Mesenchymal stem cells and their regenerative and immunomodulatory potential

Mesenchymální kmenové buňky a jejich regenerační a imunomodulační potenciál

Ph.D. Disertation

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Pilsen 2016

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 x 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 x 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.

Acknowledgement

Declaration

I declare that I have done this work independently and all sources used within this work are cited inside.

In Pilsen 30th of June 2016

MUDr. Mgr. Michaela Miklíková

Content

List of abbreviations

ANG	Angiopoietin
aP2	Acid binding protein 2
APC	Antigene – Presenting cell
BMP	Bone morphogenetic factor
CCL	CC chemokine ligand
CDMP	Cartilage-derived morphogenetic protein
CFU	Colony Forming Unit
CMCs	Cardiomyocytes
DC	Dendritic cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factro receptor
FCCP	Carbonyl Cyanide-4-(trifluoromethoxy) phenylhydrazone
FGF	Fibroblast growth factor
fMLP	N-formyl-Methionyl-Leucyl-Phenylalanine (N-formyl-Met-Leu-Phe)
FoxP3	Forkhead box P3
FPR	Formyl peptide receptor
FPRL	Formyl peptide receptor-like
GM-CSF	Granulocyte macrophage colony – stimulating factor
GVHD	Graft versus host disease
HGF	Hepatocyte growth factor
HLA-DR	Human Leukocyte Antigen – antigen D related
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3 - dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
LFA	Lymphocyte function – associated antigen
LIF	Leukemia inhibitory factor
LPL	Lipoprotein lipase
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex

MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-difenyl tetrazolium bromide
NK cell	Natural killer cell
NKG2D	Natural-killer group 2, member D
Omy	Oligomycin
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PGE2	Prostaglandin E 2
PHA	Phytohemaglutinnin
PPARy2	Peroxisome proliferation-activated receptor γ-2
Rot	Rotenon
SCF	Stem cell factor
SDF	Stromal cell-derived factor
TGF	Transforming growth factor
TH2	Helper T-lymphocytes 2
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Tregs	Regulatory T - lymphocytes
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

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.

2 Mesenchymal Stem Cells

2.1 Discovery and definition

Mesenchymal stem cells (MSCs) were identified in 1960s by Friedenstein and his colleagues who discovered a rare subpopulation of bone marrow cells that had stem cell potential, adhered to plastic, formed colonies in vitro and had fibroblast-like appearance. MSCs since then have been found in several different tissue types where proper niche is present [da Silva Meirelles et al., 2006].

Different origins and its possible effect on MSC behavior together with heterogeneity of MSCs cell population resulted in decades lasting inconsistence in nomenclature. Designation of these cells with non hematopoietic multipotency include „bone marrow (stromal) cells", „bone marrow stem cells", „stromal (stem) cells", „colony-forming-unit-fibroblasts", „mesenchymal progenitor cells", „skeletal stem cells", „mesodermal progenitor cells", „non-hematopoietic stem cells" and others [Young and Black, 2004].

Despite inconsistencies in nomenclature and definition, MSCs have been reported to support regeneration of the tissue by differentiation into target tissue cells, by paracrine signaling, which promotes growth and proliferation of neighboring cells and also with immunomodulatory effect. All together, MSCs abilities made them promising cell type for the regenerative medicine of the future.

2.2 MSCs characteristics

MSCs are very heterogeneous cell population, considering their morphology, physiology and cell markers expression.

2.2.1 MSCs morphology

First studies performed with MSCs showed phenotypically uniform population of fibroblast-like cells. Studied MSCs had symmetric spindle shape with a small cell body and few long cell processes. Observed homology was higher than 98% [Pittenger et al., 1999]. Microscopy of MSCs revealed cell body containing large, round nucleus with a prominent nucleolus surrounded by finely dispersed chromatin particles giving the nucleus a clear appearance. Transmission electron microscopy of MSCs revealed dilatated cisternae of rough endoplasmatic reticulum, lipid droplets, well developed contractile filaments with dense bodies and complex foldings of the plasma membrane [Pasquinelli et al., 2007].

Following studies discovered, that MSCs consist of minimally two phenotypically different subpopulations, smaller spindle shape cells and larger cuboideal cells. Considering their proliferation abilities, these two subpopulations were named small quickly dividing cells and large slowly dividing cells [Reyes et al., 2001].

Later on, third, very small, around 7μm in diameter, subpopulation of quickly dividing cells was observed. These cells show high nucleus to plasma ratio together with expression of specific proteins containing vascular endotelial growth factor (VEGF) receptor-2, tyrozinkinase receptor, transferrin receptor and annexin II [Colter et al., 2001]. Since then MSCs are considered to be heterogeneous cell population.

2.2.2 MSCs surface markers

Since the time of MSCs discovery, their characterisation was complicated. MSCs express variety of antigenes typical for other cell types, till now there is not known any marker specific only for MSCs.

MSCs isolated from bone marrow express following surface markers: CD29, CD44, CD73, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD117, CD166, (SH3 a SH4), CD90 (Thy-1), STRO-1 and Sca-1 and completely lack the markers typical for hematopoietic and endothelial cell lines, including CD11b, CD14, CD31, CD33, CD34, CD133 and CD45. MSCs can be also characterized by presence of STRO-1, Thy-1, CD10, CD49a, Muc18/CD146 together with presence of platelet derived growth factor receptor (PDGFR) and epithelial growth factor receptor (EGFR) [Gronthos, 2003].

MSCs also express receptors associated with adhesive interactions with matrix and other cells such as integrins αVβ 3 and αVβ 5, intercellular adhesion molecule – 1 (ICAM-1), ICAM-2, lymphocyte function-associated antigene-3 (LFA-3) and L-selectin [Pittenger et al., 1999].

Interestingly, expression of MSCs markers slightly vary between freshly isolated MSCs, MSCs in low passages and MSCs in higher passages.

Research on adipose tissue derived MSCs showed very similar antigene profile with bone marrow derived MSCs, with small differences, for example expression of CD62e and CD31 is present only on MSCs derived from adipose tissue [Gronthos, 2003].

All these findings lead in 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to publish Minimal Criteria every cell has to meet to be classified as Mesenchymal Stem Cell [Dominici et al., 2006]. According to that,

MSCs are defined as adherent fibroblast-like cells expressing CD73, CD90 and CD105, not expressing CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR with the ability to differentiate into adipocytes, osteoblast and chondrocytes [Kim et al., 2014].

2.3 MSCs in physiological conditions

MSCs, as described, are heterogeneous population of cells meeting Minimal criteria [Dominici et al., 2006] able to adhere to cultivation plastic, proliferate and form colonies in vitro.

In healthy organism MSCs rest in niche and help maintain many features of healthy organism.

2.3.1 MSCs localization

At first, MSCs were found in bone marrow, where they form small portion of cell population, approximately 0,01 – 0,001% of mononuclear cells [Pittenger et al., 1999]. Number of MSCs in bone marrow is age-depended, the highest presence of MSCs is in newborns and in the age of 80 it is possible to detect only a half of number present in individual in the time of birth [Inoue et al., 1997].

Since then, MSCs have been found in different tissues including placenta, umbilical cord, fat, muscle, tendon, ligament, skin [Murray et al., 2014], synovial fluid [Jones et al., 2004] or teeth [Nakashima and de Crombrugghe, 2003] or, as many evidence shows, in all over the body [Crisan et al., 2008], [da Silva Meirelles et al., 2006]. Despite these findings MSCs are rarely detected in peripheral blood.

Some researchers explain MSCs presence in all body tissues by claiming, that pericytes, cells resting on the abluminal surface of endothelium in the microvascular part of connective tissue, are, in fact, MSCs. Several studies reported that MSCs co-express many markers similarly to pericytes [Sarukhan et al., 2015] and isolated pericytes in culture showed similar abilities as MSCs, including ability to differentiate into osteocytes, chondrocytes and adipocytes [Crisan et al., 2012]. These works, supported by findings that, besides bone marrow, MSCs are usually found resting in perivascular niche, led researchers to thoughts that MSCs and pericytes are the same cell type. Evidence showing other, non perivascular related cells, having similar MSCs characteristics [Kaukua et al., 2014] led to conclusion that pericytes are important and widely represented, but only a subpopulation of MSCs. It has been suggested that when pericytes sense damage, they become a MSCs to provide environment for local tissue regeneration by secretion of various trophic, angiogeneic and

immunogenic factors. When the repair is done, they can convert back to pericyte stage [Caplan and Hariri, 2015].

2.3.2 MSCs niche

MSCs niche is an environment consisted of cells and cell produced molecules which regulate stem cell function together with stem cell autonomous mechanisms. It was observed that death MSCs are easily replaced when there is functional niche. In the case of niche destroyal there will be no more MSCs in surrounding area.

Niche controls balance between aging, self-renewal and differentiation of MSCs as well as engagement of specific programs in response to stress and injuries [Ehninger and Trumpp, 2011]. It has been suggested that regulation of MSCs differentiation is done by Wnt family members, who support not differentiated state of MSCs, and their inhibitors, including Dickkopf-1 (Dkk1), Frizzled b-1 (Frzb-1) or sFRP1. Wnt signalization inhibits differentiation process by increased level of oct-3/4, rex-1 and transcriptional factor Nanog [Sato et al., 2004]. Besides Wnt and Dkkl mediated signalization, differentiation process of MSCs is also controlled by Notch, Hedgehog and bone morphogenetic protein (BMP) pathways.

In bone marrow, according to STRO-1 positive cell population research, niches can be found very close to the endosteum [Gronthos, 2003]. In other tissues, MSCs are usually resting in perivascular niche, which determines the characteristics of the cells. Existence of perivascular niches may be the reason why MSCs can be found all over the body [Bouacida et al., 2012].

2.3.3 MSCs function in healthy organism

In healthy organism MSCs have many functions, but the most important described one is their involvement in hematopoiesis. Bone marrow MSCs are very closely connected to hematopoiesis from the early development of the individual, even in fetus MSCs can be found in liver and bone marrow, supporting hematopoiesis [Campagnoli et al., 2001].

In postnatal organism MSCs play very important role in creation and maintenance of microenvironment of bone marrow. MSCs create and mechanically support tissue framework and release important proteins, including fibronectin, laminin, collagen and proteoglycans [Pontikoglou et al., 2011][Nakamura et al., 2010].

MSCs in resting state or after stimulation secrete variety of growth factors, cytokines and chemokines, including IL-1a, IL-1b, IL-6, IL- 7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-

CSF), leukemia inhibitory factor (LIF), stem cell factor (SCF), fetal liver tyrosine kinase-3 (FLTrk-3), trombopoetin and hepatocyte growth factor (HGF) [Gronthos, 2003; Uccelli et al., 2008a; Pontikoglou et al., 2011]. Some of these cytokines, especially GM-CSF, SCD and IL-6, support proliferation and differentiation of hematopoietic cells [Dormady et al., 2001]. It has been proven on animal models that transplantated MSCs stained with GFP were able to integrate themselves as a functional part of hematopoiesis environment. These MSCs actively helped in development of hematopoietic cells by physical interactions and secretion of specific factors [Muguruma et al., 2006].

Besides their involvement in hematopoiesis MSCs helps in renewal of wide variety of cell types and help to maintain connective tissue all over the body.

Usually, all these functions are done and substances are released by MSCs resting in niche. Evidence suggests that MSCs leave niche only after receival of some stimuli, following problems in body [Watt and Hogan, 2000].

Migration of MSCs from niche to site of needed reparation is initiated by reception of signal from existing disharmony. MSCs express not only receptors for growth factors and other chemoatractans, but also toll-like receptors (TLR) sensitive to substances released from damaged cells and tissues, including heat shock proteins (HSP), and to molecules originating in pathogene organisms [Pevsner-Fischer et al., 2007][Raicevic et al., 2010]. Therefore migratory stimuli can be released by all mechanical injury, inflammation, infection and/or beginning of cancer transformation [Watt and Hogan, 2000].

After reception of certain stimuli, MSCs are able to leave their niche and transfer to circulatory system.

Stromal cell-derived factor (SDF)-1 and its receptor CXC chemokine receptor-4 (CXCR4) are important mediators of MSCs recruitment for variety of tissue damages. Also CCR1, overexpressed in MSCs increase migration into damaged site [Hodgkinson et al., 2010].

In murine MSCs expression of nine corresponding receptors was found including CCR2, CXCR4, IL-6-RA, E-selectin ligand, CD29, CD49d, CD49f, integrin α8, and integrin α9 [Ip et al., 2007]. Recently, several additional ligand/receptor pairs have been suggested to be important in the homing of MSCs to ischemic tissues, including hyaluronic acid/CD44, N-formyl peptide receptor (FPR) and the formyl peptide receptor-like-1 (FPRL1), platelet-derived growth factor-AB (PDGF-AB)/PDGF receptor alpha and beta, and insulin-like growth factor 1 (IGF-1)/IGF receptor [Wu and Zhao, 2012].

Movement of MSCs according to gradient of soluble molecules is similar to migration of immune cells to center of inflammation [Ponte et al., 2007].

2.4 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.

Many are known, but, so far, there is not enough of evidence about MSCs behavior in patients. Majority of the studies from which the informations are derived was done in vitro, the rest was majorly performed on preclinical level, including tests with small laboratory animals. Real patient data are lacking, but starting to appear.

2.4.1 Differentiation ability

One of the MSCs major characteristics is their ability to differentiate into cells of mesodermal origin, such as osteoblasts, chondroblasts, adipocytes, stromal cells and other connective tissue cell types, including tubular cells in kidney [Liu et al., 2015]. Recently, it was reported, that MSCs can differentiate into cells coming from all three germinal layers (Figure 1), which could be explained by their developmental origin in mesenchymal tissue, including mesoderm and also neural crest [Uccelli et al., 2008a]. In search for MSCs true origin it was found that MSCs are not a homogenous population. Analyses of their complex transcriptome showed, that a wide range of proteins is encoded, which suggest different developmental pathways and involvement in large number of diverse biological processes [Phinney et al., 2006].

It was found that MSCs can differentiate into myocytes, including cardiomyocytes [Toma et al., 2002], striated muscle cells [Schulze et al., 2005] and smooth muscle cells [Brun et al., 2015], but also to pneumocytes [Li, 2015] and epithelial cells of digestive tractus, including hepatocytes [Katagiri et al., 2015], all examples of entodermal origin cells. However, MSCs are able to differentiate also into neuroglial cells [Donega et al., 2014] and epithelial cells of ectodermal origin.

2.4.2 MSCs paracrine effect

MSCs are able to release variety of growth factors, anti-apoptotic, anti-inflammatory and trophic molecules, including FGF, HGF, IGF-1, VEGF, stromal cell-delivery factor (SDF), transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF) and

matrix metalloproteinases (MMP). All these molecules benefits for example to damaged myocardium with reduction of pathological fibrosis, by attenuating cardiomyocyte apoptosis and hypertrophy and also by increasing of neovascular formation and stimulation of endogenous stem/progenitor cells for myocardial infarction [Xiang et al., 2009].

In general MSCs paracrine effect influences many cell types including main tissue cells, such as hepatocytes, cardiomyocytes or neuroglial cells together with surrounding fibroblasts, endothelial cells, accumulated inflammatory cells, endogenous stem cells and progenitor cells [Kinnaird et al., 2004]. MSCs can also recruit local progenitors and subsequently induce the differentiation into target tissue cells, including neural cells [Munoz et al., 2005].

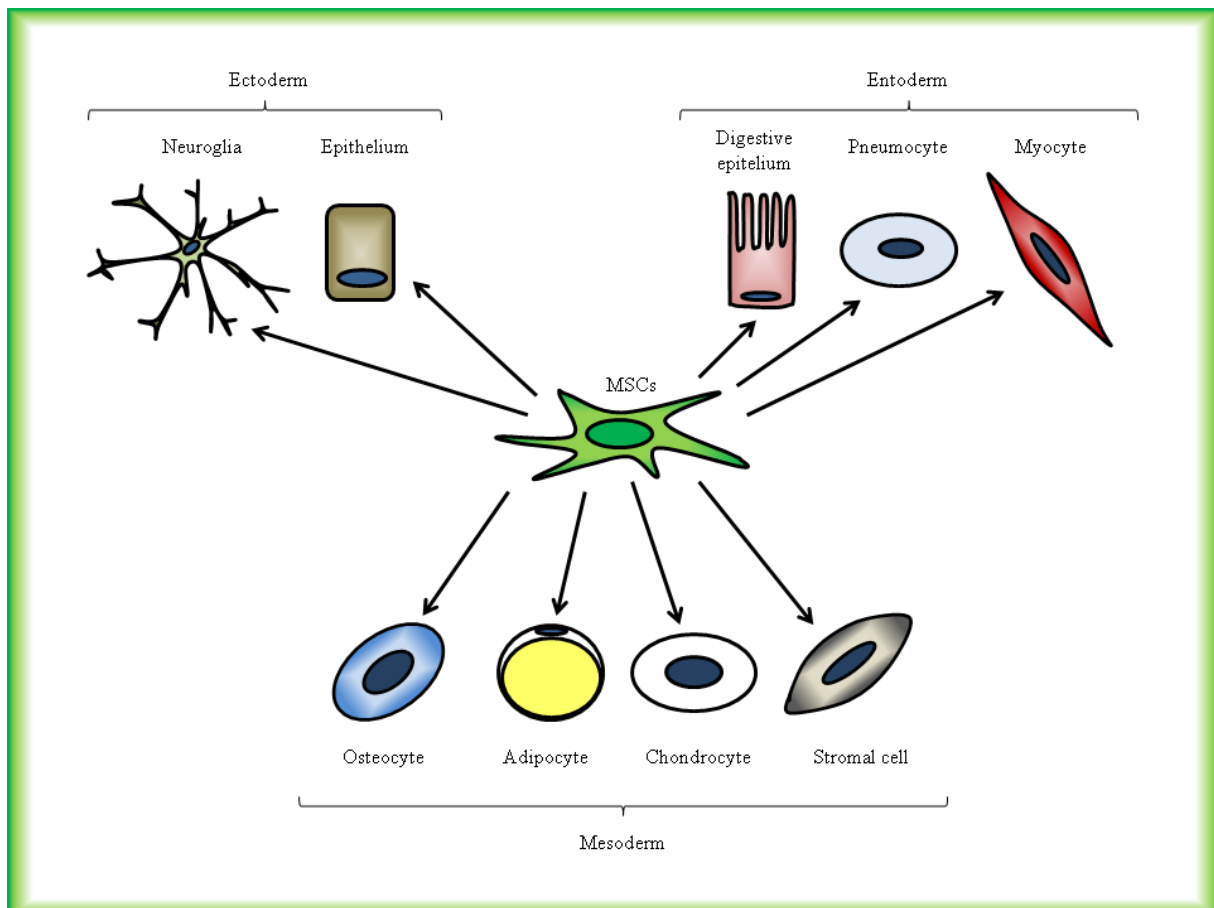**Figure 1** - Scheme of MSCs differentiation ability into different cell types together with their division according to germinal origin.

2.4.3 MSCs and immunity

MSCs are considered to be reliable and powerful immune suppressors. They influence many immune cells with paracrine production (Figure 2). It has been reported that MSCs are effective and non harmful as an autologous, allogeneic and also xenogeneic transplantation.

All these findings support evidence, that MSCs are also immunopriviledged cells, in the other case, successful allotransplantation would be complicated and xenotransplantation would not be possible at all.

2.4.3.1 MSC immunopriviledge

MSCs isolated from adults express medium levels of major histocompatibility complex class I (MHC-I) molecules, important for cell recognition, fetal MSCs express even lower levels of MHC-I [Le Blanc and Ringdén, 2005]. Both adult and also fetal MSCs do not express MHC-II, most important molecule for histocompatibility determination, on their surface. In lysate from MSCs the MHC-II molecules were detected by simple Western Blott which suggests presence of human leukocyte antigen class II (HLA-II) in intracellular deposits. When MSCs differentiate into osteocytes, adipocytes or chondrocytes all these cells express MHC-I, but not MHC-II. It shows that MSCs are non-immunogenic before and also after differentiation in vitro [Le Blanc et al., 2003].

