The influence of Plaferon LB on regenerative processes in the pancreas in an Alloxan-induced diabetes rat model

(Regeneration of β-cells during Alloxan diabetes)

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Abstract

Background: The ability of dysfunctional β-cells lesion to regulate blood glucose levels can be normalized in by increasing the mass of existing β-cells in the pancreas. Study of regenerative processes in Diabetes Mellitus and finding new ways to activate β-cells precursors is of great theoretical and practical interest.

Methods: Using the methods of light, electron-microscopic, immunohistochemistry, electron-morphometric researches pancreas was studied in the experimental model of diabetes. In the present study, the influence of anamniotic derived peptide (Plaferon LB) on renewal processes was investigated in the pancreas of rats with Alloxan-induced diabetes. From a total of 90 Wistar laboratory rats, 30 received an Alloxan injection to induce diabetes and undergo treated one month later with Plaferon LB for ten days (Alloxan/Plaferon LB-treated group), 30 received an Alloxan injection but no subsequent treatments (Alloxan-treated group), and a control group of 30 rats (control group).

Results: Dystrophic and necrotic changes were detected in the pancreatic β-cells following the induction of diabetes with Alloxan, and several alterations were observed in the vascular system of the islets. Special emphasis was made on display of extra-islet cells during the experiment. Data obtained suggest that extra-islet cells exist in the rats pancreas with Alloxan diabetes in the different degree of maturation. Immunohistochemistry researches showed, that in Alloxan/Plaferon LB-treated rats pancreas cells adjacent to the duct epithelium and islets endothelial cells strongly expressed endothelial, hemopoetic and stem cell marker CD34. Cytokeratin CK19 and insulin were manifested in proliferating duct and some acinar cells. Electron-morphometric analysis of insulin granules in the extra-islet isolated β-cells showed increased proportion of light granules which is associated with increased insulin secretion.

Results suggest that Plaferon LB: 1) promotes a decrease in the blood glucose level, 2) helps to stabilize oxidative metabolism in the vascular system and to decrease the intensity of endotheliocyte injury, 3) encourages the maturation and differentiation of precursor cells, and 4) induces the renewal of β-cells in the pancreas of rats with Alloxan-induced diabetes.

Conclusions: Experimental results gave possibility to hypothesis, that the cells which can potentially differentiate into β-cells are extra-islet cells. Immunohistochemic and electron-morphometric investigation have shown, that damaged pancreas has capacity of generating new β-cells from ducts, exocrine or stem cells after activation with external stimuli. Presumably Plaferon LB takes part in the renewal processes of β-cells. However, these effects of Plaferon LB were not found to be sufficient to compensate for insulin deficiency. (TCM-GMJ October 2016; 1(2):P4-P10)

Keywords: Pancreas, β-cells, Precursor cells, Alloxan, Plaferon LB, Rats

Introduction

Diabetes mellitus (DM) has long been recognized as one of the most serious medico-social problems throughout the world (1). An insufficient quantity and the slow renewal of β-cells during the progression of DM reduce the proliferation of β-cells, causing subsequent problems in the provision of insulin (2-4). The ability of dysfunctional β-cell lesions to regulate blood glucose levels can be normalized by increasing the mass of existing β-cells in the pancreas (5, 6). The latter approach can be achieved by the regeneration, proliferation, and replication of pre-existing β-cells, as well as by the neogenesis of β-cells from precursor or stem cells or by trans-differentiation from other differentiated cells (7, 8). Trans-differentiation of β-cells were reported in various experimental conditions associated with β-cells loss and damage (9). Neogenesis of β-cell from duct epithelium is one of the most spread hypothesis (10). Duct cells of the neonatal and adult rat pancreas actively proliferate and express high levels of specific cytokeratines (CK) proteins. CK19 serve as immunohistochemical marker of epithelial duct cells (11, 12), and have previously been shown to have role in the develop-
ment of pancreas (13). Immunohistochemic research (14, 15) showed that subpopulation of cells adjacent to the duct epithelium contained endothelial and hemopoetic cell marker CD34, which have been used as indicator of stem or precursor cells.

