MEDICAL FACULTY IN PILSEN
CHARLES UNIVERSITY IN PRAGUE

Mesenchymal stem cells and their regenerative and immunomodulatory potential

Mesenchymální kmenové buňky a jejich regenerační a imunomodulační potenciál

MUDr. Mgr. Michaela Miklíková

Self report of
Ph.D. Dissertation

Pilsen 2016

Disertation was written as a part of full-time doctoral study program in Anatomy, Histology and Embryology on Department of Histology and Embryology in Medical faculty in Pilsen, Charles University in Prague.

Student:	MUDr. Mgr. Michaela Miklíková
Head of departmental board:	doc. MUDr. Jitka Kočová, CSc. Department of Histology and Embryology,
Supervising Tutor:	doc. MUDr. Daniel Lysák, PhD. Hematology-oncology Department
Consulting Tutor:	Ing. Lucie Vištejnová, Ph.D. Department of Histology and Embryology Biomedical Center
Oponents:	doc.MUDr.Mgr.Milan Raška, PhD. Imunology Department Medical faculty, Palacky University Olomouc RNDr. Vratislav Horák, CSc. Laboratory of tumor biology Institute of Animal Physiology and Genetics AS CR

Self report was sent: .....

Dissertation defense infront of committee for defense of dissertations in Anatomy, histology and Embryology program will take a place: .....

Defense location: Department of Histology and Embryology, Karlovarská 48, Pilsen.

Dissertation is available for perusal at Headquaters of Medical faculty in Pilsen, Charles University in Prague, Husova 3, Pilsen.

Content

Abstract

Mesenchymal stem cells (MSCs) possess multidirectional regenerative ability, which, together with their immunomodulatory potential, makes them promising cell type for therapy of wide variety of diseases. Despite ongoing research, which proved MSCs application to be safe, reported effect of MSCs administration on patients is not convincingly beneficial yet.

In our work we focused on elucidation of MSCs role in regeneration of vital organs, heart and liver, where a large damage is life threatening for patients and any improvement in therapy would save many lives. Similar situation is in Graft versus host disease (GVHD), where MSCs immunomodulatory properties could be beneficial.

Role of MSCs in heart regeneration was examined *in vitro*. Primary adult swine cardiomyocytes (CMCs) were co-cultured with or without swine MSCs for 3 days and morphological and functional parameters (contractions, current, respiration) of CMCs were measured. MSCs showed supportive effect on CMCs survival, especially at day 3 of the experiment, where in co-culture was significantly higher number of viable CMCs with physiological morphology and maintained function.

Effect of MSCs on liver regeneration was observed in swine model of chronic liver disease. Piglets underwent liver lobe resection followed by MSCs administration (1×10^6 cells /kg) into portal vein. Cytokines and growth factors quantification was performed in selected time points. The morphometry of regenerated liver tissue was analyzed by quantative histology. Results showed the insignificantly increased connective tissue volume in liver parenchyma after MSCs administration and other measured parametres were not significantly influenced by MSCs.

Immunomodulatory effect of MSCs on GVHD was evaluated first *in vitro*, on mixed lymphocyte culture, where, according to metabolic activity measurement test, MSCs suppressed lymphocyte activity. Second, MSCs were administered in one dose to patients with severe GVHD and for three months levels of regulatory T – lymphocytes together with helper T-lymphocytes were measured as an evidence of MSCs immunomodulation. Statistics of obtained data showed no significance, but clinical condition of patients significantly improved.

Our work showed that MSCs have supportive and immunomodulatory effect on cells in *in vitro* culture, where conditions can be controlled easily. After MSCs administration into living organism many more variables influence results of the research, and the outcomes are usually promising, but not convincing. More experiments on large groups of participants need to be done to transfer MSCs transplantation from the research field into clinical practice.

Abstrakt

Mesenchymální kmenové buňky (MSCs) dokáží mnoha způsoby podpořit regeneraci tkání, což je, spolu s jejich imunomodulačním potenciálem, dělá slibným buněčným typem pro léčbu širokého spektra onemocnění. Probíhající výzkum prokázal, že aplikace MSCs do organismu je bezpečná, ale popisovaný efekt na pacienty není příliš přesvědčivý.

V této práci jsme se soustředili na objasnění role MSCs v regeneraci životně důležitých orgánů, srdce a jater, jejichž rozsáhlé poškození je život ohrožující pro pacienty a jakékoli zlepšení současných terapeutických možností by mohlo zachránit mnoho životů. Podobná situace je u pacientů s nemocí typu reakce štěpu proti hostiteli (GVHD), kde by se mohly prospěšně uplatnit imunomodulační vlastnosti MSCs.

Role MSCs v regeneraci srdce byla zkoumána v *in vitro* experimentech. Primární kardiomyocyty (CMCs) izolované z dospělého prasete byly ko-kultivovány s prasečími MSCs a po 3 dny, byly sledovány a měřeny jejich morfologické a funkční vlastnosti (kontrakce, vápníkové proudy, respirace). MSCs prokázaly podpůrný efekt na přežívání CMCs, což bylo obzvláště významné ve 3. dni experimentu, kdy se v ko-kultuře vyskytovalo významně více živých CMCs se zachovanými morfologickými i funkčními vlastnostmi.

Vliv MSCs na regeneraci jater byl sledován na prasečím modelu chronické jaterní nemoci. Po resekci jaterního laloku byly MSCs (1×10^6 bb /kg) aplikovány do portální žíly prasete. Ve vybraných časových intervalech pak byla prováděna kvantifikace hladiny cytokinů a růstových faktorů v periferní krvi zvířat. S využitím kvantitativní histologie pak byla provedena morfometrická analýza regenerované jaterní tkáně. Z výsledků vyplývá, že aplikace MSCs nemá významný efekt na sledované parametry, snad s vyjímkou pozorovaného nevýznamného zvýšení podílu pojivové tkáně v jaterním parenchymu.

Imunomodulační efekt MSCs na GVHD byl nejdříve zkoumán *in vitro*, kde ve smíšené lymfocytární kultuře po přidání MSCs, ukázal test metabolické aktivity, že MSCs potlačují aktivitu lymfocytů. Dále byly MSCs v jedné dávce aplikovány pacientům s těžkou GVHD. V následujících třech měsících byly měřeny hladiny regulačních T-lymfocytů a pomocných T-lymfocytů pro průkaz imunomodulačního efektu MSCs. Statistické vyhodnocení získaných dat neprokázalo významný rozdíl, ale klinický stav pacientů se výrazně zlepšil.

Naše práce ukázala, že MSCs mají podpůrný a imunomodulační efekt na buňky v *in vitro* kultuře, kde je snadné zajistit ideální experimentální podmínky. Po aplikaci MSCs do živého organismu ovlivňuje výsledný efekt mnoho proměnných, proto jsou často výsledky sice slibné, ale nepřesvědčivé. Je nutno provést další experimenty na velkých skupinách účastníků, než bude možné přenést aplikaci MSCs z laboratoří do běžné klinické praxe.

1 Introduction

1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells possessing multidirectional regenerative ability. Nowadays MSCs are in a focus of scientists and clinicians as a therapeutic option of the future for wide variety of diseases.

MSCs reported ability to differentiate into adipocytes, chondrocytes and osteocytes together with other observed differentiation abilities made them promising cell type for tissue regeneration at first. Further research of these cells showed that MSCs improve regeneration of variety tissues not only by differentiation, but, in much bigger percentage, by release of cytokines, chemokines and immunosuppressive molecules, which modulate inflammation and support cell survival together with connective tissue formation, resulting in complex tissue regeneration. In more than 40 years of MSCs research there were not reported any tumor formations or any other serious adverse effect after MSCs application in vivo.

MSCs are naturally present in developing and also developed adult organism. They can be isolated from many tissues and cultivated for further in vitro or in vivo research. From culture they can be easily harvested and administered into living organism, where they do not trigger immune response. Therefore MSCs are ideal cell type for not only autologous transplantations, but also allogeneic, or, as reported, xenogeneic.

Recently, all these findings lead researchers and clinicians to start more than 500 clinical trials, where MSCs are transplanted to patients as a treatment or co-treatment for variety of diseases. So far, results of clinical trials are not as positive and promising as it was expected. MSCs are not reported to be harmful, but sometimes it seems that their application is not having any effect. Many reasons for this observation can be found, but only more detailed research in this field can provide sufficient answers and solutions.

Therefore we performed series of experiments in vitro and also in vivo, in order to explain in detail MSCs role in heart and liver regeneration together with their immunomodulation abilities and their effect in Graft versus Host disease.

1.2 MSCs characterisation

MSCs are very heterogeneous cell population, considering their morphology, physiology and cell markers expression. Till today there was not identified marker specific only for MSCs. To prevent chaos, in 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy published Minimal Criteria every cell has to meet to be classified as Mesenchymal Stem Cell [Dominici et al., 2006]. Cell must:

1. posses fibroblast-like shape, adhere to surface, grow in field and form colonies,
2. express specific antigenes: CD73, CD90, CD105 and in the same time cannot express CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR,
3. be able to differentiate into adipocytes, chondrocytes and osteocytes.

1.3 Mechanism of MSCs therapeutical function

After reception of specific stimuli, MSCs are able to home into site of injury and affect surrounding cells with cell-to-cell contact or by release of variety of factors. Three main mechanisms how MSCs support regeneration are paracrine production of cytokines and other

molecules, immunomodulatory function and in small percentage also differentiation into target tissue cells.

1.4 MSCs and cardiac regeneration

Myocardial tissue was, at first, considered to be incapable of regeneration. But in 2001 it has been shown that injection of MSCs into heart of mice with infarction resulted in improvement of cardiac functions [Orlic et al., 2001]. Since then many more experiments was performed with more and less positive results, where MSCs proved to have beneficial effect on whole heart [Liu et al., 2015; Lee et al., 2014; Guo et al., 2007]. MSCs support cardiac repiar with three main mechanisms; immunomodulation, differentiation and paracine effect. All of them influence not only CMCs, but also connective tissue and vessels. MSCs supported vasculogenesis and cardiac tissue regeneration improves chance of successful cardiac repair

Despite the described beneficial effect, detailed metastudy of clinical researches showed that more positive results are, more errors in research design can be found. Methodically correct studies showed non or very mild effect of MSCs on patient hearts [Nowbar et al., 2014].

In our study we focused on experiments with swine cells to elucidate role of MSCs in cardiac regeneration.

1.5 MSC and liver regeneration

It has been shown that MSCs transplantation helps to partially restore liver function and decrease symptoms of the disease. MSCs support liver repair with three main mechanisms; immunomodulation, differentiation and paracine effect, all of them influence not only hepatocytes, but also connective tissue and vessels. MSCs supported vasculogenesis and hepatocyte proliferation together with suppression of inflammation and hepatocyte apoptosis improves chance of successful liver repair. All together MSCs has been reported to increase survival rate of patients with variety of liver diseases [Lin et al., 2011].

In our work, new model of chronic liver disease in pig was introduced. MSCs were administered to pigs with developed liver disease after liver lobe resection.

1.6 MSCs and GVHD

MSCs are tested as a possible treatment or co-treatment of Graft-versus-host disease (GVHD), but evidence show, that MSCs can be used also as a prevention of GVHD relapse.

Commonly used and also only effective therapy for GVHD is long term administration of high doses of corticoids. If the GVHD is resistant for this drug, it is difficult to manage the disease. It has been reported that application of MSCs for these patients can reduce symptoms of the GVHD and lower amount of relapses, if administered regularly in infusions ($1x10^6$cells/kg) [Kebriaei and Robinson, 2011].

Application of MSCs to GVHD patients helps to inhibit donor T-lymphocyte reactivity to histocompatibility antigens of normal host tissue, together with suppression of DCs maturation and suppression of NK cytotoxicity. All these findings have been observed in many studies [Muguruma et al., 2006; Le Blanc and Ringdén, 2005, 2006].

In our work we focused on elucidation of overall effect of MSCs on lymphocytes.

2 Aims

Optimize swine and human MSCs isolation and cultivation
Standardize and optimize MSCs verification protocols
Describe the role of MSCs in cardiomyocyte function repair
Describe the role of MSCs in liver regeneration
Describe the role of MSCs in GVHD

3 Materials and methods

All animal experiments were performed following the guidelines of European parlament and European Council 2010/63/EU about protection of animals used in scientific experiments. All experiments were approved by Expert comittee for work with laboratory animals of Medical faculty in Pilsen, Charles University in Prague.

3.1 MSCs isolation

In this study we used bone marrow MSCs isolated from adult organisms, pig MSCs for majority of experiments, human MSCs for immunity related experiments. MSCs were isolated with gradient centrifugation method and seeded on cultivation plastic. After attachment period, floating cells were washed and remaining cells, MSCs, were verified in flow cytometry for marker expression (positivity for CD73, CD90 and CD105 and negativity for markers of hematopoitic line) and part of the cells underwent dfferentiation to osteo-, adipo- and chondro- line for confirmation of differentiation ability.

3.2 MSCs and cardiomyocytes

Cardiomyocytes (CMCs) were isolated from left ventricles of young adult pigs (Sus Scrofa, n = 5) in anesthesia. Heart was mounted to constant pressure Langerdorff's aparathus and perfussed with warm (37°C) oxygenated solutions; Tyrode with/without calcium and collagenase solution. Isolated CMCs were seeded into cultivation plastic and evaluation of cell culture yield was performed. As CMCs, all long striated cells with rough edges were counted, the rest was considered to be dead or dying cells.

