Assessment of the connection between Alzheimer's diesase and neuronal insulin resistance in an in vitro model

Doctoral short thesis

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1. Introduction

Recently neuronal insulin resistance has been suggested to play a major role in the pathomechanism of Alzheimer's disease. Impairment of insulin signaling in the central nervous system has been linked with decreased neuroprotective pathways, increased apoptosis and enhanced activity of glycogen-synthase-kinase-3 (GSK-3). Overactivation of the kinase corresponds to hyperphosphorylation of tau-protein, which may result in increased production of neurofibrillary tangles, typical hallmarks of the neurodegenerative disease.

To better understand the precise connection between the neurodegenerative and metabolic disease and therefore to provide new therapeutical methods, numerous *in vitro* and *in vivo* models have been developed. Streptozotocin is a widely used compound for the induction of type I and type II diabetes, furthermore its local, cerebral injection impaires central insulin sensitivity and induces an Alzheimer's disease like state.

The substance has been also used in *in vitro* experiments involving neuronal cells and its concentration dependent cytotoxicity has been proven. This phenomenom can be explained by multiple cellular processes, that is mitochondrial damage, oxidative stress, disturbance in calcium homeostasis, increased production of protein aggregates like amyloid plaques and neurofibrillary tangles that are both common in Alzheimer's disease among others. Also reduced glucose uptake and GSK-3 inactivation and decreased expression of some elements of insulin signaling were observed after streptozotocin treatment. However, to the best of our knowledge there has been no studies directly aiming to investigate how insulin sensitivity is influenced by the compound and if it is an appropriate model to study the connection between Alzheimer's disease and neuronal insulin resistance *in vitro.*

2. Aim

My aim was to study the conncetion between Alzheimer's disease and neuronal insulin resistance in an *in vitro* model, therefore the following experiments were purposed:

- To study the effect of streptozotocin on the viability of non-differentiated and retinoic acid induced differentiated SH-SY5Y human neuroblastoma cells
- To study the effect of insulin on the viability of streptozotocin-treated non-differentiated and retinoic acid induced differentiated cells
- To compare the sensitivity of the non-differentiated and retinoic acid induced differentiated cells against the cytotoxic compound and protective treatment
- To evaluate if insulin resistance develops in non-differentiated and retinoic acid induced differentiated cells after streptozotocin treatment, and if insulin sensitivity might be improved by glucagon-like petide -1 (GLP-1) agoinst.

3. Methods

3.1 In our study the concentrations of the test compounds were chosen according to literature data.

3.2 In our experiments SH-SY5Y human neuroblast cell line was used, which was provided by the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in DMEM/F12 medium containing 1% stable glutamine, 1% non essential amino acid solution and 10% FBS and incubated at 37 ^{o}C, in 5% CO_2. For the induction of differentiation, the cell culture medium was freshly exchanged and supplemented by 50 μM retinoic acid on day 0 and 3. The differentiation procedure was 5-day long.

For cell viability assays one day before the experiment the cells were seeded to 24-well plates (10^4 cells/well). Twenty-four hours later the medium of the the cells was changed to low serum medium that contained only 1% FBS. Furthermore, the cells were treated with various concentrations of streptozotocin (0, 0.3, 1, 3, 5, 7, 10 mM). To study the effect of insulin on low serum or streptozotocin treated cells the medium was supplemented with various concentrations of insulin (0, 10, 30, 100, 300, 1000, 3000 nM). Streptozotocin treatment was applied once, only on the first day; however, the replacement of fresh medium and insulin treatment were daily repeated.

For Western blot and ELISA studies the cells were seeded to 10 cm Petri dishes ($6x10^5$ cells/dish) and experiments were carried out after reaching 80-90% of confluency. The cells were treated with low serum medium or streptozotocin and after 24 hours insulin treatment was applied in various concentrations (0, 10, 30, 100, 300, 1000, 3000 nM) for 30 minutes. Cells were harvested in ice cold lysis buffer containing phosphatase inhibitor. For some experiments exenatid pretreatment was also used. Directly after the low serum medium replacement and 1 hour before the streptozotocin treatment 100 nM exenatid was given to the culture medium and this treatment was repeated later parallel with the insulin treatment.