A positive effect on the regeneration of β-cell mass in diabetic animals can be achieved by administration of hormones and growth factors (glucagon-like peptides, gastrin, betacellulin, activin A, exendin-4, and epidermal growth factors) (16-21).

Plaferon LB is a placenta-derived, biologically active substance known to have hypoglycemic, antioxidant, immunomodulating, anti-inflammatory, and anti-apoptotic effects (22-25). Plaferon LB has been successfully adopted in clinical practice. Based on our earlier researches (26, 27) we used an experimental animal model with Alloxan-induced diabetes to examine the effects of Plaferon LB on regenerative processes in the pancreas and provide a more complete description of the origin and functional ability of β-cell precursors.

Methods

Animal care and maintenance

All study procedures were implemented in accordance with the Institutional Guidelines for Animal Experiments at Bioethical Board of Al. Natishvili Institute of Morphology. Ninety male Wistar laboratory rats (aged eight weeks, \( \approx 200 \) g each) were housed 2 per cage in a room with a 12 h/12 h light/dark cycle and an ambient temperature of 22 to 25 °C. Free access to food and water was provided throughout the duration of the study. The body weight of each animal was measured at the beginning and at the end of the experiment.

Alloxan and Plaferon LB treatments

Alloxan (2, 4, 5, 6-pyrimidinedione) is a toxic glucose analogue, which selectively destroys insulin producing cells in animals pancreas. It causes an insulin-dependent DM, so called Alloxan-induced diabetes with characteristics similar to type 1 diabetes in humans (28, 29). Plaferon LB is biologically active peptide which is manufactured from amniotic membranes of human placenta. Plaferon LB was developed in the Institute of Medical Biotechnology of the Academy of Sciences of Georgia and licensed for clinical use by Georgian Drug Control Agency. It was approved in 1992 by the government of the Republic of Georgia as pharmaceutical and therapeutic drug (Republic of Georgia, rifistfry” o’“Health”, Registration Number A-0001).

The animals were divided into three groups: 1. Alloxan/Plaferon LB-treated group consisted of 30 rats; 2. Alloxan-treated group consisted of 30 rats; and 3. Control group was composed of 30 rats that received no treatment. Experimental diabetes was induced in 60 rats consisting of Alloxan/Plaferon LB and Alloxan-treated groups by a single intraperitoneal injection of Alloxan (150 mg of a 10% solution in water). Alloxan-treated group of 30 rats was then treated with Plaferon LB (0.25 mg/kg of body weight) for 10 consecutive days starting one month after the development of experimental diabetes. Blood glucose levels were measured (Glucometer IME-DC, FIA Biomed GmbH, Germany) in order to assess the development of diabetes. Blood samples were collected from the tail vein prior to the Alloxan injection as well as 2 and 24 h. and 7, 14, 21, 27, 31, 32, 33, 35, 37, 39 and 40 days after Alloxan administration. After the initiation of treatment with Plaferon LB, a blood sample were collected at 1, 2, 3, 5, 7, 9, 10 days from Plaferon LB injection and at 31, 32, 33, 35, 37, 39, 40 days after Alloxan injection. In addition, glucose level was measured two hours before and two hours after Plaferon LB was administered to the animals in Alloxan-treated group. An injection of a lethal dose of a sodium pentobarbital mixture (1%) was used for cessation of the experiment.

Tissue morphological analysis

Pancreas samples from all three groups of animals were obtained at the moment of completion of the Plaferon LB treatment course for Alloxan/Plaferon LB-treated group. To prepare samples for the morphological studies, each pancreas was fixed in a Carnua mixture and a Buen solution. Paraffin sections (5 mc) were stained with hematoxylin and eosin.