Four different cultivation surfaces were used for cell culture optimisation; 1, CMCs were seeded on normal cultivation plastic without any further treatment (TPP, Switzerland), 2, CMCs were seeded on normal cultivation plastic (TPP, Switzerland) coated with laminin (mouse, Sigma-Aldrich, USA) in concentration 1,5 mg/ml, 3, CMCs were seeded on normal cultivation plastic (TPP, Switzerland) coated with 0,5 % gelatine (Sigma-Aldrich, USA), 4, CMCs were seeded on cultivation plastic specially treated for better cell attachment (Corning, Sigma-Aldrich, USA). Cell adhesivity was evaluated on all four cultivation surfaces in light microscopy (Nicon Eclipse Ti, Japan). One asigned person, experienced observer, shaked with cultivation plastic and rated cell adhesivity in each sample by number 1 – 5, where number 1 ment majority of the cells (> 90 %) was flowing in media not attached to surface and number 5 ment majority of the cells was attached to cultivation surface. For all four cultivation surfaces the results were statistically processed, regular cultivation plastic without any treatment was used as standart for comparisons.

For co-cultivation experiments were first MSCs seeded to 6-well plate (TPP, Switzerland) in concentration 54 000cells/well each and let to attach overnight in humidified incubator (37°C, 5% CO_2). Insert of, transwells, 3 microns membrane pores, Transwell® (Corning, Sigma-Aldrich, USA), into 3 wells with MSCs followed and CMCs in Complete CMC media were added to all 6 wells of 6-well plate, as a control CMCs without presence of MSCs were used. For 10 days CMCs morphology was observed and percentage of living CMCs in culture was evaluated with use of MitoTracker® (579/599) (Molecular probes, Life Technologies, USA). In day1 and day 3 of the experiment mitochondrial respiration was measured with high-resolution respirometry, oxygraph Oroboros (Oroboros, Insbruck,

Austria), and CMCs electrophysiology parameters (contractility and transient calcium currents) were evaluated with Ionoptix HyperSwitch Myocyte Calcium and Contractility System (IonOptix LLC, Westwood, USA).

For statistics analysis software STATISTICA Cz (Statsoft CR, Czech republic) was used. Parametric data were analysed with Student t-test, non parametric statistics was performed with use of Wilcoxon test and for complex data, analysis of variance with multiple factors (ANOVA) was used. Results are presented as averages ± standart error of the mean (SEM), where probability level <0,05 was considered to be significant.

3.3 MSCs in liver regeneration

For this study, not previously described model of chronic liver disease in pig (Sus Scrofa) was estabilished. Pigs in general anesthesia underwent biliary obstruction surgery, were observed for 9 weeks and resection of left liver lobe as a simulation of surgery on diseased liver in human, followed. In the same moment, liver samples for morphometry analysis were taken and administration of MSCs (1×10^6 cells / kg) into portal vein followed. After resection, peripheral blood was collected in 7 timepoints ; 0 h, 2 h, 24 h, 3 days, 7 days, 10 days and 14 days for luminex analysis, Luminex® 200™ (Luminex Corporation, USA), of IL-6, IL-8, TNF-α and TGF-β concentrations. In day 14 after resection animals were sacrificed and liver samples for morphometry analysis were taken, see Table 1.

All gained data were analysed in software STATISTICA CZ (Statsoft, Czech republic). For data with normal distribution Students t-test was used, for non parametric statistics Wilcoxon test or Mann-Whitney U test was used. As signficant $p < 0,05$ was taken.

3.4 MSCs and GVHD

For evaluation of influence of MSCs on GVHD and further implementation of these findings into regular medical practice, human cells were used. This study was approved by Ethical committe of Teaching hospital in Pilsen. All donors provided written cosent to the study.

For initial experiments, in vitro culture was chosen. As a GVDH model, mixed lymphocyte culture was used, where lymphocytes from HLA incompatible donors were mixed. For more comparisons, stimulation with nonspecific mitogenes and chemotaxis activators Phytohemaglutinnin (PHA) and N-formyl-Met-Leu-Phe (fMLP, both Sigma-Aldrich, USA), as a simulation of medicaly induced state, followed.

To the 200 µl lymphocyte mixture 50 µl MSCs in different concentrations: 4×10^5 cells/ml (MSCs/lymphocyte ratio 1:5), 4×10^4 cells/ml (1:50) and 4×10^3 cells/ml (1:500) were added and cultivated for 6 hours. Addition of MTT solution followed and after 2 h incubation, the spectrophotometric analysis was performed with Synergy HT (Biotek, Germany).

Results from 32 tests of the cells from 15 healthy donors were statistically analysed by STATISTICA CZ (Statsoft, Czech republic) with use of the Wilcoxon paired test. As significant, $p < 0,05$ were taken.

Table 1 - Quantitative parameters used in liver morphometry, their stereological principles, histological staining and sampling of photographs. The lowest possible magnification was used to maximize reference space for each parameter.

Abbreviation	Parameter (unit)	Stereological principle used for quantification	Section staining and objective magnification
A(lobule)	Mean cross-sectional area of classical morphological lobules (mm^2)	Step 1. Systematic uniform random sampling of one lobule per tissue section for quantification. Step 2. Nucleator probe in isotropic uniform random (IUR) sections.	Anilin blue and nuclear red objective 2x
V_V(connective, liver)	Volume fraction of connective tissue in the liver (%)	Step 1. Systematic uniform random sampling of microscopic image fields selected for quantification from multiple physical sections. Step 2. Point grid and Cavalieri of Delesse principle.	Anilin blue and nuclear red objective 2x
V_V(hepatocytes, liver)	Volume fraction of hepatocytes in the liver (%)	Step 1. Systematic uniform random sampling of microscopic image fields selected for quantification from multiple physical sections. Step 2. Point grid and Cavalieri of Delesse principle.	PAS objective 40x
V_V(MH,liver)	Volume fraction of mononuclear hepatocytes in the liver (%)	Step 1. Systematic uniform random sampling of microscopic image fields selected for quantification from multiple physical sections. Step 2. Point grid and Cavalieri of Delesse principle.	PAS objective 40x
V_V(PH,liver)	Volume fraction of polynuclear hepatocytes in the liver (%)	Step 1. Systematic uniform random sampling of microscopic image fields selected for quantification from multiple physical sections. Step 2. Point grid and Cavalieri of Delesse principle.	PAS objective 40x
V_V(BB,liver)	Volume fraction of blood and biliary vessels (%)	The parameter was calculated by subtracting the total liver volume the fractions of connective tissue and hepatocytes from 1.	-
V(MH)	Mean volume of mononuclear hepatocytes (μm^3)	Step 1. Systematic uniform random sampling of the lobules (at least 30 per tissue section) selected for quantification. Step 2. Nucleator probe in isotropic uniform random (IUR) sections.	PAS objective 40x
V(PH)	Mean volume of polynuclear hepatocytes (μm^3)	Step 1. Systematic uniform random sampling of the lobules (at least 30 per tissue section) selected for quantification. Step 2. Nucleator probe in isotropic uniform random (IUR) sections.	PAS objective 40x

For clinical study, only patients with severe GVHD, both accute (n = 8) and chronic (n = 10), were enrolled. Every patient received one dose of MSCs ($1 - 5 \times 10^6$/kg). Blood samples were taken in times; 0 (before the MSCs were injected), 14 days, 1 month, 2 months and 3 months after MSCs administration. The count of white blood cells (WBC), lymphocytes

and subpopulations of regulatory T-lymphocytes (Tregs) and helper T-lymphocytes were measured with flow cytometer BD FACSCanto II (BD Biosciences, USA) as markers of immunomodulation. Tregs were evaluated by two antibody sets, first set confirmed Tresgs as cells $CD4^{+}$, $CD25^{bright+}$, $CD127^{-}$ and second set confirmed Tregs as cells $CD4^{+}$, $CD25^{bright+}$, $FoxP3^{+}$. Both detection methods are mentioned in literature as efficient. Comparison of both antibody sets results for Tregs detection optimisation was performed.

Results from five different timepoints from 10 patients with chronic GVHD and 8 patients with acute GVHD were statistically analysed by STATISTICA CZ (Statsoft, Czech republic) with use of the Wilcoxon paired test. As a base for analysis, time 0 was transformed to 100%, the rest of values in different times were compared and transformed to percentage. Statistics was performed on these values, as significant, $p < 0,05$ were taken.

4 Results

4.1 MSCs isolation and culture

MSCs, both swine and human, can be isolated with described procedure. Isolated cells showed to have properties of MSCs, verification under light microscopy for MSCs phenotype (Figure 1), differentiation into three lines (Figure 2), and flow cytometry antigene determination was performed (Figure 3).

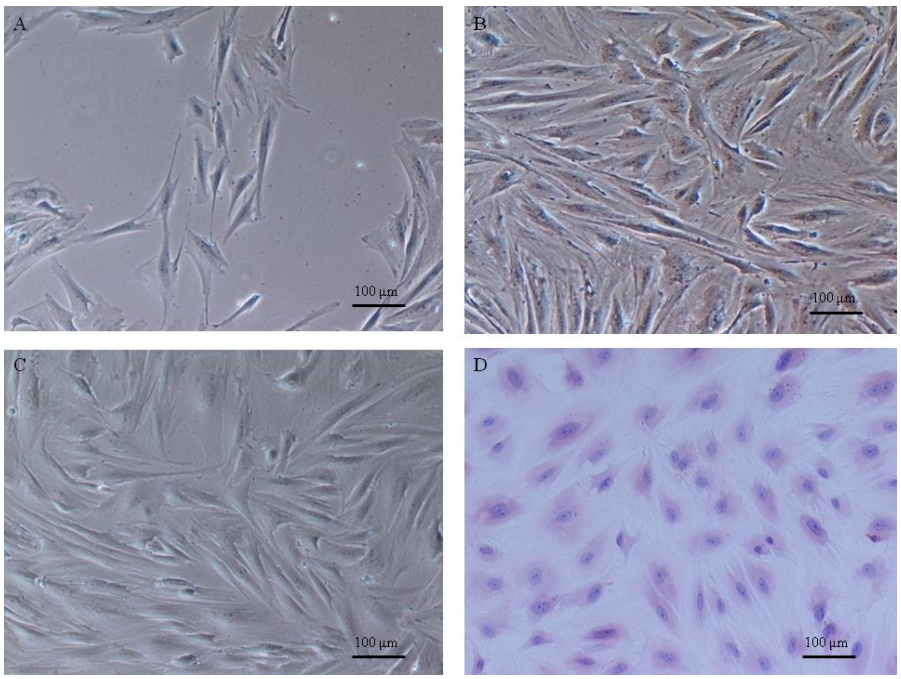

Figure 1 - MSCs morphology in light microscopy. Adhesive behaviour, spindle shape and large nucleus. A – MSCs forming colonies, B – MSCs in 100 % confluency, C – MSCs in phase contrast, D – MSCs stained with Hematoxylin-eosin.

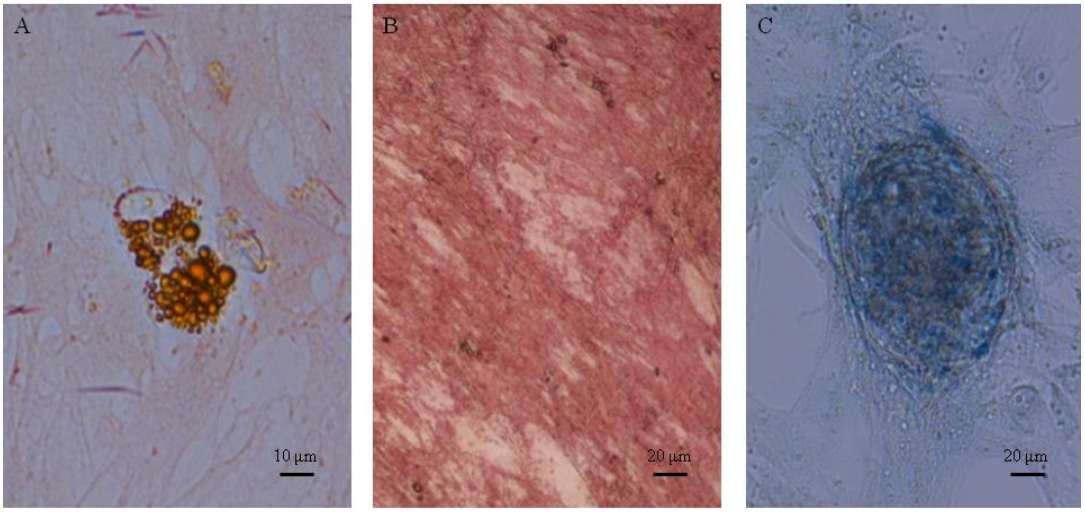

Figure 2 - Differentiation of MSCs into three lines; A – MSCs differentiated into adipocyte line – fat vacuolas stained with Oil Red O; B – MSCs differentiated into osteocyte line – calcein structures stained with Alizarin Red S; C – MSCs differentiated into chondrocyte line – blue color of mucopolysacharides stained with Alcian Blue.

