OTKA PD 109051 Final Report

1. Summary

Ischemic heart disease including myocardial infarction is a leading cause of death worldwide. Therefore, cardioprotective interventions to limit myocardial damage are of great clinical importance. The main objective of the present project was to investigate whether exosomes and microvesicles are involved in the mechanism of cardioprotective interventions such as ischemic conditioning. Furthermore, our secondary aim was to investigate whether known or novel cardioprotective miRNAs are transferred by exosomes or microvesicles.

We have successfully performed the majority of the proposed studies according to the project plan. We assessed that exosomes are required for ischemic preconditioning and we identified specific miRNA moieties in stem cell-derived exosomes which may be involved in cytoprotective and proliferative mechanisms triggered by exosomes. Furthermore, we performed several extra, unplanned methodological studies after we assessed that currently available methods to isolate exosomes from blood are not suitable for qualitative analyses of exosomes. As a result, we developed several novel exosome isolation methods, and discovered that exosomal preparations are frequently contaminated with lipoproteins. These advances enabled us only recently to investigate blood-derived exosomes, therefore, the originally proposed in-vivo experiments will be finished shortly after the end of the grant period.

The grant contributed to the training of three PhD students. Based on the results, 6 full paper (cumulative impact factor: 23.4, citations according to Google Scholar: 226) and 4 conference abstract publications have already been released, and several journal publications are in preparation. We initiated numerous international collaborations in the grant period, and by using results of these collaborations, we secured further funding and applied for international grants. The methodological results also enabled us to launch a product development program, which aims to commercialize exosome isolation equipment and consumables.

2. Aims

The originally proposed aims of the grant were:

1) To assess whether ischemic conditioning modulates the release or content of exosomes or microvesicles.
2) To assess whether remote ischemic conditioning-induced release of exosomes or microvesicles are necessary and sufficient to mediate cardioprotection.

Based on the unexpected results obtained during the grant period the above aims were supplemented with the following goals:

3) To establish exosome isolation methods suitable for the qualitative analysis of blood-derived exosomes with well-characterized and low amount of contaminants.

3. Performed experiments, major achievements

We performed an exploratory study in rats and proved that ischemic conditioning induces a release of exosomes from the heart and that exosomes are necessary ischemic conditioning. Accordingly, we were the first in the literature to show that exosomes are required for the propagation of remote

ischemic preconditioning in isolated rat heart (Giricz Z et al, J Mol Cell Cardiol. 2014). This publication has received 95 citations in less than three years according to Google Scholar, and the data was incorporated in a high-impact review publication (Pickard JM et al, Basic Res Cardiol. 2015) which shows the high interest of the research of exosomes in the field of cardioprotection.

Since our main objectives included the characterization of exosomal transfer of cardioprotective singals, in collaboration with Dr. Prof. Edit Buzás, we optimized the parameters for the isolation and storage of exosomes from plasma samples, which is essential for the downstream analyses. We assessed that ultracentrifugation results in exosomal preparations which are rich in impurities, such as albumin or microglobulins. In collaboration with Dr. Péter Nagy (National Institute of Oncology) we assessed that the most abundant proteins in ultracentrifuge-derived exosomal preparations are soluble plasma proteins, which can greatly hinder the downstream proteomic analysis. Furthermore, we hypothesized that due to these impurities, analysis of exosomal miRNA and mRNA content is not feasible from blood-derived exosomal preparations obtained by ultracentrifugation. Therefore, we concluded that ultracentrifugation is suitable for neither in-vivo nor analytical studies. To establish a standardizable method for the isolation of exosomes, which would enable us to perform our primary aims, i.e., the assessment of the role of exosomes in cardioprotective interventions, we investigated size exclusion chromatography, which has been recently implicated in exosome isolation. We evidenced that exosomes can be isolated without albumin impurity on Sepharose CL-4B and Sephacryl S-400HR columns, however, not on Sepharose 2B. Although, we also discovered that only a minor fraction of total exosomes can be separated from albumin with these techniques. Nevertheless, we concluded that exosomes isolated with size exclusion chromatography is suitable for analytical purposes. In collaboration with Dr. Samir El-Andaloussi (University of Oxford, UK) we assessed that upscaling the column does not improve yield and purity of size exclusion chromatography. Based on these data, we concluded that 10-20 mL columns are sufficient for exosome isolation, and characterized a novel, cartridge-based column system in this size, which improved between-run and between-day reproducibility of exosome isolation, significantly reduced costs of isolation, and allowed certain level of automation. The publication of these results (Baranyai T et al, PLOS ONE 2015) received 39 citations in 2 years according to Google Scholar, which shows the high interest in technical studies in the field of exosome isolation.