Immunohistochemistry

Neutral-buffer 10% of pancreatic sections (3 cm) which were formaline-fixed and paraffin-embedded was immunostained. The following primary antibodies: Mouse Monoclonal CK19 (clone RCK108, 1:50 dilution DAKO), Mouse Monoclonal Endothelial Cell Marker CD34 (clone QBEnd/10, 1:100 dilution, DAKO), and guinea pig anti-swine insulin antibody (N1542, 1:100 dilution, DAKO, Carpinteria, CA, USA) were used. Immunohistochemic research was performed according to manufacturer’s protocol using Novolink-Polimer detection system. Sections were incubated with biotinylated secondary antibody, washed in PBS and incubated with horseradish peroxidise-conjugated streptavidine. Visualisation was made by DAB chromogen (Novocastra, UK). The sections were counterstained with Harris haematoxylin to facilitate nuclear identification. The reliability of obtained results was defined using both positive and negative control. Immunohistochemic reaction was based on visual evaluation taking into account the intensity of colour.

Electron- microscopic research

The pancreas samples were fixed in 2.5% glutaraldehyde followed by a fresh 1% solution of osmium tetroxide in a colloidal buffer (pH 7.2 - 7.4) for 2 h. at + 4 °C. The samples were dehydrated in increasing concentrations of alcohol, poured over an araldite mixture, and polymerized for 24 h at +58°C. A Reichert-42 Ultramicrotome (Vienna, Austria) was used to prepare sections of
the samples, and the Reynolds method was used for the analysis of glucose content. The sections were covered with a silver-containing emulsion and observed under a microscope (Tesla-BS 500, Praha, Czech Republic, magnification of 3000-22000). The negative images were magnified 3-5 times when printed.

Electron-morphometric research

For measurement of the insulin granules Adobe Photoshop CC was used. Overall number of insulin granules, number and percentage parity, mean diameter of light and dark insulin granules were measured in isolated extra-islet \( \beta \)-cells from Alloxan/Plaferon LB-treated group, in \( \beta \)-cells from Alloxan and control groups. Insulin granules in all groups were measured in the same area of the cells. All the measurements from pixel were transferred to micrometers (41 pixel was 1 mc).

Statistical Analysis

Student t-test was used to check the reliability of the differences between blood mean of the three groups. The same test was used for two independent variables and for one independent variable to compare data in control and experimental groups followed by comparison of data before and after Plaferon LB injection, respectively. All results are given as means ±SE. Measurement of the insulin granules were statistically calculated in all three groups. All statistical procedures were conducted with IBM SPSS Statistics 20.0 for Windows (IBM Corporation, Armonk, NY, USA).

Results and discussion

One month after the administration of Alloxan, the glucose blood level in Alloxan-treated group was 222.29 ± 22.67 mg/dL. The body weight had decreased by 50-70 g and was 150-170 g compared to control group. After two hours from Plaferon LB injection glucose content in the rat’s blood was shown to decrease by 40 - 50 mg% in Alloxan/Plaferon LB-treated group compared to Alloxan-treated group. Blood glucose level in Alloxan/Plaferon LB-treated group is lower than in Alloxan treated group (P<0.000) and higher than in control group (P<0.000). Statistical analysis of changes in blood glucose levels of control and experimental groups are presented in table 1 and figure 1.

Minimal standard deviation after the injection of Plaferon LB points to the homogenous effect. Positive correlation suggests the presence of the positive effect of Plaferon LB in all cases.

Using the histological research method pancreas islets had various size, some of the islets were atrophied, other hypertrophied and hyperemic in Alloxan-treated group. The islets structure was disorganized. Necrobiotic changes of \( \beta \)-cells were observed in the central part of the islets (e.g. destruction, degranulation, and vacuolization) (Figure 2) compared with the intact control group. There was reduction of \( \beta \)-cells in the islet. Necrotized cell regions were surrounded by connective tissue. Fibrous inclusions were detected in the islet. The ultrastructure of the pancreatic islets of the rats from Alloxan-treated group was found to be markedly changed in comparison to those from control group. After one month from the beginning of Alloxan diabetes amount of apoptotic cells increased, compared to intact group.