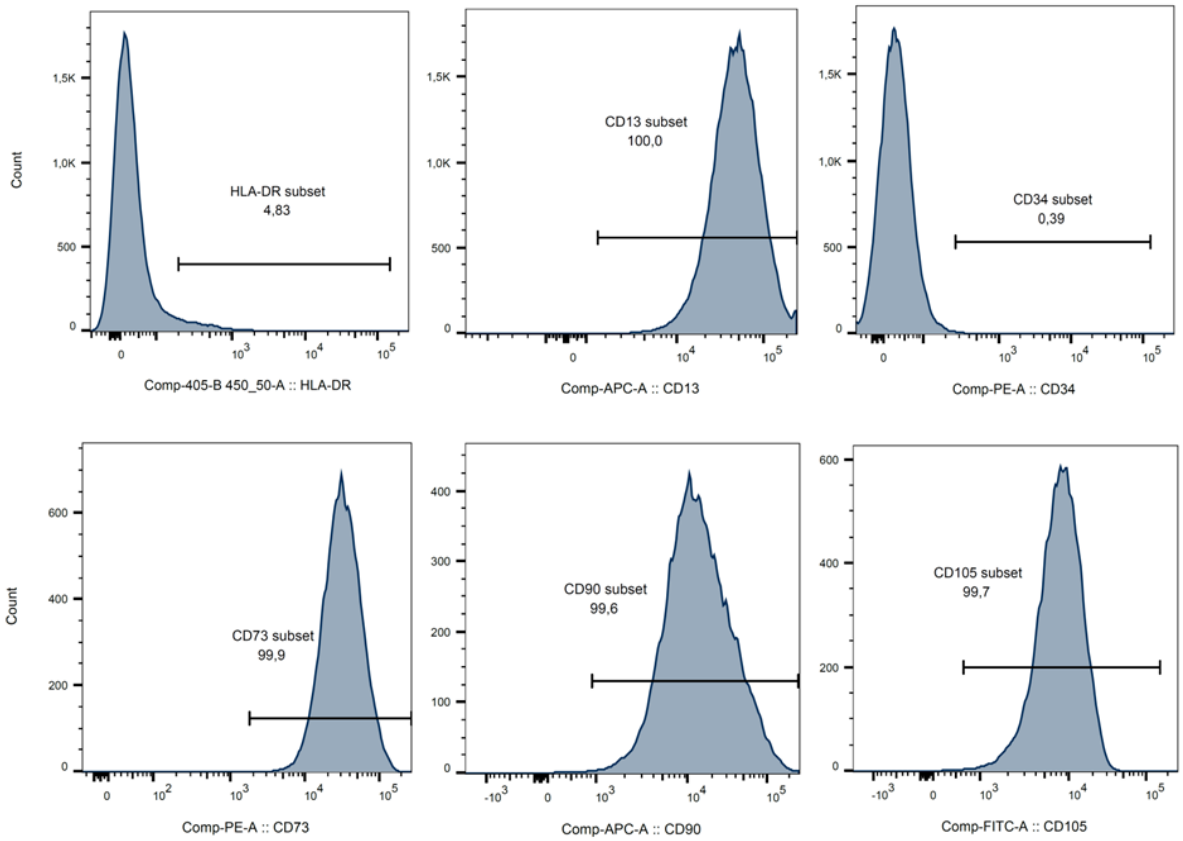

Figure 3 - Flow cytometry of human MSCs. Example of markers typical for cells; HLA-DR$^-$(95,17 %), CD34$^-$ (99,61 %), CD13$^+$ (100 %), CD73$^+$ (99,9 %), CD90$^+$ (99,6 %) and CD105$^+$ (99,7 %).

4.2 MSCs and cardiomyocytes

4.2.1 Cardiomyocyte isolation and culture

Porcine primary adult CMCs can be isolated by standart procedure. Isolated CMCs possessed characteristic phenotype, long, trabecular shape with rough edges on both sides and visible stripes. However, the percentage of live CMCs among other cells; e.g. dead, dying, stromal and debris, was low 20 % (± 10 %).

CMCs can be cultivated on all four used cultivation surfaces, with no observed negative effect on CMCs morphology or survival. CMCs cultivated on standart cultivation plastic covered by gelatine or laminin showed statistically significant higher percentage of adhesivity ($p = 0{,}00068$ for gelatine, $p = 0{,}00011$ for laminin) in comparison with CMCs cultivated on standart cultivation plastic without cover or on special cultivation plastic for higher cell adhesivity (Table 2). Together with increased adhesivity of CMCs, other cells in suspension, dead and dying cells, showed increased adhesivity too. Simple cultivation on different types of plastic did not helped to increase culture purity, to increase the ratio of living CMCs cells in culture.

Table 2 - Comparison of cell adhesivity on different cultivation surfaces with different cover.

SURFACE TYPE	COVER	ADHESIVITY OF CMC (MEAN ± SD)	ADHESIVITY OF OTHER CELLS (MEAN ± SD)
Standart cultivation plastic	-	1,8 ± 0,63	1,8 ± 0,63
Standart cultivation plastic	gelatin	3,5 ± 1,08 *	3,5 ± 1,08 *
Standart cultivation plastic	laminin	3,8 ± 1,03 *	3,8 ± 1,03 *
Superadhesive cultivation plastic	-	2,2 ± 0,92	2,2 ± 0,92

Evaluated under light microscope by experienced observer by 1 – 5 scale, where 1 stands for majority (> 90 %) of the flowing cells and 5 for majority adhered

* Statistically significant results (t–test $p < 0,001$) in comparison with regular cultivation plastic without cover.

4.2.2 Co-cultivation experiments

CMCs and MSCs cocultivation experiments showed, that CMCs in direct contact with MSCs tend to adhere to MSCs and form 3D culture phenomenon. This impeded division of CMCs from MSCs and further manipulation with cells without cell damage. According to observation it is possible to say, that this way of cultivation is beneficial for the CMCs the most, because the morphology of living CMCs was observed here for the longest time, but the observation was not possible to quantify and analyse. Therefore we did not used CMCs cocultivated with MSCs directly for further experiments. CMCs cultivated alone, without MSCs presence and CMCs co-cultivated with MSCs across the transwells were used for further comparisons.

Freshly isolated cardiomyocytes had long, trabecular, shape with sharp edges on both ends and their stripes as a sign of striated muscle were nicely visible (Figure 4A). In longer term culture the sharp edges of the cells started to round and stripes slowly dissapeared. After three days of the culture CMCs started to develop morphological pathologies (Figure 4B – E), but many cells in the culture remained phenotypically CMCs. After 9 days, CMC phenotype in culture was observed very rarely on all cell cultivation surfaces.

Fluorescent microscopy confirmed observation under light microscope. In day 1, staining with MitoTracker, which stains with red colour healthy mitochondria having sufficient membrane potential for effective oxidation functions, was nicely visible in both compared groups, in CMCs cultivated alone and in CMCs co-cultured with MSCs (Figure 5). In day 3 of observation the signal was also visible (Figure 6), but cells already showed first signs of deterioration, after this day rapid decrease in observed signal was found.

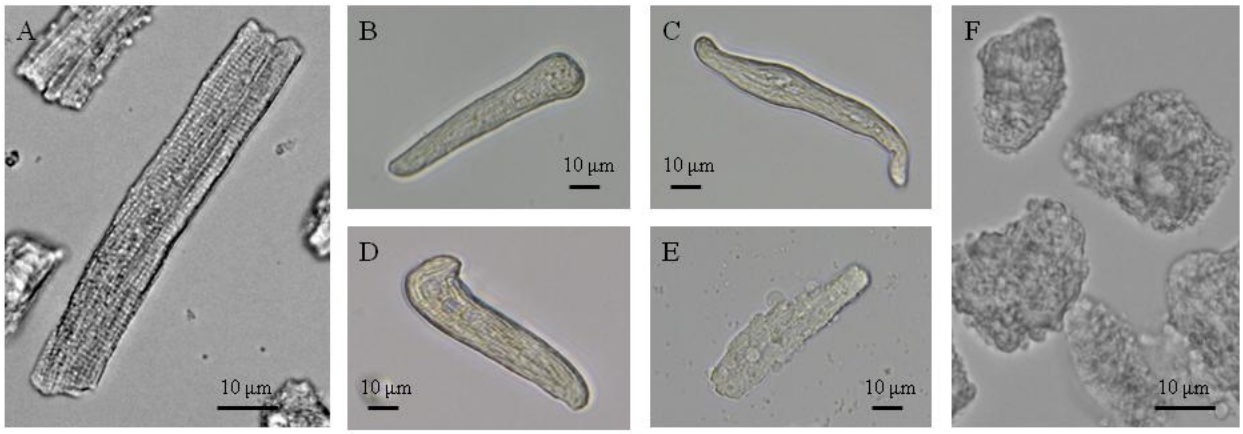

Figure 4 - Morphology of CMCs in time; A – freshly isolated CMC with long trabecular shape, rough edges and stripes, B – D – morphology pathologies of CMCs in long term culture (3 – 10 days), CMCs loose stripes, edges starts to round and cell crooks, E – dying cell, F – dead cells and cellular debris.

CMCs cultivated with MSCs in the transwells, without cell-to-cell contact, preserved CMCs morphology for aproximatelly one day longer than CMCs cultivated without MSCs. CMCs in separate culture also showed statistically significant ($p = 0{,}0196$) decrease in ratio of living cells between day 1 and day 3 of assessment. Comparison of ratio of living CMCs in culture with and without MSCs in day 1 showed diffrence, but not significant. In day 3 the difference between both CMCs groups was statistically significant ($p = 0{,}0152$). MSCs positively influenced CMCs in culture (Figure 7).

Oxygen consumption (Figure 8) of intact CMCs had tendency to decrease with cultivation time, but the decrease was not significant in both CMCs groups, cultivated with and without MSCs. Basic respiration (ROUTINE state) fluctuated from 249 ± 62 pmol/(s*10^6 cells) in CMCs cultivated for 1 day without MSCs and 126 ± 42 pmol/(s*10^6 cells) in CMCs cultivated for 3 days with MSCs. Similar characteristics showed also state LEAK. Maximal respiration induced by FCCP was nearly identical for both CMCs groups and had small trend to decrease in time.

Cell culture had detrimental effect on calcium transient and contraction of CMCs. CMCs cultured for 3 days compared to CMCs culture for 1 day showed significant decrease in the baseline values of transient and contraction amplitude and decrease in transient and contraction velocities. Cultivation of CMCs with MSCs did not prevented the changes. Moreover calcium transients of cells cultivated with MSCs after 1 day of cultivation showed significantly slower departure and return velocity, longer time to 50 % of peak, shorter time to 10 % of peak and lower peak (amplitude of the peak is also higher, but not significantly) compared to cells cultivated for 1 day without MSCs. Contraction of these cells was not significantly different. In cells cultivated for 3 days were differences in calcium transients mostly inverse (CMCs cultivated with MSCs showed better calcium transients), but significant only for sin exp amp, sin exp tau and peak. Contraction of cells cultivated for 3 days was significantly different only in the integral (CMCs cultivated without MSCs had higher integral), but contraction of the CMCs cultivated with MSCs was worse.

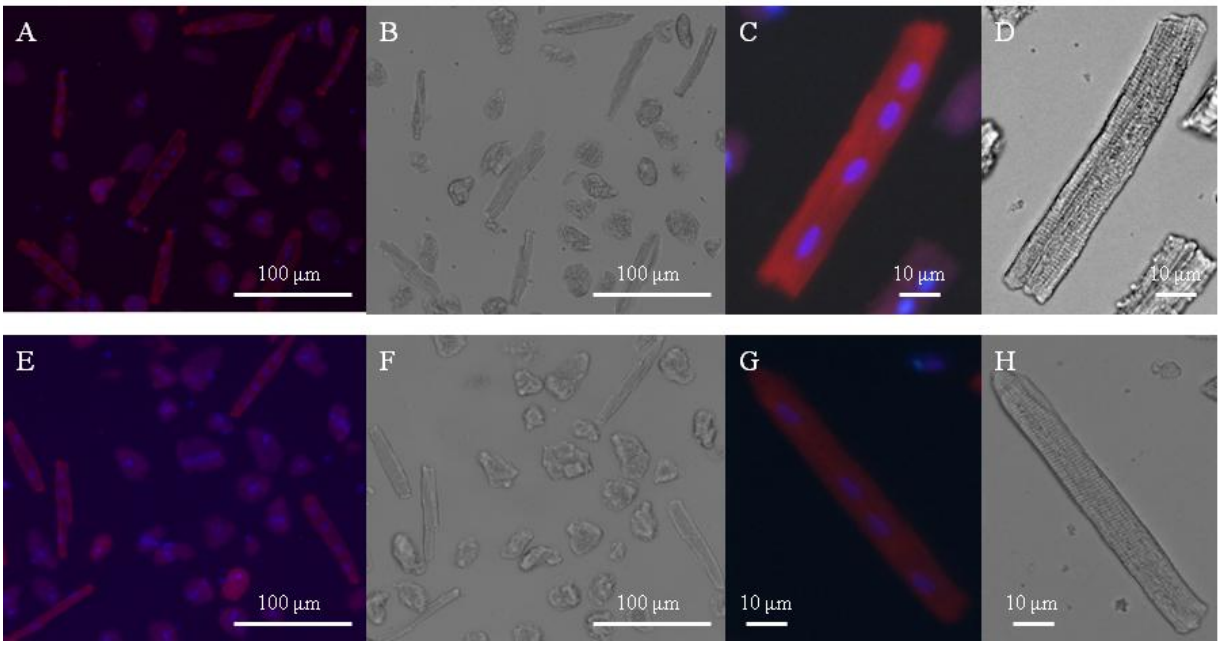

Figure 5 - Fluorescent microscopy of mitochondria in CMCs in day 1 of cultivation (red color – functional mitochondria, blue color – nuclei). Upper row CMCs cultivated alone, bottom row CMCs co-cultured with MSCs; A, E – Fluorescent microscopy of cell culture, B,F – Same frame of cell culture but taken in bright field, C,G – detail of single cardiomyocyte in fluorescence, D – detail of single cardiomyocyte in brightfield, CMC exhibits rough edges and nice stripes, H – detail of single cardiomyocyte in brightfield, CMC exhibit stripes, but edges already start to round.