3.3 For evaluating the viability of the cells reszazurin reduction assay was preformed following the instructions of the manufacturer. Briefly, after 24, 48, 72 hours the medium

of the cells were changed to new medium containing 10% reszazurin solution. Cells were incubated for 4 hours at 37 °C and after that the concentration of resorufin, the fluorescent product, was measured by Fluoroskan Ascent FL Microplate fluorimeter (Thermo Fisher Scientific, Waltham, MA USA) at 530/590 nm.

3.4 To assess the integrity of cell membrane lactate-dehydrogenase release was measured following the instructions of the manufacturer. After 24, 487, 72 hours the medium of the cells were collected to determine the amount of the enzyme. On the last day to measure the intracellular lactate-dehydrogenase concentration cells were lysated with 1% Triton X-100. The activity of the lactate-dehydrogenase was evaluated by measuring the fluorescent product at 590 nm by microplate fluorimeter following the instructions of the manufacturer.

3.5 For the Western blot assay total protein concentration of cell lysates was determined by Bradford's method. After denaturation by heating at 95 °C for 5 min in Laemmli buffer samples were separated in 10% SDS-polyacrylamide gels and then transferred onto PVDF membranes. Membranes were then blocked with 5% non-fat dry milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour. Membranes were then probed with GAPDH, anti-GSK-3 and biotinylated anti-phospho-GSK-3 primer antibody in 5% non-fat dry milk solution overnight at 4 °C. The other day membranes were washed with TBST three times for 10 minutes and then incubated with streptavidin or secondary anti-mouse antibody conjugated horseradish peroxidase for one hour at room temperature. Following the washing procedure with TBST three times for 10 minutes the specific proteins were detected on autoradiography films using Pierce ECL Western blot reagent. Our results were normalized by GPADH bands.

3.6 For quantitative assessment of GSK-3 phosphorylation ELSIA method was used according to the manufacturer instructions. Briefly 100 μL cell lysate was added to the anti-GSK-3 coated ELISA plate and incubated overnight at 4 °C. After washing the plate was probed by anti-phospho-GSK-3 antibody for 4 hours at room temperature. After the washing procedure samples were incubated with streptavidin conjugated horseradish peroxidase for 40 minutes at room temperature. After adding the substrate solution the optical density was determined at 450 and 550 nm using Multiskan Ascent ELISA reader

(Thermo Fisher Scientific, Waltham, MA USA). The results were corrected by the protein content of the lysates.

3.7 Data are expressed as mean ± standard error of mean of at least 3 parallel measurements. Our results were compared to the control damage, the low serum condition. EC50 and E_{Max} values were estimated by the concentration-response curves, which were constructed by non-linear regression method. One-way ANOVA was used for data analysis followed by Dunnett's post-hoc test for multiple comparisons. Corrected $p < 0.05$ was considered statistically significant. Data were analyzed by Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Figures contain the results of all our experiments.

4. Results

4.1 The cytotoxic effect of streptozotocin compared to low serum condition was studied on non-differentiated SH-SY5Y cells. Cells were treated with 0.3, 1, 3, 5, 10 mM streptozotocin in low serum medium and cell viability was measured by resazurin reduction and LDH release assays after 24, 48 and 72 hours.

The preformed assays showed similar results as low concentration (0.3 mM) streptozotocin caused only mild, non-significant cytotoxicity, while higher concentrations (3, 5, 10 mM) considerably decreased the cell viability even acutely after 24 hours. One mM concentration of streptozotocin caused mild but significant acute cytotoxicity that increased gradually on the second and third days so it better models the progressive neurodegenerative processes.

