Research project

Nowadays, the progress in medicine allows treatingdiseases, which previously lead to patient death. Even though the treatment often saves patient life, it does not restore the damaged tissue. One of important examples of such situation is the condition of heart after myocardial infarction (MI). Quick surgery interventions save lives but do not restore the injured tissue. For these patients the pharmacological treatment is also crucial but it is focused on prevention of subsequent events, e.g. clopidogrel andacetylsalicylic acid (ASA), or elimination of the cause in order for prevention of next episodes, e.g. statins for treatment of hypercholesterolemia, acommon cause of MI, anti-diabetic drugs or medications regulating blood pressure.Still, none of these therapies is triggered to regeneration of infarcted tissue. It is even more important as heart has very limited regenerative capacity[1] and its dysfunction affects the condition of the whole organism.

Currently there are two ways to induce the regeneration of damaged tissue. Fist one is the activation of endogenous progenitors, e.g. by usingerythropoietin (Epo). The advantage of this method is the facts that it activates endogenouscells of patients. On the other hand, this activation might not be very effective as progenitors residing within infarcted tissue might be damaged. What is more, the activation of progenitors from other tissues, mainly bone marrow (BM), might be insufficient, what can happen in older patients (due to decreased therapeutic properties of cells or reduced cell number)[2],or too late comparing to the time of injury. Second method is the delivery of progenitors directly to the place of injury. Currently, the efforts of scientists are focused on such application, using mesenchymal stem cells (MSCs). There is a big advantage of MSCs over induced pluripotent stem cells (iPS), which is lack of risk of carcinogenesis and no ethical concerns.

Thinking about delivery of cells,two groups have to be taken into consideration: autologous and allogenic transplantations. Autologous MSC can be obtained from bone marrow (BM-MSCs) or adipose tissue (AT-MSCs). In case of application of autologous progenitors,it needs time necessary for cell isolation and culture, what is a problem for improving heart condition immediately after MI. Another problem is cell quality as for older or diseased patients it might not be good enough for regenerative purposes[3].It was demonstrated for BM-MSCs isolated from patients with chronic heart failure, which exhibit more profibrotic phenotype, manifested by elevated transforming growth factor β (TGFβ) pathway, decreasedcardiac natriuretic peptides and diminished proliferation[4].Properties of another type of MSCs used for regeneration - AT-MSCs also decrease with age[5], what is important as many of MI patients are older ones. Thus, it seems autologous cells might not be the best choice for this type of patients. For allogenic transplantationalso MSCs isolated from umbilical cord Wharton's jelly (UC-MSCs) can be used. As UC-MSCs are obtained from a waste material and their obtaining does not require the special procedure on patient it is of great importance to utilize this type of cells to the maximum. Moreover, the comparison of UC-MSCs with AT-MSCs and BM-MSCs demonstrated the highest proliferation and immunomodulatory as well as immunosuppressive potential of UC-MSCs[6].Thus, UC-MSCs are the best candidatesfor utilization of regeneration of infarcted heart.

The therapeutic usage of MSCs for heart regeneration is intensively studied and there are many ongoing clinical trials utilizing them for cell therapy after MI. The reduction in the size of infarcted area and improved cardiac function caused by MSCs is due to their paracrine action, induction of the generation of capillaries and endogenous cardiomyocytes[7]. Nevertheless, the efficacy of this therapy is still relatively low. The reason of this is a very weak survival of cells after delivery into damaged heart, as the survival of cells at day 4 after delivery is less than 0.44%. This is caused by local hypoxia and lack of the restoration of blood flow in the place of injury, elevated reactive oxygen (ROS) level resulting from reoxygenation after reperfusion as well as a release of ROS by infiltrating immune cells in later stages of MI, proapoptotic signals triggered by factors released by necrotic cells, deprivation of pro-survival signals or importantly lack of intercellular connections and attachment to extracellular matrix (ECM) leading to cell death called anoikis[7].

The strategies of improvement of the MSCs survivalafter delivery into infarcted heart and ways to enhance cells therapeutic properties include cell preconditioning, using e.g. Epo, hypoxia or statins[7].Optimal preconditioning strategy depends on the situation in which cells will be after delivery into damaged tissue. In case of intramyocardial delivery immediately after MI the most crucial things are 1) the antiapoptotic/prosurvival signalling and 2) the reduction of danger of cell damage caused by ROS. Big amount of ROS are generated during reperfusion, mainly by mitochondria[8]. Importantly, elevated level of ROS was also detected in rat MSCs subjected into hypoxia and serum deprivation (HSD), a condition

mimicking the lack of blood flow restoration in infarcted heart, which caused MSCs apoptosis[9]. Hypoxia and serum deprivation applied separately[10] or together[11] also increased ROS level in human MSCs. Elevated amount of ROS in infarcted tissue is responsible for inhibited adhesion of transplanted cells and induction of cell detachment, what triggers anoikis in these cells[12]. Thus, it seems that elevation of MSCs capacity to handle enhanced ROS level is crucial for the enhancement of survival of MSCs after implantation both in hypoxic tissue and in tissue after reperfusion, thus it is important regardless of state of blood flow in the place of cell injection.

The strengthening of cell resistance to ROS can be done by applying antioxidants, such as N-acetyl-cysteine[13], desferoxamine (DFO)[14], or lycopene[11]. Another way is to overexpress thetranscription factors regulating the production of antioxidants/antioxidant enzymes, likenuclear factor E2-related factor 2 (Nrf2)[15]. Moreover, also the enzymes producing factors with antioxidants properties, such asinducible enzyme degrading heme - heme oxygenase-1 (HO-1)[16], can be overexpressed. Finally, the target of genetic upregulation can be antioxidant enzymes, like in the case of adenoviral overexpression of thioredoxin-1(Trx1)[17]. Still, the first method does not guarantee that delivered cells will survive, while the genetic overexpression approach is dangerous for the recipient patient. Therefore, the strengthening of cells themselves without their genetic modification seems to be the best option. This can be achieved by treatment with compounds with antioxidant properties or activation of the antioxidant defence system within the cells.