After MSCs administration T-lymphocytes are not triggered by MSCs presence, because MSCs do not express MHC-II or costimulatory molecules, including CD40, CD80 or CD86. In experiments, MSCs stimulated by IFN γ upregulated MHC-II, but the activation of T-lymphocytes was not reported [Chinnadurai et al., 2014]. Possible explanation is that, under inflammatory conditions, where IFN γ is present, MSCs do not express costimulatory molecules, which helps them to do not trigger T-lymphocytes [Sivanathan et al., 2014]. Many studies reported that MSCs can be applicated safely as allogeneic transplant without the risk of rejection [Aggarwal and Pittenger, 2005; Chinnadurai et al., 2014; Sivanathan et al., 2014]. MSCs can be transplanted across the species, but in this case limits were reported. In one study after application of human MSCs into organism of immunocompetent mice the late hypersensitivity leading to rejection of transplant occurred [Grinnemo et al., 2004].

But, as stated, all these findings make MSCs ideal cell type for autologous and also allogeneic transplantations.

2.4.3.2 MSCs and immunomodulatory effect

In 2002 studies describing immunomodulatory effect of MSCs have been published [Bartholomew et al., 2002; Di Nicola et al., 2002]. This redirected scientific attention from MSCs differentiation towards their possible regulatory effect on immune cells. Since then it has been reported that MSCs can influence immune system by cell-to-cell contact with immune cells, mediated by adhesion molecules, and/or by release of several soluble

immunosuppressive factors. MSCs cross-talk with target cells can also increase their production of soluble factors. [Augello et al., 2005].

It has been shown that MSCs are involved in modulation of both innate and also adaptive immunity.

2.4.3.2.1 Innate immunity

As a part of innate immunity, dendritic cells (DCs) play a crucial role in processing antigen material to naive T-lymphocytes. Innate DCs encountered with pathological stimuli express costimulation molecules, including CD40, CD80, CD86, produce interleukin-12 (IL-12) and increase expression of MHC II molecules together with CCR7 chemokine receptor, which helps to transfer from periphery to lymphatic nodules, where DCs present molecules to T-lymphocytes. MSCs has been reported to inhibit reversibly the maturation of monocytes and CD34+ progenitor cells into DCs in vitro [Nauta et al., 2006], together with inhibition of all three key elements of DCs maturation. MSCs decrease expression of costimulation molecules, decrease MHC II expression and prevent homing of DCs by decrease in expression of CCR7 and decrease in production of E-cadherin, which sustain innate DCs in periphery. Cocultivation of MSCs with mature DCs resulted in decreased interleukin-12 (IL-12) production in DCs together with decreased expression of MHC class II molecules, CD11c, CD83 and costimulatory molecules, the combination, which impaired the antigen-presenting function of DCs [Aggarwal and Pittenger, 2005; Ramasamy et al., 2007]. It was also reported that MSCs can inhibit the production of tumor-necrosis factor (TNF) and decrease the pro-inflammatory potential of DCs [Aggarwal and Pittenger, 2005]. Subpopulation of DCs in plasma, responding to microbial stimuli by production of high levels of interferon – I (IFN-I), upregulated production of the anti-inflammatory cytokine IL-10 after incubation with MSCs [Aggarwal and Pittenger, 2005].

Natural killer cells (NK) are very important part of innate immunity, playing a key role in anti-viral and anti-tumor immune responses. NK cell function is regulated by surface receptors of targeted cell, which can inhibit or activate NK-cell-mediated lysis. Low or absent expression of MHC class I molecule together with recognition of targeted cell receptors by NK cells is necessary for lysis [Moretta et al., 2001]. In resting NK cells MSCs down regulate expression of NKp30 and natural-killer group 2, member D (NKG2D), which are activating receptors involved in NK-cell activation and in killing of target cells [Spaggiari et al., 2006].

Freshly isolated, resting NK cells after culture with IL-2 or IL-15 proliferate and show strong cytotoxic activity, but when the resting NK cells are cultivated in the same culture

together with MSCs, NK cell proliferation and interferon - γ (IFN γ) production are nearly not present. Pre-activated NK cells in culture with MSCs also showed decreased proliferation, IFN γ production and cytotoxicity. However, in comparison, pre-activated NK cells were reported to be more resistant to MSCs induced decrease in NK cell activity [Spaggiari et al., 2006]. On the other hand, MSCs were reported to be killed by pre-activated NK cells in vitro [Sotiropoulou et al., 2006; Spaggiari et al., 2006]. MSCs are suscepted to NK-cell-mediated cytotoxicity according to level of their surface expression of MHC class I molecules together with expression of various ligands NK cells recognize. Incubation of MSCs with IFN-γ partially protected them against NK cells [Spaggiari et al., 2006]. These findings suggest that MSCs can inhibit NK cell activity, but NK cell ability of killing MSCs is not compromised [Uccelli et al., 2008a]. IFN-γ protects MSCs from NK-cell-mediated lysis which may be the answer, why some MSCs therapeutic applications are successfully immunomodulative and some show no difference with placebo. IFN-γ presence favoring MSCs induced NK cell inhibition instead of MSCs being killed by NK cells in absence of IFN-γ may be the reason.

MSCs were also reported to be able to inhibit neutrophil apoptosis and process so called respiratory burst, which starts when neutrophil binds to bacteria in order to destroy it. MSCs-mediated preservation of neutrophils and their increased life span may be important especially in anatomical locations where large number of neutrophils is stored [Raffaghello et al., 2008].

2.4.3.2.2 Adaptive immunity

MSCs inhibit T-lymphocyte proliferation and cytotoxicity [Aggarwal and Pittenger, 2005; Zappia et al., 2005] by arresting T-lymphocytes in the G0/G1 phase of the cell cycle [Benvenuto et al., 2007]. MSCs support survival of T-lymphocytes subjected to overstimulation, which are committed to undergo ligand-dependent activation of induced cell death [Benvenuto et al., 2007]. MSCs induced inhibition of T-lymphocyte proliferation has been reported to lead to decreased IFN-γ production both in vitro [Aggarwal and Pittenger, 2005] and also in vivo [Zappia et al., 2005] and to increased IL-4 production by T helper 2 (TH2) cells. It has been shown that MSCs can trigger maturation of regulatory T-lymphocytes (Tregs), specialized T-lymphocyte subpopulation, which is able to suppress activation of immune system [Maccario et al., 2005; Aggarwal and Pittenger, 2005].

All these findings indicate that MSCs can modulate the intensity of an immune response by inhibition of antigen specific T-lymphocyte proliferation and cytotoxicity together with promotion of maturation of regulatory T-lymphocytes.

This may mean, that MSCs transplantation could make the host vulnerable to infectious agents. Fortunately evidence about fail-safe mechanism exists. MSCs express functional Toll-like receptors (TLRs) which after interaction with pathogen ligands induces proliferation, differentiation and migration of MSCs together with their secretion of chemokines and cytokines. It has been shown that MSCs Notch signaling after triggering of TIR3 and TI4 is impaired and MSCs lose the ability to inhibit T-lymphocyte proliferation [Uccelli et al., 2008b]. Therefore in case of pathogen infiltration immune system should work properly.

MSCs has been reported to inhibit B-lymphocyte activity and proliferation in vitro together with differentiation and constitutive expression of chemokine receptors [Corcione et al., 2006]. Other in vitro studies showed that MSCs support survival, proliferation and differentiation of antibody-secreting cells [Traggiai et al., 2008]. The controversial in vitro results are not significant considering the fact that B-lymphocytes responses are mainly T-lymphocyte dependent. Which means that final outcome of MSCs influence on B-lymphocyte will be influenced by MSCs inhibition of T-lymphocytes function.

Despite MSCs immunosuppressive activities, in specific conditions, when IFN-γ concentration is low, MSCs upregulate the expression of MHC II molecules, so they can function as antigen presenting cell (APC). In the case of IFN-γ increase, MSCs loose APC abilities [Stagg et al., 2006].

Contribution of different factors mediated by MSCs to immunosuppressive reactivity varies between different studies. Therefore, MSCs mediated immunoregulation can be considered as complex system, where none of the produced molecules plays exclusive role [Uccelli et al., 2008b].
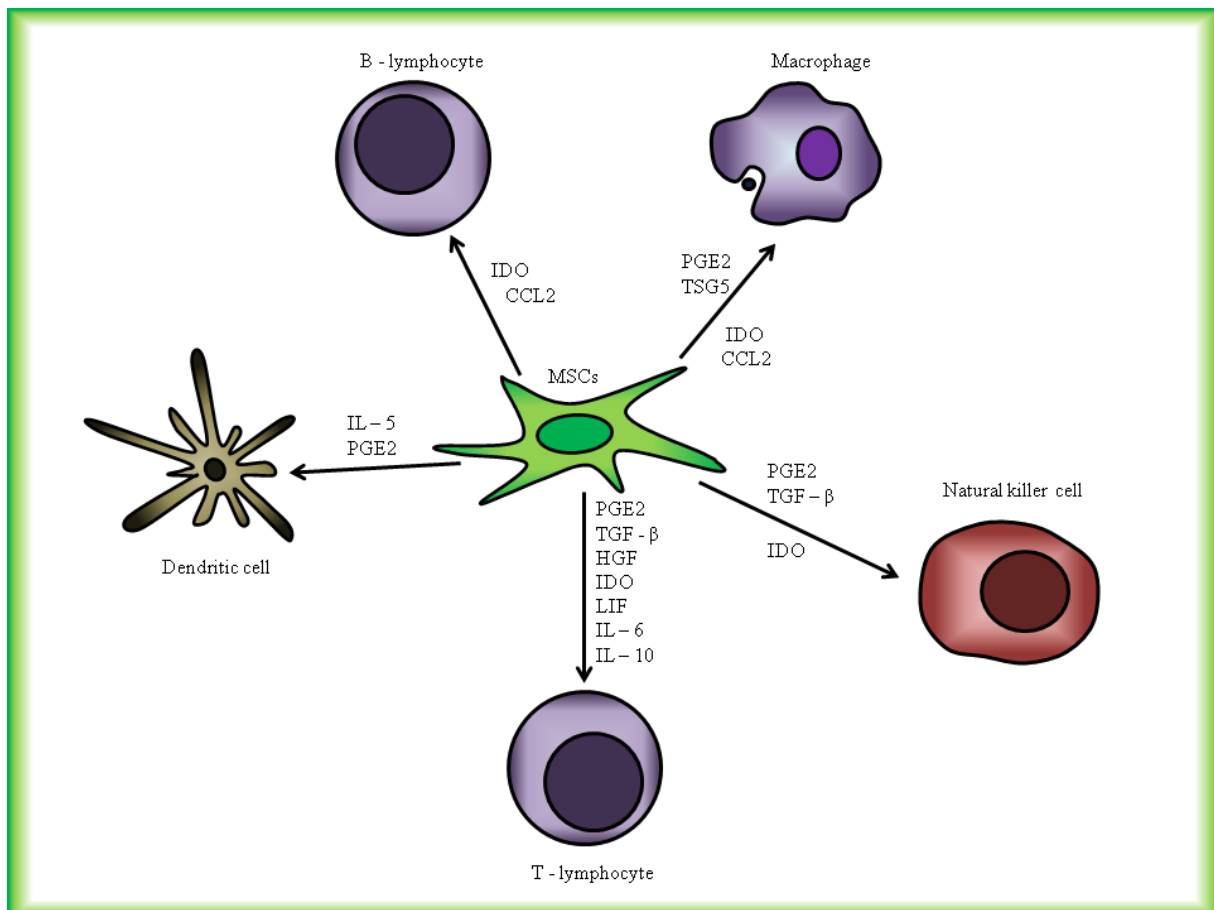
Figure 2 - MSCs influence on immune cells.
Abbreviations: CCL2 – chemokine (C-C) ligand 2, HGF – hepatocyte growth factor, IDO – Indolamine-2,3-deoxygenase, IL-5 – interleukin 5, IL-6 – interleukin 6, IL-10 – interleukin 10, LIF – leukemia inhibitory factor, PGE2 – prostaglandin E2, TGF-β – tumor growth factor.

2.5 MSC in *in vitro* conditions

In vitro studies of MSCs are still the most used way for understanding of their behavior on all the possible levels. MSCs can be cultured alone in specific growth media or in media supplemented with variety of substances or co-cultured with other cell types.

2.5.1 Isolation

MSCs can be obtained easily by separation of white blood cell fraction from bone marrow sample or from peripheral blood sample following stem cell release after stimulation. Contrary to majority of other bone marrow cells, MSCs adhere to plastic, which is used for culture purification. Non adherent cells are washed away, while adherent cells, mostly MSCs, remain in culture. Procedure for isolation of MSCs used in our studies can be found in Materials and methods section.

After isolation, confirmation of MSCs characteristics follows. As MSCs, only cells having MSCs markers (CD34$^-$, CD45$^-$, CD73$^+$, CD90$^+$, CD105$^+$) and ability to differentiate into adipocytes, osteocytes and chondrocytes are taken.

2.5.2 MSCs cultivation

MSCs are the cells able to attach to cultivation plastic without special surface coating. In culture MSCs form colonies in 5-7 days after isolation [Song et al., 2014]. These colony forming units-fibroblasts (CFU-F) show big proliferation potential in vitro, but real numbers varies between individuals from which MSCs has been isolated and also with cultivation conditions. Human MSCs proliferate the best when seeded in low densities (1x10^5 cells/cm^2). Originating seeding density influences behavior and morphology of MSCs in culture. MSCs in low density are more spindle shaped, but when the culture is confluent and MSCs are growing in layers, their morphology is flat with fuzzy edges [Tropel et al., 2004].

Interestingly, human MSCs can be cultivated without feeder cells, but cultivation of rodent MSCs requires feeder cells for achievement of maximal proliferation [Prockop et al., 2003].

Cell cycle analysis of MSCs in culture showed that 10% of the cells are in S, G2 and M phases, majority of the cells stays in G0/G1 phases.

In the scale of whole cell population, MSCs in vitro growth is characterized by three phases. First, lag phases, takes 3-4 days, following log phases lasts until confluence, when cells reach stationary phases. It has been described, that MSCs are driven by expression of Dickkopf-1 (Dkk- 1) a Wnt5a genes, which have antagonistic effect. The highest expression of Dkk-1 is in the beginning of log phases and shortens the previous lag phases by inhibition of expression of Wnt5 protein. On the other hand, production of Wnt5 is maximal during the stational phases [Gregory et al., 2003].

In optimal cultivation conditions can MSCs 20-30 times double its population and still be able to differentiate [Prockop et al., 2003].

In the moment the cells achieve 80% confluence, passage should follow in order to preserve MSCs characteristics as long as it is possible, it was mentioned than confluent cells lose their phenotype and characteristics. MSCs are not immortal cells, but their life span in culture is stable till passage 12, when MSCs posses majority of main characteristics. Later, MSCs senescence and higher percentage of apoptosis occurs.

Crucial for maintaining of good cultivation conditions is use of proper growth media. On market there is variety of prepared complete growth medias especially for MSCs, but sufficient is use of standard α-MEM or DMEM supplemented with 5-20% FBS.

2.5.3 MSCs differentiation *in vitro*

In order to confirm that isolated cells are MSCs, differentiation into adipocytes, osteoblasts and chondrocytes needs to be performed and documented. This differentiation in vitro requires special maturation cocktails, but the role of basic nutrients, cell density, mechanic influences, growth factors and cytokines is also important.

Interestingly, use of the same substance is having different effect in MSCs from various species. Dexamethason as a basic substance necessary for differentiation of human MSCs into osteoblasts, can be used in mice MSCs for differentiation into adipocytes [Dennis et al., 2002]. Low doses of recombinant human bone morphogenetic protein-2 (rhBMP-2) start differentiation of mice MSCs to osteoblasts, but in human MSCs it is necessary to use significantly higher doses to achieve similar effect [Claros et al., 2014].

Another important factor is cell density. If MSCs are seeded in low density, they proliferate and release high levels of Dkk-1, which supports non differentiated phenotype. Contrary to that, fully confluent MSCs express Wnt-2 which suppress Dkk-1 and its effect [Gregory et al., 2003].

All these factors need to be considered before differentiation test is performed.

2.5.3.1   Differentiation into osteoblasts

For differentiation of MSCs into osteoblasts it is necessary to cultivate them in media enriched with β-glycerolphosphate, ascorbic acid and dexamethason for 2-3 weeks. In formation of bone tissue the bone morphogenetic proteins (BMP), TGF-β, insulin-like growth factor (IGF), brain-derived growth factor (BDGF), FGF-2, leptins and peptides binding parathyroid hormones are involved [Shima et al., 2015]. Other transcription factors participating on osteogenesis are Cbfa1/Runx2, Osterix, FosB, Fra-1, Aj18, Osf1, Msx2, Dlx5 and TWIST [Lin and Hankenson, 2011]. Transcription factor Cbfa1/Runx2 is important for forming of osteoblasts, but only Dlx5 helps to differentiate to mineralized osteoblasts.

Process of osteogenesis can be measured by quantification of alcalic phosphatases or by visualization of accumulation of calcium inside the cells [Pittenger et al., 1999].

2.5.3.2 Differentiation into adipocytes

In vitro adipogenesis can be induced by addition of dexamethason, isobutylethylxanthine and indomethacine into media. Differentiation process usually takes 7-14 days.

Successful differentiation can be visualized by Oil Red O Staining or by validation of expression of specific proteins, including peroxisome proliferation-activated receptor γ-2 (PPARγ2), lipoprotein lipase (LPL) and acid binding protein (aP2) [Xu et al., 2015].

2.5.3.3 Differentiation into chondrocytes

Chondrogenesis can be initiated by addition of TGF-β into culture media for 2-3 weeks. Among TGF-β family the most important for chondrogenesis is BMP and cartilage derived morphogenetic proteins (CDMPs) [Dennis et al., 2002].

Chondrocyte differentiation can be verified by detection of proteoglycans in extracellular matrix or by detection of collagen type II by alcian blue [Pittenger et al., 1999].

2.5.4 MSCs visualization

MSCs like other cells are naturally colorless. In order to observe MSCs behavior in vitro proper staining is necessary.

2.5.4.1 Cytochemistry of MSCs

For basic visualization of MSCs in tissue culture the hematoxylin-eosin staining is widely used. Hematoxylin is a basic compound, originally extracted from logwood tree, which binds with acidic or basophilic structures such is nucleic acid and stains them dark blue or violet. Eosin is acidic compound which binds to basic proteins, including proteins in cytoplasm of the cells, and stains them dark red or pink [Vacek, 1995]. Cells stained with hematoxylin-eosin have blue nucleus and red cytoplasm. Hematoxylin-eosin stain is not selective, therefore, for specific visualization of MSCs only, consideration of other types of staining is necessary. These specific stainings are very often combined with addition of hematoxylin stain for visualization of nucleus.

2.5.4.2 Fluorescent visualization of MSCs

MSCs positivity for CD73, CD90 and CD105 together with negativity for CD34 and CD45 is used for visualization of MSCs only. Antibodies against these receptors conjugated with specific fluorophores can be used, flow cytometry or fluorescent microscopy follows to verify the cells. Commercionally available are also antibodies for other markers, the only limit

is species specifity. Usually all these antibody staining are fatal for the cell, often cell permeabilisation is needed.

For long term observation of cell behavior, its survival is necessary. Fortunately, commertionally available are also kits for Live cell imaging, where the staining particles are harmless for cells and cell releases them after some time period.

2.5.5 Comparison of MSCs isolated from different tissues

As it was mentioned, MSCs have different germinal origins, but resulting abilities of all MSCs in heterogeneous population in adults are very similar. In this chapter we offer comparison of characteristics of three MSCs types mostly used in current research, providing the explanation why we focused in our experiments only on MSCs isolated from bone marrow.

2.5.5.1 Bone marrow derived MSCs

Bone marrow is a rich source of cells, majority of them are hematopoietic stem cells, blood cell progenitors and blood cells necessary to satisfy the average human need of approximately one hundred billion new blood and hematopoietic cells each day [Malgieri et al., 2010]. In bone marrow MSCs represent a very small fraction, 0.001–0.01% of the total population of nucleated cells [Pittenger et al., 1999], but, considering the total number of nucleated cells in bone marrow, the density of MSCs is here one of the highest from all the body.