4.2 The protective effect of various concentrations (30, 100, 300, 1000 nM) of insulin was studied for 1-3 days on non-differentiated cells cultured in low serum medium in the presence or absence of 1 mM streptozotocin. Insulin dose-dependently attenuated cellular damage detected by resazurin reduction assay. The damage caused by low serum condition was completely reversed at all time points, furthermore on day 3 the number of viable cells was almost doubled. In case of streptozotocin treatment insulin showed only partial protection, cell viability was plateaued between 25-50 % of the control group. The insulin concentrations inducing half maximal protection, however, were not significantly different in the two groups. Similarly, insulin dose-dependently prevented the loss of cell membrane integrity measured by LDH release assay. Insulin entirely prevented LDH release induced by LS medium, while in streptozotocin treated cells only an incomplete protection was observed (Fig. 1). Moreover, the concentration response curves of insulin shifted to the right on day 1 and 2 with significant difference in the concentration inducing half maximal protective effect.

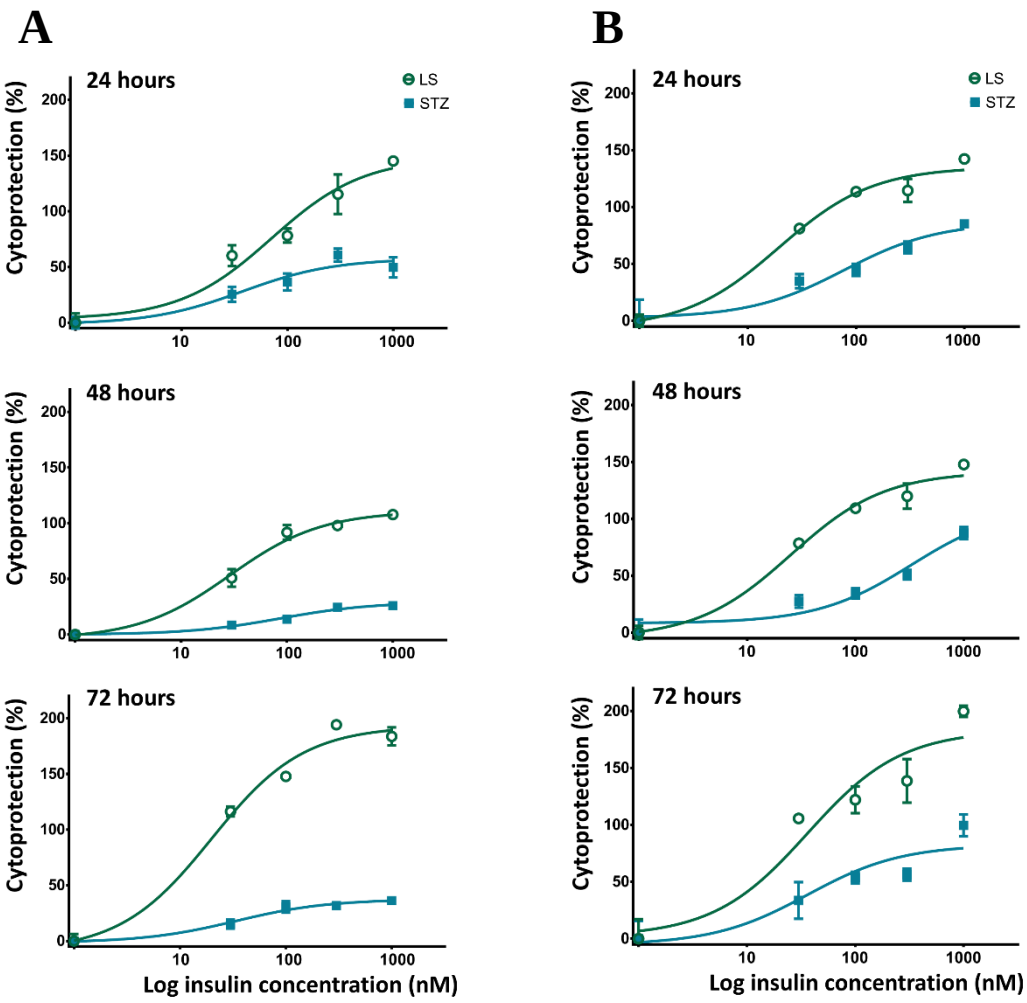

