Final report on the OTKA-PD-128480 project
„Blood-brain barrier changes in acute pancreatitis: a study on animal and culture models"

In our study we aimed to explore the mechanisms, factors and cellular interactions involved in the dysfunction of the blood-brain barrier (BBB) during acute pancreatitis. Our preliminary *in vivo* data on the non-invasive acute pancreatitis model induced by the intraperitoneal injection of the cationic amino acid L-ornithine showed that BBB morphology and barrier functions are damaged in these animals. We hypothesized that BBB dysfunction and barrier damage may play a major role in the development of pancreatic encephalopathy during severe acute pancreatitis. Therefore, we aimed to study the direct effects of L-ornithine on brain endothelial cells and BBB function. In this project we used an *in vitro* static cell culture insert and a dynamic lab-on-a-chip BBB model with the co-culture of rat primary endothelial, pericyte and astroglia cells.

During the first year of our study we described the effects of different concentrations of L-ornithine on the viability of primary brain endothelial cells. We observed, that the 20 mM treatment concentration reduced the impedance of the cell monolayers, but did not change the metabolic activity significantly. We found that BBB integrity was decreased after 24 h treatment with 20 mM L-ornithine both on the static cell culture insert and the biochip model. These observations were corroborated with immunofluorescent staining, where the discontinuity of tight junction and adherens junction proteins were seen, along with the upregulation of cell adhesion molecules ICAM-1 and VCAM-1. Our observations helped us to identify the optimal L-ornithine treatment concentration and time point for further studies, we evaluated the barrier integrity changes of the *in vitro* BBB model after L-ornithine treatment and we identified the junctional protein and adhesion molecule morphology changes potentially causing the barrier integrity changes after L-ornithine treatment.

During the second year we explored the background mechanisms for the first year's observations. We found, that after 24 h 20 mM L-ornithine treatment reactive oxygen species (ROS) release was elevated within the cells, but this was not derived from nitric oxide production. We also observed mitochondrial network discontinuity, but no change was found in the mitochondrial membrane potential. We also confirmed, that there was no nuclear translocation of the NF-κB transcription factor after 1 h 20 mM L-ornithine treatment. A direct treatment with L-ornithine also did not change the intracellular Ca^{2+} levels. Therefore, we suggest, that in the observed BBB changes reactive oxygen stress and mitochondrial damage involvement is probably key to brain endothelial dysfunction in L-ornithine treatment.

In the course of the third year the role of the brain endothelial surface glycocalyx was investigated in the model. Glycocalyx on the luminal surface of brain endothelial cells provides one of the physiological barriers of the BBB. We found, that while there was no change in the sialic acid and n-acetyl-neuraminic acid composition of the glycocalyx after L-ornithine treatment, ultrastructural changes occurred in the glycocalyx of brain endothelial cells showing discontinuous surface pattern. Along with this observation we found, that acute L-ornithine treatment elevated the surface potential of cultured brain endothelial cells reflecting to a surface charge alteration. These observations were one of the key findings in the study.

Short description of the results according to the workplan:

First Year

1. The direct effect of L-ornithine was tested on the cell viability of cultured primary brain capillary endothelial cells.

- We measured the real time effect of L-ornithine on cell adherence, junctional tightness and viability of brain endothelial cells with the use of the xCelligence real time cell impedance measurement. We found that by testing the concentration range 1-40 mM L-ornithine slightly but significantly reduced the impedance of the cells at the concentration of 20 mM, but not as drastically as the 40 mM treatment. Below 20 mM L-ornithine did not have an effect on cell layer integrity.
- The data from the xCelligence E-plate measurements showed the real time effect of L-ornithine treatment on brain capillary endothelial cells. We were aiming for the *in vivo* treatment timepoint (24 h). 1-20 mM L-ornithine treatment only influenced cell viability mildly at this timepoint, therefore we tested the metabolic activity of brain endothelial cells after L-ornithine treatment for 24 h with the MTT dye conversion assay. With this endpoint measurement we observed a slight decrease in the metabolic activity of brain endothelial cells while the 40 mM treatment had a more prominent decreasing effect.
- We did not perform the LDH test in this case, because in these experiments 24 h treatment with serum-free medium is needed. It is important to indicate that primary endothelial cells are very sensitive to serum deprivation therefore we concluded that the results with this assay are not to be involved in the study.
- Besides the L-ornithine treatments we always treated cells with its inactive form, D-ornithine as a reference molecule.

2. After determining the appropriate treatment concentration and timepoint – 20 mM and 24 h L-ornithine treatment – we evaluated the effect of L-ornithine on barrier integrity.