Bone marrow aspiration is a painful process, but the success rate of isolation of MSCs is nearly 100% [Kern et al., 2006]. These MSCs are capable of proliferation in vivo and also in vitro, where their growth is reported to be arrested around 11-12 passage. According to colony forming unit-fibroblast assay (CFU-Fa), average colony forming ability of bone marrow isolated MSCs is (16.5 ± 4.4) [Jin et al., 2013].

It was found that bone marrow MSCs express a large spectrum of cell adhesion molecules and exhibit high expression of integrins that also play role in homing to site of injury and in binding to specific matrix molecules [Malgieri et al., 2010].

As they were discovered first [Friedenstein et al., 1966], majority of global MSCs characteristics were found while experimenting with this type of MSCs. Those characteristics represent standards for comparison up to date.

2.5.5.2 Adipose tissue derived MSCs

Adipose tissue consists of adipocytes and other connective tissue cells. It contains 500 times more stem cells in 1g of fat than in 1g of bone marrow. Among these cells are present also MSCs, meeting all Minimum criteria markers and characteristics, considered to be the most similar to bone marrow MSCs than all MSCs from other sources.

Considering the fact that routine liposuction performed by beauty clinics, which many people voluntarily undergo willing to even pay for, is good and reliable source of MSCs, it is easier to obtain this type of MSCs than bone marrow derived MSCs. This simple fact favors use of adipose tissue derived MSCs for scientific purposes.

On the other hand it has been found that proliferation potential of adipose tissue derived MSCs are lower as same as growth rate and culture time. Performed cell growth experiments were arrested around passage 11, also colony forming ability of these MSCs was only 6.4 ± 1.6 [Jin et al., 2013]. Other disadvantage is that even adipose derived MSCs from different tissue locations posses different abilities. Comparison of MSCs from abdominal fat, mesodermal origin, and eyelid adipose tissue, ectodermal origin, showed different phenotype of the cells together with variety in CD90 expression suggesting higher abdominal fat MSCs response to angiogeneic factors [Kim et al., 2013]. But not only adipose derived MSCs from different germinal origin vary. Comparison of cardiac adipose tissue MSCs and abdominal fat MSCs, both mesodermal origins, showed that cells are phenotypically identical, but their characteristics and abilities are different. Cardiac tissue MSCs constituted intrinsic properties toward myogenesis and vasculogenesis in significantly higher percentage and therefore had much better regenerative potential, especially for cardiac therapy [Wang et al., 2014].

All these findings suggest that adipose tissue MSCs can be promising cell type for regenerative medicine, but their use should be tissue specific, which makes them inappropriate for scientific experiments in general.

2.5.5.3 Umbilical Cord Blood derived MSCs

Umbilical cord blood is a rich source of variety blood precursors and stem cells. MSCs are present here in bigger percentage compared with other tissues they can be obtained from. Umbilical cord blood is very easy to obtain, the procedure is painless and non invasive unlike other MSCs types acquirement, placenta and umbilical cord are considered to be medical waste.

MSCs from umbilical cord also posses all Minimal criteria markers and characteristics, but in comparison to other sources of MSCs their isolation and cultivation is much more complicated, success rate of isolation is 63% [Kern et al., 2006]. If successful, their culture lasts for long periods, their proliferation is arrested at passage 14-16 and their clonogenic ability is enormous 23.7 ± 5.8 compared to others [Jin et al., 2013], but in culture they usually have very low proliferation activity [Musina et al., 2007].

On the other hand, it was shown that umbilical cord MSCs together with amniotic membrane origin MSCs posses higher immunomodulatory capacity than bone marrow MSCs based on gene expression profiling [Wegmeyer et al., 2013], which could be useful in therapy of many diseases.

All MSCs from all three sources mostly used in research possess promising abilities for regenerative medicine, but as it was mentioned they all have limits. Low-yielding isolation and complicated cultivation of umbilical cord MSCs made them, for us, source not reliable enough to be used in our experiments. Tissue specific variety in characteristics and behavior of adipose tissue derived MSCs made them, for us, not suitable for wide range of experiment we performed.

We chose bone marrow MSCs as experimental subpopulation, because their isolation is well described with high-yield and their characteristics are stable and suitable for variety of comparisons. Therefore, in further text, we will refer about bone marrow derived MSCs only.

2.5.6 MSCs aging

It is known, that MSCs are aging, but this factor is often neglected in MSCs research. MSCs can age in vivo together with individual and also in vitro in long term culture.

2.5.6.1 In vitro aging

MSCs are a heterogeneous population of cells, which is reported to mature in cultivation conditions. Expression of surface markers change, longer cultivation time MSCs undergo, more their characteristics and abilities differ [Dazzi et al., 2006].

It was found that MSCs in culture, shortly after isolation (1-3days), showed 100% positivity for CD73 and CD49a, but only 45,4%, respectively 49%, of cultivated MSCs showed positivity for CD105 and CD90 respectively [Boiret et al., 2005]. Marker CD44 is

present on freshly isolated and low passaged MSC, but slowly disappears in long term MSCs culture [Fibbe and Noort, 2003].

Similar results can be seen on example of expression of chemokine receptors. MSCs in second passage produced CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6, but from twelve passage up there was no expression of these molecules found and MSCs were also unable to react on chemokine attractants. Loss of these receptors was accompanied with decreased production of adhesive molecules ICAM-1, ICAM-2, VCAM-1 and CD157 [Honczarenko et al., 2006]. Also multiple apoptotic pathway inductions were observed [Liu et al., 2015].

2.5.6.2   In vivo ageing

It is well known that older organism is not capable of renewal as young one.

Diversity of MSCs population varies with age. In MSCs from older donor the number of MSCs from neuroepithelial and non-mesodermal origin decreases, while the MSCs from mesodermal origin are the most present subpopulation. That may be the reason, why MSCs from older individual do not differentiate into other than connective tissue cells often [Takashima et al., 2007].

Isolated from elder people, in culture aged MSCs are bigger, broad, flatten and show no spindle-formed morphology contrary to younger spindle shaped MSCs [Stenderup et al., 2003]. Aged MSCs contain more stress actin fibres and form small colonies [Liu et al., 2004]. More importantly, young MSCs are 30-40 times capable of maximal population doubling, aged MSC have significant decline in replicative lifespan[Baxter et al., 2004].

Aged MSCs also express different levels of various regulatory substances. For example in human the MSCs emission of IL-6, interleukin capable of regulating proliferation, differentiation and activity of various cell types, is increased with age [Cheleuitte et al., 1998] contrary to that, production of IL-11, cytokine with protective properties and anti-inflammatory function, is decreasing in human aged MSCs [Kuliwaba et al., 2000].

All these findings about MSCs aging should be considered while using long term cultivated MSCs for scientific experiments as well as in use of MSCs for therapy. In clinical practice it may semm to be better to use patients own cells for therapy, but when patients is old, young donor MSCs should be considered as a better therapeutic option.

2.6 MSCs in tissue regeneration

More than 50 years of research showed that MSCs transplantation and application is safe, there is no evidence of MSCs supporting tumor formation. The only reported exception was tumor formation in mice, for which the possible explanation can be chromosomal instability in this species [Miura et al., 2006], which is not related to MSCs application itself.

MSCs are considered to be promising cell type for cellular therapy of the future for variety of diseases and tissue damages. MSCs were reported to differentiate into variety of cell lines, to have ability to immunomodulate inflammatory response and to release variety of cytokines and chemokines.

2.6.1 MSCs application and migration into site of injury

The use of MSCs as a treatment for different tissue damages requires ability to travel across the body and find site of injury, where help is needed. So far, outcomes from different experiments are contradictory. While it was reported that systematically administered MSCs, into non-human primate, preferentially homed to the site of injury and supported there functional recovery [Devine et al., 2003], it was also reported, that systematical application of MSCs into rat with ischemic heart disease showed very poor homing ratio. Up to 70% applicated cells were trapped in lungs, the rest was spread in variety of tissues and to the heart, finally, homed only 4,6 – 6,3% [Assis et al., 2010]. Nevertheless, beneficial effect of MSCs application is reported in majority of performed studies regardless the ratio of MSCs homed into site of damage.

The detailed study of MSCs homing mechanism showed that MSCs coordinately roll and adhere to endothelial cells with a p-selectin and vascular cell adhesion molecule 1 (vCAM1) dependent manner [Rüster et al., 2006]. As a result of the expression of adhesion molecules on MSCs surface, they can extravasate from the blood vessels. MSCs migrate in response to several chemokines that bind to receptors expressed on their cell surface [Sordi et al., 2005] which leads to activation of matrix metalloproteinases that degrade the basal membrane and allow subsequent extravasation [Son et al., 2006].

It has been suggested that many receptors and variety of chemical substances are involved in homing process. In heart infarction, or ischemia in general, stromal cell-derived factor (SDF)-1 and its receptor CXC chemokine receptor-4 (CXCR4) are important mediators of stem cell recruitment. Also CCR1, overexpressed in MSCs increase migration into damaged site [Hodgkinson et al., 2010]. Genes that are significantly up-regulated following

infarction of myocardium include CC ligands 2, 6, 7 and 9, CXC ligands 1, 2, and 12, cytokines including IL-1 and IL−6, transforming growth factor β1 (TGF - β1), TGF - β2, tumor necrosis factor receptor II (TNFRII), and cell adhesion molecules including fibronectin-1, laminin-1, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, thrombospondin 1, and tenascin C [Wu and Zhao, 2012]. In murine MSCs, in addition, expression of following was found; E-selectin ligand, CD29, CD49d, CD49f, integrin α8, and integrin α9 [Ip et al., 2007].

2.6.2 MSCs influence on target tissue

In vitro and also in vivo differentiation of MSCs into wide variety of cells from different embryonic origin was reported, but the real contribution of MSCs differentiation into target tissue cells in regeneration process remains unclear [Uccelli et al., 2008a].

On example of the myocardial infarction it is clearly visible that application of MSCs can contribute to heart regeneration, but differentiation of MSCs into cardiomyocytes is minimal or not functionally contributing at all. Many studies showed that MSCs injected into mice with myocardial infarction are able to differentiate into cardiomyocytes, are positive for myocardial markers and also loses MSCs markers [Garikipati et al., 2014; Yannarelli et al., 2014], but other studies reported that MSCs injected into mouse peripheral blood, migrated into heart infarct area, differentiated into cardiomyocytes, but after 14 days there was no evidence of functional cardiomyogenic differentiation [Siegel et al., 2012].

Based on this evidence it is not surprising, that, although it was proposed that wide differentiation ability of MSCs can be used for regeneration of almost any tissue, MSCs differentiation is, so far, proved to have very important role only in treatment of bone defects [Uccelli et al., 2008a].

In regeneration of target tissue paracrine and immunomodulatory effect of MSCs are reported to be main reason of improved regeneration. MSCs can influence damaged cells by cell to cell contact and also by secretion of various cytokines and chemokines. MSCs do not help only to affected cells, but also create supporting environment, where it is easier for damaged cells to survive and repair.

2.6.3 MSCs clinical application

In order to use MSCs as a treatment option for variety of diseases, application mean and route need to be optimized. Despite all the ongoing research, there was not enough of attention focused on this aspect.

2.6.3.1 MSCs application considerations

For application into patient, MSCs need to be isolated precisely, identified reliably and cultivated under special conditions. In some trials, isolation of the cells is followed by transplantation, so no cultivation is needed.

Nevertheless, it has been shown that MSCs therapy is the most effective, when 1 or 2 millions of MSCs per kilogram of the host are applicated. Such number is possible to achieve only with cultivated MSCs. Laboratories for these purposes need to have special permission and setup of the cultivation room itself preventing contamination of the environment and cell culture. In order to prevent adverse events, MSCs are cultivated in specified Cultivation media without any unnecessary supplements, such as antibiotics, widely used in preclinical research. Purity of the cell culture needs to be regularly tested in order to do not contaminate organism of the host by bacterias or other pathogens. Right before the application itself MSCs need to be gently harvested, regular Trypsin-EDTA use is questioned, it was shown that it can destroy the surface proteins used by MSCs for attachment in vivo. MSCs also need to be purified from the culture media and transferred into physiological or other solution suitable for patients. Sometimes MSCs tend to form clusters hard to dispense, in which case, microembolism of the patient microvessels can be expected as reported [Janowski et al., 2013].

In the moment of application, in situ or peripheral, sterile application conditions need to be secured. In case of application into blood stream, MSCs should be applicated very slowly. In rat it has been shown that speed 0,2ml/min and lower are the only suitable for good result achieval[Janowski et al., 2013].

2.6.3.2 MSCs route of application

MSCs application route is very important factor for MSCs facilitated regeneration, nevertheless it was not clarified which of them is the best.

Application route of first choice is usually injection of MSCs directly into site of injury/damage, in situ. This way is preferred when there are concerns about MSCs being

trapped in other organs, usually in the lungs, and/or when the larger number of MSCs at once is necessary for having beneficial effect. With this application route there is a maximal chance that the cells will stay and attach in the site of injury. This application cannot be used in cases when anatomical location of damages tissue is difficult to access, such as in central nervous system, or when the nature of illness is systemic, such as in autoimmune diseases [Uccelli et al., 2008a].

Other application route is administration of MSCs into peripheral blood stream. This way is preferred in cases where minimal invasivity is the key aspect. Also the price of this application is very low, no special equipment, skills or environment is needed, which favors this application route for use in routine medical practice. Unfortunately, it was reported that with this way of application only very low percentage of the MSCs migrate into site of injury, majority of the cells is trapped in lungs [Barbash et al., 2003]. Despite this evidence the beneficial effect of MSCs application is reported also with this application route, which suggests that systemic influence of MSCs can be more important than it was thought.

For many conditions, the ideal treatment should be use of both application routes, bolus of MSCs into site of damage and one more applications of MSCs into peripheral blood stream.

2.6.4 MSCs in clinical trials

Encouraging results from preclinical research provided the basics for the first clinical trials. Currently there are more than 70 clinical sites all over the world, where efficiency and safety of MSCs therapy for wide variety of illnesses is tested. On the largest clinical trial platform called Clinical Trials (http://clinicaltrials.gov/) there are registered more than 500 different clinical trials where MSCs are transplanted to patients as main therapy or as a co-treatment following regular therapy.

Several of these trials undergone early termination or have failed to meet primary end-points, but many more trials continue and the list of evidence of MSCs facilitated repair in patients enlarges [Malgieri et al., 2010].

Here we offer some MSCs application examples, which are currently in focus of scientists. Applications we focused on in our research are described in separate chapters.

2.6.4.1 Orthopedic application

One of the essential MSCs abilities is differentiation into osteoblasts and chondroblasts, which put MSCs into focus of researchers in bone and cartilage field.

Besides proved MSCs ability to improve bone healing [Huang et al., 2015], it was shown that MSCs administered to children with osteogenesis imperfecta, genetic disorder, where osteoblasts synthesize defective collagen type I, give rise to healthy osteoblasts producing non defective collagen, resulting in increase of bone mineral content and reduction of bone fracture frequency [Horwitz et al., 2002]. As visible on website for Clinical trials (ClinicalTrials.gov), encouraging results gave rise to many clilnical trials, but long time follow-up of patients showed that application of MSCs may not be curative as it was hoped .

Another application of MSCs in orthopedic field is treatment of cartilage lesions. Many studies showed positive results for MSCs in cartilage regeneration [Bornes et al., 2015; Fujie et al., 2015]. So far more than 30 clinical trials was announced, but none of them was convincing enough to use MSCs application as a treatment option. Issues about correct application way, including site of administration, way of administration and/or use of scaffold, together with number of applied cells need to be solved first.

2.6.4.2 Skin defects

MSCs posses many abilities, which could be used in repair of skin defects. In variety of animal models it was shown, that MSCs improve healing of skin defects [Basiouny et al., 2013; Chen et al., 2008]. Some studies also show possible future use of MSCs conditioned media in pharmacology/cosmetics industry [Kwon et al., 2015] .

The proper delivery way of MSCs to wounded patients is not fully assessed yet, considering the route of application and/or scaffold use [Steffens et al., 2015]. Therefore more research in this topic is needed for establishment of MSCs application as a new therapeutic option.

2.6.4.3 Neuronal diseases

MSCs are not proven to be able to pass liquor barrier, but when applied into site of lesion or into intraventricular space, they are able to differentiate into neuroglial cells and release the trophic factors in rat model and benefit in variety of neuronal diseases, including Parkinson's disease, Huntington's disease, hypoxic-ischemic neural damage and retinal injury [Bouchez et al., 2008; Muñoz-Elías et al., 2003; Rossignol et al., 2011].

Nowadays 5 clinical trials are announced on ClinicalTrials.gov where MSCs were/are applicated into different parts of brain of patients with Parkinson's disease. One example for

all, MSCs were successfully transplanted by stereotactic surgery into the sublateral ventricular zone of seven patients with advanced Parkinson's disease. Application was proved to be safe and long term observation (3 years after application) did not shown any adverse effect [Venkataramana et al., 2010]. Although the results were promising, the improvement of patient functions was not significant as expected. More research in this field need to be done, before the MSCs application will became routine treatment option.

2.6.4.4 Kidney disorders

In several preclinical studies, including acute renal ischemia, experimental glomerulonephritis and acute tubular epithelial injury, it was demonstrated that MSCs application can ameliorate renal injury and accelerate repair [Liu et al., 2015; Qian et al., 2008].

According to ClinicalTrials.gov there are more than 20 clinical trials where autologous or allogeneic MSCs are administered to patients with wide variety of kidney problems. Current focus is on lowering the number of organ rejection and further transplantation complications in patients with kidney transplantate. Majority of clinical studies is in process, so it is soon to make conclusions, but promising fact is, that non of the mentioned trials was canceled for severe patients complications or due to loss of interest of the clinicians, which suggests that results may be positive.

2.6.4.5 Systemic disorders

Systemic application of MSCs can benefit to whole organism, which is tested in clinical trials focused on treatment of systemic diseases. In patients with cancer, who underwent high-dose chemotherapy, systemic allogeneic application of MSCs showed acceleration in bone marrow recovery [Koç et al., 2000].

The immunomodulatory potential of MSCs is currently tested also on variety of autoimmune diseases, including Crohn's disease, where MSCs modulate immune response and also contribute to regeneration of gastrointestinal epithelial cells [Okamoto et al., 2002].

2.7 MSCs and cardiac regeneration

The role of MSCs in treatment of variety of cardiac diseases is investigated. Many work has been done in vitro and on animal models, recently also clinical trials in this field has been designed and started [Hare et al., 2012; Chullikana et al., 2014].

2.7.1 Cardiac diseases

Cardiac diseases are a major cause of worldwide morbidity and mortality, in America every 34 second somebody suffers a coronary event [Go et al., 2013]. Cardiac function of patients is increasingly compromised with the progression of adverse ventricular remodeling, many eventually develop fatal end-stage heart failure [Sutton and Sharpe, 2000].

Cardiac insufficiencies are caused by long term overload of cardiac muscle by large volumes and pressures or by acute oxygen and nutrient deficiency. In both cases cardiomyocyte function is compromised by lack of nutritions and oxygen not sufficient to cover cell needs. Some cells die directly, other enter apoptotic cycle.

Progress in cardiovascular pharmacotherapy, cardiosurgery and interventional cardiology decreased mortality of cardiac diseases, but patients still remain at increased risk of development of adverse cardiac remodeling, mostly in cases when damaged territory is large and reperfusion therapy is suboptimal [Psaltis et al., 2014].

2.7.2 MSCs in 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].

It has been shown that MSCs, in very small percentage differentiate into cardiomyocytes, but in much larger percentage the immunomodulation effect is present. Largest influence of MSCs on cardiomyocytes (CMC) is done by expression of variety of cytokines and chemokines which support survival of CMC itself, but also supports vasculogenesis and neoangiogenesis in myocardial muscle, which further improves survival and regeneration of heart tissue (Figure 3).

Encouraged by positive results, in last two years more than 5 clinical trials administering MSCs into patients with variety of heart diseases has been open.

Despite the described beneficial effect of MSCs on cardiac tissue, detailed metastudy of clinical research 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].

One of the reasons maybe that majority of the studies was performed on small animal models. More similar subject to human, such is pig, need to be used in order to obtain results valid also for human application. Therefore in our studies we focused on work with pigs.