Figure 1: **Insulin dose-, and time-dependently improved metabolic activity (A) and membrane integrity (B) in low serum (LS) and 1 mM streptozotocin (STZ) treated non-differentiated cells. Effect of various concentrations (30, 100, 300, 1000 nM) of insulin on resazurin reducing activity of low serum and streptozotocin treated cells after 24, 48, and 72 hours is shown. Data are expressed as percent protection against damage.**

4.3 Insulin induced phosphorylation of GSK-3 was evaluated to assess the activity of insulin signaling in low serum and streptozotocin treated non-differentiated cells. Western blot analysis revealed maintained response to insulin in the streptozotocin treated cells. Phospho-GSK-3 levels were similar in both groups after stimulation by 100, 300, 1000 nM insulin. For quantitation of GSK-3 phosphorylation ELISA analysis was carried out using phospho-GSK-3 specific antibody. Insulin caused similar concentration-

dependent increase in the level of phosphorylated enzyme in both low serum and streptozotocin treated groups (Fig. 2). The dose response curves were similar with no difference in neither the efficacy nor the potency of insulin.

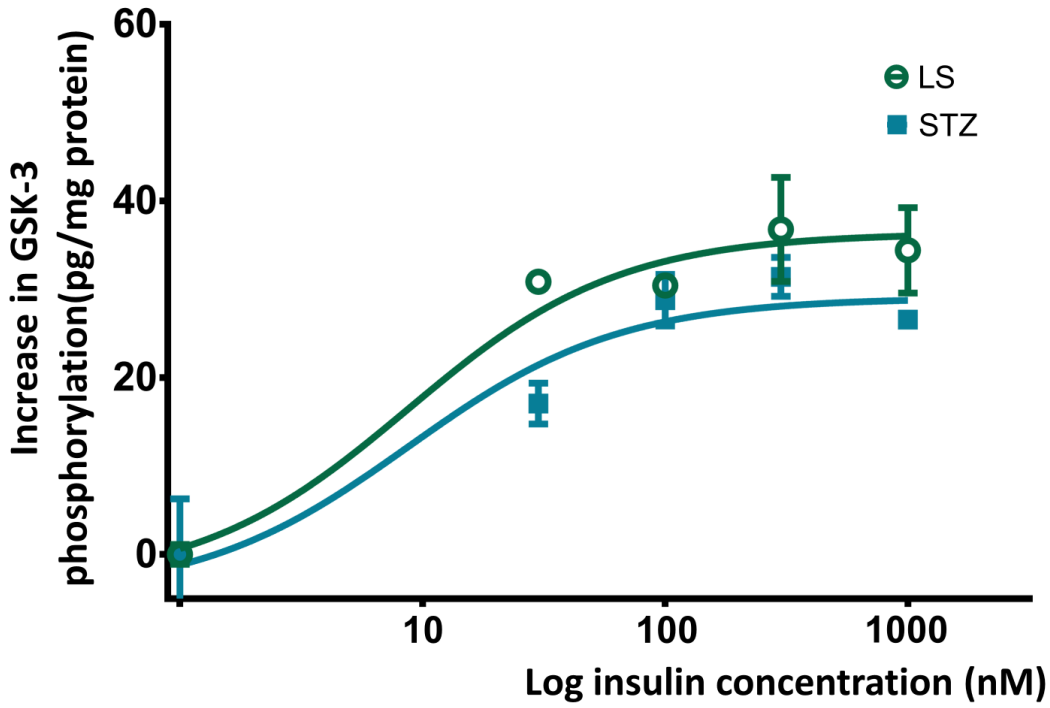

Figure 2: **Insulin dose-dependently increased GSK-3 phosphorylation in low serum (LS) and 1 mM streptozotocin (STZ) treated non-differentiated SH-SY5Y cells. Effect of various concentrations (30, 100, 300, 1000 nM) of insulin on GSK-3 phosphorylation was measured with ELISA method. Insulin stimulation was used for 30 minutes before harvesting the cells.**

