rats were treated with Olimpiq® StemXCell SL for 4 weeks, whereas controls were fed with standard lab chow. Permeability of blood-retinal barrier was measured by the extravasation of fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA).

**Results:** Six weeks subsequently to Alloxan treatment, a significantly elevated tissue fluorescence, vascular leakage, and BRB breakdown could be demonstrated in the diabetic group, compared to the non-diabetic group. Olimpiq® StemXCell SL treatment significantly decreased the BRB breakdown, tissue fluorescence, and vascular leakage, compared with the control, non-treated group. Long-term Olimpiq® StemXCell SL treatment significantly decreased tissue fluorescence, vascular leakage, and the BRB breakdown. The mechanism for these effects may involve retinal vascular regeneration induced by stem cell stimulation. Blood glucose values were decreasing gradually, without significant differences between groups, therefore insulin secreting beta cell regeneration could not be demonstrated.

**Conclusions:** The results suggest that Olimpiq® StemXCell SL would be useful for treatment of ocular diseases associated with BRB leakage, such as diabetic macular edema and retinopathy.

**Keywords:** diabetic retinopathy, experimental diabetes, alloxan, FITC-BSA, stem cell stimulation

## Introduction

Diabetes mellitus (DM) is currently a major global epidemiological problem due to the high number of people involved: approximately 7,000,000 new patients are registered annually worldwide [1].

At this point, worldwide about 246 million diabetic patients are diagnosed, mostly suffering from type 2 diabetes. By 2025 this number is expected to grow to 380 million, representing 7.1% of the adult population of the planet.

In Romania, the prevalence of diabetes reaches 4.2% of the total population. According to the Eurodiab study, the incidence of diabetes for the age group of 0–14 years in our country, is approximately 3/100.000 people. According to recorded data provided by the Diabetes Mellitus Center in Mures County, the number of diabetic patients are (until 2009) [2]:

- ▶ 8794 patients with DM;
- ▶ 1271 insulin dependent diabetes (type I DM);
- ▶ 7523 insulin independent diabetes (type II DM).

These data suggest that diabetes and its complications demand both more effective and less expensive biological treatments.

Breakdown of the blood-retinal barrier (BRB) is the most characteristic change in diabetic retinopathy, and is responsible for macular oedema, the most common cause of visual morbidity in diabetic patients [3].

The BRB is located on two levels, forming an outer barrier in the retinal pigment epithelium and an inner barrier in

the endothelial membrane of the retinal vessels. Both these membranes have tight, “non-leaky” type junctions, without fenestrations however the thyroid and kidney vessels have very thin endothelial cells with numerous cytoplasmic discontinuities and the basement membrane is also very thin and irregular. The structure of the retinal capillaries is very similar to that of the brain capillaries [4,5].

BRB breakdown has been demonstrated by fluorescein angiography and vitreous fluorophotometry in diabetic humans [6,7] and rats [8]. Vinoses et al. [9,10] reported that immunohistochemical staining for albumin was useful in localising BRB breakdown in human diabetic retinas because, as albumin is one of the serum proteins, its extravascular localisation signifies breakdown of the BRB [11,12].

Recent studies in streptozotocin (STZ) diabetic rats have shown that 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 increases in VEGF expression [13,14,15].

However, the mechanisms by which diabetes increases VEGF expression and causes BRB breakdown are not yet understood.

Recently, promising results have been published on the beneficial effect of G-CSF (granulocyte colony-stimulating factor) or stem cell factor-induced increase in the number of circulating stem cells in mice or in human patients with myocardial infarction [16]. Cytokine-induced stem cell

mobilization has been found to be beneficial in other conditions like: kidney failure [17], bone fracture [18], cerebral ischemia [19], and various neurological diseases [20].

Bone marrow stem cells have been found to differentiate into several cell types like cardiac muscle cells [21], neurons, [22], liver cells [23] etc. Regeneration of the pancreas and formation of beta-cells after local or systemic application of stem cells is also under investigation [24]. There haven't been published any results yet, on retinal regenerative effect of stem-cell stimulation.

Several authors have hypothesized that modulation of stem cell number or their properties represents the background mechanism of the effect of diet on aging or carcinogenesis [25,26,27].

A complex mixture (Olimpiq® StemXCell [Crystal Institute Ltd., Eger, Hungary], a dietary supplement) was found to statistically significantly increase the number of circulating CD34+ cells [28]. There is also evidence of certain benefits in functional improvement of the pancreas in beta-cell destruction-based experimental diabetes [29,30,31].

Several studies confirm that stem cell mobilization or stem cell production enhancement through dietary means is a realistic possibility to achieve health benefits. It also seems very reasonable that chronic, moderate stimulation of stem cell production will result in similar effects and might be preventive against several chronic degenerative diseases, in accordance with the experience of traditional herbal medicine [29].

Olimpiq® StemXCell SL is a product variant of Olimpiq® StemXcell, specifically designed for diabetes mellitus treatment. Its active components seem to have a role in improving the lives of people with diabetes and ensure balanced blood sugar levels, preventing also the occurrence of complications in both types of diabetes, improving the body's regenerative capacity [24,26,29,32,33,34].