One of the compounds exhibiting antioxidant properties is N-acetyl-cysteine (NAC), which contains cysteine residues and plays a role in glutathione maintenance and metabolism[18]. Pretreatment of human MSCs with NAC reduced cellular ROS level with concomitant elevation of cellular glutathione level. It also increased cell adhesion and spreading of MSCs under oxidative stress *in vitro* and improved conditions of mice and MSCs cell retention in bleomycin-induced lung injury. NAC decreased lung inflammation and fibrosis, numbers of apoptotic cells andneutrophils as well as inflammatory cytokines in bronchoalveolar lavage fluid[19]. Moreover, cotreatment of MSCs with NAC and H_2O_2 rescued the diminishment of cell adhesion and spreading caused by H_2O_2. Additionally, concomitant injection of NAC and MSCs into infarcted heart of rat increased MSCs adhesion and reduced infarct size and fibrosis compared to MSCs-injected heart. Delivery of NAC*in vitro* also rescued decrease of FAK and Src kinases, diminishment of expression ofadhesive proteins: vinculin and paxillinas well as drop in Rac-1 level evoked by 20 μM H_2O_2.Furthermore, NAC restored the distribution and localization of focal adhesion proteins impaired by H_2O_2[13].Also my own studies demonstrated potent antioxidant properties of NAC *in vitro* in porcine proximal tubular cells treated with ochratoxin A(OTA), Moreover, in this study 24h delivery of 1mM NAC alone elevated expression of TGFβ2, indicating that it can also affect gene expression[20]. Still, the usage of NAC for preconditioning of MSCs for heart regeneration has never been investigated before.

Redox balance in the cells is mainly regulated by nuclear factor E2-related factor 2(Nrf2), a transcription factors activated in oxidative or electrophilic stress conditions. Under the basal condition Nrf2 is kept in cytoplasm bound to Keap1 protein, which regulates Nrf2 ubiquitination and subsequent protosomal degradation. Upon oxidative stress complexes Keap1-Nrf2 are disrupted, Nrf2 is stabilized and translocated to the nucleus, where it binds to antioxidant response elements (ARE) sequences and activates expression of many genes encoding proteins with antioxidant properties, such as enzymes involved in synthesis and metabolism of glutathione, like glutathione reductase (GR) and catalytic and theregulatory subunits of γ-glutamyl cysteine ligase (GCLC and GCLR, respectively), enzymes essential for the reduction of oxidized protein thiols: Trx, thioredoxin reductase 1 (TrxR1) and sulfiredoxin, antioxidant enzyme catalase as well as HO-1[2122]. Adenoviral overexpression of Nrf2 in human BM-MSCs protected these cells against H_2O_2, serum deprivation as well as hypoxia induced apoptosis[15]. Additionally, plasmid transient transfection of rat BM-MSCs with human HO-1 protected these cells from apoptosis evoked by HSD and H_2O_2. Still, genetic manipulation of cells carries big risk upon delivery to humans, thus better solution seems to be the pharmacological activation of Nrf2.

One of compounds activating Nrf2 is sulforaphane (SFN), one of isothiocyanates widely present in cruciferous vegetables[23]. SFN is beneficial in cardiovascular diseases (CVDs) protecting patients from hypertension, artherosclerosis, cardiac ischemia/reperfusion injuries and diabetes-related complications thanks to its antioxidant and anti-inflammatory properties. The crucial role in this effect plays the activation of Nrf2 directly by inhibition of its proteosomal degradation and indirectly via modulating the activity of intercellular kinases: mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC), what leads to changes in phosphorylation of Nrf2 altering its nuclear-cytoplasm trafficking and modulating its stability[23]. In case of the effect of SFN on MSCs, low doses of this compound

reduced MSCs basal ROS level (0.25 μM SFN) as well as ROS level upon treatment with H_2O_2 (0.25 and 1 μM SFN) while higher doses of SFN (20 μM) even enhanced H_2O_2-elevated ROS level. Moreover, 20 μM SFN was toxic for MSCs cells, what can be connected with inhibition of histone deacetylase enzymes (HDAC) activity.Still, low doses of SFN induced MSCs proliferation and protected from senescence, the properties which might result from its antioxidant action[24]. This compound also inhibits adipogenic differentiation of MSCs and increase MSCs self-renewal[25].The effect of preconditioning of MSCs with Nrf2 activator –SFN on the response to HSD as well as paracrine signalling from dying cardiomyocytes has not been investigated as of yet. Furthermore, the complete effect of SNF on therapeutic properties of UC-MSCs, including comparison of their survival in different MI *in vitro* models as well as the analysis of their paracrine activity remains to be elucidated.In my own studies intraperitoneal delivery of SFN induced HO-1 expression in kidney of mice and diminished OTA-induced expression of IL-1β, IL-6 and p53-upregulated modulator of apoptosis (PUMA)[26]. The effect of SFN on PUMA is apromising one as it indicates SFN antiapoptotic action, what is also crucial in the context of MI.

Nrf2 is a transcription factor activated by oxidative stress and electrophilic toxicants, for example by H_2O_2. H_2O_2 has already been used for improving the therapeutic properties of MSC. Preconditioning of UC-MSCs with 200 μM H_2O_2 for 2h diminished myocardial fibrosis and increased neovascularization in mouse MI model. Treatment of MSCs with 200 μM H_2O_2 increased paracrine activity of these cells upregulating the production of IL-6 and hepatocyte growth factor (HGF). Supernatants from preconditioned MSCs promoted endothelial cell (HUVEC) proliferation and migration[27]. Concomitantly, 2h-prestimulation of UC-MSCs with 200 μM H_2O_2 resulted in cellgrowth arrest in 3-4 passages after treatment with H_2O_2[28].