4.4 The effect of streptozotocin on cell viability was compared to that of low serum condition on retinoic acid-differentiated SH-SY5Y cells exposed to 0.3, 1, 3, 5, 7, 10 mM streptozotocin in low serum medium. Resazurin reduction assay was carried out after 24, 48 and 72 hours.

Compared to the cytotoxic effect of low serum treatment streptozotocin further decreased the number of the metabolically active cells in a concentration-dependent manner; however, cells were less sensitive to the treatment. Lower concentrations, 0.3, 1 mM of

streptozotocin caused non-significant or mild decrease in cell viability, respectively, while higher concentrations (7, 10 mM) induced considerable decline in the number of viable cells. A significant but still moderate cytotoxicity could be observed after 3 or 5 mM streptozotocin treatment indicating the eligibility of both concentrations to model the chronic neurodegenerative processes. For the further experiments 5 mM streptozotocin treatment was chosen.

4.5 The impact of insulin was studied on both low serum and 5 mM streptozotocin induced cytotoxicity. Cells were treated by various concentrations of insulin (0, 10, 30, 100, 300, 1000, 3000 nM) and their viability was estimated after 24, 48 and 72 hours by using resazurin assay. The protective, concentration-dependent effect of insulin was also seen in case of the differentiated cells, however to a lesser extent. In case of low serum treatment insulin increased cell viability by 25-40%, which was significantly higher than seen in case of streptozotocin treated cells where the maximal achieved protection was between 10-25% (Fig. 3). The potency of insulin, however, was similar in both groups (Fig. 2).