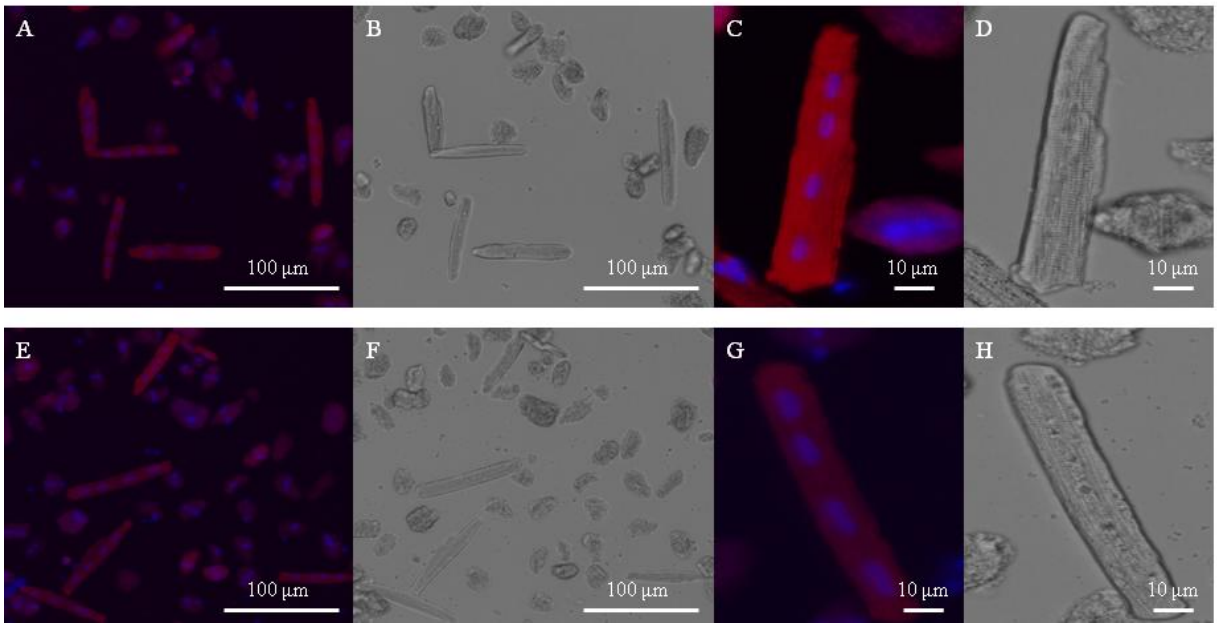

Figure 6 - Fluorescent microscopy of mitochondria in CMCs in day 3 of cultivation (red color – functional mitochondria, blue color – nuclei). Upper row CMCs cultivated alone, bottom row CMCs co-cultured with MSCs; A, E – Fluorescent microscopy of aging cell culture, B,F – Same frame of cell culture but taken in bright field, more cell pathologies and less stripes is visible in comparison with day 1, C,G – detail of single cardiomyocyte in fluorescence, D – detail of single cardiomyocyte in brightfield, edges are round and stripes are hardly visible, H - detail of single cardiomyocyte in brightfield, edges are round, stripes hardly visible and inside the cell apoptotic vacuole formation is visible.

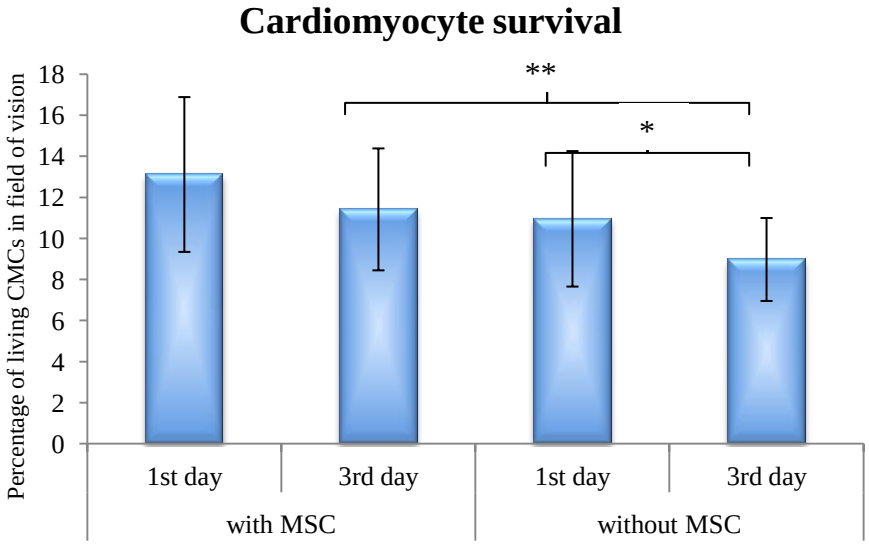

Figure 7 - Comparison of percentage of living CMCs in culture with/without MSCs, Count of living CMCs in both culteres decrease in time.
* Decrease in number of living CMCs cultivated without MSCs, while comparing 1st and 3rd day of cultivation, according to Wilcoxon test, was statistically significant (p = 0,0196).
** Survival of CMCs was significantly higher in CMCs cultivated with MSCs in day 3, according to Wilcoxon test p = 0,0152.

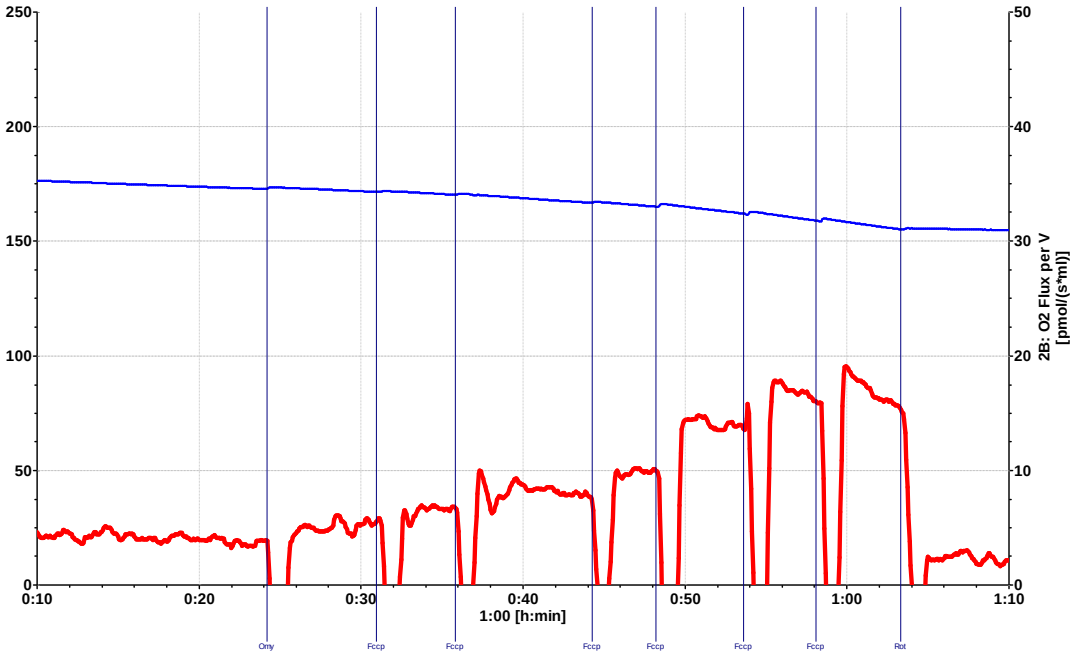

Figure 8 - Typicall curve of oxygen consumption by cultivated CMCs (in 3rd day of culture without MSCs). Red line shows oxygen consumption, blue line shows oxygen concentration in chamber. Oxygen consumption in chamber in pmol/(s*ml) is immediatelly transferred to pmol/(s*10^6 cell). Vertical lines mark application of variety of chemicals (Omy = oligomycin; FCCP = karbonylkyanid-p-trifluoromethoxyfenylhydrazon; Rot = rotenon).

4.3 MSCs and liver regeneration

4.3.1 Concentration of IL-6, IL-8, TNF-α, TGF-β

Plasma samples from 7 timepoints of chronic liver disease experiment were analysed with Luminex for concentrations of IL - 6, IL - 8, TNF – α and TNF – β (Figure 9). Statistics of the results showed no significant differences (Mann Whitney U test, $p < 0,05$).

Yet, concentration of IL – 6 showed increasing trend since the first evaluated timepoint (0 h) in both groups, from the third timepoint (1 day) concentration of IL – 6 continued to rise in group treated with MSCs, meanwhile NO MSCs group showed decrease in IL – 6 concentration. Trend was visible, however, there was not statisticall significance in measured concentrations. Concentration of IL – 8, of pro-inflammatory interleukin, together with concentration of TNF – α, cytokine of acute phase of inflammation, showed no specific trend in all measured times in both groups. Concentration of TNF – β had increasing trend, but similar in both measured groups.

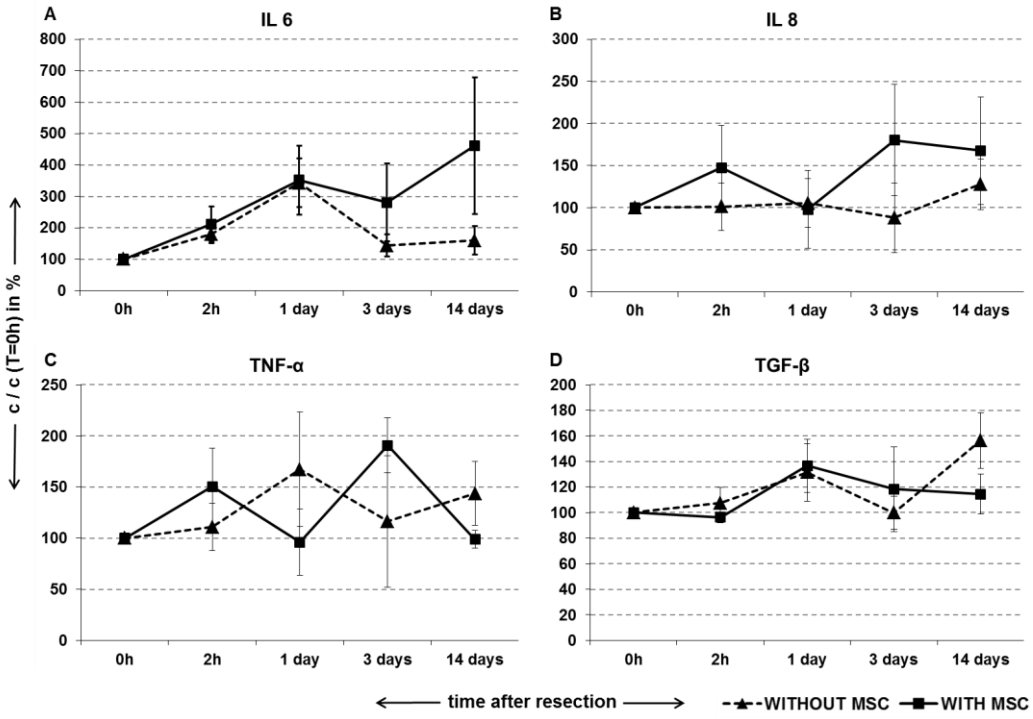

Figure 9 - Quantification of pro-inflammatory cytokines and TGF-β in plasma of pigs. Values are expressed as % of marker level in time point T = 0 h and standard error means are shown.

4.3.2 Liver morphology and morphometry analysis

Quantitative histology analysis of liver morphology (Figure 10) showed no significant differences between both, MSCs and NO MSCs, groups and also comparison of samples before and after regeneration period (14 days) did not show any significant (Figure 11).

Regenerated samples after application of MSCs had a smaller V_V*(hepatocytes, liver)* (59.5±10.1%, mean ±SD) than the samples of the same individuals before regeneration (70.2±5.5%) (Wilcoxon matched pairs test $p = 0.013$), retaining their percentage division to individual groups in the same animals before and after applying MSCs.

After MSCs application, the volume fraction of connective tissue within the regenerated liver was negatively correlated with the mean volume of hepatocytes (Spearman R=-0.78 in mononuclear hepatocytes and -0.70 in polynuclear hepatocytes), so the regions with more connective tissue contained smaller hepatocytes (Table 3). This correlation was absent in animals in NO MSCs group. After MSCs application, the volume fraction of both mononuclear and polynuclear hepatocytes within the regenerated liver was strongly correlated with the volume fraction of hepatocytes (R=0.69), so the regions containing more regenerated hepatocytes were populated by mainly mononuclear hepatocytes. This correlation was much weaker (R=0.23) in animals in NO MSCs group.