Nevertheless, in case of H_2O_2 it is crucial to remember that the effect is strongly dose-dependent.H_2O_2 at very low levels 3-15 μM induces cell proliferation. Doses 120-150 μM of H_2O_2 evokes a temporary growth arrest which protects cells from excess usage of energy and damage of DNA (4-6h). After 4-6h the antioxidant protection and damage repair is highly increased while 18h after adding of H_2O_2 the antioxidant protection is at a maximum. Moreover, 36h after the stimulation cell will return to original sensitivity to H_2O_2. Concentrations 250-400 μM of H_2O_2 cause permanent growth arrest with cells performing normal cell functions but never proliferating again. Doses 0.5-1 mM of H_2O_2 evoke cell apoptosis with nuclear condensation, loss of mitochondrial transmembrane potential, degradation of mitochondrial RNAs and laddering of both nuclear and mitochondrial DNA. Further increase in the dose of H_2O_2 to 5-10 mM and higher triggers disintegration of cell membrane and denaturation of proteins and nucleic acids leading to necrosis[29].

As so far for the preconditioning of MSCs only short-time pretreatment with high doses of H_2O_2 has been used, it is of great importance to testdifferent schemes of preconditioning and importantly to try out longer preconditioning with lower doses of this compound, which can enhance both antioxidant protection and repairing capacity of cells.Finding proper dose and length of prestimulation seems to be crucial as too high dose might cause cell senescence or even cell death, whereas too low stimulation or too short/long one might not stimulate the antioxidant response and DNA repair system enough for protection in MI. Moreover, H_2O_2 might be beneficial over SFN and NAC as it corresponds to the induction of cell defence triggered specifically to factors harmful in MI and in this way the response might be the most complete.

Taken together, in this project 3 different compounds affecting cell antioxidant capacity are planned to be used for UC-MSCs preconditioning in order to improve therapeutic properties of these cells and their survival in MI *in vitro* models: NAC, compound with antioxidant properties affecting gluthatione metabolism, SFN, an activator of Nrf2, which in this way may exert more complete response, and H_2O_2, which corresponds to the situation in infarcted tissue, which proper usage might trigger the response necessary to prevent toxicity of higher doses of ROS. Thus the prestimulation of UC-MSCs will be done with novel compounds (NAC), novel schemes of stimulation (H_2O_2) or using all MI *in vitro* models (SFN).

In this Project the effect of UC-MSCs preconditioning on their therapeutic properties, including their adhesion, paracrine activity and survival, will be assessed using *in vitro* modelsmimicking the situation in the infarcted heart. In these *in vitro* models the causes of low survival of cells after delivery into infarcted hearts, which are mentioned above, are taken into consideration.Thus these models resemble various types of injuries present in heart after MI:1) hypoxia serum deprivation (HSD)[30] corresponding to the lack of blood flow, 2) H_2O_2 as a model of increased level of ROS resulting from reoxygenation[27]3) and conditioned media from injured cardiomyocytes (ICM) equivalent to paracrine proapoptotic signalling present in dying tissue[31].Prominently, these models will be improved in this project as new aspects will be taken into the

consideration – cell adhesion after delivery to the patient and the presence of anticoagulant treatment as well as statins in the circulation of patients after MI.

During the analysis of the therapeutic properties of MSCs the important factor, which has to be taken into consideration, iscell adhesion after delivery. After preconditioning cells have to be harvested, stored and then delivered to the patients. Thus, it is important to include the aspect of harvesting corresponding to the need for cell attachmentduring the assessment of the effect of preconditioning on UC-MSCs therapeutic properties in different *in vitro*MI models. It is of even greater importance as ROS inhibit adhesion of MSCs on plastic plates as well as on dishes with various matrices, such as fibronectin and cardiac fibroblast-derived three-dimensional matrix (cardiogel). Delivery of 20 μM H_2O_2 diminished the level of focal adhesion-related molecules, such as phospho-FAK and p-Src, as well as of integrin β1[13]. Thus, it is important to compare the therapeutic properties of cells with consideration ofthe need for cell adhesion between preconditioning and MI *in vitro* models.

Another substantial aspect, which has to be taken into account, is the presence in the circulation the anticoagulants: ASAand clopidogrel, which are crucial for treatment of patients with acute coronary syndrome[32,33]. Both compounds are part of standard treatment for patients after MI and delivered together they are marked as dual therapy (DT)[32]. ASA is an irreversible inhibitor of cyclooxygenase-1, an enzyme generating thromboxane A2 (TXA2) from arachidonic acid. In this way ASA inhibits platelet activation mediated by the TX and prostaglandin endoperoxide receptors[32].Clopidogrel is an antagonist of the adenosine diphosphate receptor $P2Y_{12}$.It is currently most widely used $P2Y_{12}$ receptor antagonist and the only one of this class approved for patients with stable coronary artery disease treated withpericutaneus coronary intervention[32]. P2Y receptors are ubiquitously present and mRNA for all P2Y receptors were found on human MSCs cells[34,35].The therapeutic effect exerted byclopidogrel is connected with reducedinside-out activation of integrin αIIbβ3[36]. As integrins are important in cell-cell adhesion, it is especially important to assess the effect of clopidogrel of MSC attachment in different MI *in vitro* models.

There are some studies investigating the impact of ASA and clopidogrel, respectively, on MSC cells. ASA inhibited proliferation of rat MSCs *in vitro*[37]. Further studies of the same group demonstrated that ASA induced apoptosis in rat MSCs by downregulation of Wnt/beta-catenin signalling pathway leading to disregulation of mitochondrial function – increased cytochrome c release and caspase-3 activation[38]. Moreover, ASA impaired MSC ability to inhibit the production of TNFα, IL-6 and IL-12p70 and induce theproduction of IL-10 in macrophages stimulated with LPS (in co-culture studies)[39]. Clopidogrel but not ASA induced *in vitro* proliferation of human BM-MSCs and C3H10T1/2 cells, a murine MSC line, at day 6 after stimulation. Moreover, in rat model of periodontitis clopidogrel but not ASA enhanced MSCs proliferation, increased the number of osteoblasts and diminished the inflammatory infiltrate after but not before induction of periodonitis[40]. ASA delivered systemically blocked MSC impairment and tendency towards malignant transformation in ovarictomized mice[41]. Local application of ASA is also considered as a novel strategy to improve MSC-based tissue regeneration via diminishing IFN-γ/TNF-α-induced MSC apoptosis[42].Still, the effect of ASA and clopidogrel on MSCs properties in the context of the usage of these cells for heart regeneration has never been addressed before. Importantly, the effect of these compounds on therapeutic cells should also be included in currently used MI *in vitro* models as they might affect the properties of MSCs. Additionally, the combinatory effect of ASA and clopidgrel, as in DT, should also be taken into consideration. In order to address this issue novel models will be developed. In case of HSD, there is no restoration of the blood flow therefore the cells will not be under the influence of DT in this model. However, in the case of H_2O_2, assessment of cell detachment-reattachment andICM the effect of these medications should be verified.