- During the evaluation of BBB tightness we tested the passage of two fluorescent marker molecules across the endothelial monolayer: sodium fluorescein (SF, paracellular marker molecule) and Evans-blue labeled albumin (EBA, transcellular marker) after L-ornithine treatment. While the inactive form of the amino acid did not have an effect on the permeability of the tight endothelial layer, L-ornithine treatment caused a significant permeability increase for both markers. Along with the permeability studies we also measured the transendothelial electrical resistance reflecting to the ionic permeability of the layer showing that L-ornithine lowered the resistance of the endothelial cells for the passage of sodium ions.
- Parallel with these studies our experiments were repeated on the BBB lab-on-a-chip model under microfluidic circumstances. In these experiments we corroborated our results we gained during our Transwell insert model experiments. During these studies we also started to optimize the cell suspension surface charge measurements after 1 h ornithine treatment and the zeta potential measurements on the cell monolayer after short-term L-ornithine treatment. These experiments are the preliminary studies for later stages of our work, and testing and optimization enhances our effectivity in describing the direct changes caused by the L-ornithine on the endothelial surface glycocalyx.

3. Morphology analysis of brain endothelial cell junctional molecules and cell adhesion molecules after L-ornithine treatment.

- After the permeability experiments we fixed the cells and used them for fluorescent immunohistochemistry. We performed Claudin-5, Occludin, ZO-1 and β-catenin immunostainings. We observed that after 24 h, 20 mM L-ornithine treatment morphology of the cells changed, the continuity of intercellular junctions was disrupted and small holes appeared between endothelial cells correlating with the toxicity, permeability and barrier integrity studies.

- Besides the junctional proteins we also stained for two cell adhesion molecules, ICAM-1 and VCAM-1 which are expressed at a low level on healthy brain endothelial cells. These molecules are upregulated when cells are under stress. We observed, that both ICAM-1 and VCAM-1 staining is more intense when treated with L-ornithine for 24 h.

During the first year's work we fulfilled all work stages planned:
1. We identified the optimal L-ornithine treatment concentration and timepoint for further studies.
2. We evaluated the barrier integrity changes of brain endothelial cells after L-ornithine treatment in the co-culture models.
3. We identified junctional protein and adhesion molecule morphology expression causing barrier integrity changes after L-ornithine treatment.

Second Year

1. Mechanisms contributing to the observed effects of ornithine on brain endothelial cells were investigated.
First, changes in the translocation of the NFκB transcription factor to the nucleus was measured by immunofluorescence on both the lab-on-a-chip device and culture inserts. We found that a short, 1 hour-long L-ornithine treatment did not change the cytoplasm-nucleus ratio of the transcription factor. Thus we can suggest, that the early nuclear translocation of the NFκB does not play a major role in the BBB integrity decreasing phenomenon. Further studies are needed to characterize the involvement of this pathway in the process after a longer treatment period.

We were also monitoring the acute changes in the intracellular Ca^{2+} levels after treatment with L-ornithine. This phenomenon was detected using live cell imaging with fluorescent microscopy with the Fluo-4-AM specific marker. Primary brain endothelial cells were cultured on special glass bottom Petri dishes under static conditions. Earlier results on pancreatic acinar cells suggested that acute treatment with L-ornithine might modify the level of the intracellular calcium production. We found that a quick treatment with L-ornithine did not change the Ca^{2+}-release, but cells reacted with a strong, positive signal to the Ca^{2+}-ionophore Calcimycin. Therefore, we can conclude, that the quick release of Ca^{2+} within the cells probably does not contribute to the observed effects.

2. Identification the role of oxidative stress and mitochondrial function in BBB integrity changes at the brain endothelial level after L-ornithine treatment.
First we measured the effects of L-ornithine treatment on the release of different reactive oxygen species (ROS) in brain endothelial cells. During our experiments we used two fluorescent probes: **(i)** specific for total ROS amount (DCFDA) or **(ii)** specific for nitric oxide release (DAF-FM). We found, that shorter (8-hour) treatment did not change the level of the produced nitric oxide or the amount of the total ROS found in brain endothelial cells. On the other hand, 24-hour L-ornithine treatment elevated the level of ROS, but not the nitric oxide release amount. This shows, that potential BBB integrity damage derived from ROS being present in the system is relevant, but is not derived from the release of nitric oxide.

When investigating reactive oxygen stress, to observe the role of the mitochondria in the phenomenon is crucial. First, we were studying the changes of the mitochondrial membrane potential after acute and chronic treatment with the L-ornithine. Changes in the membrane potential were detected using the TMRM dye with fluorescent microscopy. We found, that neither the acute, nor the 24-hour treatment changed the mitochondrial membrane potential. We further evaluated the effect of L-ornithine on mitochondrial morphology inside the cells with the Mitotracker staining. We found, that after 24-hour L-ornithine treatment the intracellular mitochondrial networks break up, get discontinuous, similarly to the toxic decoupling agent, cyanide.