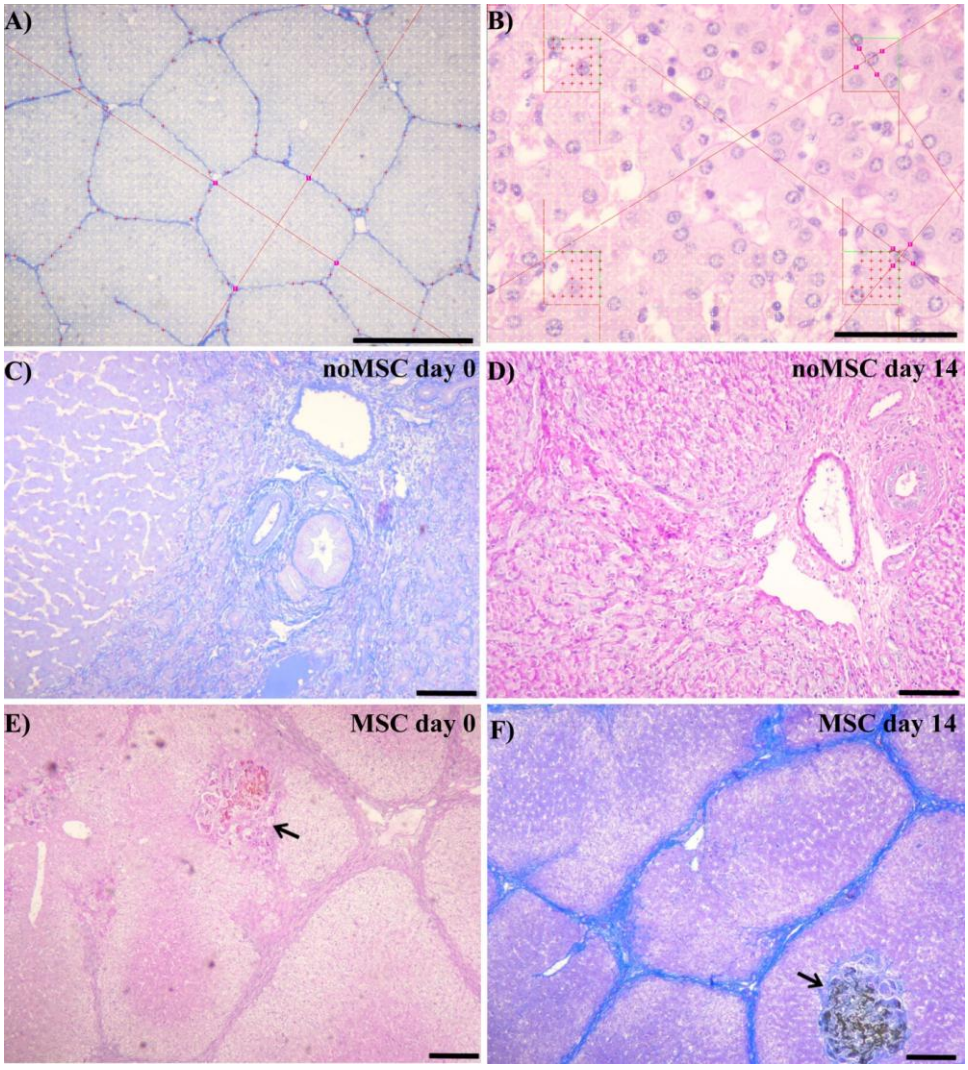

Figure 10 - Quantitative histological analysis and examples of liver morphology in the selected experimental groups. A – The volume fraction of connective tissue within the liver was estimated using a point grid (yellow). Cross-sectional area of hepatic lobules was estimated using a two-dimensional nucleator probe (red). B – In hepatocytes selected with the counting frames, the volume fraction of hepatocytes within the liver was quantified using the point grid (red marks). The mean volume of individual hepatocytes was estimated using the nucleator probe (red lines with intercepts on the edges of the hepatocytes). The areas with portal triads in animals with MSCs transplantation at resected (C) and regenerated (D) groups are showed. Overall morphological pictures of liver structure are shown in animals without MSCs transplantation at day 0 (E) and day 14 (F) groups without apparent differences in compared areas. Isolated areas of bile obstruction (arrows) are shown on E and F. Alcian blue and nuclear red stain (A,C,F), PAS stain (B,D,E). Scale bars 500 μm (A), 50 μm (B), 100 μm (C,D), 200 μm (E,F).

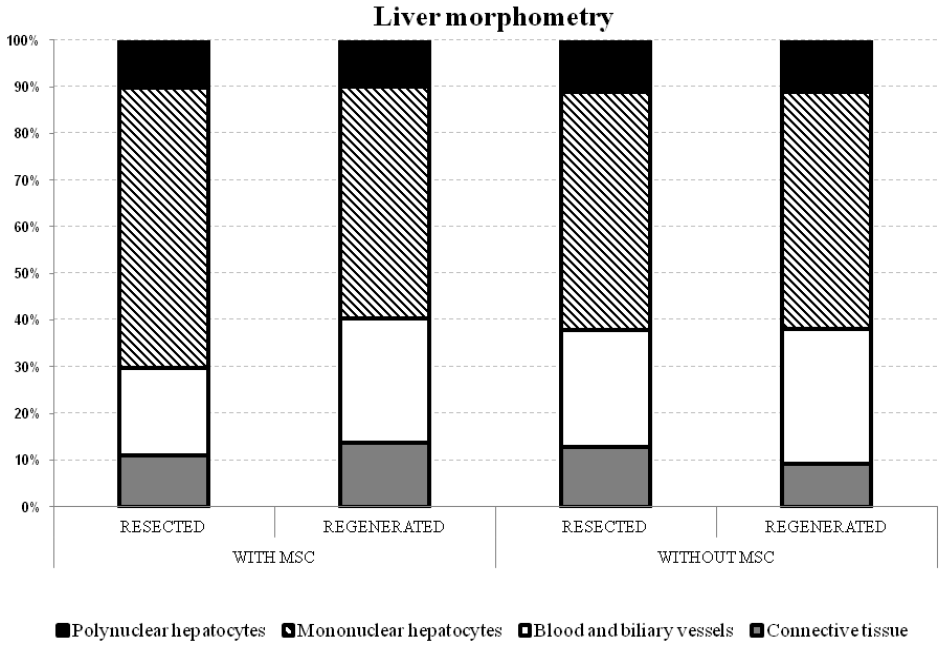

Figure 11 - Results of the histological morphometry comparing day 0 (RESECTED) and day 14 (REGENERATED) for two groups; with MSCs administration and without MSCs. No significant differences were found when comparing the volume fractions of mononuclear $V_V(MH,liver)$ and polynuclear hepatocytes $V_V(PH,liver)$, connective tissue $V_V(connective,liver)$, and blood and billiary vessels $V_V(connective,liver)$.

Table 3 - Correlations between histological morphometric parameters specified in the first and second columns in separate experimental groups. Spearman coefficients in significant correlations ($p < 0.05$) are presented.

Parameter 1	Parameter 2	WITH MSCs		WITHOUT MSCs	
		day 0	day 14	day 0	day 14
V_V (hepatocytes, liver)	V_V (BB,liver)	-0.76	-0.71	-0.82	-0.93
	V_V (MH,liver)	-	0.69	0.66	0.23
	V_V (PH,liver)	-	-0.69	-0.66	-0.23
V (MH)	V (PH)	0.65	0.72	0.78	0.61
V_V (MH,liver)	V_V (PH,liver)	-0.96	-	-	-
V_V (connective, liver)	V (MH)	-	-0.78	-	-
	V (PH)	-	-0. 70	-	-

4.4 MSCs and GVHD

4.4.1 In vitro GVHD model

Stimulation of lymphocytes with alloantigens or PHA and fMLP enabled to create in vitro GVHD model sufficient to analyse immunosuppresive effect of MSCs co-cultured with these lymphocytes. In total 32 experiments were peformed. The results were obtained by MTT test, where absorbance level represents the metabolic and proliferative activity of cocultured lymphocytes.

Positive control, lymphocytes stimulated with HLA incompatible inactivated lymphocytes, showed increase in metabolic activity with an increment of absorbance by 0,24

($p < 0,001$) compared to negative control, unstimulated lymphocytes. In contrast to this observation, in co-cultivation experiments, where MSCs were present, the decline of absorbance was observed. The average decline of absorbance for MSCs/lymphocyte ratio 1:5 was by 0,23 ($p < 0,01$), in 10 x diluted MSCs (1:50) the decline was 0,10 ($p < 0,01$) and in 100 x diluted MSCs (1:500) the average decline by 0,02 ($p=0,043$) was measured.

The presence of MSCs led to an absorbance decline in all tests in comparison with positive control. The effect of MSCs was dose dependent, the higher amounts of MSCs added into lymphocytes, the better immunosuppresive response was measured; in co-culture with MSCs/lymphocyte ratio 1:5 the absorbance level decrease by 62%, in ration 1:50 the decrease was by 26% and in ratio 1:500 the decrease was 6% only (Figure 12).

Also comparison of groups of lymphocytes stimulated with PHA or fMLP showed significant differences in absorbance. Both groups were co-cultured without and with MSCs (1:5 ratio). The presence of MSCs decreased lymphocyte activity. The absorbance was reduced by 0,17 ($p < 0,01$) in samples stimulated with fMLP and by 0,31 ($p < 0,001$) in samples treated with PHA, which corresponded to decline by 42% and 67% .

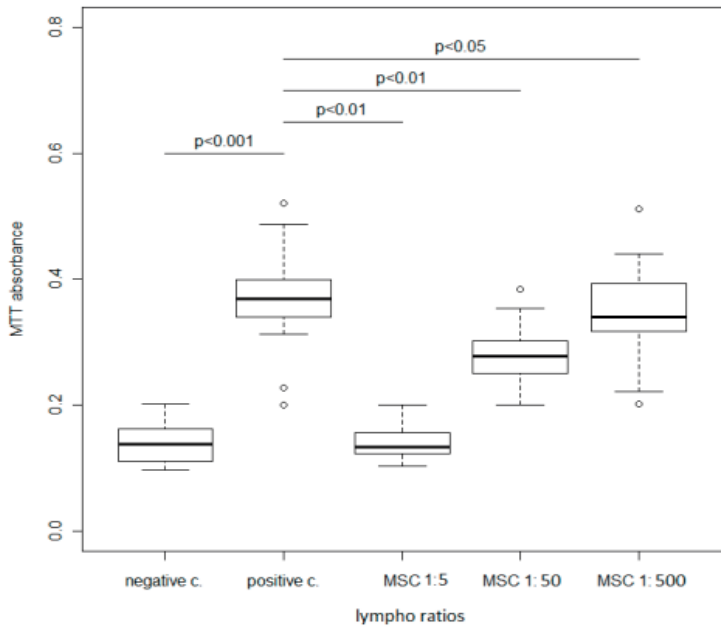

Figure 12 - Reduction of stimulated lymphocytes metabolic activity after MSCs addition (stimulation with alloantigenes). The co-cultivation with MSCs reduces the metabolic activity of stimulated lymhocytes in comparison with positive control. The higher dilution of MSCs i suspension, less effective the suupression is, the effect is highly dose dependent. MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max).

4.4.2 MSCs and GVHD patients

MSCs were administered to 8 patients with acute GVHD and to 10 patients with chronic GVHD. Blood samples from 5 timepoints (0 h, 14 days, 1 month, 2 months, 3 months) were processed and Tregs presence together with helper T-lymphocytes were seeked and evaluated.

For Tregs detection two approaches were compared (Figure 13). It was shown, that there is no significant difference in number of Tregs as $CD4^{+}$, $CD25^{bright+}$, $CD127^{-}$ and Tregs

with markers $CD4^+$, $CD25^{bright+}$, $FoxP3^+$. Any of these antibody sets showed to be alone sufficient enough to show Tregs population.

MSCs administered to patients with chronic GVHD showed to have supportive effect on number of Tregs ($CD4^+/FoxP3^+$ population), the trend was visible in each blood draw, with the biggest influence in 3 months after application of MSCs into patients. As visible in Figure 14, other measured parameters varied, for example production of IL – 17 was decreased after MSCs application, but in the 3 months check (T= 100) it was nearly in the same level as before MSCs application.

MSCs administered to patients with acute GVHD showed to have no influence on number of Tregs, whose number decreased in time. The levels of IL – 4 and IL – 17 in blood of patients increased with time. IFN – γ levels in patients blood was decreased after MSCs administration and remained low in all experimental time points suggesting long term effect of MSCs application on Tregs. However non of the showed result was statistically significant. If the extreme values would be removed, for acute and also chronic GVHD data, differences would be more prominent, but still not significant, more patient need to be enrolled in study to obtain statistically significant results.

Despite the insignificant changes in measured parameters, clinical improvement of GVHD in patients was satisfactory. In 90 % of patients enrolled in study dose of corticosteroids was decreased. In three patients with acute GVHD total remision occured, in other three partial remission was observed. Chronic GVHD is harder to manage, but two patients reached partial remission of disease. However, clinical observation and data were not part of this work.