Additionally, MI is frequently caused by hypercholesterolemia, thus patients after MI very often also receive medications lowering cholesterol level – statins, such as rosuvastatin (RSV)[43,44]. Statins are inhibitors of HMG-CoA reductase. They exhibit pleiotropic effects including the most important -the diminishment of cholesterol level, but also the protection of endothelial function, enhanced nitric oxide bioavailability, antioxidant and anti-inflammatory effects and stabilization of atherosclerotic plaques[45]. RSVin dose of 1 μM protected AT-MSCs from HSD (1% O_2, 6h) when applied concomitantly, increasing cell viability, proliferation and reducing cell apoptosis and caspase-3 activity with the effect mediated by activation of PI3K and MEK1/2. Moreover, RSV elevated paracrine activity of MSCs as it elevated the production of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and HGF.Furthermore, RSV increased FoxO3a phosphorylation and nuclear exports well as level of anti-apoptotic proteins Bcl-xL and Bcl-2 with concomitant downregulation of pro-apoptotic proteins Bim and Bax. Additionally, combination therapy with AT-MSCs and RSV improved myocardial function in murine model of MI with elevated survival of AT-MSCs and diminished cardiomyocyte apoptosis and

fibrosis[46].The effect of RSV is crucial in models corresponding to restoration of blood flow, thus it has to be applied in models of reoxygenation and ofproapoptptic signal from dying cardiomyocytes with respect to the need of cell adhesion, what has not been investigated before.As patients after MI very often exhibit elevated cholesterol level and have to receive statins, therefore statins should be also included in *in vitro* models of MI. Additionally, as all MI patients receive anticoagulant therapy after MI, it is important to assess the combinatory effect of RSV and DT (TT, triple therapy) on MSCs therapeutic properties as well as to include TT in *in vitro* MI models.Additionally, I have participated in the studies demonstrating that atorvastatin elevated hypoxia-diminished expression of Ref-1 in human endothelial cell line (HMEC-1)[47]. It is important as Ref-1 is a protein with DNA repair activities. Therefore the usage of statins might boost the DNA repair capacity of MSCs, what might be beneficial in the surviving during *in vitro* MI models.

Concluding the aim of this project is to find the bestnovel preconditioning method altering antioxidant capacity of UC-MSCs improving cell propertiesfor regeneration of heart after MI. Preconditioning will be done using compounds with different mechanisms of action: NAC, an antioxidant also stimulating GSR production, SFN, a Nrf2 activator, and low dose of H_2O_2 as oxidative stress. Verification of the effect of preconditioning on UC-MSCs therapeutic properties will be done using*in vitro* models of MI. HSD will be used as a model correspondingto the lack of blood flow in infarcted tissue, H_2O_2 will mimic the elevated generation of ROS resulting from reperfusion and ICM will provide the proapoptotic paracrine signals present in the injured tissue. These *in vitro* models will be improved with utilization of the necessity of cell adhesion as well as pharmacological treatment present in patients - anticoagulant treatment as well as RSV.Using novel *in vitro* MI models the therapeutic properties of UC-MSCs will be assessed, focusing on cell viability, cell death, cell adhesion, intracellular ROS level, antioxidant and apoptotic response as well as DNA repair and paracrine activity. The properties of UC-MSCs in different models will be investigated, what will allow to choose the preconditioning method improving UC-MSCs properties depending on the restoration of the blood flow in infarcted tissue and on the pharmacological treatment of patient.

Plan of the research project

1. **Firstly, the effect of different types of preconditioning affecting UC-MSCs antioxidant capacity (NAC, SFN and H_2O_2) on cell therapeutic properties will be verified.**

In the project following cell features important in their therapeutic action will be analysed: UC-MSC viability, adhesion, paracrine activity and cell survival in typical MI models: HSD, H_2O_2, ICM also with regards to the need of cell attachment. This will allow to choose the best preconditioning method: working regardless of restoration of blood flow in patients in the place of injury (the protection in all models: H_2O_2, HSD and ICM) or optimal for one situation (H_2O_2 vs HSD), what is important in the context of the success of reperfusion (pharmacological or by coronograpy) in patients. Additionally, the effect of preconditioning on the expression of apoptosis-related proteins antioxidant enzymes, enzymes participating in DNA repair and adhesion proteins as well as cellular ROS level, DNA damage as well as cell apoptosis, proliferation and morphology will be measured. This will allow to predict the possible mechanisms of observed alteration.

2. **Concomitantly, the effect of RSV, clopidogreland ASA on UC-MSCs properties will be assessed**.

In the analysis of the impact exerted by RSV, clopidogrel as well as its active metabolite (clopidogrel-AM) and ASA and its metabolite salicylic acid will be verified. This analysis will allow the verification of the effect exerted by separate compounds on UC-MSCs properties as well as compare the action of drug and its metabolite. Furthermore,the effect of combination of clopidogrel and ASA (DT) as well as DT + RSV (TT) will be assessed, as this situation corresponds to patients after MI without or with hypercholesterolemia, respectively.The effect of these compounds on UC-MSC viability, necrosis, adhesion, proliferation, paracrine activity, apoptosis, ROS production, DNA damage and cell survival in models: HSD, H_2O_2, ICM also with regards to the need of cell attachment will be verified. Moreover, the expression apoptosis-related factors, antioxidant and DNA-repair enzymes as well as adhesion protein will be tested for more complete verification of the effects exerted by tested compounds. Cell morphology will be also verified.