## Material and methods

Male Whistar rats (250–300 g) 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) – a specifically pancreatic beta cell-toxic substance (Alloxan monohydrate, Alfa Aesar GmbH & Co KG), and the remaining control group received vehicle. After 24 hours, blood sugar was measured and rats were considered to be diabetic when the fasting blood sugar exceeded 13.7 mmol/l. Blood glucose was measured weekly using an automated blood-glucometer (Accu-Chek® – Roche, Darmstadt, Germany) with blood obtained by tail vein puncture. Bodyweight was also measured at the beginning and end of the experiment.

Drug treatment administered via food intake was initiated 2 weeks later. The Alloxan-induced diabetic rats were treated with Olimpiq® StemXCell (Crystal Institute Ltd., Eger, Hungary) for 4 weeks (7.14 mg/kg/day dose, calcu-

lated for each rat, based on the recommendation of the producer company), whereas controls were fed with standard lab chow.

Blood glucose (weekly), weight and fluorescein extravasation were then measured.

Permeability of blood-retinal barrier was measured by the method described by Antonetti et al. [11], partially modified.

After general anesthesia with pentobarbital, 100 mg/kg bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA – Sigma-Aldrich) was injected via the tail vein. After 10 minutes, the animals were decapitated, and after eye enucleation (n = 3×10) retinal 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, (pH = 7, 4% concentration) for further histological processing.

Collected blood samples were processed by 2000 G centrifugation for 10 minutes; the supernatant plasma was assayed with a UV-160°-Shimadzu fluorescence spectrophotometer with excitation at 433±2 nm and emission at 455±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.

Samples were viewed with a fluorescence microscope fitted with a spot camera (Nikon Eclipse E800, DN100 Network Camera). Images were analyzed using Image J, at 10 different retinal areas (200 µm<sup>2</sup>) in each section (2 avascular spots, at 5 different retinal layers: external nuclear, external plexiform, internal nuclear, internal plexiform and ganglionar). The average retinal fluorescence intensity was calculated and normalized to the plasma fluorescence intensity for each animal using the formula:

$$\frac{(Tf_1+Tf_2+\dots+Tf_{10})}{Pf \times 10}$$

Tf = tissue fluorescence  
Pf = plasma fluorescence

Statistical analysis was made with GraphPad Prism, using one-way Anova and Tukey's multiple comparison test.

## Results

Retinal fluorescence was significantly higher in the untreated diabetic group. Figure 1 represents the non-diabetic group, Figure 2 the non-treated diabetic and Figure 3 the treated diabetic.

The fluorescence, assayed with image J, was significantly different between groups. The control non-diabetic and the treated diabetic group presented lower fluorescence intensity, with no statistically significant difference between these two groups. The untreated diabetic group presented the highest fluorescence intensity, with significant difference between treated and untreated diabetic groups. Figure 4 represents the comparative retinal fluorescence between groups, normalized to the plasma fluorescence.