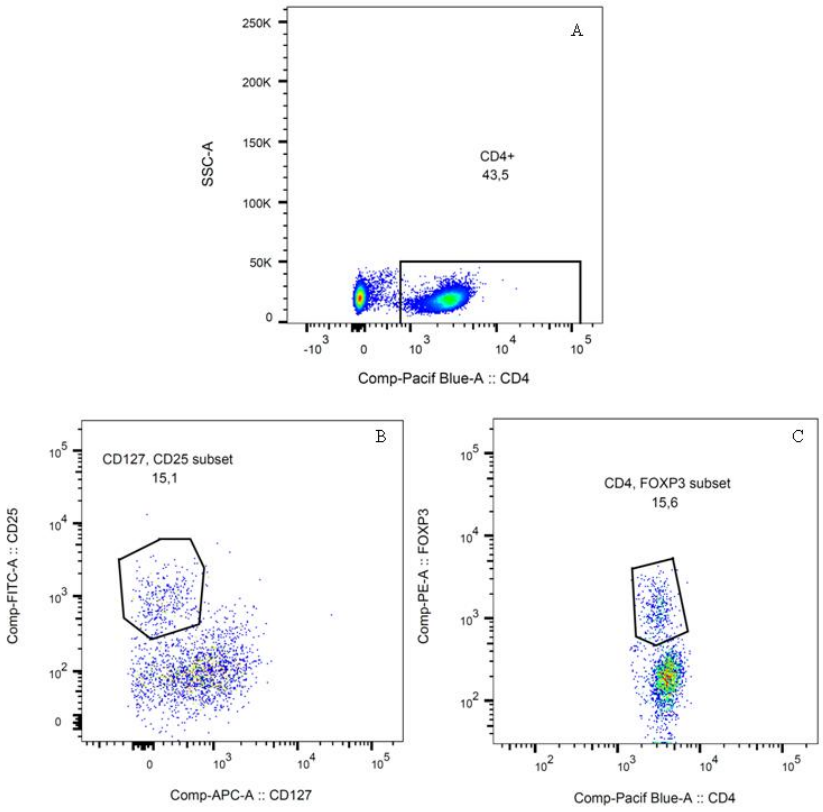

Figure 13 - Example of Tregs gating strategy in flow cytometry. Comparison of two different antibody sets (B,C) A – subpopulation of lymphocytes positive for CD4 (43,5 %). B – Tregs positive for CD25 and also CD127 (15,1 %). C – Tregs positive for CD4 and FOXP3 (15,6 %).

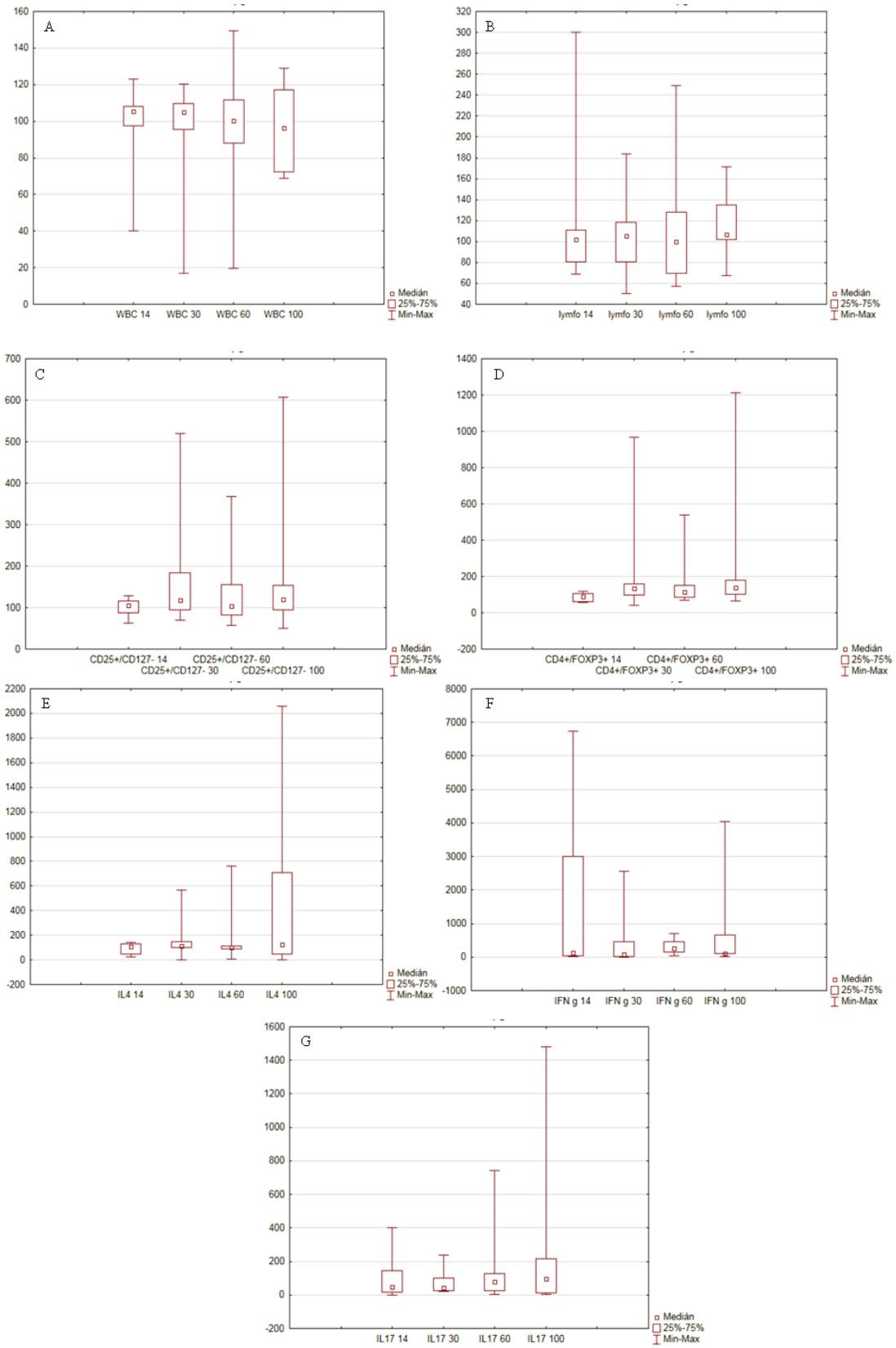

Figure 14 - Chronic GVHD after MSCs administration, changes of numbers of Tregs and helper T-lymphocytes in timepoints (14 days, 30 days, 60 days, 100 days). No significant differences were found. A – count of white blood cells (WBC), B – count of all lymphocytes, C – Tregs ($CD25^{+}$,$CD127^{-}$) count development in time, D - Tregs ($CD4^{+}$,$FOXP3^{+}$) count development in time, E – levels of IL-4 in time, F – levels of IFN-γ in time, E – levels of IL-17 in time.

5 Disscussion

Mesenchymal stem cells are promissing cell type for cellular therapy for wide variety of diseases of the future [Bouchez et al., 2008; Qian et al., 2008; Basiouny et al., 2013]. Since their discovery, their isolation and cultivation was well described [Reyes et al., 2001], together with exact criteria for their verification [Dominici et al., 2006]. In our work we isolated swine and also human MSCs from bone marrow. Standart procedure was sufficient to gain more than 98 % purity of MSCs culture.

Morphology observation showed that cell possesed spindle shape with several spurs and large nucleus. With commercialy accesible media we confirmed that our cells were able to differentiate into adipocytes, osteocytes and chondrocytes, flow cytometry confirmed MSCs antigenes on the surface of these cells.

Series of experiments with swine MSCs was performed to elucidade their role in cardiac and liver regeneration. Human MSCs were used for determination of role of MSCs in immunomodulation, specifically in GVHD.

5.1 MSCs and cardiomyocytes

Influence of MSCs on CMCs has been studied in many works [Silva et al., 2014; Psaltis et al., 2014; Narita and Suzuki, 2015], but application of these findings into clinical practice is not satisfactory yet [Nowbar et al., 2014]. Majority of the studies has been performed on small animal models, but large animals, such is pig, are more similar to human [Reardon, 2015] and transfer of obtained data into clinical practice should be easier.

For our experiments, isolated primary adult swine CMCs were chosen, because of their similarity to CMCs in functional heart [Graham et al., 2013]. Primary CMCs, as fully differentiated cells, are unable to proliferate and their life span is short, so experiments need to be performed within few days [Xu and Colecraft, 2009].

Isolation of primary adult swine CMCs showed to be very difficult, process needed a lot of optimisation experiments and yet the results were not satisfactory. Isolated CMCs were easily distinguishable from surrounding cells according to the long shape with rough edges and stripes on the cell body. In our experiments, we gained low yield of living CMCs in cell suspension from each isolation, contrary to isolation results published in smaller animals [Ellingsen et al., 1993].

In the literature can be found that CMCs are able to adhere to cultivatuíon surface, which helps with culture purification, because other cells can be washed away [Mitcheson et al., 1998; Louch et al., 2011]. We tryied 4 different cultivation surfaces, where we found that CMCs adhered only to coated (laminin or gelatine) cultivation surface but nearly not to normal non treated cultivation plastic. Contrary to literature [Louch et al., 2011], in our experiments also other cells, dying and dead, adhered to cultivation surface proportionally to CMCs. Cultivation itself did not helped to increase percentage of living CMCs in culture. Magnetic separation of living CMCs from other cells was more efficient, but expensive and time consuming.

Adult CMCs live in culture for approximatelly 3 days, later dedifferentiation and progressive cell death follows [Banyasz et al., 2008; Mitcheson et al., 1998]. Our experiments are in agreement with these findings. Co-cultivation with MSCs improves CMCs survival, especially when cell-to-cell cross-talk is enabled [Plotnikov et al., 2008]. In our experiments

CMCs cultured directly with MSCs maintained their morphological integrity for the longest time, but also adhered to each other and precluded further manipulation without cellular damage.

CMCs cultivated across the transwells with MSCs showed statistically longer survival in culture then CMCs cultivated alone. While CMCs without MSCs showed significant decrease in percentage of living CMCs in culture between day 1 and day 3, difference between day 1 and day 3 for CMCs cultivated with MSCs were not significant. In day 3 comparison of CMCs cultivated with and without MSCs resulted in statistically significant bigger percentage of living CMCs in co-culture.

High-resolution oxygraphy of cell suspension showed decrease in mitochondrial respiration of CMCs during the cultivation time in both groups. Co-cultivation of CMCs with MSCs was not significantly harmful or beneficial.

Electrophysiological values of calcium currents and CMCs contractility on single cell level showed deterioration in functions of CMCs in time. Cultivation of CMCs lead to decrease in the resting values of intracellular calcium, deceleration of calcium release from the sarcoplasmic reticulum, decrease in calcium transient amplitude and deceleration of calcium decrease during relaxation. These changes were accompanied with deceleration of contraction and relaxation speed and decrease in contraction amplitude. Co-cultivation of CMCs with MSCs did not prevent these undesirable changes. After 1 day of cultivation CMCs co-cultivated with MSCs showed significantly worse results than cells cultivated without the MSCs. After 3 days of cultivation the calcium transients were less impaired in cells co-cultivated with MSCs, but not significantly. Moreover, contraction parameters remained worse in MSC co-cultivated cells. Therefore co-cultivation of the cardiomyocytes with MSCs was not significantly beneficial for calcium handling and contraction of the isolated CMCs.

Our results showed statistically significant improvement of percentage of living CMCs in 3rd day of culture in CMCs co-cultured with MSCs. Contrary to that, mitochondrial functions and electrophysiology parameters of CMCs seemed not to be influenced by MSCs co-culture. Possible explanation could be that MSCs have trully beneficial effect on CMCs, so the cells lived longer in better condition. High-resolution oxygraph results of mitochondrial respiration were related to number of cells in chamber. Therefore the higher number of living CMCs in sample of co-cultured CMCs showing similar results as the strongest survivor cells in CMCs culture only shows beneficial effect of MSCs, which in results related to cell count, was hidden. Similarly to that, electrophysiological parameters were measured on single cells, so not significant differences between both groups may mean that MSCs helps CMCs to maintain not only morphological parameters, but also functional parameters for longer time.

5.2 MSCs and liver regeneration

Influence of MSCs on liver tissue has been studied intensively [van Poll et al., 2008]. Many of the studies are performed on small animal models, but it is known, that large animals, such is pig, are closer to people in many ways [Reardon, 2015].

Our surgeon team introduced new, clinicaly relevant, model of chronic liver disease in swine due to biliary obstruction. After nine weeks of biliary obstruction effect, resection of left lobe was performed. This model mimics similar situation in human patients, who suffer

from biliary obstruction due to malignancies or other leasions [Barbier et al., 2014], whose liver is damaged and who need resection of affected liver tissue [Govil and Ramaswamy, 2012]. These patients are in high risk of severe life threatening complications. Improved regeneration by MSCs could be very beneficial to them.

MSCs have documented ability to support liver regeneration via production of different cytokines and chemokines [Lin et al., 2011]. In our work MSCs suspension was administered to 10 pigs after liver lobe resection, 11 pigs received saline solution only. Production of cytokines was evaluated in plasma of pigs for 14 days, but no significant differences between MSCs and NO MSCs group were found. Group which received MSCs showed higher concentrations of IL – 6, which is pro inflammatory cytokine, but it has been reported to have important role in stimulation of hepatocyte proliferation [Wang et al., 2015; Lin et al., 2011], however, the difference was not statistically significant. All other measured parameters IL – 8, TNF – α and TNF – β showed inconsistend results with no visible trends and no statistically significant results.