3. **Finally, the investigation of the effect of DT (clopidogrel and ASA) and TT (RSV + DT) on the alterations in UC-MSCs therapeutic properties evoked by UC-MSCs preconditioning will be conducted.**

In this final step, the effects of DT and TT on the protective effect exerted by preconditioning will be tested. This situation resembles the most the actual situation in patients. In order to achieve that goal, DT and TT will be added into H_2O_2 and ICM also taking into consideration the need of cell attachment, as these models corresponds to the situation after reperfusion, creating novel MI *in vitro* models.

The effect of UC-MSCs preconditioning on cell therapeutic properties, including cell viability, necrosis, apoptosis, adhesion, proliferation and paracrine activity will be verified in novel MI *in vitro* models. The analysis of the expression apoptosis-related factors, antioxidant factors, DNA-repair enzymes and adhesion protein as well as intracellular ROS level, DNA damageand cell proliferation will be tested.

Based on steps 1-3 the best preconditioning methodimproving therapeutic properties of UC-MSCsfor the improvement of heart regeneration after the delivery into infarcted tissue will be chosen, depending in the reperfusion of infarcted heart and including the presence of DT and TT in recipient.

Application of different models of tissue injury, performing transfer assay using media from injured cardiomyocytes and including the presence of DT and TT in the patients will provide the most complete information about therapeutic potential of preconditioned MSCs.

Combining the information from 1-3 will also allow to predict the possible mechanisms of observed effects with respect to changes in levels of apoptosis-related factors, antioxidant proteins, DNA-repair enzymes and adhesion molecules.

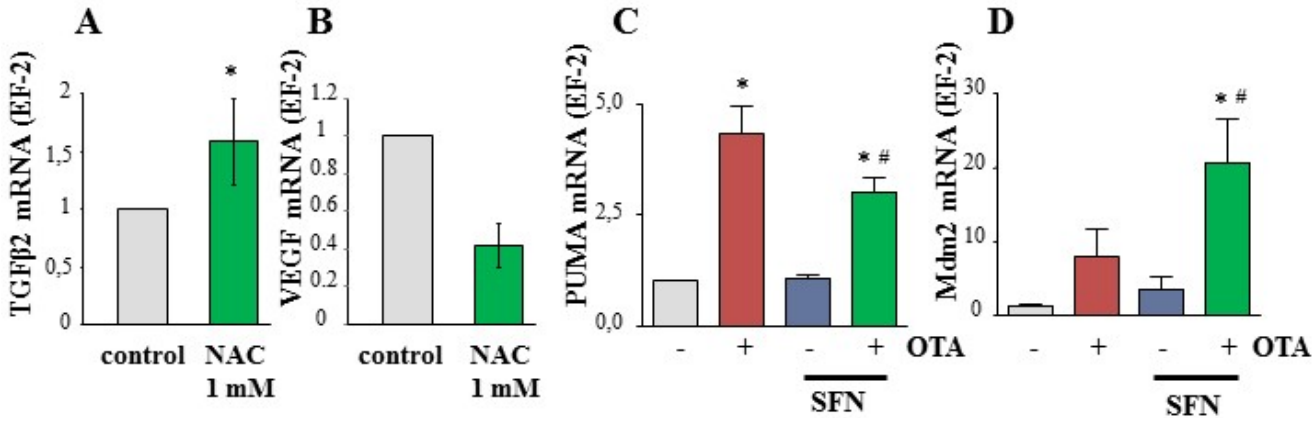
Rationality of the plan of the project

1. The analysis of the effect of H_2O_2, NAC and SFN on UC-MSCs properties

In this step the doses of compounds and time of stimulation will be chosen for next experiments (part 3 of the project). It is especially crucial for H_2O_2 and SFN, as the effect exerted might depend greatly on the dose and length of stimulation.

In case of H_2O_2 the aim is to find the concentration which will elevate the antioxidant capacity and induce the DNA repair system. High induction of both antioxidant system and DNA repair enzymes is important as it will allow not only to get rid of ROS but also to improve cell correction mechanisms against damages caused by ROS. In order to achieve that the dose cannot be too low, because it will generate too weak response, or too high, as it will cause cell senescence or cell death. The length of stimulation is important to achieve high antioxidant capacity, as too short incubation might not induce DNA repair enzymes, and too long incubation might allow cells to come back to their original state.

As far as NAC is concerned, it is a compound which is converted to cysteine and then delivered to cells where serves as glutathione precursor[48].Therefore, in order to confirm its action, the GSH level should be verified. Moreover, in my previous studies I have demonstrated that 24h stimulation with 1 mM NAC elevated TGFβ2 expression in porcine proximal tabular epithelial cells (LLC-PK1)[20] (Ryc. 1A). Moreover, the tendency in downregulation of VEGF after 24h delivery of 1mM NAC was observed in LLC-PK1 cell line (Ryc. 1B). This effect is important as VEGF is important paracrine factor known to be involved in therapeutic properties of MSC[46]. The therapeutic action of MSCs greatly involves elevated angiogenesis, and VEGF, being one of the most important inductors of this process, is also necessary in this action[46,49,50]. Therefore, in order to verify the therapeutic outcome of NAC it is crucial to assess the effect of paracrine activity of UC-MSCs upon NAC delivery.



Ryc.1 The effect of modulators of antioxidant capacity on gene expression. LLC-PK1 were stimulated for 24h with 1 mM NAC (A-B). $Nrf2^{+/+}$ mice received OTA (10 doses every second day 2.5 mg/kg bw) and SFN (10 doses every second day, 7.5 mg/kg bw) i.p. Expression of genes in kidney was analysed(C-D). Analysis of TGFβ2 (A), VEGF (B),

PUMA (C) and Mdm2 (D) mRNA level was done using real-time PCR. n=5 (A), 2 (B), 6-8 (C), 5-8(D); * $p<0.05$ vs control in each group; # $p<0.05$ vs OTA

The usage of the optimal treatment is also important for SFN as too high doses might cause cell death and even enhance the toxicity of H_2O_2[24]. In my previous studies intraperitoneal (i.p.) delivery of SFN attenuated OTA-elevated expression of PUMA in kidney of $Nrf2^{+/+}$ mice[26] (Ryc. 1C). Moreover, it strongly induced OTA-elevated Mdm2 expression[26] (Rys. 1D), what indicates the SFN-exerted inhibition of p53 signalling. The antiapoptotic action of SFN might be crucial in the context of elevation of UC-MSCs survival under stressful conditions present in infarcted heart.