Along necrotic changes in the \( \beta \)-cells were observed cells with highly condensed or fragmented nuclei, and cells without nuclei. Type1 DM is characterized by a significant deficit in pancreatic \( \beta \)-cell mass presumably caused by \( \beta \)-cell necrosis and apoptosis (30, 31). According to researchers (32, 33) Alloxan-induced diabetes in pancreatic \( \beta \)-cells manifests by alkylation of DNA and formation of toxic compounds such as superoxide anion peroxinitrite, and nitric oxide. Damage to DNA and intracellular structures causes necrosis and activates apoptosis of pancreatic \( \beta \)-cells. In Alloxan/Plaferon LB-treated group single hypertrophied islets (Figure 3), mitosis in some cells and decrease of apoptotic bodies were observed compared to Alloxan-treated group. We suppose that Plaferon LB acts as a protector of insulin-producing \( \beta \)-cells, reduces intensity of apoptosis, restores potential of mitochondria and stimulates \( \beta \)-cell proliferation, by increasing its mitogenic activity.

Changes such as dystrophy and necrosis were apparent not only in the \( \beta \)-cells but also in the vascular system of the pancreatic islets. Pancreatic islets endothelial cells play an important role in the early phase of type 1 DM. It is well known that the endothelial layer represents a barrier to blood cells; \( \beta \)-cells and the islet micro-vascular system cooperate in the maintenance of glucose homeostasis (34-37). During the development of Alloxan-induced diabetes, the endothelium underwent alterations (dystrophy and desquamation of the endothelium) followed by the transendothelial migration of immune and inflammatory cells into the pericapillary space and the endocrine tissues, with consequent progressive injury of the \( \beta \)-cells. Fibrous inclusions were revealed in pericapillary space. The endothelial lining of capillaries adjacent to cells in the islet of Alloxan/Plaferon LB-treated group were thin and well fenestrated. Within islets newly arisen capillaries were observed. Pericapillary space was found widened and without fibrous inclusions, collagen fibres in pancreatic islets become thin compared to the morphological changes which developed in sinusoidal network in Alloxan-treated group. Based on our findings, we suggest that Plaferon LB exerts antioxidant and anti-inflammatory effect, stabilizing oxidative metabolism in the vascular system and decreasing the intensity of injury in the endothelium of sinusoidal capillaries.

Based on immunohistochemical analysis, in Alloxan/Plaferon LB-treated group strong expression of CD34 was observed in the endothelial cells cytoplasm of islet capillaries (Figure 4). Immunoreactivity of CD34 in the cells adjacent to ducts and islets were high. CD34 positive cells scattered throughout the pancreas (Figure 5), whereas expression of CD34 in Alloxan-treated and control group were limited (Figure 6). According to this finding we hypothesized that Plaferon LB activates processes of endothelial proliferation and initiates formation
of new capillaries. Immunohistochemic researches showed that subpopulations of cells adjacent to the ductal epithelium contained CD34-positive precursor cells and perhaps giving rise to β-cells. Therefore Plaferon LB might activate angiogenic processes and mobilizes bone marrow derived cells in pancreas of the diabetic rats.

Substantial changes were detected in the extra-islet cells in Alloxan/Plaferon LB-treated group. In Alloxan/Plaferon LB-treated group expression of CK19 of the area around the islet, and throughout the pancreas was detected (Figure 7). In the islets expression of CK19 was weak. CK19 mainly expressed in the pancreatic ducts, with slight expression in acinar cells. Weak and negative expression of CK19 has been observed in cells near the pancreas islets in Alloxan-treated and control groups. In addition, in Alloxan/Plaferon LB-treated group some ducts and single acinar cells were including insulin granules. The intensity of staining on anti-insulin varied and usually is stronger in ducts nearby islets (Figure 8).