All of our questions were answered with our experiments during the second year as well:

1. We explored several signaling pathways in cultured brain endothelial cells, which take part in the pathomechanism of acute pancreatitis in the *in vivo* model. We found that in the case of the *in vitro* model neither the NFκB, nor the Ca^{2+}-level dependent signaling pathways are involved in the acute effect of L-ornithine. Further studies are needed to identify the exact background mechanisms.

2. We evaluated the effects of oxidative stress after short term and long term L-ornithine treatments on brain endothelial cells. We found that while the level of the nitrogen monoxide did not change, 24-hour treatment elevated the total ROS production and created a disturbance in the mitochondrial network, while not changing neither acutely nor after 24 h the mitochondrial membrane potential.

Third Year

1. Changes in the endothelial surface glycocalyx after ornithine treatment

Besides the tight junctions between brain endothelial cells and the specialized (efflux) transport systems, glycocalyx on the luminal surface of brain endothelial cells provides an additional protection at the BBB. Our goal here was to investigate whether the glycocalyx is affected in brain endothelial cell damage induced by the cationic amino acid L-ornithine. First we performed wheat germ agglutinin lectin staining (WGA-A488) using the triple co-culture BBB model. We found, that L-ornithine elevated the staining intensity WGA, which does not corroborate other findings. We think, that the cationic amino acid might interact with the stain and causes an unspecific binding. Therefore, we did not carry on our experiments with this particular stain. We performed additional morphological analysis using transmission electron microscopy and found discontinuous glycocalyx labeling on the surface of cultured brain endothelial cells which observation was similar to the *in vivo* findings.

2. Measurement of brain endothelial cell surface charge after ornithine treatment

After the lectin staining we performed zeta potential measurements to confirm whether the observed BBB changes are reflected in endothelial surface charge difference. The surface potential evaluations were performed by Malvern Zetasizer Nano equipment using single cell suspensions in folded capillary cuvettes. We found, that direct L-ornithine treatment turned the basal very negative cell surface potential to more positive, which reflects to the direct interaction of ornithine with this very negative defense layer on the surface of brain endothelial cells. This effect was also concentration-dependent. While 5 and 10 mM treatments with L-ornithine did not change the zeta potential of the cells, the 20 mM treatment concentration elevated the surface potential compared to the untreated group indicating a direct interaction with brain endothelial cells.

3. Analysis of the effects of blood sera from rats suffering from acute pancreatitis

We obtained blood sera from rats with confirmed acute pancreatitis induced by the intraperitoneal injection of L-ornithine. Blood sera from a control untreated group was also collected (Animal experimental protocol authorization number: XVI./3554/2020.). Effects of these sera on cultured brain endothelial cell viability, morphology and barrier integrity was measured using mono- and co-cultures. We compared the rat serum treatments (20%) with the regular cell culture serum (plasma-derived bovine serum, 20%).

We found that treatment with healthy serum and serum from rats with pancreatitis did not change the viability of the primary rat brain endothelial cells (impedance measurement and MTT test). Along with this observation barrier integrity (resistance and permeability) was also not compromised compared to the regular cell culture serum treated group. Blood sera from rats with acute pancreatitis did not elevate ROS or nitric oxide levels and did not alter the mitochondrial network within the cells.

After finishing the third year's work proposed in my grant we can conclude that we fulfilled the planned work stages:

1. We obtained new data on glycocalyx changes following ornithine treatment on cultured brain endothelial cells. We found, that direct treatment with L-ornithine elevated the surface zeta potential turning the originally very negative surface charge to more positive. This observation might indicate, that L-ornithine interferes with the physiological defense mechanism of the glycocalyx at the surface of brain endothelial cells which might contribute to the observed BBB damage.

2. According to previous observations cytokine and enzyme levels in the blood of the animals with L-ornithine-induced pancreatitis rise. Therefore, our hypothesis was, that blood sera from animals with pancreatitis could also induce damage on cultured brain endothelial cells. In our experiments we observed that serum from rats with acute pancreatitis did not change viability, barrier integrity or reactive oxygen stress of cultured brain endothelial cells compared to the control cell culture serum treated groups, therefore we could not confirm our original hypothesis.

Fourth Year

The experiments were finished by the end of the third research year. A 1-year-long grant deadline extension was requested due to the COVID-19 pandemic: we faced difficulties both with research and article publishing deadlines. This request was granted by the NKFIH. The article writing and publishing was carried out in the fourth year. The project lead is on maternity leave since the beginning of 2022.