Therefore firstly the cytotoxicity of NAC, H_2O_2 and SFN (different doses and time of stimulation) will be verified and only non-toxic doses will be chosen for further analysis. Then the UC-MSCs survival (viability and necrosis), adhesion and paracrine activity in MI *in vitro* models will be investigated: HSD, H_2O_2 and ICM also in regards will cell adhesion. Then the effect on gene expression, ROS level, apoptosis, cell morphology, GSR level and Nrf2 activity, cell proliferation and DNA damage will be verified.

In the analysis of the survival of UC-MSCs cell will be stimulated with NAC, SFN and H_2O_2 and then put into HSD conditions, treated with H_2O_2 or ICM with or without cell detachment between preconditioning and MI *in vitro*model. In HSD protocol 1% O_2 will be applied, as this situation reflects lack of proper blood flow. In reoxygenation model H_2O_2 will be used as it better corresponds to reoxygenation of whole tissue. Significant part of the analysis will be the utilization of media from injured cardiomyocyte cell line, what allows evaluating the response of UC-MSCs to proapoptotic signal coming from dying cells. Apart from verificationof UC-MSCs survival also the therapeutic properties of cells cultured in these models will be evaluated. This will allow to assess how the conditions of injured heart might affect the therapeutic properties of UC-MSCs.

2. The analysis of the impact of ASA, clopidogrel and RSV on UC-MSCs properties.

In this step the effect exerted on UC-MSCs properties by pharmacological doses of medications listed above used during or immediately after MI or their metabolites, corresponding to concentrations of these compounds in the blood of patients after MI, will be verified.

The highest dose of RSV for patients is 40 mg per day, what corresponds to plasma concentration of RSV 9.8 ng/mL[51]. RSV is included in pharmacological therapy of patients immediately after MI in case of high hypercholesterolemia, thus it is usually prescribed in daily dose of 40 mg. Thus UC-MSCs will be stimulated with 9.8 ng/ml (20 nM) RSV for 24h in order to assess the effect of pharmacological dose of RSV exerted on UC-MSCs therapeutic propertiesproperties.

During MI, ASA is delivered in dose of 300 mg. Normal pharmacological daily dose is usually 75 mg. For clopidogrel the loading dose is 300 to 600 mg and the maintenance daily dose is 75 mg. After oral delivery of clopidogrel in dose of 600 mg the plasma concentration of its active metaboliteis ~ 6 ng/ml while of clopidogrel is ~ 40 ng/ml, whereas for 300 mg clopidogrel is ~ 3 ng/ml and 15 ng/ml, respectively[52]. Delivery of 300 mg of ASAp.o resulted in maximal plasma concentration (cmax) 3.01 mg/l of ASA in plasma and for its metabolite salicylic acid (SA)cmax was 13.31 mg/l[53]. After oral intake of 80 mg of ASAthe maximum level of ASAwass 1 μg/ml (5.55 μM) and of SAwass 4 μg/ml (20 μM) in healthy, young male subjects[54]. Oral delivery of ASA (up to 460 mg cumulative dose) resulted in concentration of 2.9-33.3 μM for ASA and 18.1-245 μM for SA in plasma of patients[55].

Concluding, in order to mimic the physiological concentrations of used medications UC-MSCs will be stimulated with 9.8 ng/mL of RSV (Sigma-Aldrich), 3 and 6 ng/ml of clopidogrel (S-(+)-Clopidogrel Hydrogen Sulfate, Toronto Research ChemicalsInc), 15 and 40 ng/ml of clopidogrel active metabolite – clopidogrel-AM(trans-Clopidogrel Thiol Metabolite Hydrochloride, Toronto Research Chemicalsinc.), 3 mg/l of ASA (Sigma-Aldrich) and 13.3 mg/l of SA (Sigma-Aldrich) for 24h. The effect exerted by drugs and their metabolites will be compared. Furthermore, the effect of prestimulation with one compound (ST, single therapy) or combinatory effect of tested compounds - DT and TT on therapeutic properties of UC-MSCs will be analysed. In order to verify the effects exerted by tested compounds more accurately, ST, DT or TT will be added into UC-MSCs during plating and then cell properties will be investigated. The effect of these compounds on UC-MSC viability, necrosis, adhesion, proliferation, paracrine activity, apoptosis, ROS production, DNA damage and cell survival in MI*in vitro* models as well as the expression apoptosis-related, antioxidant and DNA-repair-involved factors and adhesion protein will be tested.

Moreover, the effect of concomitant delivery of ST, DT or TT and stress conditions: H_2O_2 (in dose mimicking MI) or ICM on UC-MSCs viability, death, adhesion and paracrine activity as well as gene expression, ROS level and DNA repair, cell morphology and apoptosis will be assessed. Therefore, the effect

of tested compounds on intact cell properties as well as on the properties of cells under stress conditions will be assessed.

3. The investigation of the effect of DT (clopidogrel and ASA) and TT (RSV + DT) on the alterations in UC-MSCs therapeutic properties evoked by UC-MSCs preconditioning

The final step will be to utilize new *in vitro* MI models for verification of the protective effect exerted by modulators of antioxidant capacity in UC-MSCs. It is of great importance to assess the combinatory effect of all therapies applied in patients as one medication might affect the action of other. Statins have already been demonstrated to impair the antitumor effect of rituximab, what was discovered by the team of prof. Jakub Gołąb[56]. The modification of the anti-cancer action of rituximab by statins is mediated via depletion of cholesterol level leading to changes in the conformation of CD20[56]. Therefore it is crucial to access the effect of statins on UC-MSCs therapeutic properties, especially on cell adhesion.