To better characterize impurities of blood-derived exosomes, in collaboration with Prof. Edit Buzás, we analysed the lipoprotein content of our preparations. We described that low-density lipoprotein associates with exosomes and it is not possible to separate them by either ultracentrifugation or conventional size exclusion chromatography. Since lipoproteins carry a high number of various bioactive molecules, including microRNA, we concluded that careful evaluation and quality control steps should be taken when one uses such preparations to assess analytes possibly associated with lipoproteins. We published these results in 2016 (Sódar B et al, Sci. Rep. 2016).

The focus of our investigation was to prove that extracellular vesicles are primarily responsible for the transfer of cardioprotective signals in-vivo. In this project, we performed 85 in-vivo rat heart surgeries evidencing that remote ischemic perconditioning protected the heart from ischemia/reperfusion injury. In these experiments, we discovered that in animals with hyperglycemia cardioprotection was less pronounced. Therefore, we rendered rats hyperglycemic by infusing glucose intravenously for 25 min, and studied the infarct size limiting effect of remote ischemic conditioning. To allow us to perform our in-vivo experiments at a high standard, we planned the purchase of major equipment by the help of this grant. Since our department managed to finance the proposed animal ventilator after submitting the grant application, we requested a change in the budget that funds were to be

transferred to consumable costs, which was necessary for the development of our novel exosome isolation methods.

From our above mentioned in-vivo experiments, we prepared plasma samples and performed exosome isolation. We found that exosomes from animals subjected to hind limb ischemia (a form of remote ischemic conditioning) did not decrease infarct size ($p=0.7$, $n=9$). We speculate that the inefficient and inappropriate exosome isolation methods might be responsible for these findings. Therefore, we concluded that the current isolation protocols, including the industry-standard ultracentrifugation-based methods, are not suitable for the preparation of large enough quantities of blood-derived exosomes with high purity, biocompatibility and intact structure to be able to utilize them in in-vivo rodent models.

Therefore, to achieve the major aims of the current project, we had to modify our model system and we applied for an extension of the grant period. In 2016, we started using myocyte and fibroblast cell cultures as sources of exosomes and as recipients of isolated exosomes. Utilizing cell cultures enabled us to use ultracentrifugation and ultrafiltration as isolation procedures, since cell culture medium does not contain as much possible contaminating agents as blood plasma. In these studies, we first discovered that certain materials used for filter membranes specifically remove exosomal subpopulations with a distinctive pattern of exosomal markers, the significance of which is high, since the use of different filters may significantly alter qualitative analyses of exosomes and since all methods for exosome isolation require filters. We plan to publish these results in late 2017. Furthermore, we established circumstances for exosome isolation from various cell types to model in-vivo ischemic conditioning, including H9c2, 3T3, and neonatal cardiomyocytes, and we established parameters for experiments where we will apply purified exosomes and assess cellular damage due to hypoxia/reoxygenation. Since we successfully applied for further research funds in this topic, we will be able to assess which cell types release exosomes that can limit cellular damage induced by hypoxia/reoxygenation in these model systems. We expect to publish the results of these experiments in early 2018.

We further characterized if exosomes transfer cardioprotective signals in cellular systems. In collaboration with Dr. Rosalinda Madonna (Università degli Studi “G. d'Annunzio, Chieti, Italy), we discovered that in the pro-anginogenic and antiapoptotic effects of mesenchymal stem cells transfected by myocardin and telomerase exosomes play a significant role. We identified 3 miRNA moieties the expression of which changes in exosomes released by transfected stem cells very dynamically, and which are related to the induction of angiogenesis and apoptosis. Therefore, we showed that exosomes are able to transfer cytoprotective signals from stem cells to neighboring tissues. Since stem cell-based therapy is a very intensively researched field nowadays, we expect a high impact of these results, being published later in 2017.