Figure 3 - MSCs influence on cardiac repair. Picture shows three main mechanisms of MSCs effect on damaged heart. 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.
Abbreviations: Ang-2 – Angiopoietin 2, CMC – cardiomyocytes, CSC – cardiac stem cells, FGF-2 – fibroblast growth factor 2, HGF – hepatocyte growth factor, IGF – insulin-like growth factor, IL-6 – interleukin 6, IL-10 – interleukin 10, MSCs – mesenchymal stem cells, PDGF – platelet-derived growth factor, SDF - stromal cell-derived factor, TGF-β – transforming growth factor beta, VEGF – vascular endothelial growth factor.

2.8 MSC and liver regeneration

It has been shown that MSCs transplantation helps to partially restore liver function and decrease symptoms of the disease. All together MSCs has been reported to increase survival rate of patients with variety of liver diseases [Lin et al., 2011].

2.8.1 Liver diseases

Liver play an important role in metabolism of lipids and carbohydrates together with synthesis of proteins, detoxification and storage of various substances, including vitamins. This all makes liver the vital organ of the human body. Main liver cells, hepatocytes, are organized around blood vessels in order to access all mentioned substances. Any pathology of hepatocytes, or of the vascular or connective tissue, leads to liver damage and compromises its function.

2.8.1.1 Chronic liver disease

Chronic liver disease is a long term condition consisting of progressive destruction and regeneration of liver parenchyma by alcohol abuse, high fatty diet, tumors or viral infections and many more [Perz et al., 2006]. When hepatocytes and surrounding parenchyma are destroyed, connective tissue compensates the loss and liver fibrosis starts. If not treated, progression in hepatocyte depletion and connective tissue enlargement can lead to next stage of disease, liver cirrhosis, where morphological defect are more prominent and liver functions may be critically compromised.

2.8.1.2 Liver cirrhosis

Cirrhotic liver are bigger with irregular surface. Percentage of connective tissue is higher in cirrhotic liver, often, fibrosis preventing proper liver function, can be found between nodules and in perivascular space. Hepatocyte nodules can be enlarged also. This kind of liver damage is nearly impossible to repair, healthy life style and drugs can stop the progression, but only currative option is liver transplant [Povýšil et al., 2007].

2.8.2 MSCs in liver regeneration

It has been reported that MSCs can support liver regeneration in many ways (Figure 4).

At first, MSCs are capable of differentiation into hepatocyte-like cells in vivo and also in vitro after stimulation with combination of HGF, FGF, EGF and other cytokines [Lin et al., 2011; Manzini et al., 2015]. But it has been found that contribution of MSCs differentiation into hepatocytes to overall regeneration process is low [Zhou et al., 2009].

Secondly, MSCs were observed to have direct antiapoptotic effect on hepatocytes. Transplantation of MSCs or MSCs conditioned media significantly, in correlation with

decrease in number of apoptotic hepatocytes, reduced mortality of rats with acute liver injury [van Poll et al., 2008].

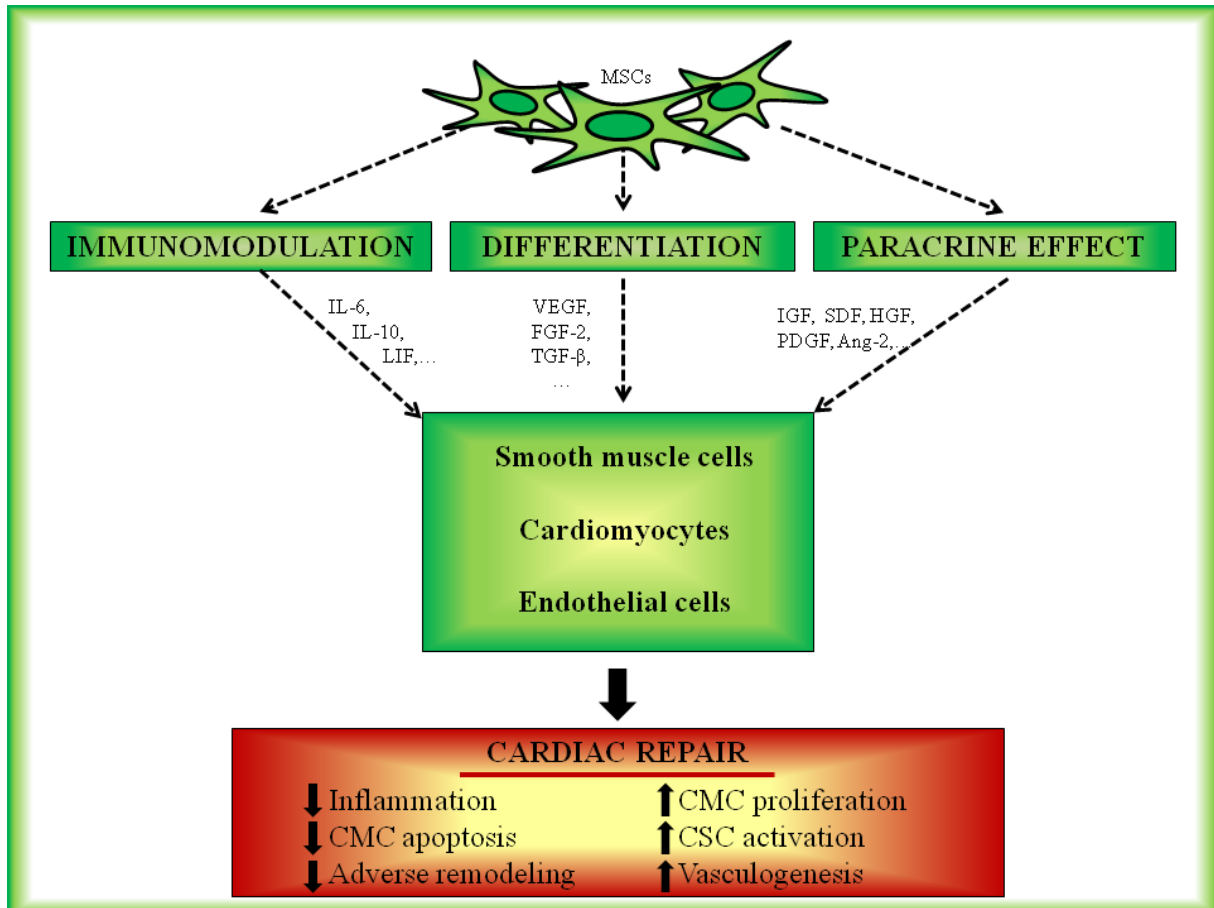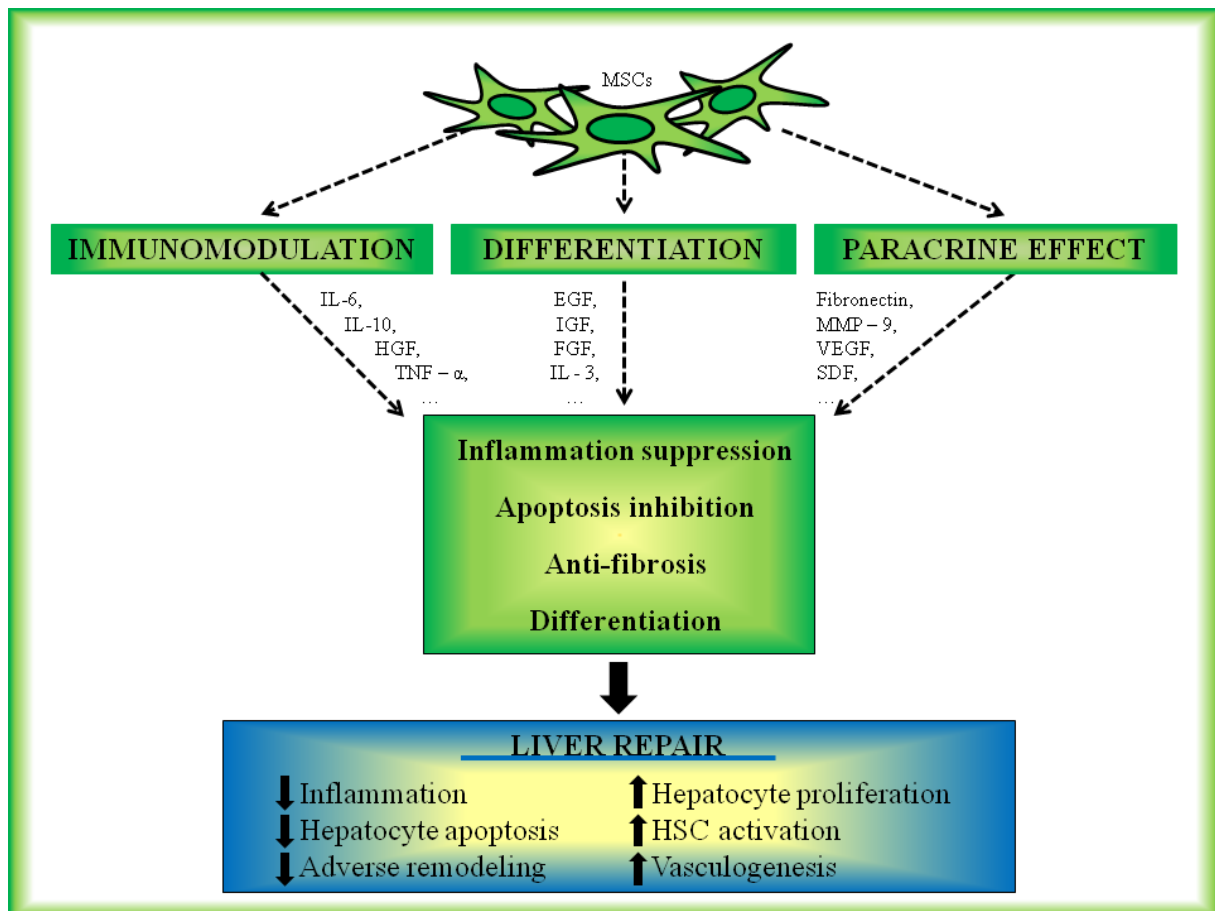**Figure 4** - MSCs influence on liver repair. Picture shows three main mechanisms of MSCs effect on damaged liver. All of them influence not only hepatocytes, but also connective tissue and vessels. MSCs supported vasculogenesis and hepatocyte proliferation together with suppression of inflammation and hepatocyte apoptosis improves chance of successful liver repair.
Abbreviations: EGF – epidermal growth factor, FGF – fibroblast growth factor, HGF – hepatocyte growth factor, HSC – hepatocytes stem cells, IGF – insulin-like growth factor, IL-3 – interleukin 3, IL-6 – interleukin 6, IL-10 – interleukin 10, MMP-9 – matrix metallopeptidase 9, MSCs – mesenchymal stem cells, SDF - stromal cell-derived factor, TNF-α – tumor necrosis factor alfa, VEGF – vascular endothelial growth factor.

Third, MSCs secrete several substances, including HGF, EGF, IL-6 and TNF-α, stimulating hepatocyte proliferation and improving hepatocyte function [Wang et al., 2015; Lin et al., 2011].

Last, but not least, liver injury is always accompanied with lymphocyte infiltration. MSCs immunomodulatory abilities influence lymhocytes together with NK-cells by inhibition of their cytotoxic activity, which has positive effect on liver tissue regeneration in general [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. Morphometry parameters of the liver of animals with MSCs administration and controll group were measured, together with levels of cytokines in swine blood.

2.9   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.

2.9.1   Graft versus host disease

Graft-versus-host disease (GVHD) is serious adverse effect following allogeneic bone marrow transplantation, when transplanted immune cells do not recognize hosts cells and attack them as a pathological threat. Small reaction of donor cells against remaining host immune cells is necessary to prevent relapse of the leukemia. But if the GVHD is serious and untreated, it results in the dead of the individual by multiple fatal organ failure.

When host APCs activate donor T-lymphocytes by discrepancies in MHC-I and MHC-II antigens, acute GVHD develops. T-lymphocytes play important role in chronic GVHD also, but recent findings suggest that B-lymphocytes are involved and contribute to tissue destruction too [Sarantopoulos and Ritz, 2015].

GVHD can be treated with high doses of corticoids, which themselves have a lot of side effects. Despite improvements in treatment and supportive care, overall posttransplatation survival and the incidence of relapses did not changed for last decades [Vasu et al., 2015].

2.9.2   MSCs in treatment of GVHD

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].

One or another, unfortunatelly, immunosuppressive effect of MSCs is not selective, therefore MSCs lower also antibacterial, antiviral and antifungal immunity. It was reported that application of MSCs into patient with GVHD affecting mainly gastrointestinal tractus lead to improvement in gastrointestinal symptoms, but, on the other hand, cytomegaloviral gastritis appeared in patient [Le Blanc et al., 2004].

It is also very important question, whether immunosuppressive effects of MSCs, inhibiting GVHD, do not rise the risk of relapse of original disease [Le Blanc and Ringdén, 2005].

In our work we focused on elucidation of overall effect of MSCs on lymphocytes. In vitro we investigated lymphocyte activity and survival after co-cultivation with MSCs. In vivo our work was focused on blood samples of patients with MSCs administered for severe GVHD. Levels of regulatory T-lymphocytes were determined together with levels of helper T-lymphocytes, all as a sign of immunomodulatory effect of MSCs.

2.9.3   MSCs application as a prevention of GVHD

Role of MSCs as a preventive therapy for GVHD is being investigated.

It has been shown that after bone marrow transplantation, infusion of MSCs can help donor cells to inhabit the bone marrow tissue of a host by restoration of its environment damaged by radiotherapy. Faster restoration of proper bone marrow function follows [Muguruma et al., 2006; Le Blanc and Ringdén, 2006]. The improvement was observed in faster restoration of all cell lines, including myeloid, lymphoid and also megakaryocytic cell line [Le Blanc and Ringdén, 2006, 2005].

In clinical study performed by Le Blanc et al. it was shown that infusion of MSCs after bone marrow transplantation can significantly improve engraftment, lower the risk of GVHD and improve survival of patients [Le Blanc and Ringdén, 2005].

Despite the amount of published data, more clinical studies on larger patient population and observation of long term effect of this cellular therapy, which would document successful use of MSCs, are needed.

3 Aims

1. Optimize swine and human MSCs isolation and cultivation
2. Standardize and optimize MSCs verification protocols
3. Describe the role of MSCs in cardiomyocyte function repair
4. Describe the role of MSCs in liver regeneration
5. Describe the role of MSCs in GVHD

4 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.

4.1 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.

4.1.1 Swine MSCs isolation

Swine MSCs were obtained from bone marrow of adult pigs (Sus Scrofa) in general anaesthesia.

Swine bone marrow from tuberositas tibiae was obtained by aspiration to syringe with mixture of 1 ml Heparin (GE Healthcare, United Kingdom) and 1ml Phosphate Buffered Saline (PBS; Sigma-Aldrich, USA). Resulting bone marrow solution was placed into sterile tube and mixed. Further processing continued in tissue cultivation lab in laminar flow hood, where 1:1 PBS (Sigma-Aldrich, USA) was added. Final mixture was layered on Ficoll-PaqueTM Plus (GE Healthcare, United Kingdom), 4ml of Ficoll Paque per 6ml of diluted bone marrow was used. Centrifugation (435xg, 30min, break 6, RT) followed. Opalescent layer of mononuclear cells was removed, placed into new tube and resuspended in PBS, centrifugation (1000xg, 8min, RT) followed. Supernatant was discarded and pellet was resuspended in 1ml of prewarmed Complete Cultivation media consisting of α-modified Eagle's medium (αMEM; Hyclone, GE Healthcare, United Kingdom) supplemented with 10% Foetal Bovine Serum (FBS; Biosera, France) and 100 IU/ml penicillin and 100 mg/ml streptomycin (Biosera, France). Cells were placed on 175 cm^2 flasks (TPP, Switzerland) and cultivated in humidified incubator (37 °C and 5% CO_2). After 48h non-adherent cells were discarded together with old media and fresh media was added, change of media every 2-3 days followed until the cells reached 80- 90% confluence. Cells were harvested with Trypsin-EDTA (Biochrom, UK), counted, and subcultured at 1:3 dilutions in culture flasks or mixed with DMSO (Sigma-Aldrich, USA) and freezed in liquid nitrogene. For all experiments MSCs from 2nd to 8th passage were used.

4.1.2 Human MSCs isolation

Human MSCs were obtained from iliac crest bone marrow aspirate from healthy donors under general anaesthesia. All donors provided written informed consent for MSCs donation.

Bone marrow aspirate was diluted 1:1 with HBSS (PAA, Austria) and layered 1:1 over LSM 1077 solution (PAA, Austria), centrifugation (1000xg, 15min, break 6, RT) followed. The opalescent layer of mononuclear cells was collected into new tube and resuspended in 20 ml of PBS (PAA, Austria), centrifugation (1000xg, 10min, RT) followed. Supernatant was discarded and pellet was resuspended in 1 ml PBS. All cells were placed into 175 cm^2 flask (Corning, USA) containing 30 ml of Complete Culture Medium (α-MEM; PAA, Austria; 10% pooled human platelet lysate, local source) and placed into humidified incubator (37 °C and 5% CO$_2$). After 48h of cultivation non-adherent cells were removed and fresh media was placed. Cultivation followed, with media exchange every 2-3 days, until the cells reached 80% confluence. The cells were then detached with TrypLE Select solution (Invitrogen, USA) and seeded in a concentration 1x10^6 per 175 cm^2 flask or mixed with DMSO (Sigma-Aldrich, USA) and deep-frozen in liquid nitrogene. For all experiments MSCs from 2nd to 4th passage were used.

4.2 MSCs phenotype

Light microscopy, multidirectional differentiation and flow cytometrical analysis were used to verify that isolated cells were MSCs.

4.2.1 Light microscopy of MSCs

Isolated cells plated on cultivation flasks and dishes were, before any manipulation, checked under light microscopy (Nicon Eclipse Ti, Japan). Adherent fibroblast-like shaped cells were seeked.

For detailed morphology observation, hematoxylin-eosin (HE) staining was performed. Cells were washed with PBS (Sigma-Aldrich, USA) and fixed with 4% paraformaldehyde (Diapath, Italy) for 15min, wash with PBS followed. Gill Hematoxylin (Kulich Pharma, Czech republic) was added for 5 min, cells were 2 x washed with PBS and staining with eosin (Merck Millipore, USA) for 1 min followed. After 3 x wash with PBS pictures under light microscope were taken.

4.2.2 Differentiation into three lines

One of the basic characteristics of MSCs, which is used for their verification, is their ability to differentiate into adipocytes, osteocytes and chondrocytes.

Harvested MSCs (as described previously) were seeded on 12-well cultivation dishes (TPP, Switzerland) with seeding density $3,8 \times 10^4$ cells/well for adipogeneic and chondrogeneic differentiation and $1,9 \times 10^4$ cells/well for osteogeneic differentiation respectively. After 24 hour attachment period cultivation media was discarded and replaced with 3 ml of specific differentiation media.

4.2.2.1 Adipogeneic differentiation

For adipogeneic differentiation StemPro® Adipogenesis Differentiation Kit (Life technologies, USA) was used. Mixture of 2,7 ml StemPro® Adipogenesis Differentiation Media and 0,3 ml StemPro® Adipogenesis Differentiation Supplement was added to each well and changed every 3 days. Cells were cultivated in humidified incubator (37 °C and 5% CO_2) for 14 days, followed by Oil Red O staining (Sigma-Aldrich, USA). In brief, cells were washed with PBS (Sigma-Aldrich, USA) and fixed with 4% paraformaldehyde for 30min. Rinse with distilled water and addition of 60% isopropanol for 5 min followed. Finally Oil Red O solution was added for 3 minutes and then three times washed.

Presence of lipid droplets inside of the cells, in red color, indicating adipocyte transformation, was verified under light microscope. Pictures were taken.

4.2.2.2 Chondrogeneic differentiation

For chondrogeneic differentiation StemPro® Chondrogenesis Differentiation Kit (Life technologies, USA) was used. Mixture of 2,7 ml StemPro® Chondrogenesis/Osteogenesis Differentiation Media and 0,3 ml StemPro® Chondrogenesis Differentiation Supplement was added to each well and changed every 3 days. Cells were cultivated in humidified incubator (37 °C, 5% CO_2) for 18 days, followed by Alcian Blue (Sigma-Aldrich, USA) staining. In brief, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Rinse with distilled water followed. Alcian Blue (1% solution in 0,1nHCL) was added for 30 min, rinse with 0,1nHCl and PBS followed.