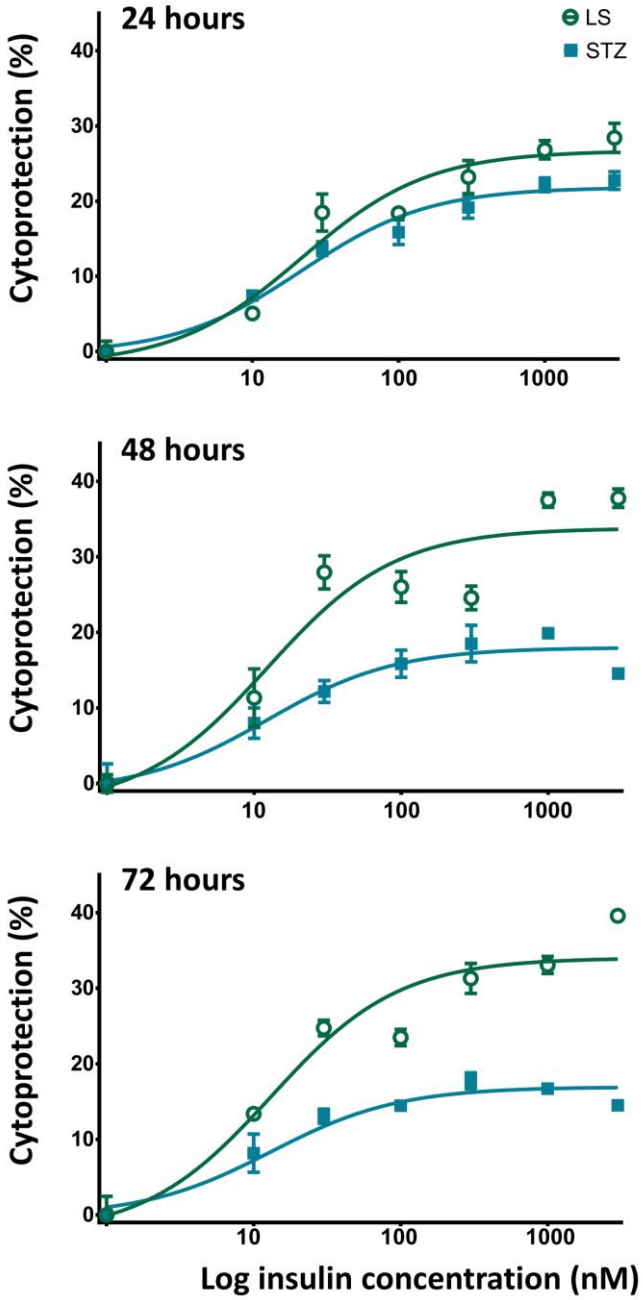

Figure 3: **Insulin dose-, and time-dependently improved metabolic activity in low serum (LS) and 5 mM streptozotocin (STZ) treated cells differentiated with retinoic acid. Effect of various concentrations (10, 30, 100, 300, 1000, 3000 nM) of insulin on resazurin reducing activity of low serum and streptozotocin treated cells after 24, 48, and 72 hours is shown. Data are expressed as percent protection against damage.**

4.6 Similarly to that seen in non-differentiated cells insulin concentration-dependently elevated the level of phosphorylated GSK-3 in both low serum and streptozotocin treated

cells. While the efficacy of insulin was similar, its potency was significantly different in the two groups. In the streptozotocin treated cells the effective concentration of insulin was significantly higher as its dose response curve shifted to the right. These data indicate the development of insulin resistance in the streptozotocin treated cells compared to the low serum group (Fig. 4).