Based on novel findings presented on the 2017 ISEV meeting, we started using gradient ultracentrifugation followed by SEC to isolate exosomes from the blood and we moved forward with the qualitative analysis of exosomes isolated from the blood of rats underwent 3×5 min limb ischemia and reperfusion. We initiated a collaboration with Dr. Rune Isak Dupont Birkler, and Dr. Hans Erik Bötker (Aarhus University, Denmark) to perform proteomic profiling of exosomes in cardioprotective procedures. The analyses are currently ongoing. Furthermore, currently we explore the microRNA patterns of exosomes isolated from blood to assess which miRNAs are required for cardioprotection by exosomes. We expect to publish our results later in 2017.

We also initiated a collaboration with Dr. Mateja Manček-Keber (National Institute of Chemistry, Ljubljana, Slovenia) who assessed that remote ischemic conditioning increases the amount of oxidized

phospholipids in exosomal preparations that exosomes isolated from animals that received remote ischemic conditioning, activation of TLR-2 receptors is elevated. These results demonstrate a possible role of oxidative modifications in the vesicle-mediated cardioprotective mechanisms. In these investigations a PhD student of Dr. Zoltan Giricz, Csilla Nagy, spent 3 months in the partner's laboratories funded by an EU COST action. The international cooperation allowed us to apply for a bilateral TéT grant in 2017 successfully, which allows us to further our knowledge in this field. Furthermore, we submitted a Slovenian-Hungarian OTKA bilateral grant application in 2017 based on these results.

4. Further experiments

The financial resources of this and further secured grants, and the high number of international collaborations made possible to design additional experiments where we further explore vesicular mechanisms of cardioprotective interventions by transferring microRNA, proteins, oxidized phospholipids or redox enzymes. Furthermore, to link exosomes to our other area of expertise, the treatment of cardiac injuries in metabolic comorbidities, we will test our hypothesis that cardioprotection against ischemia/reperfusion injury elicited by extracellular vesicles is not blunted in metabolic derangements such as diabetes or obesity.

5. Education, involvement of early carrier investigators

We recruited PhD students who are involved in the investigations of the current project. From 2013 Dr. Tamas Baranyai, from 2014 Csilla Nagy works primarily on projects funded by this grant. In 2015, Dr. Gábor Brenner, an outstanding medical professional from Serbia with a Hungarian nationality joined our research team. He works in part on the role of exosomes in cardioprotection in small- and large animal studies. Their contributions have greatly contributed to the success of the project.

We also welcomed PhD students from our international collaborators, e.g., in 2016 Dr. Tomas Rajtik (Comenius University, Bratislava, Slovakia), in 2017 Kristina Ferenczyova (National Academy of Sciences of Slovakia), spent 3-6 months in our laboratories working on collaborations. These collaborations will further increase the scientific output of the current project and may lead to multiple new grant applications.

Results obtained from experiments funded partly by this grant served as a basis for a Bolyai Fellowship (Hungarian Academy of Sciences) application, which Zoltan Giricz received a second time in 2017, that will greatly contribute to the advancement of his scientific career.

6. Utilization of results

Some of the findings of this program have a potential for commercial utilization. We described in our methodological projects that exosomes can be obtained from blood plasma with the use of size exclusion chromatography methods by using specific chromatographic column filling matrices. Since there is no cost efficient and user friendly equipment on the market, with which it is possible to perform these techniques in a reproducible, automated manner, we ventured to develop and commercialize a novel exosome isolation platform in collaboration with the Pharmahungary 2000 Ltd. with the financial support of a consortial NVKP_16-1-2016-0017 grant. We also started developing and

characterizing novel immunaffinity- and size exclusion chromatographic columns and accessories, which we also plan to commercialize.

7. Research output

The majority of goals set in our grant proposal have been achieved by our scientific programs, and several further aims have been investigated. The grant has contributed to a number of journal publications (6), conference presentations (4) and to three PhD theses. The output exceeded initial expectations, in part, due to the high number of international and Hungarian collaborations. The number of publications is going to be further increased since manuscripts of numerous experiments will be submitted in the near future due to additional experimental needs.