As MI patients usually receive both ASA and clopidorel (DT), therefore the effect of DT added during MI*in vitro* models: H_2O_2 and ICM (including need for cell adhesion) on preconditioned UC-MSCs will be verified. In another group, RSV will be added to DT during *in vitro* MI models (H_2O_2 and ICM), as this situation corresponds to MI patients with hypercholesterolemia. Here cell preconditioning with the most promising dosesof NAC, H_2O_2 and SFN from part 1 of the Project will be conducted.

Moreover, based on the data from part 2 of the Project also the effect of preconditioning with RSV, ASA and clopidogrel will be assessed as they might exert beneficial effect. RSV in dose of 1 μM increased paracrine activity of AT-MSC, exerted antiapoptotic action and protected MSCs from HSD[46]. Therefore, it is important to assess the effect of preconditioning using pharmacological doses of tested compounds.

Then the effect of UC-MSCs preconditioning on cell therapeutic properties, including cell viability, necrosis, apoptosis, adhesion, proliferation and paracrine activity as well as ROS intracellular level, DNA damage and gene expression in novel *in vitro* MI models including DT and TT will be tested.

Methodology of the research project

UC-MSCs will be bought from ATCC. Cells between passages 5-7 will be used for all experiments. Cells from 4-6 different donors will be obtained. For the studies UC-MSCs on passage 5-7 will be used.The analysis of the presence of human MSCs (hMSCs) markers such as CD29, CD90, CD105 as well as hematopoietic antigens CD34 and CD45 will be conducted using flow cytometry in order to confirm cell phenotype. Human Cardiomyocytes Immortalized using SV40will be bought from abm.

Firstly, **the effect of H_2O_2, NAC and SFN on UC-MSCs therapeutic properties will be analysed**. UC-MSCs on passage 5-7 will be stimulated with 0.5 and 1mM NAC[13] (Sigma-Aldrich), 10-1 000 μM H_2O_2[29] (Sigma-Aldrich) and 0.25-25 μM SFN[24] (LKT Laboratories) for 2-24h. Then the medium will be removed, cells will be washed with PBS and then cultured in medium without serum in 1% O_2 (HSD)incubator chamber with regulated O_2 concentration for cell cultureor 600 μM H_2O_2followed by analysis of UC-MSCs parameters. For the experiments using conditioned media, cardiomyocytes will be cultured in ischemic conditions – 1% O_2 with the lack of serum (HSD) for 2h and then reoxygenated for 22h. Then the medium will be transferred to UC-MSC (modified from[31]). In case of including the effect of cell attachment, UC-MSCs after preconditioning will be harvested using trypsin and then seeded in different MI *in vitro* models.

Concomitantly, **the impact exerted by RSV, ASA and its metabolite SA, clopidogrel and clopidogrel-AM will be investigated**. UC-MSCs on passage 5-7 will be treated with 20 nM of rosuvastatin calcium (Sigma-Aldrich), 3 and 6 ng/ml of clopidogrel (S-(+)-Clopidogrel Hydrogen Sulfate, Toronto Research Chemicals Inc), 15 and 40 ng/ml of clopidogrel-AM (trans-Clopidogrel Thiol Metabolite Hydrochloride, Toronto Research Chemicals inc.), 3 mg/l of ASA (Sigma-Aldrich) and 13.3 mg/l of SA (Sigma Aldrich) for 24h alone or in combinations: DT or TT. Then medium will be removed, cells will be washed with PBS and then put in MI models described above: H_2O_2, HSD and ICM with or without cell harvesting. Moreover, ASA, SA, clopidogrel, clopidogrel-AM, RSV, DT and TT will be added into UC-MSCs without prestimulation concomitantly with 600 μM H_2O_2 or ICM with cells attached to the plate or immediately after plating.

Finally, **the effect of DT and TT on alterations in UC-MSCs therapeutic properties evoked by UC-MSCs preconditioning** will be tested. For this purpose UC-MSCs on passage 5-7 will be treated with H_2O_2, NAC, SFN, RSV, ASA and clopidogrel-AM with doses and time of stimulation based on previous

experiment conducted within this Project. Then the medium will be removed and cells with be put under ICM or 600 μM H_2O_2 for 24h with or without cell harvesting between prestimulation and MI *in vitro*models. Additionally, DT (ASA 3mg/l, clopidogrel-AM 40 ng/ml) and TT (TT + RSV 9.8 ng/ml) will be added into H_2O_2 or ICM, respectively.

Cell viability will be assessed using Thiazolyl Blue Tetrazolium Bromide (MTT) reduction (Sigma-Aldrich). Cell death will be tested analysing LDH release (Pierce LDH Cytotoxicity Assay kit, Life Technologies). ROS production will be measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Cell proliferation will investigated using BrdU incorporation assay (Roche).Cell phenotype will be verified using standard markers for UC-MSCs characterization (CD29, CD90, CD105, CD34 and CD45) by flow cytometry using flow cytometry.Expression of apoptosis-related factors (p53, PUMA, Mdm2, Bcl2, Bax), adhesive proteins (vinculin and paxillin), antioxidant enzymes (Nrf2, HO-1, catalase, NQO1, GSR, SOD) as well as enzymes participating in DNA repair (glutathione S-transferases – GSTs, glutathione peroxidase - GPx)[57]will be quantified on RNA level using Real-time PCR and on the protein level (western blot, immunofluorescent staining). Nrf2 activity will be measured usingTransAM® Nrf2 Transcription Factor ELISA Kit (Active Motif). GSH level in cells will be tested withGSH-Glo™ Glutathione Assay (Promega). Paracrine activity of UC-MSCs will be assessed measuring VEGF,bFGF, HGF, IGF-1 and Epo in the culture medium by ELISA (R&D Systems). Cell apoptosis will be tested with Anexin V staining using flow cytometry. For the analysis of cell adhesion, UC-MSCs will be added to wells of 6-well plate and allowed to attach for 1h at 37°C. Then cells will be washed 3 times with PBS, 4 separate fields will be photographed with a phase-contrast microscope and cells will be counted[13]. Adhesion of UC-MSCs will be analysed on Primaria plates and plates coated with fibronectin.