Presence of glycoproteins, in blue colour, indicating chondrocyte transformation was verified under light microscope. Pictures were taken.

4.2.2.3 Osteogeneic differentiation

For osteogeneic differentiation StemPro® Osteogenesis Differentiation Kit (Life technologies, USA) was used. Mixture of 2,7 ml StemPro® Chondrogenesis/Osteogenesis Differentiation Media and 0,3 ml StemPro® Osteogenesis Differentiation Supplement was added to each well and changed every 3 days. Cells were cultivated in humidified incubator (37 °C and 5% CO_2) for 21 days, followed by Alizarin Red S (Sigma-Aldrich, USA) staining. In brief, cells were washed with PBS (Sigma-Aldrich, USA) and fixed with 4% paraformaldehyde for 30 min. Rinse with distilled water followed. Finally Alizarin Red S solution (4% solution in water) was added for 3 minutes and then three times washed.

Presence of calcium formations, in red color, indicating osteocyte transformation was verified under light microscope. Pictures were taken.

4.2.3 Flow cytometry phenotype confirmation

Fraction of MSCs, about 100 000 cells trypsinised with use of TrypLE^{TM} Select (Gibco, Life Technologies, Denmark) was used for flow cytometer verification of proper immunetype possesion.

4.2.3.1 Swine MSCs

Cells were washed with PBS (Sigma-Aldrich, USA), resuspended in 100 µl PBS and mixed with 3 µl of antibodies; anti-CD44 – DAPI (Biolegend, USA), anti-CD45 – FITC (Bio-Rad, USA), anti-CD73 – PE-A (RnD Systems, Canada) and anti-CD90 – APC (Biolegend, USA) incubation followed (15 min, dark, room temperature; RT). Cell suspension was washed with PBS and resuspended in 300 µl of PBS. Cytometry analysis was performed using BD FACS Aria Fusion and BD FACS Diva 8.0.1 software (both Becton Dickinson, USA). As MSCs, all cells showing CD45$^-$, CD44$^+$, CD73$^+$ and CD90$^+$ were taken.

4.2.3.2 Human MSCs

Cell suspension was washed twice with PBS (Sigma-Aldrich, USA) and centrifuged (200xg, 10min, RT). After supernatant disposal, pellet was resuspended in 100 µl of PBS. Mix of antibodies; anti-CD3 – PerCP (Exbio, Czech republic), anti-CD13 – APC (Immunotech, USA), anti-CD14 – APC-Cy7 (BD Bioscience, USA), anti-CD19 – PC7 (Immunotech, USA), anti-CD34 – PE (Immunotech, USA), anti-CD45 – Krome Orange (Beckman Coulter, USA), anti-CD34 – PECy7 (Immunotech, USA), anti-CD73 – PE, anti-CD90 – APC and anti-CD105 – FITC (all Biolegend, USA), HLA-DR – Pacific Blue

(Immunotech, USA) was added, incuvation followed (20 min, dark, RT). Cells were washed with PBS (Sigma-Aldrich, USA) and resuspended in 500 µl volume. The flow cytometry acquisition and data analysis were performed by using BD FACS Canto II (BD Biosciences, USA) flow cytometer. An analysis was performed in FlowJo software (TreeStar, USA). As MSCs, all cells showing CD13, CD73, CD90 and CD105 positivity, together with CD3, CD14, CD19, CD34 and CD45 negativity were taken.

4.3 MSCs and cardiomyocytes

4.3.1 Cardiomyocyte isolation

Cardiomyocytes (CMCs) were isolated from left ventricles of young adult pigs (Sus Scrofa, n = 5) in anesthesia.

In brief, after animal sacrifice the heart was removed and placed into bowl with ice cold Ca^{2+} free Tyrode solution (composition of solution in mmol/l: NaCl 137; KCl 4,5; $MgCl_2$ 1; $CaCl_2$ 2; glucose 10; HEPES 5; with use of NaOH pH was adjusted to 7,4; all Sigma-Aldrich, USA). After cannulation of the anterior descending branch of the left coronary artery, the heart was mounted to constant pressure Langerdorff's aparathus and perfussed with warm (37°C) oxygenated solutions; 1, Tyrode solution without Ca^{2+} (5 min), 2, Tyrode solution with Ca^{2+} (0,5 µM), collagenase (1 mg/ml; Roche Diagnostics, Germany) and bovine serum albumin (BSA, 0,5 mg/ml, Sigma-Aldrich, USA) (30 min), 3, Tyrode solution without Ca^{2+} (5 min). Midmyocardial cells, cardiomyocytes (CMCs), were harvested from the wall of left ventricle after endocardial tissue removal and placed into Tyrod solution without Ca^{2+} (37°C). Filtration through gauze followed. Calcium concentration was gradually increased in several steps (1; 5; 10; 100; 200 µmol/l) each 10 min after another to the resulting 0,2 mM Ca^{2+} concentration.

4.3.2 Cardiomyocyte culture optimisation

Isolated CMCs were let to sediment, supernatant was removed and CMCs were resuspended in Complete CMC Cultivation media consisting of Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% Foetal Bovine Serum (FBS; Biosera, France), Glucose (4 500mg/l, Sigma-Aldrich, USA), L-Glutamine (4mmol/l, Biosera, France), and 100 IU/ml penicillin and 100 µg/ml streptomycin (Biosera, France). Resulting cell suspension was used for following experiments.

4.3.2.1 Evaluation of purity

Following each isolation, evaluation of percentage of living CMCs among all other cells in suspension using light microscopy (Nicon Eclipse Ti, Japan) was performed by experienced observer. All long striated cells with rough edges were counted as living CMCs, the rest was considered to be dead or dying cells.

4.3.2.2 Cardiomyocyte culture

In order to optimize culture conditions of CMCs and also to find the best way how to increase CMCs culture purity, four different culture surfaces for CMCs attachment were used; 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 suspension was resuspended in complete CMC cultivation media, divided into groups and seeded on mentioned cultivation plastic, cultivation in humidified incubator under standart cultivation conditions followed (37°C, 5% CO_2).

4.3.2.3 Cell adhesivity evaluation

Evaluation of cell adhesivity on all four used cultivation surfaces was perfomed for 10 samples 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 and evaluated, regular cultivation plastic without any treatment was used as standart for comparisons.

4.3.2.4 Magnetic separation

In order to increase cell culture purity, to have bigger percentage of living CMCs in culture, magnetic separation of live cells using manual magnetic cell separator MACS^{TM} (Miltenyi Biotec, Germany) and Dead Cell Removal Kit (Miltenyi Biotec, Germany) was performed. Kit contained magnetic microbeads capable to bind with dead and dying cells.

Cell suspension was centrifuged (300 x g, 10 min), supernatant discarded and pelet was resuspended in 200 µl of buffer with magnetic microbeads. After 15 min of cultivation

cell suspension was transfered into columns of separator, where the strong magnetic field was generated. When the cell suspension went through the column, all cells with binded magnetic particles, dead or dying cells, were trapped inside of the column and only non binded, living, cells were allowed to leave column into new clean tube.

Before and also after magnetic separation experienced observer under ligh microscope (Nikon Eclipse Ti, Japan) evaluated percentage of live CMCs in cell suspension, results were compared.

4.3.3 Co-cultivation experiments

MSCs in Complete MSC media were 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) (Figure 5).
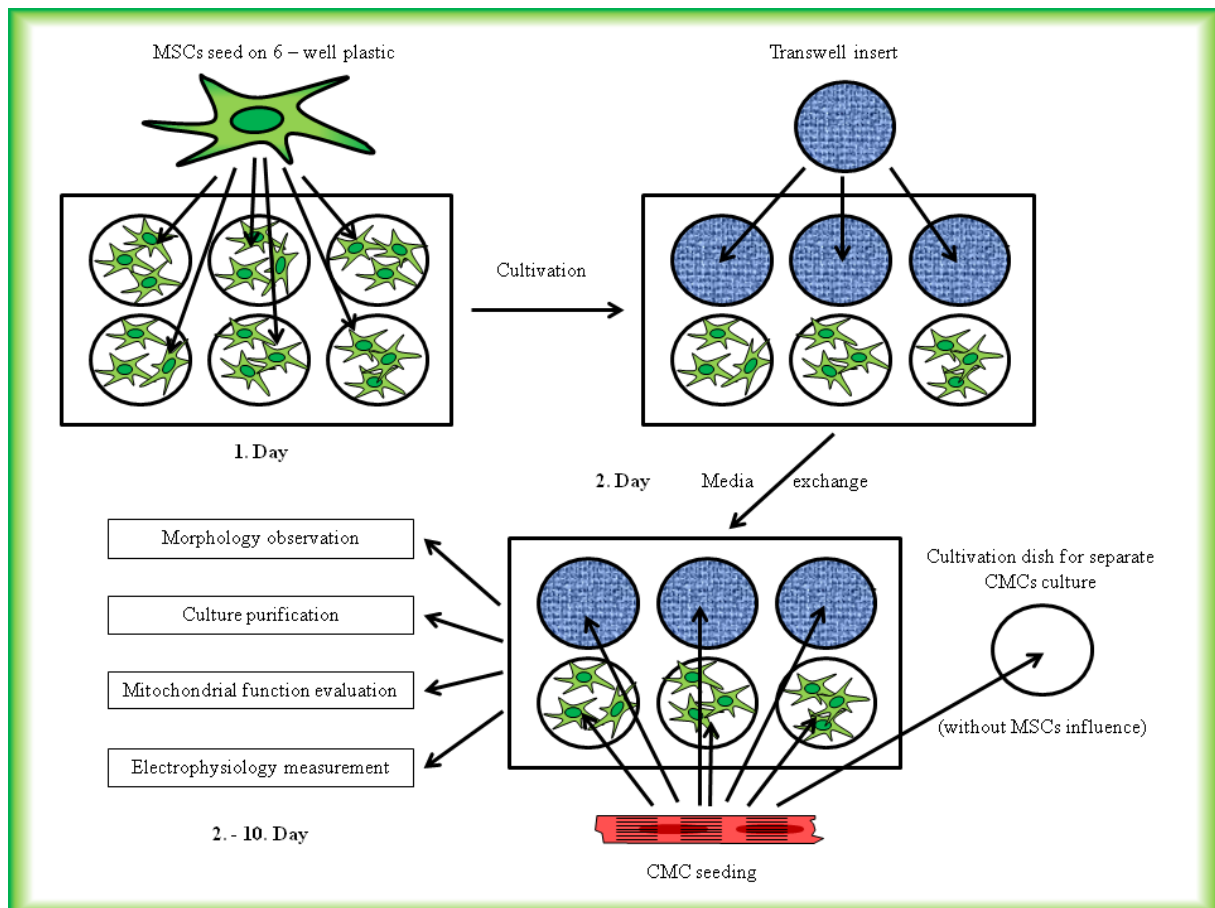

Figure 5 - CMCs co-cultivation with MSCs experiment scheme.

After MSCs media removal, transwells, cupcakes with 3 microns membrane pores, Transwell® (Corning, Sigma-Aldrich, USA), were placed into 3 wells with MSCs 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.

4.3.3.1 Cell morphology observation

CMCs cultivated in all three cultivation conditions (without MSCs, with direct contact with MSCs and with MSCs without direct contact) were observed under light microscopy (Nicon Eclipse Ti, Japan) every day of experiment untill 10th, last day. Their shape was documented.

4.3.3.2 Cell survival evaluation

In the first and the third day of cultivation, simple observation under light microscope was quantified by experienced observer for five animals. On cultivation plastic (n=20 per animal), were counted all living CMCs, long striated cells with rough edges, in field of vision (magnification 10x), and counting of all other cellsin field of vision followed.

Resulting percentages of living CMCs in suspensions in all three types of CMCs cultivation conditions were statistically evaluated.

4.3.3.3 Cardiomyocyte mitochondria staining

For visualisation of mitochondrial network as a sign of living cell, mitochondrial dye MitoTracker® (579/599) (Molecular probes, Life Technologies, USA) was used. After adition of the dye into media and 30 min incubation, dye was temporarily attached to the functional mitochondria, showing red signal. Nuclei was stained by NucBlue® (360/460) (Molecular probes, Life Technologies, USA). This staining was performed in all CMCs cultivation conditions in day 1 and day 3 of the cultivation.

4.3.3.4 CMCs mitochondrial function measurement

For assesment of CMCs mitchondrial functions, high-resolution respirometry was used. Mitochondrial oxygen consumption in isolated CMCs in day 1 and 3 of cocultivation was measured by oxygraph Oroboros (Oroboros, Insbruck, Austria) in 37°C in chambers with 2 ml volume. Cultivation media was placed into oxygraph chambers and let to equilibrate for 40 min. In the end of equilibration period, concentration of oxygen in chamber coresponded to actual concentration of oxygen in atmospheric air and its solubility in the medium (0,89). Chambers were closed and CMCs were injected in. Respiration activity of intact nonpermeabilised cardiomyocyteswas measured.

In titration protocol, where different substrates and disruptors of oxidation chain were added, following respiration statuses, routinelly present in intact cells, were measured; ROUTINE (R – basic oxygen consumption necessary for physiologic control of substrate transport of intermedial metabolism and energetic switch), LEAK (L – status after inhibition of ATP synthasis by oligomycin, when phosphorylation is stopped and oxygen consumption corresponds only to electron transport, which is necessary for compensation of proton leak across the inner mitochondrial membrane), ETS (E – electrontransport capacity of respiration system, which corresponds to maximal oxygen consumption when oxydation and phosphoration are disrupted) and ROX (R – residual oxygen consumption after rotenon, inhibitor of Complex I., addition).

After state R measurement, 2,5 µmol/l of oligomycin (Sigma-Aldrich, USA) was added to chambers and measurement of state L followed after oxygen consumption stabilisation. By titration of protonofor carbonylcyanid-p-trifluoromethoxyphenylhydrason (FCCP; 0,05 – 0,40 µmol/l) (Sigma-Aldrich, USA) the ETS state was induced. Finally addition of 0,5 µmol/l of rotenon (Sigma-Aldrich, USA), inhibitor of Complex I., the ROX state was induced, measurement followed.

CMCs oxygen consumtion was analysed by on-line software DatLab (Oroboros Instruments, Innsbruck, Austria) as negative time derivation of decrease in oxygen concentration in chamber, expressed in pmol $O_2/(s*10^6)$ cells and corrected to ROX.

4.3.3.5 Cardiomyocyte electrophysiology

In order to evaluate change of CMCs electrophysiology properties in time, together with differences between CMCs cultivated alone or with MSCs, contractility and transient calcium currents were measured in day 1 and 3 of the experiment.

CMCs were stored in low calcium Tyrode solution (composition of solution in mmol/l: NaCl 137; KCl 4,5; $MgCl_2$ 1; $CaCl_2$ 0,2; glucose 10; HEPES 5; with use of NaOH pH was adjusted to 7,4; all Sigma-Aldrich, USA) and for experiment transferred to normal Tyrode solution (composition as stated above, only difference was $CaCl_2$ 2 mmol/l) .

CMCs electrophysiology parameters were measured by Ionoptix HyperSwitch Myocyte Calcium and Contractility System (IonOptix LLC, Westwood, USA), where Sarclen sarcomere length acquisition module was used to measure cell contractions. Cells were loaded with Fura-2 (Molecular probes, Invitrogen, USA), stock solution Fura-2-am powder was dissolved in DMSO (Sigma-Aldrich, USA) to reach final concentration of 1 mmol/l. Cells were incubated for 20 minutes in low calcium Tyrode solution with 2 µM Fura-2-am and then

repeatedly washed with low calcium Tyrode solution. After 20 min of incubation, measurements followed.

For analysis of obtained data IonWizard 6.5 software (IonOptix LLC, Westwood, USA) was used. Baseline, peak, amplitude (peak - baseline), time to peak, times to 10 % and 50 % of peak, times to 10 % and 50 % of recovery, max velocities (the maximum of first derivative of transient during the deflection and recovery phases of the transient), rate constants (the exponential rate constants associated with recovery phase of the transients: sin exp amp and sin exp off), time constants (the exponential time constants associated with recovery phase of the transients: sin exp tau) and integral (the area under the transient relative to baseline) were analysed together with Fura-2 ratio and sarcomere length.

4.3.4 Statistics

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.

4.4 MSCs in liver regeneration

4.4.1 Liver cirhosis model in swine

For this study, new, not previously described, model of chronic liver disease in pig (Sus Scrofa) was estabilished. All experimental animals were 2 month old females with approximate weight 20 kilograms, with no significant differences between individuals. 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 brief, after general anesthesia induction, bone marrow collection from tuberositas tibiae was performed, middle laparotomy followed. Via retrograde cholecystectomy Hepatic Artery and Ductus Choledochus were disrupted and Fogarthy's catheter was inserted. End of cathether was lead to subcutaneous area on the right side of the pig, baloon of the cathether was adjusted to obliterate the bile duct properly. Pigs with stitches were moved to heated hutches with free access to water and food, everyday USG monitoration of Fogarthy's catheter position and volume followed for 9 weeks.

4.4.2 MSCs cultivation and preparation for application

MSCs were isolated from swine bone marrow according to protocol in chapter 5.1.1 and cultivated in order to have MSCs culture with high purity. When the cells reached 80% confluency in 2nd passage, trypsinisation with Trypsin-EDTA (Biochrom, UK) followed, cells were mixed with DMSO and freezed in liquid nitrogene. Two weeks before MSCs application, cells were thawed and recultured. In the day of application were MSCs trypsinised, counted and resuspended in physiological solution. Individual number of cells was used for each pig according to its weight (average weight of pigs was 25 kg), number of MSCs in application dose was 1 x 10^6 cells / kg.

4.4.3 Liver resection and MSCs application

After nine weeks with biliary obstruction, pigs underwent second surgery in general anesthesia. Fogarthy's catheter was removed, bile ducts were reconstructed and left liver lobe was resected. In the same time blood samples and liver biopsy were collected. At the end of the surgery randomly chosen group of pigs received MSCs suspension (10^{6}cells/kg) to vena portae, control group (NO MSCs) was injected with saline in the same volume. The laparotomy was closed and pigs were moved back to warmed hutches with free access to food and water. In the day 14 animals were sacrificed and liver parenchyma samples were taken for histological analyses.

4.4.4 Plasma analysis

Peripheral blood was collected in 7 time points; 0 h, 2 h, 24 h, 3 days, 7 days, 10 days and 14 days, after resection into tubes with EDTA and placed into centrifuge (1000 x g, 10 min, RT). Plasma was collected and deeply freezed for further Luminex assay of IL-6, IL-8, TNF-α and TGF- β concentrations. In the day of Luminex$^®$ 200^{TM} (Luminex Corporation, USA) analysis, plasma samples were thawed and concentrations of IL-6, IL-8 and TNF-α were measured with use of MILLIPLEX MAP Porcine Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, USA), concentration of TGF-β was measured with use of MILLIPLEX MAP TGFβ1 Single Plex Magnetic Bead Kit (Merck Millipore, USA).

Samples were processed using manufacturer protocols. In brief, 25μl of plasma was added to wells of 96 – well plate in triplets and mixed with 25 μl of kit Buffer. Addition of 25 μl of Luminex microbeads followed. Plates with samples were sealed and gently shaked overnight at 4 °C. After incubation wells were 3 x washed with Wash Buffer, 50 μl of Anibody Detection solution was added and incubation followed (2 hrs, RT). After incubation

50 µl of Streptavidin-Phycoerythrin was added to each well and let to incubate (30 min, RT). Finally, wells were 3 x washed with Wash Buffer, 100 µl of Sheat fluid was added to each well and Luminex analysis of processed samples was performed. Data were collected and statistics was performed.

4.4.5 Liver processing

Liver samples (n=10 for MSCs, n=11 for NO MSCs) from two timepoints; the day of resection (day 0) and the final day (day 14), were processed with experienced histologists.