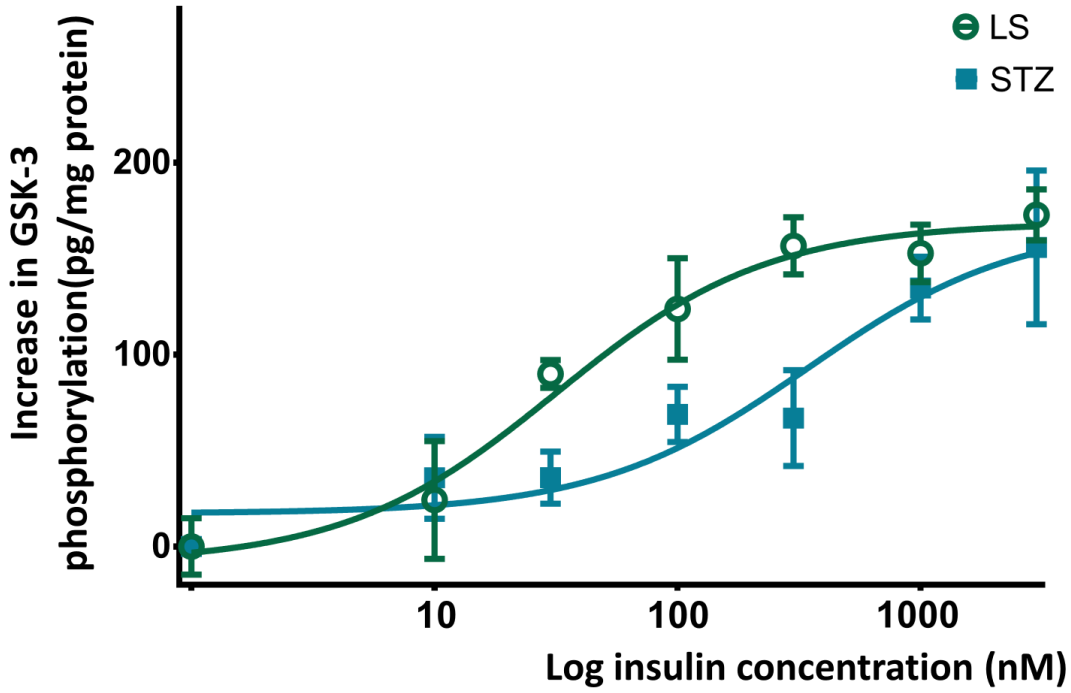

Figure 4: **Insulin concentration-dependently increased the level of GSK-3 phosphorylation in low serum (LS) or 5 mM streptozotocin (STZ) treated cells differentiated with retinoic acid after 24 hours with lower potency in the streptozotocin group. Insulin stimulation in various concentrations (10, 30, 100, 300, 1000, 3000 nM) was used for 30 minutes before harvesting the cells for measurement of GSK-3 phosphorylation.**

4.7 To confirm our hypothesis we examined how pretreatment with exenatid influences the insulin induced GSK-3 phosphorylation in differentiated cells. Pretreatment with 100 nM exenatid for 24 hours had also a different impact on the insulin-induced GSK-3 phosphorylation in low serum and streptozotocin treated cells. In the low serum group

exenatid pretreatment did not alter significantly the 100 nM insulin induced phosphorylation of GSK-3. However, in the streptozotocin treated cells it significantly potentiated the effect of 100 nM insulin on GSK-3 phosphorylation (Fig. 5).

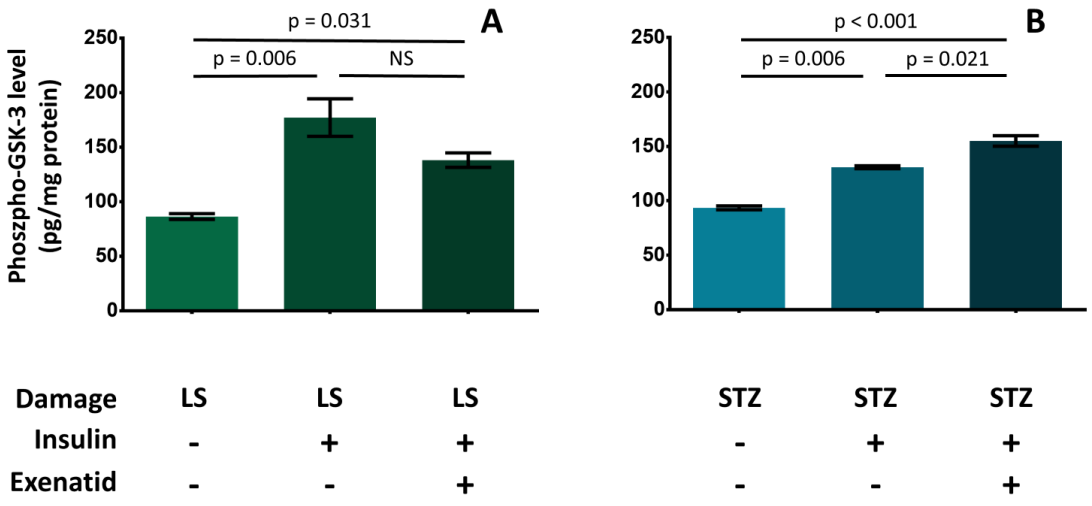

Figure 5: **Pretreatment with 100 nM exenatid for 24 hours did not increase the 100 nM insulin induced GSK-3 phosphorylation in low serum (LS) treated, differentiated cells (A); however a significant increase could be observed in the streptozotocin (STZ) treated group (B). Insulin stimulation was used for 30 minutes before harvesting the cells for measurement of GSK-3 phosphorylation, NS: non-significant.**

5. Conclusions

In our present study we aimed at examining the cytotoxic effect of streptozotocin on SH-SY5Y human neuroblastoma cells, furthermore, to determine if insulin resistance is involved in its mechanism.