Equipment necessary for conducting this project is available for the applicant in the Department of General Biochemistry at the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University. Equipment not present in the Department of General Biochemistry but necessary for conducing this project: flow cytometry and incubator chamber with regulated O_2 concentration for cell culture are accessible in other Departments at the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University and available for the applicant.

Consequences of the project

The issues addressed in this Project will have very strong impact on the progress both in heart regeneration and in regeneration field in general. This project utilizes different methods for boosting cell antioxidant capacity in order to improve UC-MSCs therapeutic properties. Moreover, it stresses the need to analyse different aspects of cell therapeutic features, such as their adhesion, paracrine activity and survival in the tissue of delivery. Importantly, it points out the divergence among conditions under which cells will be placed after transplantation, e.g. HSD vs H_2O_2 or presence of anticoagulants and RSV in the circulation of patients, as well as the need of cell attachment after delivery.

The results of the Project will give a new product – preconditioned UC-MSCs with boosted antioxidant capacity for usage in regeneration of infarcted heart. Another product of the project will be new *in vitro* models for the investigation of the therapeutic properties of UC-MSCs in regeneration of the infarcted heart utilizing pharmacological treatment of recipient patient. This Project is highly applicate not only by cardiologists and transplantologists, but in all MSCs-based regenerative therapies as it stresses out the need of investigation of the pharmacological treatment of cell recipient on the efficacy of regeneration obtained using MSCs. Additionally, this Project points out the need of more complete understanding of the regenerative properties of MSCs and their survival in different *in vitro* models in order to mimic in the best possible way the conditions under which cells will be put after delivery into injured myocardial tissue. Currently the regenerative properties of MSCs are investigated using one model in each publication but it is important to apply all of them to get the most complete information. In will also allow personalized optimization of cell preconditioning for the usage in regenerative therapies, as individual medications of patients, which corresponds also to other diseases present in individuals, should be taken into consideration during the choosing of the most optimal strategy of such regenerative therapy.

The results of the Project will increase scientific knowledge in different fields: stem cells, antioxidant response, regeneration of injured tissues as well as the effect of statins and anticoagulants. Therefore obtained data can be presented on conferences about these divergent topics. The presentation of project results on conferences and publishing them in prestigious, specialized journals will make this Project results available for the scientists worldwide.

The impact of the Project on the field of regenerative therapies is important not only for scientists but also for physicians and patients as well as patients' friends and families. In this way it will have a great impact on the society. It will affect the economy, as the effective heart regeneration therapy is also important for employers, government and social security system. Mentioned above are the groups interested in this project results. Moreover, it is also of interest for private clinics offering new regenerative therapies for patients.

The results of the project are planned to be published in 2 original papers in prestigious scientific specialized journal: 1) about the effect of anticoagulant treatment and RSV during *in vitro* analysis of the therapeutic properties of UC-MSCs and 2) concerning the effect of different modulators of cell antioxidant capacity with divergent mechanism of action on UC-MSCs therapeutic properties. Furthermore, the review article about the effect of MSCs preconditioning on therapeutic properties of MSCs for their usage in ischemic diseases is planned to bewritten within the project to further increase the knowledge of the Applicant. The project results are also planned to be presented on scientific conferences.The results of the part of the project conducted by PhD student will be incorporated into Master thesis. The planned topic of Master thesis: The effect of Nrf2 activation by sulforaphane on UC-MSCs therapeutic properties. The results of the Project will create the foundation for next projects: Starting grant from European research Council (ERC), Iuventus Plus from the Ministry of Science and Higher Education, Frist Team from Foundation for Polish Science or Opus or Sonata from National Science Centre.

Importantly, as the applicant has never been a PI in the projectbefore, this will boost drStachurska experience and allow Her to develop as a PI. Within the project drStachurska will supervise Master Student, what will also add to Her skills. This is important as in next steps of drStachurska scientific carrier. She will supervise more students, both master and PhDs. Utilizing the knowledge of drStachurska about antioxidant response within new field: usage of UC-MSCs for regenerative therapies, which is really important task nowadays, will also allow Her to grow as a scientist. Thus it will enable drStachurska to improve her R&D potential.Additionally, conducting this research will allow drStachurska to start working in the Department of General Biochemistry at the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University. It is of even greater importance as drStachurskais currently unemployed. Getting this funding will get drStachurska the possibility to start and the collaboration between the Applicant and prof. Jolanta Jura, giving permission to conduct the studies within this Project in the Department of General Biochemistry at the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University, of which prof. Jolanta Jura is a Head. The Research Partner of the Applicant is prof. Jakub Goląb from the Department of Immunology of the Medical University of Warsaw and winning this fundingwill also give the chance to the Applicant to start the scientific collaboration with prof. Gołąb. The result of the project will be also an increase in the R&D potential of drStachurska, what will be achieved via using the knowledge of drStachurska in new field of tissue regeneration as well as obtaining by drStachurska experience as PI of the project and the supervisor of Master thesis. Additionally, participation of drStachurska in trainings about intellectual property and flow cytometry will increase Her R&D potential.

R&D potentialof Master Student, who will participate in this Project, will also be improved. The part of the Project planned for the Master student concerns the usage of SFN for improving the therapeutic properties of UC-MSCs as it is a consistent and complete topic suitable for master thesis. Within this Project Master student will learn how to culture and to conduct the experiments with UC-MSCs as well as get to use many laboratory techniques, such as analysis of cell viability (MTT reduction) and mortality (LDH release), isolation of RNA, reverse transcription, analysis of gene expression by real-time PCR, investigation of intracellular ROS level (DCFH-DA), etc. Therefore the Student will get to know many laboratory techniques and will be also a part of bigger study. Moreover, the Student will get the theoretical knowledge in the topic. The Master Student will also participate in scientific conferences, importantly on students' conference. Furthermore, taking part in trainings about intellectual property and flow cytometry will boost R&D potential of the Student. Defending the Master thesis will also be great developing activity for the Student.

Concluding, results of this project with great social and economic impact pointing out important novel aspects (novel preconditioning methods and novel in vitro tests) in the usage of UC-MSCs in the regenerative therapy of the heart will have an impact not only on science but also on the practical usage of these cells with the wide range of potential customers.

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