In total 42 tissue blocks were fixed in 10% paraformaldehyde (Diapath, Italy). Histological cutting plane of each sample was randomized using the orientator [Mattfeldt et al., 1990; Gundersen et al., 1988]. Samples dehydratation, embedding to paraffin blocks and cut to 5 µm-thick histological sections followed. Hematoxylin (Kulich Pharma, Czech republic) - eosin (Merck Millipore, USA) staining was used for overall morphology assessment and Anilin Blue (Diapath, Italy) together with Nuclear Fast Red (Merck Millipore, USA) staining were used for connective tissue contrasting. The outlines of individual hepatocytes were stained by combination of Alcian Blue (Sigma-Aldrich, USA) with Periodic Acid Schiff (PAS; Merck Millipore, USA) reaction.

In total, 398 sections were used for quantitative analysis. In order to quantify the volume fractions of connective tissue and area of lobules, one field of view with a random position was recorded using the PlanC N 4x/0.1 microscope objective (Nicon, Japan) from each section stained with Alcian Blue. In sections stained with PAS, systematic random unit sampling [Marcos et al., 2012] of each 15th field of view was done using PlanC N 40x/0.65 lens. This sampling resulted in 2113 micrographs for morphometry analyses, done by our histology specialists.

4.4.6 Quantitative morphometric analysis of liver

For quantitative analysis, histological parameters in Table 1 were used.

The *A(lobule)*, *V(MH)* and *V(PH)* were estimated using the stereological probe on the two-dimensional images [Gundersen and Jensen, 1985]. Briefly, this method approximates the area or volume of spatial structures with geometrical circles and spheres, therefore estimates their cross-sectional areas by multiple measures of their radiuses. The measurement was done within the central regions of cells with clearly visible nuclei and nucleoli. Only cells selected

by the unbiased counting frames were measured. From these measurements of cross-sectional areas, the volume of hepatocytes was estimated.

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

The volume fractions $V_V(connective\_tissue,liver)$, $Vv(hepatocytes,liver)$, $V_V(MH,liver)$ and $V_V(PH,liver)$ were estimated using the point grid method [Howard et al., 1985]. All quantitative techniques were done using stereological software Ellipse (ViDiTo, Košice, Slovakia).

4.4.7 Statistics

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.

4.5 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 studies.

4.5.1 In vitro GVHD model

For initial experiments, in vitro culture was chosen. As a GVDH model, mixed lymphocyte culture was used.

4.5.1.1 Lymphocyte culture

Lymphocytes were isolated by gradient centrifugation, Histopaque − 1077 (Sigma-Aldrich, USA), washed and diluted in cultivation media RPMI 1640 (Lonza, Belgium) to final concentration 1 milion cells/ml. Half of the prepared lymphocytes were inactivated by cultivation in 1% solution of paraformaldehyde in PBS for 5 min. The mixture of 100 μl of living and 100 μl of inactivated lymphocytes from different HLA incompatible donors as a simulation of GVHD disease was prepared. 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.

4.5.1.2 Mesenchymal stem cells

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. Cultivation for 6 hours in 96-well plates according to standart cultivation conditions in humidified incubator (37 °C , 5% CO_2) followed.

4.5.1.3 MTT test

MTT test results correspond to mitochondrial activity and cell viability. Test is based on the reduction of soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-difenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) to insoluble formazan on the mitochondrial membrane of living cells. After addition of solvent, DMSO (Sigma-Aldrich, USA) and detergent, SDS (Sigma-Aldrich, USA) formazan dissolves and the arising color is detected by spectrophotometry with a wavelenght 540 nm, MRXII (Dynex, Czech republic). Level of absorbance indicates the number of living, metabolic active, cells.

The MTT solution was added to cells and incubated for 2 h. The spectrophotometric analysis was performed after this time by Synergy HT (Biotek, Germany).

4.5.1.4 Statistics

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.

4.5.2 MSCs and GVHD patients

4.5.2.1 Patients

For the study only patients with severe GVHD, both accute (n = 8) and chronic (n = 10), were enrolled. For classification of GVHD as severe, following criteria were used; 1, disease did not responded to corticosteroids (minimal dose 1 mg/kg) administered at least 2 weeks or 2, GVHD progressed despite the mentioned treatment or 3, disease was corticosteroid-dependend (GVHD responded to corticosteroids, but the drugs needed to be administered permanently in minimal dose 0,1 mg/kg/day).

4.5.2.2 Human MSCs preparation for human application

Human MSCs, from healthy donors, were prepared according to protocol, approved by State Institute for Drug Control, for ongoing clinical study (EudraCT 2013-003626-88) on Hematology-oncology department. The details of the protocols are nonpublic essential part of the study, therefore I cannot describe them here.

4.5.2.3 MSCs application and sample collecting

Every patient enrolled in study received one dose of MSCs ($1 - 5$ x 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 application. The count of white blood cells (WBC), lymphocytes and subpopulations of regulatory T-lymphocytes (Tregs) and helper T-lymphocytes were measured with flow cytometry as markers of immunomodulation.

4.5.2.4 Detection of regulatory T- lymphocytes

The peripheral blood ($100\mu l$) was mixed with primary antibodies anti-CD45 – Krome Orange (Beckman Coulter, USA), anti-CD4 – Pacific Blue (Exbio, Czech Republic), anti-CD127 – APC (BD Bioscience, USA), anti-CD25 – FITC (Beckman Coulter, USA) and incubated (15 min, dark, RT). Then, the incubation with 3 ml of Lysing solution (eBioscience, USA; 12 min, dark, RT) followed. Unbound antibodies and lysed erythrocytes were washed away by centrifugation (350 x g, 5 min). The supernatant was discarded and cell pellet was washed with Flow Cytometry Staining Buffer (SB; 350 x g, 5 min). After centrifugation, pellet was resuspended in 1 ml of 1X Fixation/Permeabilization solution (eBioscience, USA) and cells were permeabilized for 1 hour at 4°C. Then, cells were washed by centrifugation (450 x g, 5 min) with SB two times. The cell pellet was resuspended in 100 μl SB and 5 μl of Anti-Human Foxp3 – PE (clone PCH101, eBioscience, USA) was added. After 30 min incubation (4°C), unbound antibody was washed away with SB (350 x g, 5 min), cell pellet was resuspended in 300 μl of SB and immediately measured on BD FACSCanto II (BD Biosciences, USA) flow cytometer. An analysis was performed in FlowJo software (TreeStar, USA). Tregs were evaluated by two antibody sets, firts 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.

4.5.2.5 Detection of helper T - lymphocytes

The peripheral blood was collected into tubes with Heparine (GE Healthcare, United Kingdom), shaked and layered on Histopaque 1063 (Sigma-Aldrich, USA) solution for gradient separation by centrigugation (500 x g, 20 min, RT). The opalescent layer of mononuclear cells was collected into new tube and washed with RPMI, dilution on final concentration 1 x 10^6 cells / ml followed. Resulting suspension was mixed with 4 μl of GolgiStop with momensin (BD Bioscience, USA), inhibitor of Golgi complex, and incubated (24 h, 37 °C, 5 % CO_2). Fixation Solution BD Cytofix (BD Bioscience, USA) was added

according to manufacturer instructions and permeabilisation with BD Perm/Wash solution (BD Bioscience, USA) followed. Finally, cell suspension was mixed with 5 μl of antibodies; anti-IFN – γ – FITC, anti-IL17A – PeCy7, anti-IL4 – PE (all Biolegend, USA), anti-CD45 – ECD and anti-CD4 – PeCy5 (both Immunotech, Beckman Coulter, USA) and incubated for 20 min. Cell suspension was washed and sample analysis on flow cytometer FC500 (Beckman Coulter, USA) was performed. Helper T-lymphocytes were evaluated as $CD45^+$ and $CD4^+$. Helper T-lymphocytes positive for IL-4 were considered to be Th1 cells, cells positive for IFN – γ were considered to be Th2 cells and cells positive for IL-17 were considered as Th17 lymphocytes.

4.5.2.6 Statistics

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.

5 Results

5.1 MSCs isolation and culture

MSCs, both swine and human, can be isolated with described procedure. Different numbers of cells were gained in each isolation, depending on quality of bone marrow collection. After non adherent cell wash, percentage of MSCs in culture was higher than 98 %.

5.2 MSCs phenotype

Isolated cells showed to have properties of MSCs, verification under light microscopy for MSCs phenotype, differentiation into three lines (adipo-, osteo-, chondro-) and flow cytometry antigene determination was performed.

5.2.1 Morphology of MSCs

Light microscopy of MSCs showed their adhesivity to cultivation plastic, spindle shape with several spurs and large nucleus (Figure 6). After seeding on cultivation plastic, MSCs started to form colonies and filled up the cultivation surface, when the surface was full (100 % confluency), cells started to form 3D structured and lost their MSCs phenotype.

5.2.2 Differentiation into three lines

Cultivation of MSCs with differentiation media confirmed ability of cells to differentiate into three basic lines. Cells differentiated into adipocyte line produced fat particles, which were showied red color after Oil Red O addition. Cells in chondrodifferentiation media produced mucopolysacharides, which were stained blue by Alcian Blue. Cells cultivated in osteodifferentiation media produced calcein molecules, which were stained red by Alizarin Red S (Figure 7).
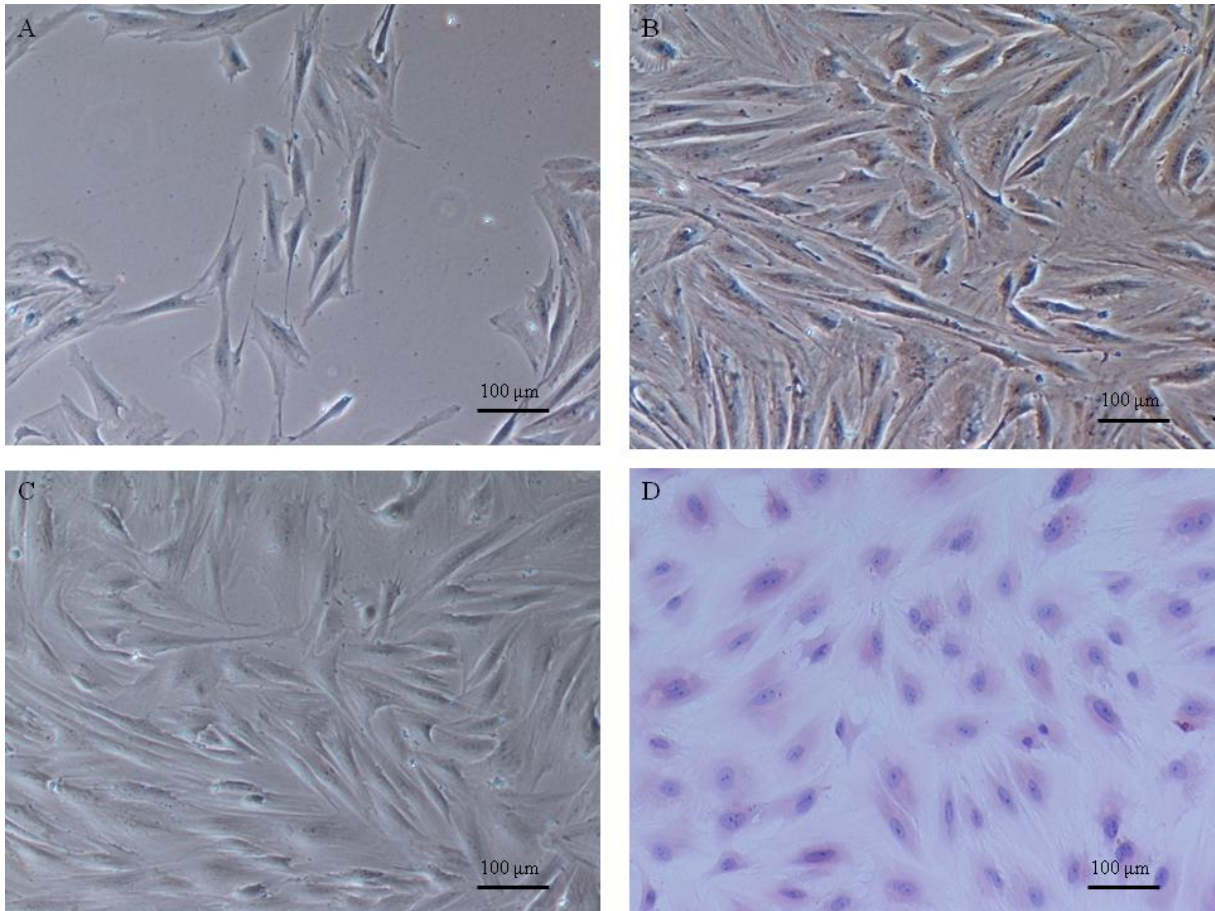
Figure 6 - MSCs morphology in light microscopy. Adhesive behaviour, spindle shape and large nucleus. A – MSCs forming colonies, B – MSCs in 100 % confluency, C – MSCs in phase contrast, D – MSCs stained with Hematoxylin-eosin.
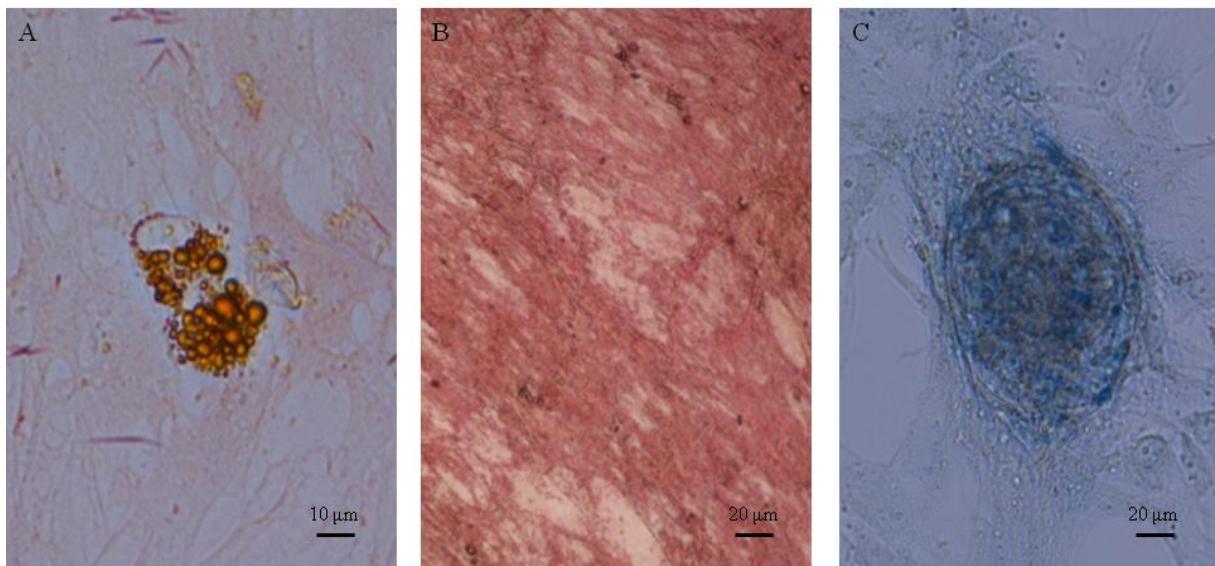

Figure 7 - Differentiation of MSCs into three lines; A – MSCs differentiated into adipocyte line – fat vacuolas stained with Oil Red O; B – MSCs differentiated into osteocyte line – calcein structures stained with Alizarin Red S; C – MSCs differentiated into chondrocyte line – blue color of mucopolysacharides stained with Alcian Blue.

5.2.3 Flow cytometry phenotype confirmation

Flow cytometry showed that processed cells are MSCs. In samples of human MSCs, more than 98 % of the cells showed negativity for blood lineage markers (CD3, CD14, CD19, CD34, CD45 and HLA-DR) together with positivity for MSCs markers (CD13, CD73, CD90 and CD105) (Figure 8). For swine MSCs, more than 98 % cells in culture was positive for MSCs typical markers (CD44, CD73 and CD90) together with negativity for markers of blood lineage (CD45) (Figure 9).
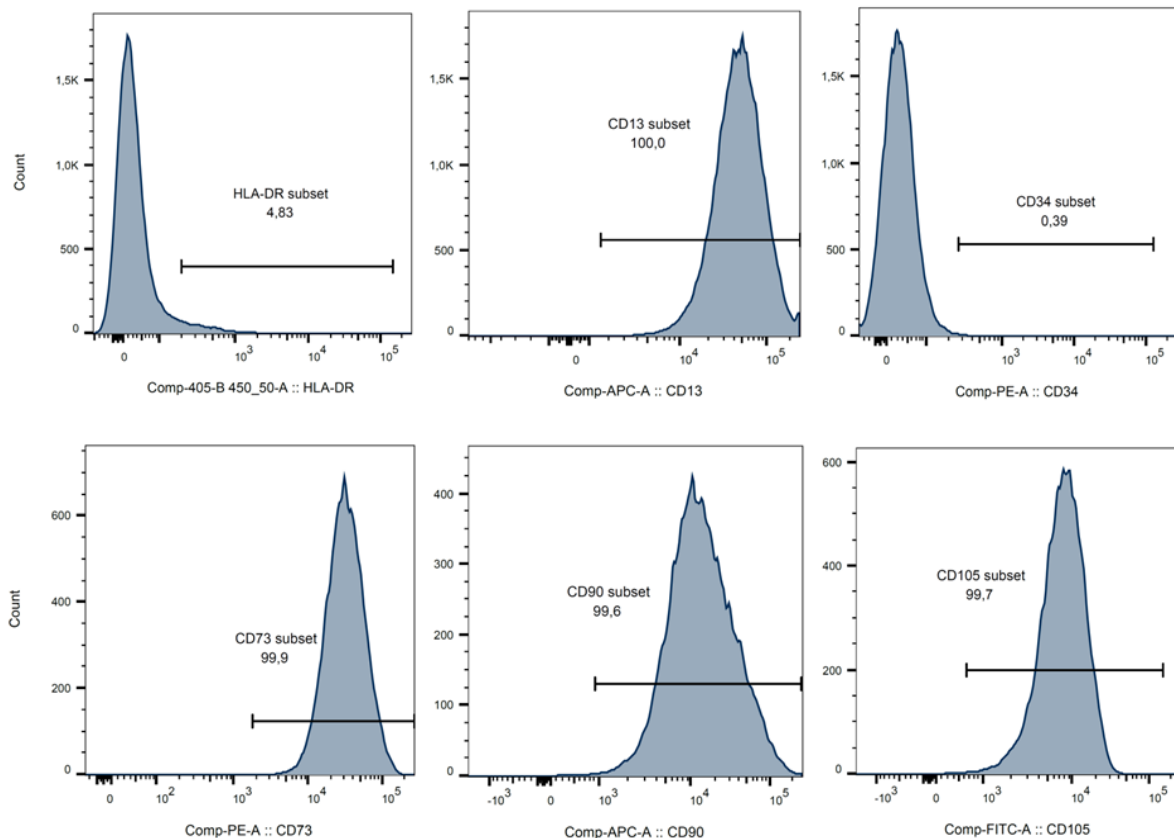

Figure 8 - Flow cytometry of human MSCs. Example of markers typical for cells; HLA-DR$^-$ (95,17 %), CD34$^-$ (99,61 %), CD13$^+$ (100 %), CD73$^+$ (99,9 %), CD90$^+$ (99,6 %) and CD105$^+$ (99,7 %).