Analysis of histological morphometry parameters showed difference between liver samples from animals with MSCs administration and from animals from NO MSCs group.

Different correlation patterns were found in day 14 between group MSCs and NO MSCs in relation between V_V *(hepatocytes, liver)* and the fractions of mononuclear and polynuclear hepatocytes. Although the mononuclear and the polynuclear hepatocytes are frequently mentioned and separately classified in many studies about liver regeneration [Nakatani et al., 1997; Gorla et al., 2001], the interpretation of importance of nuclei count is still insufficient. Binucleated hepatocytes are reported to be common in healthy liver, in human it is approximately 20 – 30 % of hepatocytes [Nadal and Zajdela, 1967], on the other hand, increase of binucleation in hepatocytes can be linked to hepatic recovery process following injury as a late result of oxidative stress [Nakatani et al., 1997]. Therefore, our work results may indicate an inclination towards a better microenvironment for hepatocyte regeneration with increased fraction of mononuclear hepatocytes in regions of hepatocytes regeneration in animals with MSCs transplantation. However, more experiments need to be performed to elucidate the biological role of mononuclear and polynuclear hepatocytes during liver regeneration.

Another difference between MSCs and NO MSCs group was in reduced volume fraction of hepatocytes in the liver tissue of a group with MSCs on day 14, which was accompanied with insignificant increase of volume of connective tissue and blood and biliary vessels. The possible explanation of this finding might be that MSCs transplantation can stimulate proliferation of connective tissue [Forbes et al., 2004], which reduces the space for parenchymal hepatocytes.

We showed that MSCs transplantation do not have significant beneficial effect on liver regeneration in animals with liver damage caused by biliary obstruction after liver resection, which is contrary to literature [Adas et al., 2016]. Cytokine analysis showed no significant difference between groups and morphometry analysis results are not convincing either. The importance of different ratio between mononuclear and polynuclear hepatocytes in day 14 between both groups needs to be verified by further studies, while increased connective tissue volume at the expense of proper hepatic tissue is not beneficial for sure.

One of the reasons of not convincingly beneficial results may be that evaluation time period was too short. Longer time observation could show more promissing results, especially in the animal of pig size. Other reason could be that dose of MSCs was too small, either our pigs needed more than 1 x 10^6 cells / kg or MSCs after application were trapped somewhere else than in liver. In general condition as chronic liver disease is, many organs may express stress signals causing MSCs homing elsewhere than to liver.

5.3 MSCs and GVHD

GVHD is a serious condition generalized to whole body. Many cells have a role in development of GVHD, but lymphocytes are the most important. MSCs are able to affect immune reaction by inhibition of inflamatory cytokine production and by increase of expression of suppressive cytokines.

Many studies showed that MSCs suppress T – lymphocyte proliferation in culture [Maccario et al., 2005; Di Nicola et al., 2002] and our results are consistent with these findings. In our study MSCs showed the capacity to significantly affect nonspecific activation of lymphocytes. MTT test showed significant decrease (60 %) in absorbance, corresponding to reduction in metabolic and proliferation activity, of lymphocytes co-cultured with MSCs in comparison with the positive control. The immunosuppresive effect was present in lymphocytes stimulated with all used methods, alloantigene stimulation, PHA and fMLP stimulation. Therefore our analysis confirmed that MSCs can regulate lymphocytes stimulated with HLA incompatible lymphocytes, which is the situation corresponding to severe forms of chronic GVHD after allogeneic hematopoietic stem cell transplantation. The immunomodulatory effect of MSCs is dose-dependent. As it was described previously [Ramasamy et al., 2008], optimal dose is ranging from 1:1 to 1:10 MSCs / lymphocyte ratio. In our study the higher ratio of MSCs, bigger immunosuppressive effect was observed. As ideal, ratio 1:5 shown, however, the exact dose and frequency needs to be optimised in larger study.

As mentioned in this work, administration of MSCs into patients with severe GVHD started in Hematology-oncology department. MSCs isolated from healthy donors, cultivated, freezed and recultivated again were used, despite the findings that freezing of the cells may innactivate some of their immunomodulatory properties due to heat-shock response [François et al., 2012]. In another work our team confirmed that recultivation of MSCs after thawing helps to restore original MSCs properties [Holubova et al., 2014].

In the time of MSCs application, first blood draw for determination of Tregs and helper T-lymphocyte population was taken from patients. In day 14, day 30, 1 month and 3 months more samples were collected.

Optimisation of Tregs detection in flow cytometry was performed. Two sets of antibodies were tested as the options for regulatory Tregs detection. Population of cells $CD4^+$, $CD25^{bright+}$ and $FoxP3^+$ is considered to be true Tregs [Valencia and Lipsky, 2007], but staining with FoxP3 requires cell permeabilisation and another washing steps, which can cause unfortunate cell loss. According to some authors, determination of Tregs according to $CD4^+$, $CD25^{bright+}$, $CD127^-$ is sufficient [Yu et al., 2012], cell processing is shorter and chance to wash cells away is smaller. In our work we compared results and counts of cells determined with both antibody sets. We found that both antibody sets are equal, counts of Tregs identified

with both antibody sets were similar. Therefore, for further experiments, detection of Tregs with anti-CD4, anti-CD25 and anti-CD127 can be used only.

Application of MSCs into patients with chronic and also acute GVHD is considered to be possible treatment option of the future, which is tested in clinical trials [Rizk et al., 2016; Miyamura, 2016]. According to preliminary study results, MSCs administration is more beneficial for patients with acute GVHD, in chronic GVHD fibrotic changes are already present, damage is large and irreversible [Herrmann and Sturm, 2014].

In our work MSCs transplantation did not showed statistically significant effect on patients, both with acute and chronic GVHD. However in chronic GVHD disease, results of MSCs transplantation were more prominent.

Inconclusive results in both patient groups could be caused by small size of study group and by heterogenity of patients, depending on disease behaviour. In the moment of enrollment into study, patients had different immunosuppression levels, severity of disease itself varied and appropriate timepoint of MSCs administration was seeked. Neverthless, obtained data suggest that repetitive administration of MSCs could fortify MSCs immunomodulatoy effect. This information is taken to consideration now and new study design is prepared.

Despite the laboratory results, majority of patients experienced significant clinical improvement of their condition. In 3 month check more than 90 % of patients received lower corticosteroid doses, in average 59 % of initial dose (21 – 100 %). In acute GVHD patient group even 3 patients reached complete remission, in 3 patients partial remission occured. In chronic GVHD patient group clinical improvement was not so obvious, but also here 2 patients reached partial remission.

Although this study was focused on laboratory results, which were not satisfactory, clinical improvement of patient condition was clear and encouraging, showing MSCs administration into patients is meaningful and beneficial. More patients will be enrolled in this study to gain sufficient set of data for valid statistics analysis, to confirm clinical observation also on laboratory data.

6 Conclusion

This work focused on elucidation of role of MSCs in regeneration of vital organs, heart and liver, together with MSCs immunomodulatory properties in GVHD.

MSCs showed to have significant regenerative and immunomodulatory properties, when they supported CMCs survival and supressed lymphocyte activity in *in vitro* experiments.

Similar significance was not found after MSCs administration into organism. Many variables influenced the results there. MSCs possess ability to support regeneration of damaged tissues, but in our liver experiments MSCs showed tendency to support connective tissue preferably, which may not be always beneficial for regeneration of the organ. MSCs immunomodulatory properties were visible in patient condition improvement, but obtained immune parameters analysis results were not significant, which show issue of proper validation criteria of MSCs influence. Necessity of large number of individuals enrolled in studies remains the biggest challenge.

Our work indicated new perspectives, which need to be considered in future research. Only results obtained from large data sets will provide significant and meaningful information necessary for transfer of MSCs administration into clinical practice.

7 Reference

ADAS, Gokhan, Bora KOC, Mine ADAS, et al., (2016). Effects of mesenchymal stem cells and VEGF on liver regeneration following major resection. *Langenbeck's Archives of Surgery / Deutsche Gesellschaft Fur Chirurgie* [online]. 19.4. ISSN 1435-2451. Retrieved z: doi:10.1007/s00423-016-1380-9

BANYASZ, Tamas, Ilya LOZINSKIY, Charles E. PAYNE, et al., (2008). Transformation of adult rat cardiac myocytes in primary culture. *Experimental Physiology* [online]. 3., vol. 93, no. 3, pp. 370–382. ISSN 0958-0670. Retrieved z: doi:10.1113/expphysiol.2007.040659

BARBIER, L., R. SOUCHE, K. SLIM and P. AH-SOUNE, (2014). Long-term consequences of bile duct injury after cholecystectomy. *Journal of Visceral Surgery* [online]. 9., vol. 151, no. 4, pp. 269–279. ISSN 1878-7886. Retrieved z: doi:10.1016/j.jviscsurg.2014.05.006

BASIOUNY, Hend Shafik, Nagla Mohamed SALAMA, Zeinab Mohamed El MAADAWI and Eman Abas FARAG, (2013). Effect of bone marrow derived mesenchymal stem cells on healing of induced full-thickness skin wounds in albino rat. *International Journal of Stem Cells*. 5., vol. 6, no. 1, pp. 12–25. ISSN 2005-3606.

BOUCHEZ, Gaëlle, Luc SENSEBÉ, Patrick VOURC'H, et al., (2008). Partial recovery of dopaminergic pathway after graft of adult mesenchymal stem cells in a rat model of Parkinson's disease. *Neurochemistry International* [online]. 6., vol. 52, no. 7, pp. 1332–1342. ISSN 0197-0186. Retrieved z: doi:10.1016/j.neuint.2008.02.003

DI NICOLA, Massimo, Carmelo CARLO-STELLA, Michele MAGNI, et al., (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*. 15.5., vol. 99, no. 10, pp. 3838–3843. ISSN 0006-4971.

DOMINICI, M., K. LE BLANC, I. MUELLER, et al., (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* [online]. vol. 8, no. 4, pp. 315–317. ISSN 1465-3249. Retrieved z: doi:10.1080/14653240600855905

ELLINGSEN, O., A. J. DAVIDOFF, S. K. PRASAD, et al., (1993). Adult rat ventricular myocytes cultured in defined medium: phenotype and electromechanical function. *The American Journal of Physiology*. 8., vol. 265, no. 2 Pt 2, pp. H747-754. ISSN 0002-9513.

FORBES, Stuart J., Francesco P. RUSSO, Virginia REY, et al., (2004). A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology*. 4., vol. 126, no. 4, pp. 955–963. ISSN 0016-5085.

FRANÇOIS, Moïra, Ian B. COPLAND, Shala YUAN, et al., (2012). Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-γ licensing. *Cytotherapy* [online]. 2., vol. 14, no. 2, pp. 147–152. ISSN 1477-2566. Retrieved z: doi:10.3109/14653249.2011.623691

GORLA, G. R., H. MALHI and S. GUPTA, (2001). Polyploidy associated with oxidative injury attenuates proliferative potential of cells. *Journal of Cell Science*. 8., vol. 114, no. Pt 16, pp. 2943–2951. ISSN 0021-9533.

GOVIL, Sanjay and Vasudevan K. RAMASWAMY, (2012). Major liver resection without preoperative biliary drainage in jaundiced patients with hilar biliary obstruction. *Indian Journal of Gastroenterology: Official Journal of the Indian Society of Gastroenterology* [online]. 12., vol. 31, no. 6, pp. 333–336. ISSN 0975-0711. Retrieved z: doi:10.1007/s12664-012-0249-2

GRAHAM, Evan Lee, Cristina BALLA, Hannabeth FRANCHINO, et al., (2013). Isolation, culture, and functional characterization of adult mouse cardiomyoctyes. *Journal of Visualized Experiments: JoVE* [online]. no. 79, p. e50289. ISSN 1940-087X. Retrieved z: doi:10.3791/50289

GUO, Jun, Guo-sheng LIN, Cui-yu BAO, et al., (2007). Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. *Inflammation* [online]. 8., vol. 30, nos. 3–4, pp. 97–104. ISSN 0360-3997. Retrieved z: doi:10.1007/s10753-007-9025-3

HERRMANN, Richard and Marian STURM, (2014). Adult human mesenchymal stromal cells and the treatment of graft versus host disease. *Stem Cells and Cloning: Advances and Applications* [online]. 2., p. 45. ISSN 1178-6957. Retrieved z: doi:10.2147/SCCAA.S37506

HOLUBOVA, Monika, Daniel LYSAK, Tomas VLAS, et al., (2014). Expanded cryopreserved mesenchymal stromal cells as an optimal source for graft-versus-host disease treatment. *Biologicals* [online]. 5., vol. 42, no. 3, pp. 139–144. ISSN 10451056. Retrieved z: doi:10.1016/j.biologicals.2014.01.003

KEBRIAEI, Partow and Simon ROBINSON, (2011). Mesenchymal Stem Cell Therapy in the Treatment of Acute and Chronic Graft Versus Host Disease. *Frontiers in Oncology* [online]. vol. 1 [accessed. 11. January 2016]. ISSN 2234-943X. Retrieved z: doi:10.3389/fonc.2011.00016

LE BLANC, Katarina and Olle RINGDÉN, (2005). Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation* [online]. 5., vol. 11, no. 5, pp. 321–334. ISSN 1083-8791. Retrieved z: doi:10.1016/j.bbmt.2005.01.005

LE BLANC, Katarina and Olle RINGDÉN, (2006). Mesenchymal stem cells: properties and role in clinical bone marrow transplantation. *Current Opinion in Immunology* [online]. 10., vol. 18, no. 5, pp. 586–591. ISSN 0952-7915. Retrieved z: doi:10.1016/j.coi.2006.07.004

LEE, Jun-Won, Seung-Hwan LEE, Young-Jin YOUN, et al., (2014). A randomized, open-label, multicenter trial for the safety and efficacy of adult mesenchymal stem cells after acute myocardial infarction. *Journal of Korean Medical Science* [online]. 1., vol. 29, no. 1, pp. 23–31. ISSN 1598-6357. Retrieved z: doi:10.3346/jkms.2014.29.1.23

LIN, Hu, Ruonan XU, Zheng ZHANG, et al., (2011). Implications of the immunoregulatory functions of mesenchymal stem cells in the treatment of human liver diseases. *Cellular & Molecular Immunology* [online]. 1., vol. 8, no. 1, pp. 19–22. ISSN 2042-0226. Retrieved z: doi:10.1038/cmi.2010.57

LIU, Nanmei, Huiling WANG, Guofeng HAN, et al., (2015). Alleviation of apoptosis of bone marrow-derived mesenchymal stem cells in the acute injured kidney by heme oxygenase-1 gene modification. *The International Journal of Biochemistry & Cell Biology* [online]. 12., vol. 69, pp. 85–94. ISSN 1878-5875. Retrieved z: doi:10.1016/j.biocel.2015.10.007

LOUCH, William E., Katherine A. SHEEHAN and Beata M. WOLSKA, (2011). Methods in cardiomyocyte isolation, culture, and gene transfer. *Journal of Molecular and Cellular Cardiology* [online]. 9., vol. 51, no. 3, pp. 288–298. ISSN 00222828. Retrieved z: doi:10.1016/j.yjmcc.2011.06.012

MACCARIO, Rita, Marina PODESTÀ, Antonia MORETTA, et al., (2005). Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica*. 4., vol. 90, no. 4, pp. 516–525. ISSN 1592-8721.