Presentations:

During all the 3 years of the original workplan **annually at least two first author presentations** were held from the results of the current OTKA-PD grant fulfilling the plans proposed in my submitted workplan.
In the first year **poster presentations** were held at **one local conference** (Straub days 2019, Biological Research Centre, Szeged) and at **one international conference** (22nd International Symposium on Signal Transduction at the Blood-Brain Barriers, Würzburg, Germany). Results and findings were discussed with experts of the BBB field. Additionally, an oral presentation was also held to communicate the results in the form of a seminar at the University of Nagasaki, Japan in 2019 July. The neurosurgeon experts were highly interested in the presented topic and the connection between pancreatitis and blood-brain barrier impairment.

During the second year one **poster presentation** was held at an **international conference** (2020 IBRO Workshop 29-30. January 2020, Szeged, Hungary) and I also presented at one **institute seminar** at the Institute of Biophysics, Biological Research Centre, Szeged. At the international conference results and findings were discussed with experts of the neurobiology and physiology field. At the local institute seminar my results were shared with the local community of researchers in an online presentation form. The biophysicist colleagues were highly interested in the presented topic, especially the experiments related to the biochip modeling. Three additional conferences (one local and two international) were canceled due to the COVID-19 pandemic.

During the third year of the project I held **two first author poster presentations** (3rd Blood-brain barrier summit, 28-30th June, 2021; Brain Barriers Virtual Conference, Cold Spring Harbor, 7-9th April, 2021, 2021) and **two first author oral presentations** (3rd Mini-Symposium on The Blood-Brain Barrier from Basic to Clinical Research, 26-27th of March, 2021; Virtual Symposium "Signal Transduction at the Blood-Brain Barriers", Sept 22-24, 2021, 2021) about the results of the current OTKA-PD grant. All of my presentations were held at virtual international conferences due to the

ongoing pandemic. I was happy that I had the chance to discuss my results with experts of the brain barriers field, with biologists, neurologists, physicians and biophysicists.

I also presented our own lab-on-a-chip device and modeling data at **two online seminars** in the form of oral presentation: on the 24th of June 2021 I was an invited speaker at the Engineering Brain Barriers seminar series and on the 18th of May 2021 at the CellME Berlin Online Tech Days (3D cell culture and organoids).

To conclude, I successfully presented the results of the present grant in a **total of 8 first author oral and poster presentations** at international or local meetings. I also was first or co-author of additional 35 poster and oral presentations about results directly or not directly linked to the current proposal.

Publications:

At the beginning of 2022 we **published the results of the OTKA PD grant** in one of the most prestigious journal of the BBB research, in the **Fluids and Barriers of the CNS** journal (**IF 2021: 6.961**, category **D1** in Medicine). Also during the grant period **I was first author of 5 additional publications** (NKFIH support indicated, total IF: 19.17, 3 Q1 journals, 2 D1 journals). Also **I was a co-author in additional 8 publications**, where NKFIH support was indicated (total IF: 41.2, 4 D1 journals, 2 Q1 and 2 Q2 journals). This adds up to a **total of 14 publications** where the NKFIH support was included with a **total IF of 60.37.**

Summary

With the results obtained during the grant period we provide useful data to understand BBB changes in the L-ornithine induced acute pancreatitis model. By studying BBB and brain endothelial cell-related changes, we could shed light on the mechanism of brain microvasculature-related damage in the L-ornithine-induced AP model. We fulfilled all the aims proposed in the original grant: we (i) characterized the permeability and morphological changes in brain capillary endothelial cells after treatment with L-ornithine; (ii) we described the mechanisms contributing to BBB changes after treatment with L-ornithine and (iii) we elucidated glycocalyx and surface charge changes in brain endothelial cell damage. Our present findings together with literature data suggest a general, AP model independent BBB damage in pancreatitis, that might be important to understand CNS complications in this disease. We revealed BBB and glycocalyx alterations in this rat AP model, for the first time. Our data on L-ornithine-induced changes on a culture model of the BBB suggest a direct interaction of the cationic amino acid L-ornithine with brain endothelial cells. This direct effect can be related to mitochondrial injury, oxidative stress, changes in barrier integrity and BBB morphology. Endothelial surface glycocalyx injury was revealed both in vivo and in vitro, as an additional novel component of the BBB-related pathological changes in AP. Our results are basis for further investigations of BBB damage and glycocalyx changes in mild, moderate and severe acute pancreatitis patients using human sera.

Szeged, 27th October 2022

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