In our experiments we confirmed that streptozotocin in the right dose exerts slow, progressive damaging effect on non-differentiated cells and on cells that are differentiated with retinoic acid. The damage could be reversed or attenuated with insulin treatment. To study the involvement of insulin signaling our results were compared to a control damage where the development of insulin resistance is unlikely. Based on these we came to the following conclusions:

- In non-differentiated cells insulin significantly reduced the damage induced by streptozotocin treatment. Although the EC50 value of the protective and GSK-3 phosphorylation inducing effect of insulin were not different to that seen in the control damage. Therefore, we can conclude that insulin resistance is not developed after streptozotocin treatment.
- Supposingly for the cytotoxic effects of streptozotocin non-specific mechanisms are responsible in case of non-differentiated cells.
- Retinoic acid induced differentiation remarkably reduced the sensitivity of the cells against the toxic compound so presumably the more mature cells are less vulnerable to the streptozotocin induced cytotoxic effects.
- Insulin was found to be also protective in differentiated cells, however to a lesser extent that seen in non-differentiated cells. Therefore, we assume that differentiation may alter insulin signaling.
- Compared to the control damage there is no difference in the protective effect of insulin; however, for the maximal phosphorylation of GSK-3 higher insulin concentration was required indicating the development of insulin resistance.
- Exenatid improved insulin induced GSK-3 phosphorylation in the streptozotocin group that confirms the decline in the insulin sensitivity.

Our results suggest that streptozotocin is suitable for *in vitro* modeling of neurodegenerative diseases due to its slowly developing cytotoxicity, as well as for studying the role of insulin resistance in differentiated cells; however, the role of non-specific cell damage mechanisms cannot be ignored.

6. Publications

Publications in the topic of dissertation

Bagaméry F, Varga K, Kecsmár K, Vincze I, Szökő E, Tábi T.

The impact of differentiation on cytotoxicity and insulin sensitivity in streptozotocin treated SH-SY5Y cells

NEUROCHEMICAL RESEARCH. 2021 Jun; 46(6): 1350-1358

DOI: 10.1007/s11064-021-03269-2

IF: **3.038** (2019)

Bagaméry F, Varga K, Kecsmár K, Vincze I, Szökő E, Tabi T.

Lack of insulin resistance in response to streptozotocin treatment in neuronal SH-SY5Y cell line

JOURNAL OF NEURAL TRANSMISSION. 2020 Jan; 127(1): 71-80

DOI: 10.1007/s00702-019-02118-5

IF: **3.505** (2019)

Publications not in the topic of dissertation:

Lakatos P. P, Vincze I, Nyariki N, **Bagaméry F**, Tábi T, Szökő E.

The effect of L-theanine and S-ketamine on D-serine cellular uptake

BIOCHIMICA ET BIOPHYSICA ACTA-PROTEINS AND PROTEOMICS. 2020 Oct; 1868(10): 140473 (Pages)

IF: **2.371** (2019)

Vincze I, Lakatos P. P, **Bagaméry F**, Tábi T, Szökő E.

Characterization of a cell line model for D-serine uptake

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS. 2020 Aug; 187: 113360 (Pages)

IF: **3.209** (2019)

Bába László-István, Kolcsár M, Kun Imre Z, Ulakcsai Zs, **Bagaméry F**, Szökő E, Tábi T, Gáll Zs.

Effects of Cariprazine, Aripiprazole, and Olanzapine on Mouse Fibroblast Culture: Changes in Adiponectin Contents in Supernatants, Triglyceride Accumulation, and Peroxisome Proliferator-Activated Receptor-γ Expression

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IF: **1.205**

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The role of autophagy induction in the mechanism of cytoprotective effect of resveratrol

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IF: **3.532**

Ulakcsai Zs, **Bagaméry F**, Vincze I, Szökő E, Tábi T.

Protective effect of resveratrol against caspase 3 activation in primary mouse fibroblasts.

CROATIAN MEDICAL JOURNAL. 2015 Apr; 56(2): 78-84

IF: **1.483**

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