5.3 MSCs and cardiomyocytes

5.3.1 Cardiomyocyte isolation

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 %).
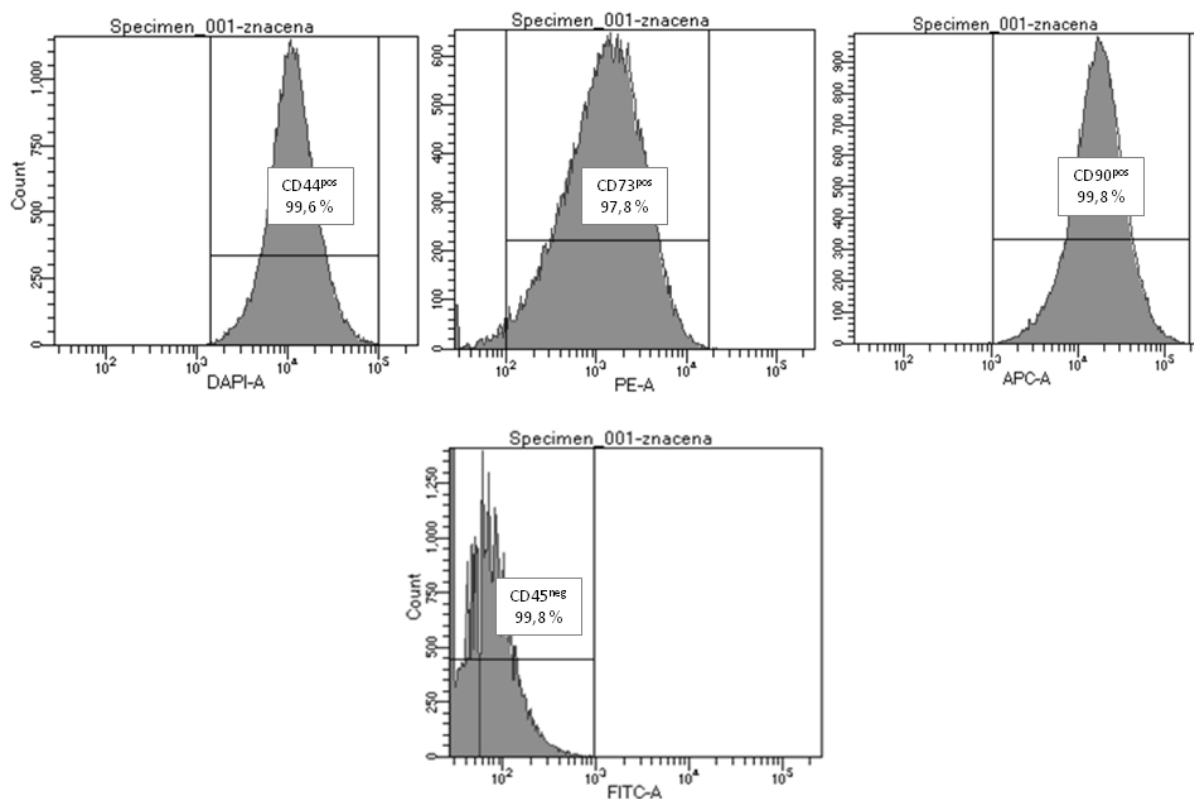
Figure 9 - Flow cytometry of swine MSCs. Cells showed to be positive for CD44 (99,6 %), CD73 (97,8 %), CD90 (99,8 %) and negative for CD45 (99,8 %).

5.3.2 Cardiomyocyte culture optimisation

CMCs can be cultivated on all four used cultivation surfaces, with no observed negative effect on CMCs morphology or survival.

5.3.2.1 Cardiomyocyte adhesivity

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 lamini) 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.

5.3.2.2 Magnetic separation of dead cells

Magnetic separation of CMCs from cell suspension was time consuming and expensive, but enabled to increase ration of living CMCs in culture from initial 20 % (± 10 %) to resulting 75 % (± 15 %), with small loss of living CMCs (15 % ± 5 %).

5.3.3 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.

5.3.3.1 Cardiomyocyte morphology

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 10A). 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 10 B – 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 11). In day 3 of observation the signal was also visible (Figure 12), but cells already showed first signs of deterioration, after this day rapid decrease in observed signal was found.
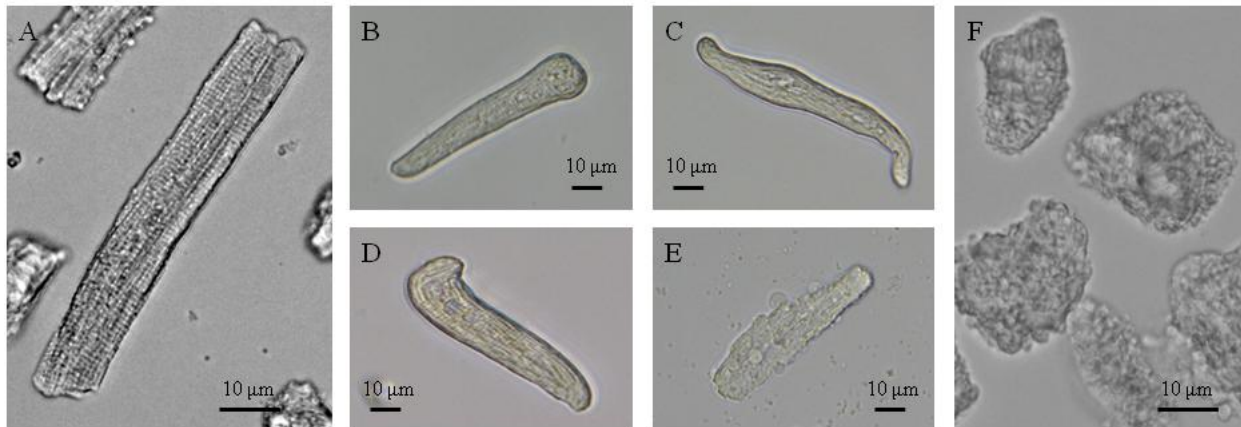

Figure 10 - Morphology of CMCs in time; A – freshly isolated CMC with long trabecular shape, rough edges and stripes, B – D – morphology pathologies of CMCs in long term culture (3 – 10 days), CMCs loose stripes, edges starts to round and cell crooks, E – dying cell, F – dead cells and cellular debris.
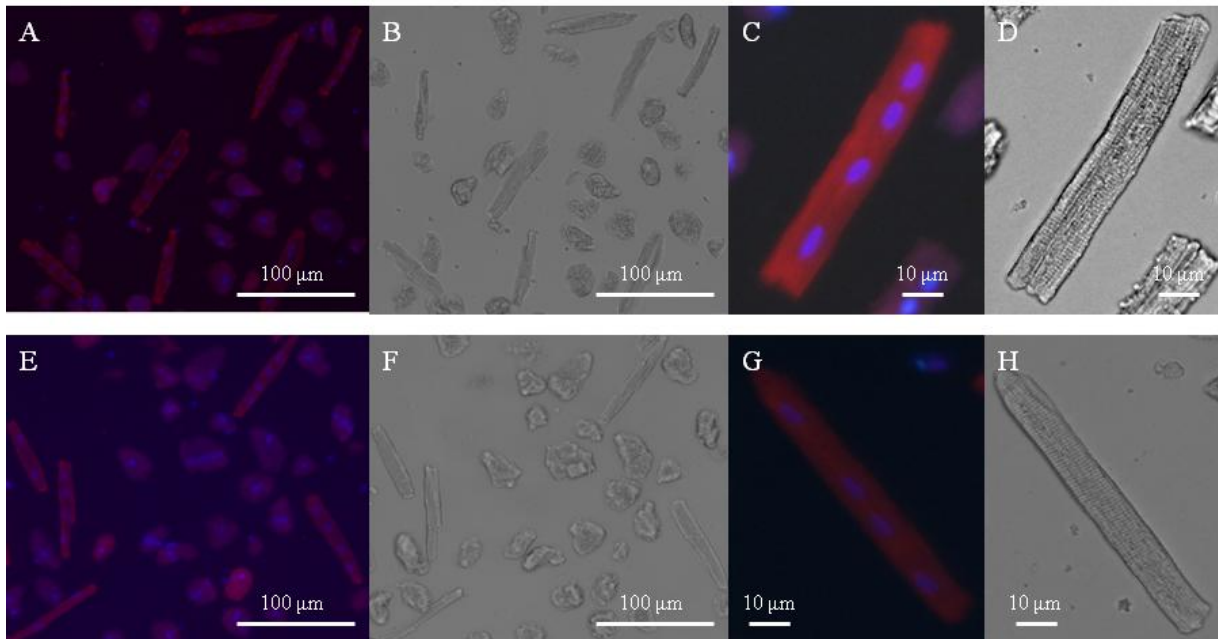
Figure 11 - Fluorescent microscopy of mitochondria in CMCs in day 1 of cultivation (red color – functional mitochondria, blue color – nuclei). Upper row CMCs cultivated alone, bottom row CMCs co-cultured with MSCs; A, E – Fluorescent microscopy of cell culture, B,F – Same frame of cell culture but taken 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.
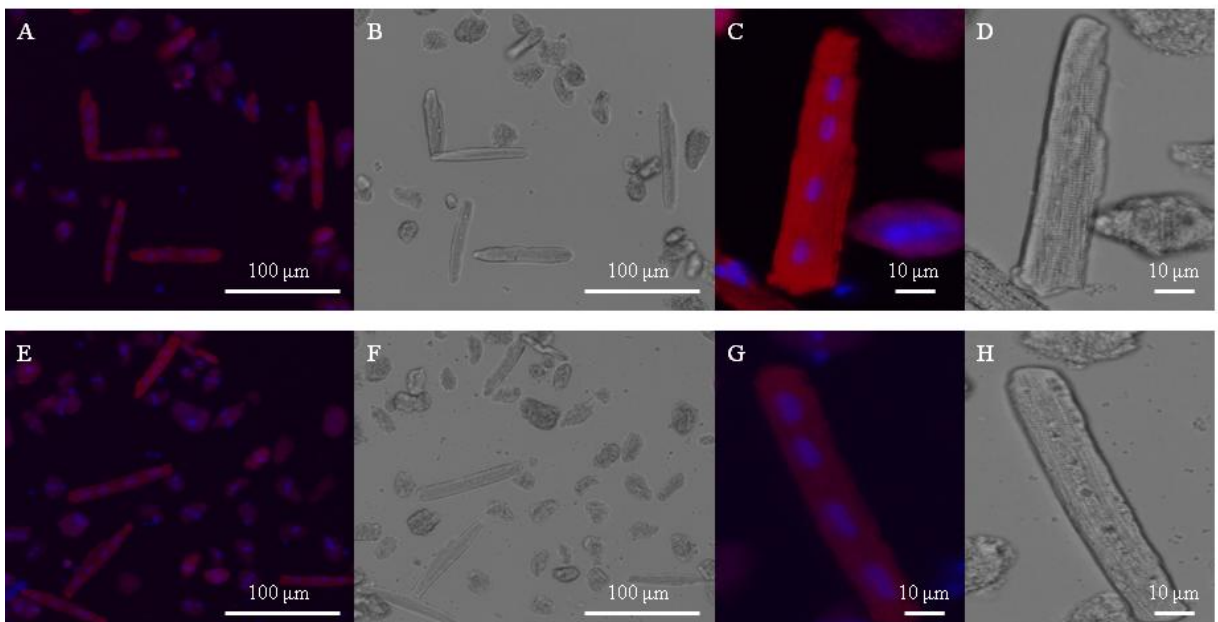

Figure 12 - Fluorescent microscopy of mitochondria in CMCs in day 3 of cultivation (red color – functional mitochondria, blue color – nuclei). Upper row CMCs cultivated alone, bottom row CMCs co-cultured with MSCs; A, E – Fluorescent microscopy of aging cell culture, B,F – Same frame of cell culture 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.

5.3.3.2 Cardiomyocyte survival evaluation

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. Quantification of results by experienced observer showed uneven distribution of data, so non parametric statistics, Wilcoxon test, was used. CMCs cultivated without MSCs 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 13).
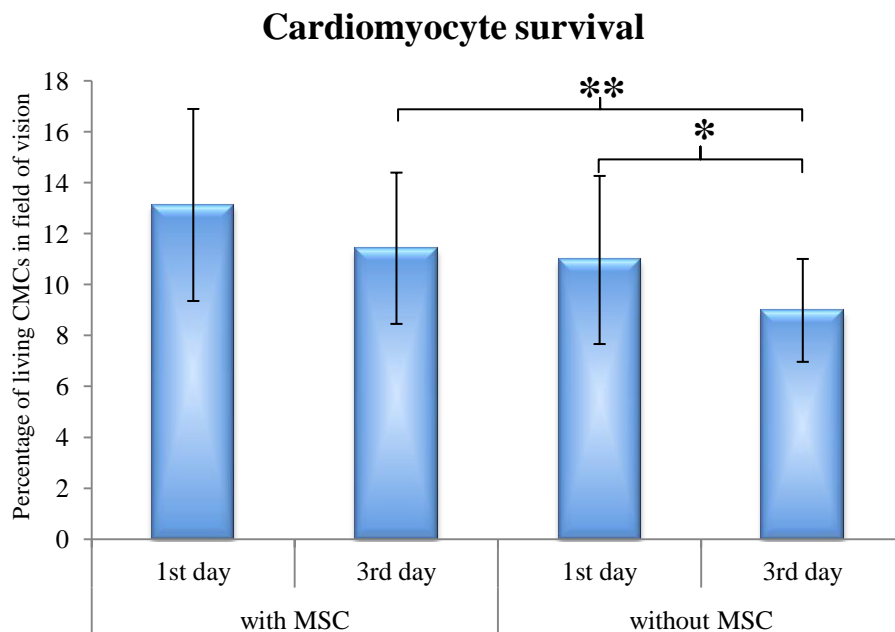
Figure 13 - Comparison of percentage of living CMCs in culture with/without MSCs, Count of living CMCs in both culteres decrease in time.
* Decrease in number of living CMCs cultivated without MSCs, while comparing 1st and 3rd day of cultivation, according to Wilcoxon test, was statistically significant (p = 0,0196).
** Survival of CMCs was significantly higher in CMCs cultivated with MSCs in day 3, according to Wilcoxon test p = 0,0152.

5.3.4 Cardiomyocyte mitochondrial functions

Oxygen consumption (Figure 14) 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 (Figure 15A).

Similar characteristics showed also state LEAK (Figure 15B). Maximal respiration induced by FCCP was nearly identical for both CMCs groups and had small trend to decrease in time (Figure 15C).

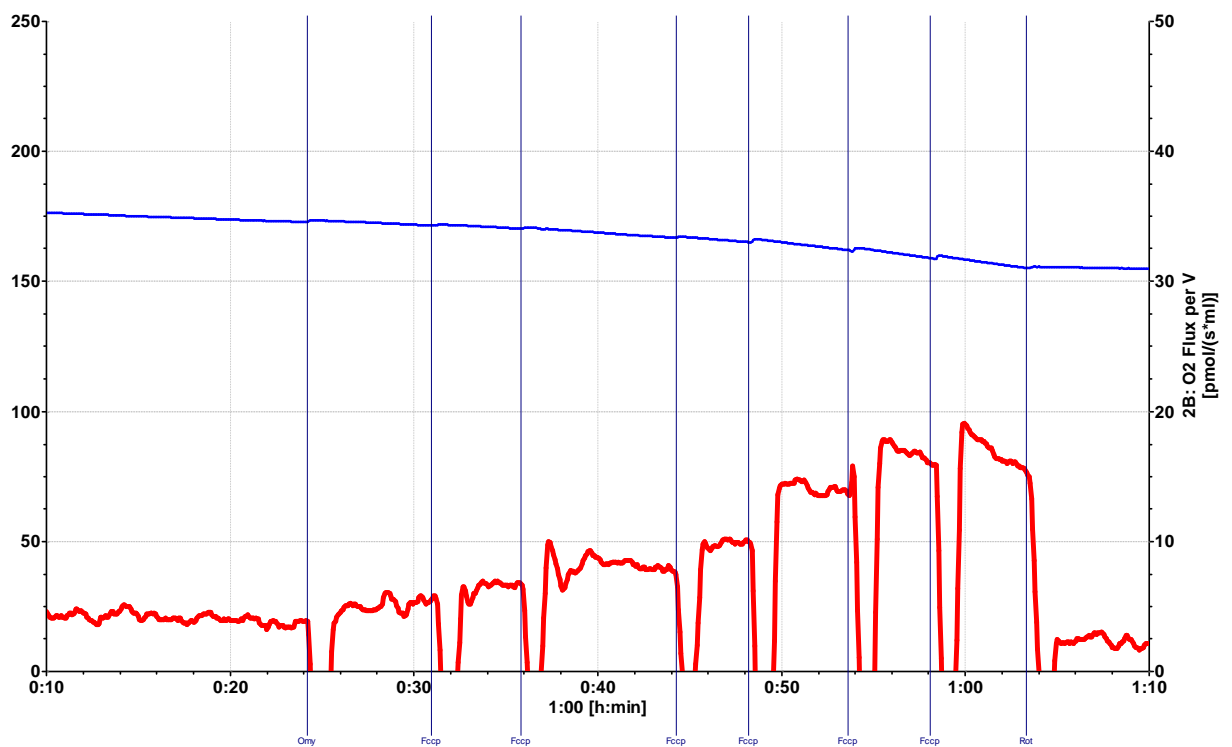**Figure 14** - Typicall curve of oxygen consumption by cultivated CMCs (in 3rd day of culture without MSCs). Red line shows oxygen consumption, blue line shows oxygen concentration in chamber. Oxygen consumption in chamber in pmol/(s*ml) is immediatelly transferred to pmol/(s*10^6 cell). Vertical lines mark application of variety of chemicals: Omy = oligomycin; FCCP = karbonylkyanid-p-trifluoromethoxyfenylhydrazon; Rot = rotenon.
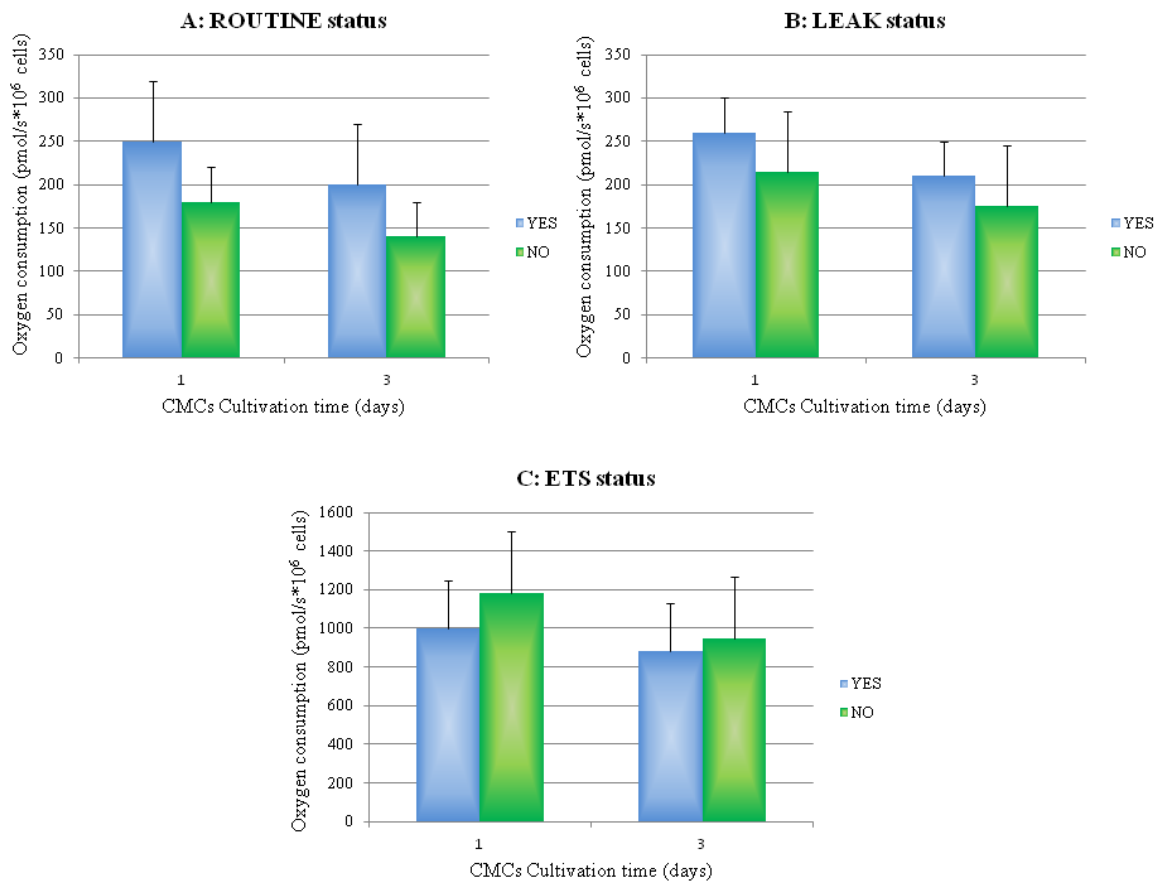
Figure 15 - Oxygen consumption in states: ROUTINE (A), LEAK (B) and ETS (C) in intact CMCs measured in day 1 and day 3 of cultivation with (YES) or without MSCs (NO). Oxygen consumption is corrigated for residual oxygen consumtion and showed in pmol per second per milion cells.