MITCHESON, J. S., J. C. HANCOX and A. J. LEVI, (1998). Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties. *Cardiovascular Research*. 8., vol. 39, no. 2, pp. 280–300. ISSN 0008-6363.

MIYAMURA, Koichi, (2016). Insurance approval of mesenchymal stem cell for acute GVHD in Japan: need of follow up for some remaining concerns. *International Journal of Hematology* [online]. 2., vol. 103, no. 2, pp. 155–164. ISSN 1865-3774. Retrieved z: doi:10.1007/s12185-015-1930-x

MUGURUMA, Yukari, Takashi YAHATA, Hiroko MIYATAKE, et al., (2006). Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood* [online]. 1.3., vol. 107, no. 5, pp. 1878–1887. ISSN 0006-4971. Retrieved z: doi:10.1182/blood-2005-06-2211

NADAL, C. and F. ZAJDELA, (1967). [Hepatic polyploidy in the rat. IV. Experimental changes in the nucleolar volume of liver cells and their mechanisms of regulation]. *Experimental Cell Research*. 12., vol. 48, no. 3, pp. 518–528. ISSN 0014-4827.

NAKATANI, T., M. INOUYE and O. MIROCHNITCHENKO, (1997). Overexpression of antioxidant enzymes in transgenic mice decreases cellular ploidy during liver regeneration. *Experimental Cell Research* [online]. 10.10., vol. 236, no. 1, pp. 137–146. ISSN 0014-4827. Retrieved z: doi:10.1006/excr.1997.3715

NARITA, Takuya and Ken SUZUKI, (2015). Bone marrow-derived mesenchymal stem cells for the treatment of heart failure. *Heart Failure Reviews* [online]. 1., vol. 20, no. 1, pp. 53–68. ISSN 1573-7322. Retrieved z: doi:10.1007/s10741-014-9435-x

NOWBAR, A. N., M. MIELEWCZIK, M. KARAVASSILIS, et al., (2014). Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis. *BMJ* [online]. 28.4., vol. 348, no. apr28 1, pp. g2688–g2688. ISSN 1756-1833. Retrieved z: doi:10.1136/bmj.g2688

ORLIC, D., J. KAJSTURA, S. CHIMENTI, et al., (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* [online]. 5.4., vol. 410, no. 6829, pp. 701–705. ISSN 0028-0836. Retrieved z: doi:10.1038/35070587

PLOTNIKOV, E. Y., T. G. KHRYAPENKOVA, A. K. VASILEVA, et al., (2008). Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture. *Journal of Cellular and Molecular Medicine* [online]. 10., vol. 12, no. 5A, pp. 1622–1631. ISSN 1582-1838. Retrieved z: doi:10.1111/j.1582-4934.2007.00205.x

PSALTIS, Peter J., Daniel B. SPOON, Dennis T. L. WONG and Rajiv GULATI, (2014). An Update on Stem Cell Therapies for Acute Coronary Syndrome. *Current Cardiology Reports* [online]. 9., vol. 16, no. 9 [accessed. 11. January 2015]. ISSN 1523-3782, 1534-3170. Retrieved z: doi:10.1007/s11886-014-0526-z

QIAN, Hui, Huan YANG, Wenrong XU, et al., (2008). Bone marrow mesenchymal stem cells ameliorate rat acute renal failure by differentiation into renal tubular epithelial-like cells. *International Journal of Molecular Medicine*. 9., vol. 22, no. 3, pp. 325–332. ISSN 1107-3756.

RAMASAMY, Rajesh, Chih Kong TONG, Heng Fong SEOW, et al., (2008). The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function. *Cellular Immunology* [online]. 2., vol. 251, no. 2, pp. 131–136. ISSN 1090-2163. Retrieved z: doi:10.1016/j.cellimm.2008.04.009

REARDON, Sara, (2015). New life for pig-to-human transplants. *Nature* [online]. 10.11., vol. 527, no. 7577, pp. 152–154. ISSN 0028-0836, 1476-4687. Retrieved z: doi:10.1038/527152a

REYES, M., T. LUND, T. LENVIK, et al., (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood*. 1.11., vol. 98, no. 9, pp. 2615–2625. ISSN 0006-4971.

RIZK, Mina, Madeline MONAGHAN, Risa SHORR, et al., (2016). Heterogeneity in Studies of Mesenchymal Stromal Cells to Treat or Prevent Graft-versus-Host Disease: A Scoping Review of the Evidence. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation* [online]. 27.4. ISSN 1523-6536. Retrieved z: doi:10.1016/j.bbmt.2016.04.010

SILVA, Daniela Nascimento, Bruno Solano DE FREITAS SOUZA, Carine Machado AZEVEDO, et al., (2014). Intramyocardial transplantation of cardiac mesenchymal stem cells reduces myocarditis in a model of chronic Chagas disease cardiomyopathy. *Stem Cell Research & Therapy* [online]. vol. 5, no. 4, p. 81. ISSN 1757-6512. Retrieved z: doi:10.1186/scrt470

VALENCIA, Xavier and Peter E. LIPSKY, (2007). CD4+CD25+FoxP3+ regulatory T cells in autoimmune diseases. *Nature Clinical Practice. Rheumatology* [online]. 11., vol. 3, no. 11, pp. 619–626. ISSN 1745-8390. Retrieved z: doi:10.1038/ncprheum0624

VAN POLL, Daan, Biju PAREKKADAN, Cheul H. CHO, et al., (2008). Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology (Baltimore, Md.)* [online]. 5., vol. 47, no. 5, pp. 1634–1643. ISSN 1527-3350. Retrieved z: doi:10.1002/hep.22236

WANG, Jun, Lijuan XU, Qianqian CHEN, et al., (2015). Bone mesenchymal stem cells overexpressing FGF4 contribute to liver regeneration in an animal model of liver cirrhosis. *International Journal of Clinical and Experimental Medicine*. vol. 8, no. 8, pp. 12774–12782. ISSN 1940-5901.

XU, Xianghua and Henry M. COLECRAFT, (2009). Primary culture of adult rat heart myocytes. *Journal of Visualized Experiments: JoVE* [online]. no. 28. ISSN 1940-087X. Retrieved z: doi:10.3791/1308

YU, Ning, Xiaomei LI, Weiya SONG, et al., (2012). CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. *Inflammation* [online]. 12., vol. 35, no. 6, pp. 1773–1780. ISSN 1573-2576. Retrieved z: doi:10.1007/s10753-012-9496-8

8 Publications of author

LYSAK, Daniel, Tomas VLAS, Monika HOLUBOVA, **Michaela MIKLIKOVA**, Pavel JINDRA, (2015). *In vitro* testing of immunosupressive effects of mesenchymal stromal cells on lymphocytes stimulated with alloantigens. *Biomed Pap*; 159(2):215-219. DOI: 10.5507/bp.2013.072 , **IF_{2015} = 1,2.**

LYSAK, Daniel, Linda KOUTOVA, Monika HOLUBOVA, Tomáš VLAS, **Michaela MIKLIKOVA**, Pavel JINDRA, (2016). The Quality Control of Mesenchymal Stromal Cells by *in Vitro*Testing of Their Immunomodulatory Effect on AllogeneicLymphocytes. *Folia Biologica*; 62, 120-130. **IF_{2015} = 1.**

MIKLIKOVA, Michaela, Dagmar JARKOVSKA, Miroslava CEDIKOVA, Jitka KUNCOVA, Jitka SVIGLEROVA, Lukas NALOS, Alena KORINKOVA, Vaclav LISKA, Daniel LYSAK, Milena KRALICKOVA, Lucie VISTEJNOVA, Milan ŠTENGL. Vliv mesenchymálních kmenových buněk na *in vitro* kulturu dospělých prasečích kardiomyocytů. *Plzeňský lékařský sborník*, accepted for print in 2017.

MIKLIKOVA, Michaela, Jana-Aletta THIELE, Daniel LYSAK, Monika HOLUBOVA, Milena KRALICKOVA, Lucie VISTEJNOVA. Mesenchymal stem cells as the near future of cardiology medicine – truth or wish? Submitted to Stem cell reviews and reports (IF_{2015} = 2,768).

CEDIKOVA, M., **M. MIKLIKOVA,** M. GRUNDMANOVA, N. H. ZECH, M. KRALICKOVA and J. KUNCOVA. Sperm mitochondrial function in men with normozoospermia and asthenozoospermia. *Ceska Gynekol*. 2014, Vol. 79, No. 1, pp. 22-28. ISSN 1210-7832.

CEDIKOVA, Miroslava, **Michaela MIKLIKOVA,** Lenka STACHOVA, Martina GRUNDMANOVA, Zdeněk TUMA, Vaclav VETVICKA, Nicolas ZECH, Milena KRALICKOVA and Jitka KUNCOVA. Effects of Czech propolis on sperm mitochondrial function. *Evidence-Based Complementary and Alternative Medicine* 2014, Vol. 2014, 10 pages. ISSN 1741-4288. Retrieved from doi: 10.1155/2014/248768. **IF_{2013} = 2,175**

KOCHOVA, P, J KUNCOVA, J SVIGLEROVA, R CIMRMAN, **M MIKLIKOVA,** V LISKA and Z TONAR. The contribution of vascular smooth muscle, elastin and collagen on the passive mechanics of porcine carotid arteries. *Physiological Measurement* [online]. 2012, **33**(8), 1335-1351 [cit. 2016-06-30]. DOI: 10.1088/0967-3334/33/8/1335. ISSN 0967-3334. Retrieved from: http://stacks.iop.org/0967-3334/33/i=8/a=1335? key=crossref.1fcf2c9fa 4c80cc2637a0aca1e42e2b0.

HOLUBOVA, Monika, **Michaela MIKLIKOVA,** Peter KRUZLIAK, Rachele CICCOCIOPPO, Martin LEBA, Daniel GEORGIEV, Pavel JINDRA, Daniel LYSAK. Cryopreserved NK cells in the treatment of Haematological malignancies – pre-clinical study. – submitted

9 Acknowledgement

I would like to thank to my tutors doc. MUDr.Daniel Lysák, Ph.D. and Ing. Lucie Vištejnová, Ph.D. for professional guidance, supervision, support and valuable advices, to prof. MUDr. Milena Králíčková, PhD. for motivation and support, to MUDr. Miroslava Čedíková, PhD. and Mgr. Monika Holubová, PhD. for the infinite amount of valuable advices, support and positive approach to all problems, to histology team, namely to doc. MUDr., Mgr. Zbyněk Tonar, PhD. and Mgr. Yaroslav Kolínko, PhD. for histology processing and analysis of liver samples, to physiology team, namely to doc. MUDr. Milan Štengl, PhD., doc. MUDr. Jitka Kuncová, PhD., doc. Jitka Švíglerová, PhD., Ing. Dagmar Jarkovská and MUDr.Lukáš Nalos, PhD. for cardiomyocyte isolation, help with data acquisition of cardiomyocyte functions and many valuable advices, to hematology-oncology team and surgeon team for their good work and finally, special thanks to my family and my fianceé Josef for understanding and support.

This work was supported by the project CZ.1.05/2.1.00/03.0076 from European Regional Development Fund, by National sustainability program I (NPU I) no. LO1503 provided by Ministry of Education, Youth and Sports, by project of Specific University Research SVV 266802/2013 and 260048/2014, by Program for development of science on Charles University (no. P36), by grant from Charles University Grant Agency no. 2090214 and by Fulbright committe (Fulbright-Masaryk stipend).