Published articles:

1. Giricz Z, Varga ZV, Baranyai T, Sipos P, Pálóczi K, Kittel Á, Buzás EI, Ferdinandy P: ***Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles***, J Mol Cell Cardiol. 2014;68:75-8, 2014
2. Baranyai T, Herczeg K, Onodi Z, Voszka I, Modos K, Marton N, Nagy G, Mager I, Wood MJ, El Andaloussi S, Palinkas Z, Kumar V, Nagy P, Kittel A, Buzas EI, Ferdinandy P, Giricz Z: ***Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods***, PLOS ONE 10: (12), 2015
3. Baranyai T, Nagy CT, Koncsos G, Onodi Z, Karolyi-Szabo M, Makkos A, Varga ZV, Ferdinandy P, Giricz Z: ***Acute hyperglycemia abolishes cardioprotection by remote ischemic perconditioning***, CARDIOVASC DIABETOL 14: (1), 2015
4. Pickard JM, Bøtker HE, Crimi G, Davidson B, Davidson SM, Dutka D, Ferdinandy P, Ganske R, Garcia-Dorado D, Giricz Z, Gourine AV, Heusch G, Kharbanda R, Kleinbongard P, MacAllister R, McIntyre C, Meybohm P, Prunier F, Redington A, Robertson NJ, Suleiman MS, Vanezis A, Walsh S, Yellon DM, Hausenloy DJ.: ***Remote ischemic conditioning: from experimental observation to clinical application: report from the 8th Biennial Hatter Cardiovascular Institute Workshop.***, Basic Res Cardiol. 2015 Jan;110(1):453., 2015
5. Sodar BW, Kittel A, Paloczi K, Vukman KV, Osteikoetxea X, Szabo-Taylor K, Nemeth A, Sperlagh B, Baranyai T, Giricz Z, Wiener Z, Turiak L, Drahos L, Pallinger E, Vekey K, Ferdinandy P, Falus A, Buzas EI: ***Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection***, SCI REP 6:, 2016
6. Giricz Z, Varga ZV, Koncsos G, Nagy CsT, Görbe A, Mentzer RM, Gottlieb RA, Ferdinandy P.: ***Autophagosome formation is required for cardioprotection by chloramphenicol***, Life Sci., 2017

Conference Presentations:

1. Zoltán Giricz: ***Are extracellular vesicles mediators of remote conditioning?***, ESC Congress 2015, 2015
2. Zoltán Giricz, Tamás Baranyai, Kata Herczeg, István Voszka, Károly Módos, Nikolett Marton, György Nagy, Ágnes Kittel, Edit Irén Buzás, Péter Ferdinandy: ***Exosomes in the blood plasma: characterization, isolation and stability***, International Society for Extracellular Vesicle 2015 Meeting, 2015
3. Zoltán Giricz, Tamás Baranyai, Kata Herczeg, István Voszka, Károly Módos, Nikolett Marton, György Nagy, Ágnes Kittel, Edit Irén Buzás, Péter Ferdinandy: ***Efficient isolation of high-purity***

exosomes from blood plasma, International Society for Heart Research XXXIII Annual Meeting of the European Section, 2015

4. Csilla Nagy, Krisztina Pálóczy, Ágnes Kittel, Zsófia Onódi, Rosalinda Madonna, Edit Buzás, Péter Ferdinandy, Zoltán Giricz: ***Isolation of exosomes from large volumes of cell culture media by ultrafiltration is superior to ultracentrifugation for the analysis of exosomal RNA***, International Society for Extracellular Vesicles, 6th Annual Meeting, Toronto, Canada, 2017

PhD Theses:

1. Dr. Tamas Baranyai has received his PhD absolutorium in 2016 and he is in the preparation of his thesis with the title: Remote ischemic conditioning and its molecular mechanism. He is going to defend his thesis in 2017.
2. Csilla Nagy has received her PhD absolutorium in 2017 and she is in the preparation of her thesis with the title: Mechanisms of remote cardioprotection in metabolic diseases. She is going to defend her thesis in 2018.
3. Dr. Gábor Brenner will finish his PhD training in 2018. One of his research topics is to describe the role of exosomes in cardioprotection in large animal studies.