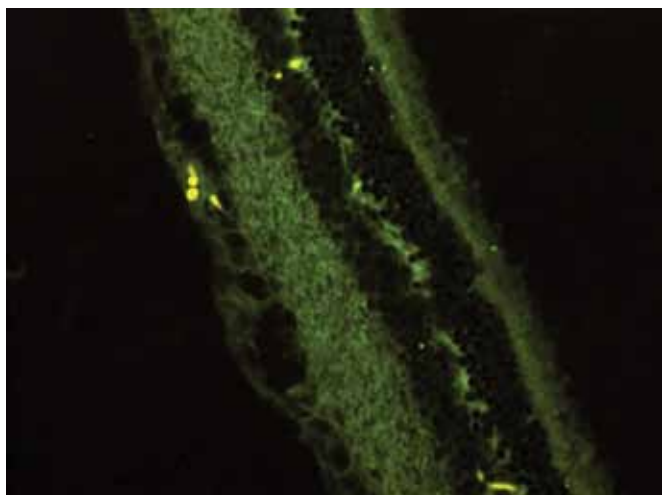


Fig. 1. Retinal fluorescence in the non-diabetic group

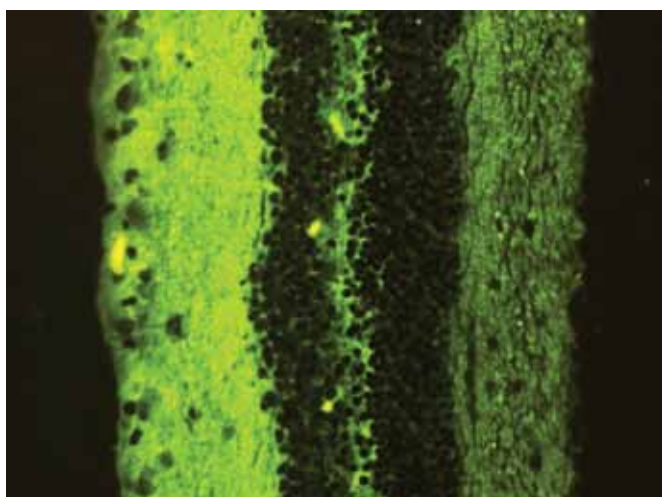


Fig. 2. Retinal fluorescence in the untreated diabetic group

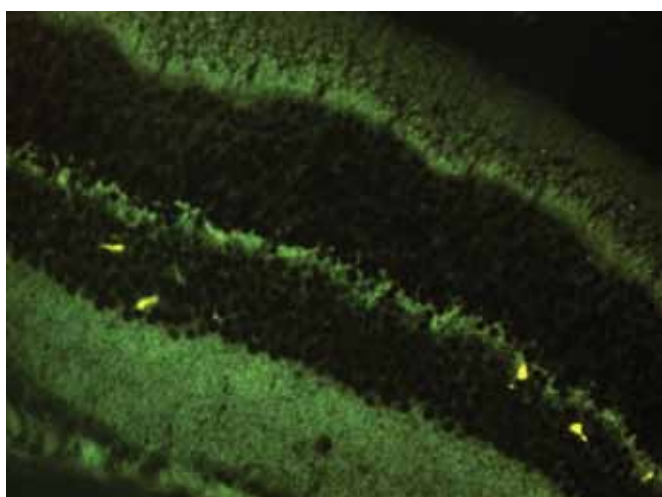
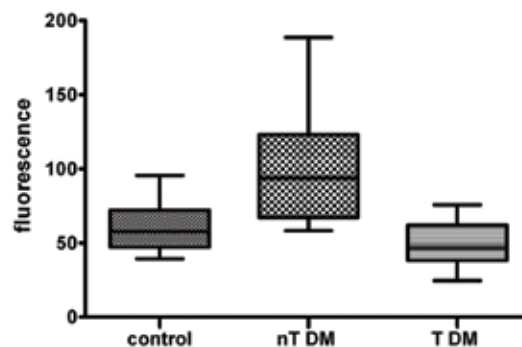


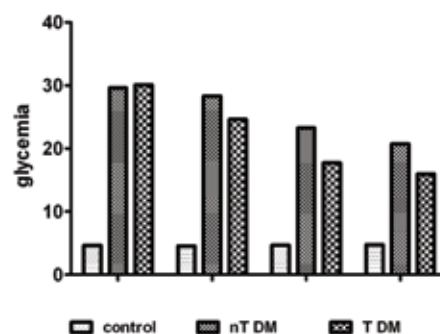
Fig. 3. Retinal fluorescence in the treated diabetic group

In the control group glycemic values presented non-significant changes, whereas both diabetic groups presented decreasing values. The difference between the non-treated and treated group was not statistically significant. Figure 5 compares the glycemic values between these groups.



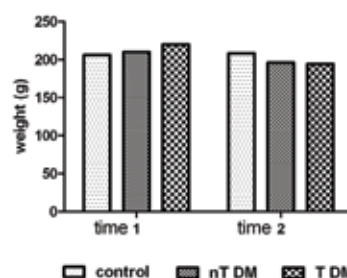
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?
control vs nT DM □	-41.44	8.501	Yes
control vs T DM □	10.58	2.171	No
nT DM vs T DM □	52.03	10.67	Yes

Fig. 4. Retina fluorescence comparison between groups



Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?
control vs nT DM M	-20.819	12.351	Yes
control vs T DM M	-17.438	10.345	Yes
nT DM vs T DM M	3.3813	2.0060	No

Fig. 5. Comparison of glycemia between groups



Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?
control vs nT DM M	4.250	0.6102	No
control vs T DM M	0.1250	0.01795	No
nT DM vs T DM M	-4.125	0.5923	No

Fig. 6. Comparison of bodyweight changes

At the end of the experiment, the body weight was increased in the control non-diabetic group, decreased in the diabetic treated group and slightly decreased in the non-treated group, compared to starting weight, without statistical significant differences between groups (Figure 6).

## Discussions

This review mainly focuses on the role of general stem cell stimulating therapies, especially, in the management of diabetic retinopathy.

We have demonstrated for the first time that stem cell stimulation can reduce BRB breakdown in diabetic rats in retinal tissue. An easy reproducible technique was presented, which is appropriate for demonstrating the effect of different pharmacologically active substances on vascular permeability changes.

Long-term Olimpiq® StemXCell SL treatment significantly decreased tissue fluorescence, vascular leakage and the BRB breakdown, compared with the control, untreated group.

Theoretically, two mechanisms may induce these changes: vascular structural regeneration through stem cell activation and lower glycemic values, through the regeneration of insulin-secreting cells. Gradually decreasing values of glycemia could be demonstrated in all groups, with no statistically significant differences between them, therefore the regenerative mechanism of insulin-secreting cells could not be demonstrated in this experiment.

## Conclusions

The results of this study suggest that Olimpiq® StemXCell SL would be useful for the treatment of ocular diseases associated with BRB leakage, such as diabetic macular edema and retinopathy.

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