The origin and nature of β-cell precursors are controversial. One of the potential reasons could be the regeneration and replication of pre-existing cells, de-differentiation, proliferation, and re-differentiation of facultative precursor cells residing within the islet (38-41). In Alloxan/Plaferon LB-treated group immunohistochemistry analysis revealed strong expression of CK19 in the duct cells nearby pancreas islets. We support the idea that β-cell progenitors go through a ductal phase from an immature to an adult β-cell (42-45). Plaferon LB appeared to activate neogenesis of β-cells from duct cells. However, new finding suggest (12, 46, 47) that β-cell precursors are not restricted to only pancreatic ducts, and generate through acino-insular transdifferentiation. In the present study by electro-microscopic research single acinar cells including insulin granules were manifested. We agree with opinion above mentioned authors specifying on a possibility of acino-insular transdifferentiation from exocrine tissue.

Extra-islet cells of various origins and with varying degrees of maturation were revealed. Some extra-islet cells, which displayed features of β-cells, were isolated and not organized into islets. We considered endocrine cells as extra-insular component, when appeared isolated. For definition of functional ability of isolated extra-islet β-cells with mature insulin granules has been lead electron microscopic morphometry. Insulin granules were counted only in extra-islet cells where nucleus was visible. Light and dark insulin granules were observed in such cells: light granules with electron lucent core and white halo, while dark granules were with an electron-dense core and a broad white halo. Most of insulin granules were observed in the perinuclear space. Large nuclei with a prevailing content of euchromatin were detected in several of the extra-islet cells. Only extra-islet cells with mature insulin granules and visible nucleus in Alloxan/Plaferon LB-treated group (Figure 9), and insulin granules in β-cells of Alloxan-treated and control group were examined by electro-morphometric method. The number of insulin granules in extra-islet β-cells in Alloxan/Plaferon LB treated-group were lower than the number of insulin granules in β-cells of pancreas islets (P<0.000) in Alloxan-treated and control group. Percent age of light insulin granules in extra-islet β-cells of rats pancreas in Alloxan/Plaferon LB treated group were higher than percentage of dark insulin granules (p<0.000) in Alloxan-treated and control group. The percentage of dark insulin granules in β-cells in Alloxan-treated and control group were higher (p<0.000) whereas the percentage of dark insulin granules in extra-islet cells were lower (P<0.000) in Alloxan/Plaferon-treated group. The area of dark insulin granules was smaller, than area of light insulin granules in all groups. No significant difference could be found between mean diameter of light and dark insulin granules in three distinct groups (Table 2).

Knowledge of these measurements is important for understanding functional capacity of these cells (48-51). Analysis showed differences in number, percentage parity, mean diameter between insulin granules in extra-islet isolated β-cells in Alloxan/Plaferon-treated group and β-cells in islet of Alloxan-treated and control group. Research showed that extra-islet cells in Alloxan/Plaferon LB-treated group were included mature and functionally active insulin granules. An increased proportion of light granules in the extra islet β-cells in Alloxan/Plaferon LB-treated group might be associated with increased insulin secretion. We share the opinion by Ogneva et al. (52) that light granules might be responsible for immediate insulin secretion whereas the dark granules restore the insulin secret for a longer period.

### Table 1. Glucose level in the blood of control, Alloxan-treated and Alloxan/Plaferon LB-treated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>SD*</th>
<th>t**</th>
<th>p***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>80.86</td>
<td>8.51</td>
<td>15.483</td>
<td>0.000</td>
</tr>
<tr>
<td>Alloxan-treated group</td>
<td>222.29</td>
<td>22.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>80.86</td>
<td>8.51</td>
<td>23.024</td>
<td>0.000</td>
</tr>
<tr>
<td>Alloxan/Plaferon LB-treated group</td>
<td>172.29</td>
<td>6.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SD = Standard Deviation

**t = Student t-test

***p = p-value

Difference is statistically reliable (t=15.483; p<0.000)

Difference is statistically reliable (t=23.024; p<0.000)
Table 2. Insulin granules in isolated extra islet β-cells in Alloxan/Plaferon-treated group, in β-cells of pancreas islet in Alloxan-treated and control group

<table>
<thead>
<tr>
<th>Group</th>
<th>Area (µm²) ± SE</th>
<th>Number of granules</th>
<th>Number of dark granules</th>
<th>Number of light granules</th>
<th>Percent of dark granules</th>
<th>Percent of light granules</th>
<th>Diameter of dark granules (µm) ± SE</th>
<th>Diameter of light granules (µm) ± SE</th>
<th>Area of dark granules (µm²) ± SE</th>
<th>Area of light granules (µm²) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Extra-islet isolated β-cells in Alloxan/Plaferon LB treated group</td>
<td>42.74 ± 0.57*</td>
<td>115 ± 9.55*</td>
<td>40 ± 5.05*</td>
<td>75 ± 5.09*</td>
<td>33.92 ± 1.90*</td>
<td>66.07 ± 1.90*</td>
<td>0.404 ± 0.007*</td>
<td>0.143 ± 0.009*</td>
<td>0.447 ± 0.01*</td>
<td>0.165 ± 0.007*</td>
</tr>
<tr>
<td>II - β-cells in Alloxan treated group</td>
<td>42.65 ± 0.61*</td>
<td>136 ± 4.74*</td>
<td>95 ± 3.84*</td>
<td>41 ± 2.51*</td>
<td>69.86 ± 1.55*</td>
<td>30.14 ± 1.55*</td>
<td>0.304 ± 0.006*</td>
<td>0.076 ± 0.03*</td>
<td>0.334 ± 0.007*</td>
<td>0.095 ± 0.04*</td>
</tr>
<tr>
<td>III - β-cells in control group</td>
<td>42.45 ± 0.48*</td>
<td>344 ± 0.51*</td>
<td>276 ± 0.35*</td>
<td>68 ± 0.71*</td>
<td>80.22 ± 1.25*</td>
<td>19.78 ± 1.25*</td>
<td>0.391 ± 0.063*</td>
<td>0.06 ± 0.05</td>
<td>0.397 ± 0.68*</td>
<td>0.124 ± 0.008</td>
</tr>
</tbody>
</table>

Values are means ± SE within an observation  *(p<0.000)

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### Fig1. Distribution of blood glucose mean in all experimental groups

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### Fig2. Alloxan-treated group. Rats’ pancreas. Necrobiotic changes in the central part of islet. Degranulation. Vacuolization of β-cells. Shown magnification 10x40 (H&E)

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### Fig3. Alloxan/Plaferon LB-treated group. Rats’ pancreas. Hypertrophic islet. Mitosis in β-cells in the islet. Shown magnification 10x20 (H&E)

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### Fig4. Alloxan/Plaferon LB-treated group. Rats’ pancreas. CD34 positive cells scattered throughout the pancreas and in the cells adjacent to ducts. Shown magnification 10x40.
Fig 5. Alloxan/Plaferon LB-treated group. Rats’ pancreas. Immunoreactivity of CD34 in the islets were observed. Alloxan-treated group. Shown magnification 10x40.

Fig 6. Alloxan-treated group. Rats’ pancreas. Weak expression of CD34 in the pancreas. Shown magnification 10x40.

Fig 7. Alloxan/Plaferon-treated group. Rats’ pancreas. Strong expression of CK19 in the area around islet and throughout the pancreas. Shown magnification 10x20.

Fig 8. Alloxan/Plaferon LB-treated group. Rats’ pancreas. Single acinar cells in the islet peripheral part and near the islet stain with anti-insulin. Shown magnification 10x40.


References