5.3.5 Cardiomyocyte electrophysiology

Cell culture had detrimental effect on calcium transient and contraction of CMCs (Table 3). 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,

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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.

Table 3 - Data for calcium transients and contraction in CMCS cultivated with or without MSCs for 1 day and 3 days.

Parameter	Transient D1		Transient D3		Contractions D1		Contractions D3	
	no MSCs	with MSCs	no MSCs	with MSCs	no MSCs	with MSCs	no MSCs	with MSCs
baseline (bl)	0,49 ± 0,05	0,52 ± 0,11	0,44 ± 0,05	0,46 ± 0,06 #	1,82 ± 0,08	1,8 ± 0,09	1,74 ± 0,14 #	1,69 ± 0,27 #
dep v	20,23 ± 9,01	13,63 ± 7,13 *	4,84 ± 2,97 #	5,24 ± 2,48 #	-0,75 ± 0,5	-0,56 ± 0,48	-0,24 ± 0,17	-0,19 ± 0,14 #
ret v	-1,44 ± 0,55	-0,97 ± 0,46 *	-0,34 ± 0,19 #	-0,4 ± 0,19 #	0,69 ± 0,5	0,4 ± 0,27	0,11 ± 0,08 #	0,09 ± 0,05 #
peak	0,85 ± 0,1	0,79 ± 0,13 *	0,54 ± 0,08 #	0,59 ± 0,07 *#	1,73 ± 0,09	1,74 ± 0,11	1,71 ± 0,14	1,66 ± 0,27
peak h	0,36 ± 0,09	0,27 ± 0,09	0,1 ± 0,04 #	0,12 ± 0,05 #	0,094 ± 0,043	0,064 ± 0,039	0,029 ± 0,017 #	0,023 ± 0,012 #
peak t	0,079 ± 0,047	0,066 ± 0,031	0,164 ± 0,15 #	0,154 ± 0,14 #	0,331 ± 0,111	0,336 ± 0,151	0,376 ± 0,141	0,345 ± 0,154
t to peak 10,0%	0,008 ± 0,021	0,005 ± 0,002 *	0,005 ± 0,006	0,006 ± 0,009	0,051 ± 0,075	0,037 ± 0,018	0,033 ± 0,011 #	0,075 ± 0,1
t to peak 50,0%	0,013 ± 0,007	0,014 ± 0,004 *	0,014 ± 0,003	0,016 ± 0,004	0,108 ± 0,037	0,107 ± 0,045	0,101 ± 0,041 #	0,113 ± 0,064
t to bl 10,0%	0,14 ± 0,06	0,16 ± 0,08	0,27 ± 0,17 #	0,24 ± 0,18 #	0,42 ± 0,15	0,43 ± 0,19	0,48 ± 0,16	0,44 ± 0,17
t to bl 50,0%	0,35 ± 0,08	0,37 ± 0,11	0,51 ± 0,18 #	0,51 ± 0,13 #	0,51 ± 0,18	0,54 ± 0,24	0,65 ± 0,14	0,62 ± 0,19 #
sin exp amp	0,31 ± 0,15	0,3 ± 0,14	0,08 ± 0,05 #	0,14 ± 0,07 *#	-0,054 ± 0,031	-0,03 ± 0,023	-0,021 ± 0,013 #	-0,016 ± 0,011 #
sin exp tau	0,28 ± 0,21	0,51 ± 0,33	0,37 ± 0,31	0,61 ± 0,42 *	0,11 ± 0,08	0,11 ± 0,1	0,26 ± 0,24	0,25 ± 0,24 #
sin exp off	0,44 ± 0,08	0,43 ± 0,13	0,42 ± 0,05	0,43 ± 0,07	1,82 ± 0,08	1,81 ± 0,09	1,75 ± 0,51	1,69 ± 0,27 #
integral	0,12 ± 0,03	0,096 ± 0,046	0,047 ± 0,016 #	0,058 ± 0,02 #	0,036 ± 0,022	0,028 ± 0,021	0,016 ± 0,01 #	0,012 ± 0,006 *#

* Statistically significant results to time matched cells cultivated without MSCs;
\# statistically significant to cells cultivated for 2 days (cultivation matched).

5.4 MSCs and liver regeneration

5.4.1 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 16). 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.
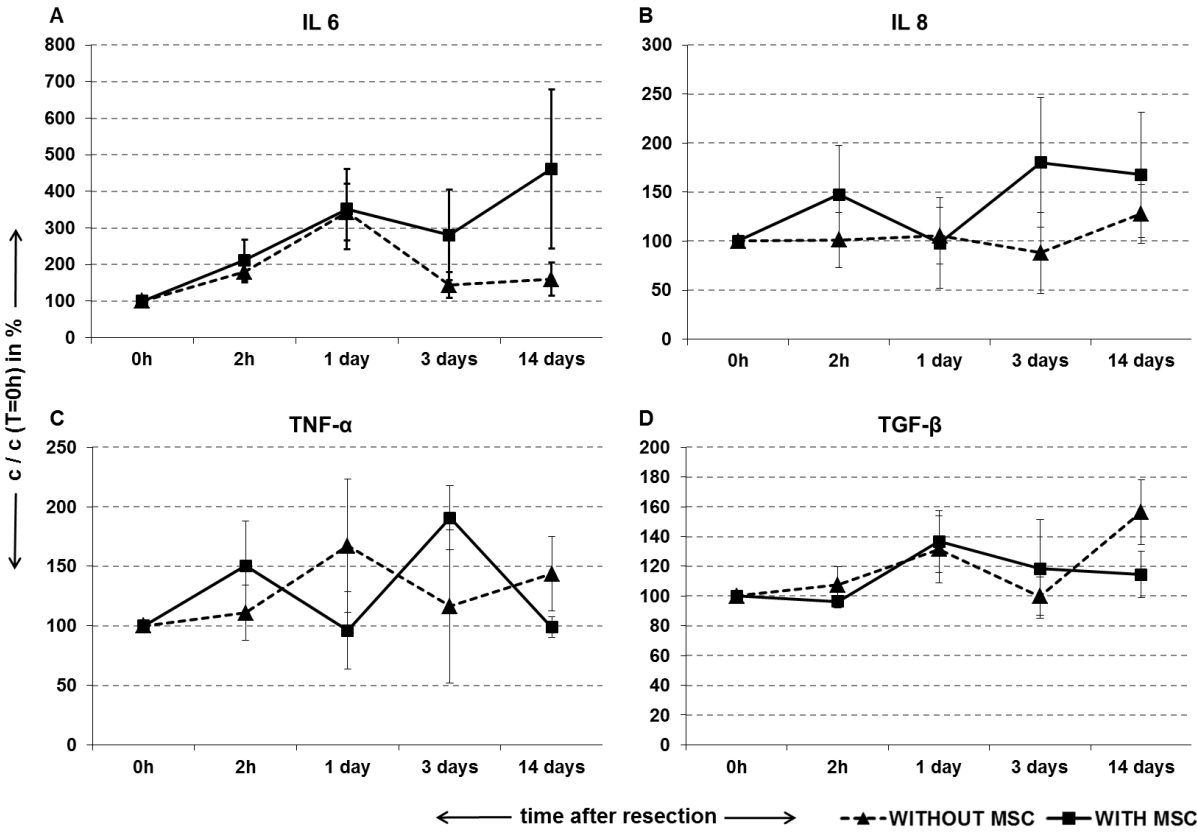

Figure 16 - Quantification of pro-inflammatory cytokines and TGF-β in plasma of pigs. Values are expressed as % of marker level in time point T = 0 h and standard error means are shown.

5.4.2 Liver morphology and morphometry analysis

Quantitative histology analysis of liver morphology (Figure 17) 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 18).

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 4). 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.
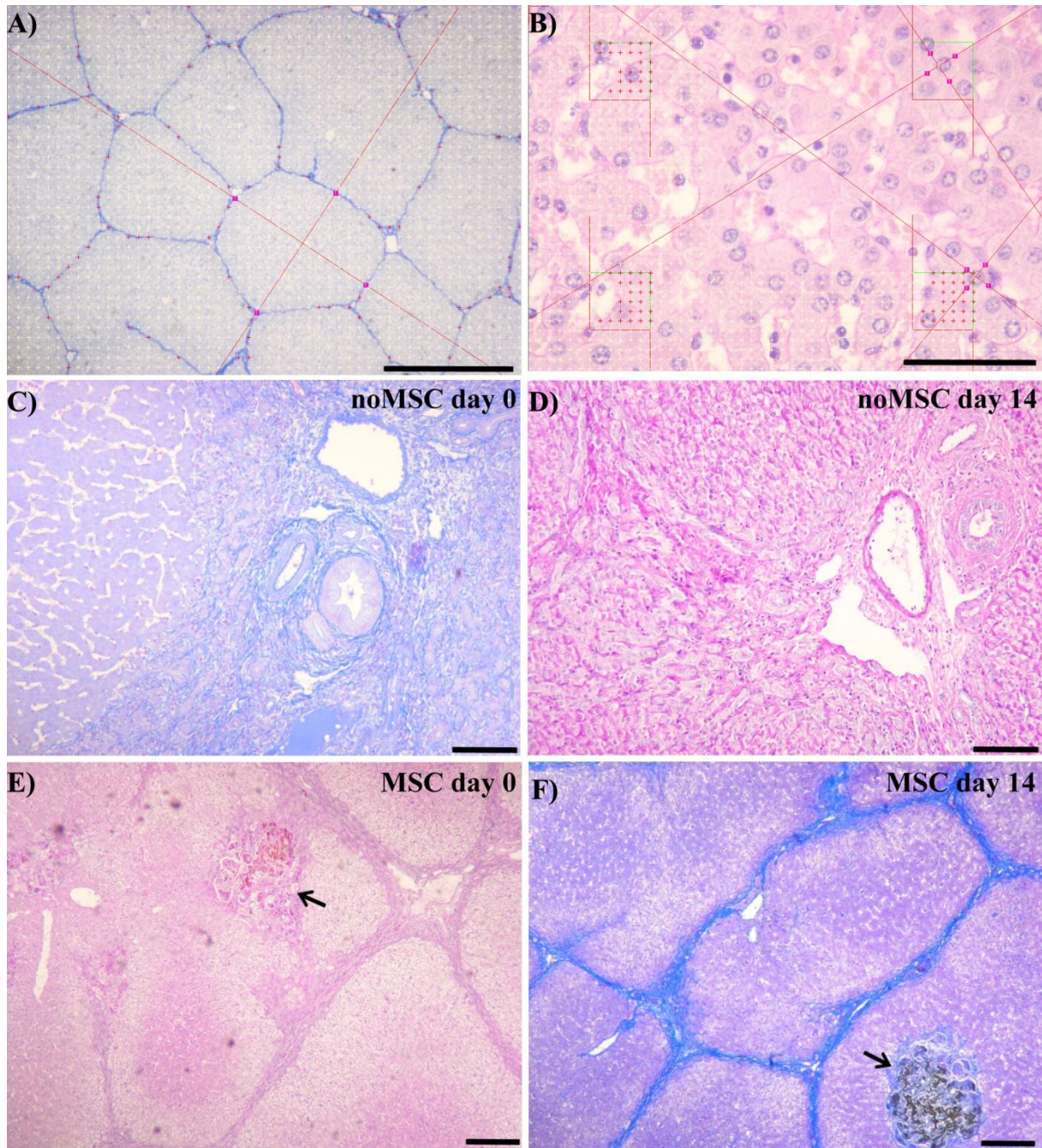
Figure 17 - Quantitative histological analysis and examples of liver morphology in the selected experimental groups. A – The volume fraction of connective tissue within the liver was estimated using a point grid (yellow). Cross-sectional area of hepatic lobules was estimated using a two-dimensional nucleator 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).
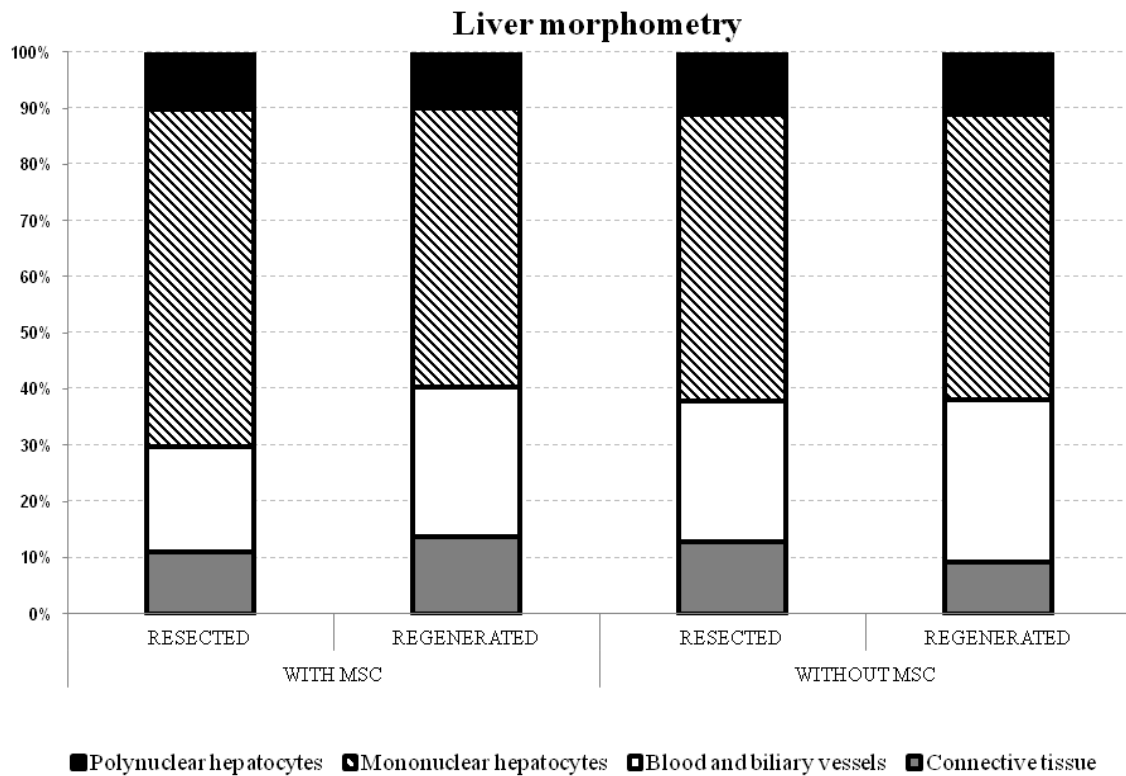
Figure 18 - Results of the histological morphometry comparing day 0 (RESECTED) and day 14 (REGENERATED) for two groups; with MSCs administration and without MSCs. No significant differences were found when comparing the volume fractions of mononuclear $V_V(MH,liver)$ and polynuclear hepatocytes $V_V(PH,liver)$, connective tissue $V_V(connective,liver)$, and blood and billiary vessels $V_V(connective,liver)$.

Table 4 - 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	-	-

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5.5 MSCs and GVHD

5.5.1 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 19).

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 20).
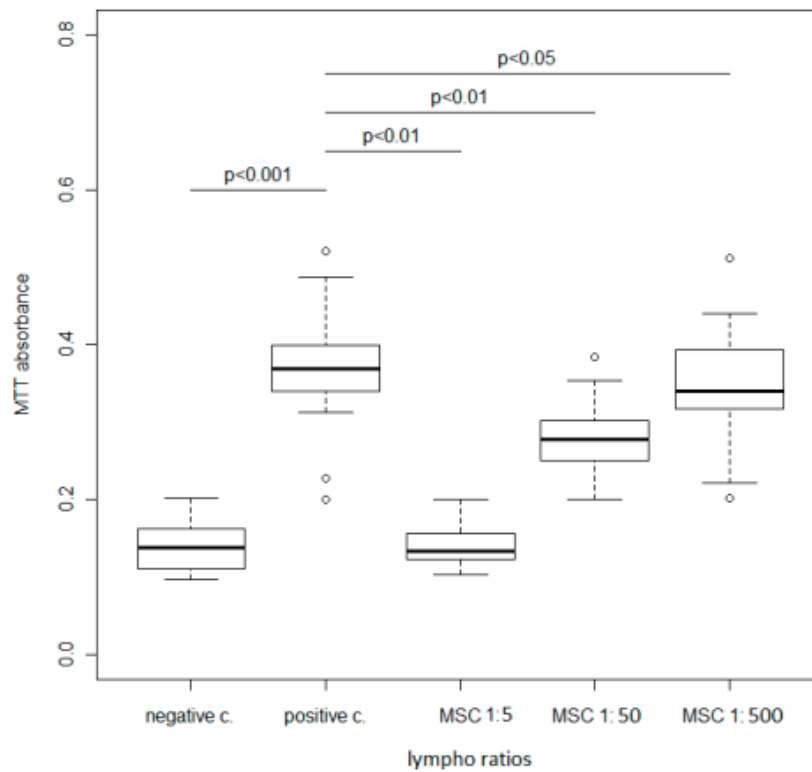
Figure 19 - Reduction of stimulated lymphocytes metabolic activity after MSCs addition (stimulation with alloantigenes). The co-cultivation with MSCs reduces the metabolic activity of stimulated lymhocytes in comparison with positive control. The higher dilution of MSCs i suspension, less effective the 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).

Figure 20 - Reduction of metabolic activity of stimulated lymphocytes after MSCs addition (nonspecific stimulation). Cocultivation with MSCs reduces significantly the metabolic activity of lymphocytes (p < 0,001) both stimulated with fMLP (A) and PHA (B).
Abbreviations: fMLP – N-formyl-Met-Leu-Phe, PHA – phytohemagglutinin.
MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max)

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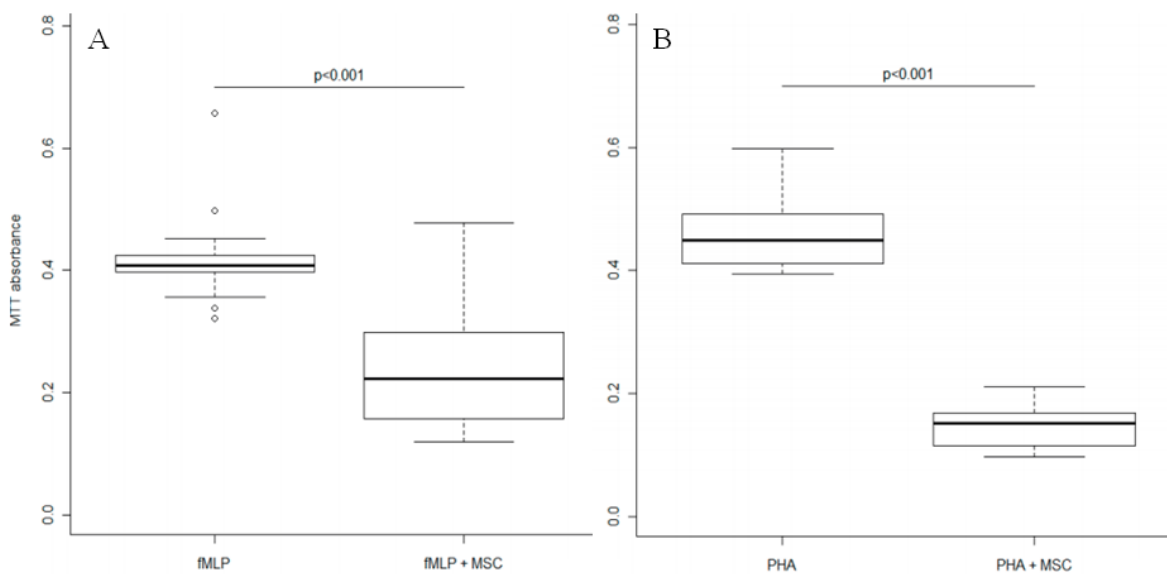
MSCs were administered to 8 patients with acute GVHD and to 10 patients with chronic GVHD. Blood samples from 5 timepoints (0 h, 14 days, 1 month, 2 months, 3 months) were processed and Tregs presence together with helper T-lymphocytes were seeked and evaluated.

For Tregs detection two approaches were compared (Figure 21). 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 22, 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, as visible in Figure 23. 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 remission 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.
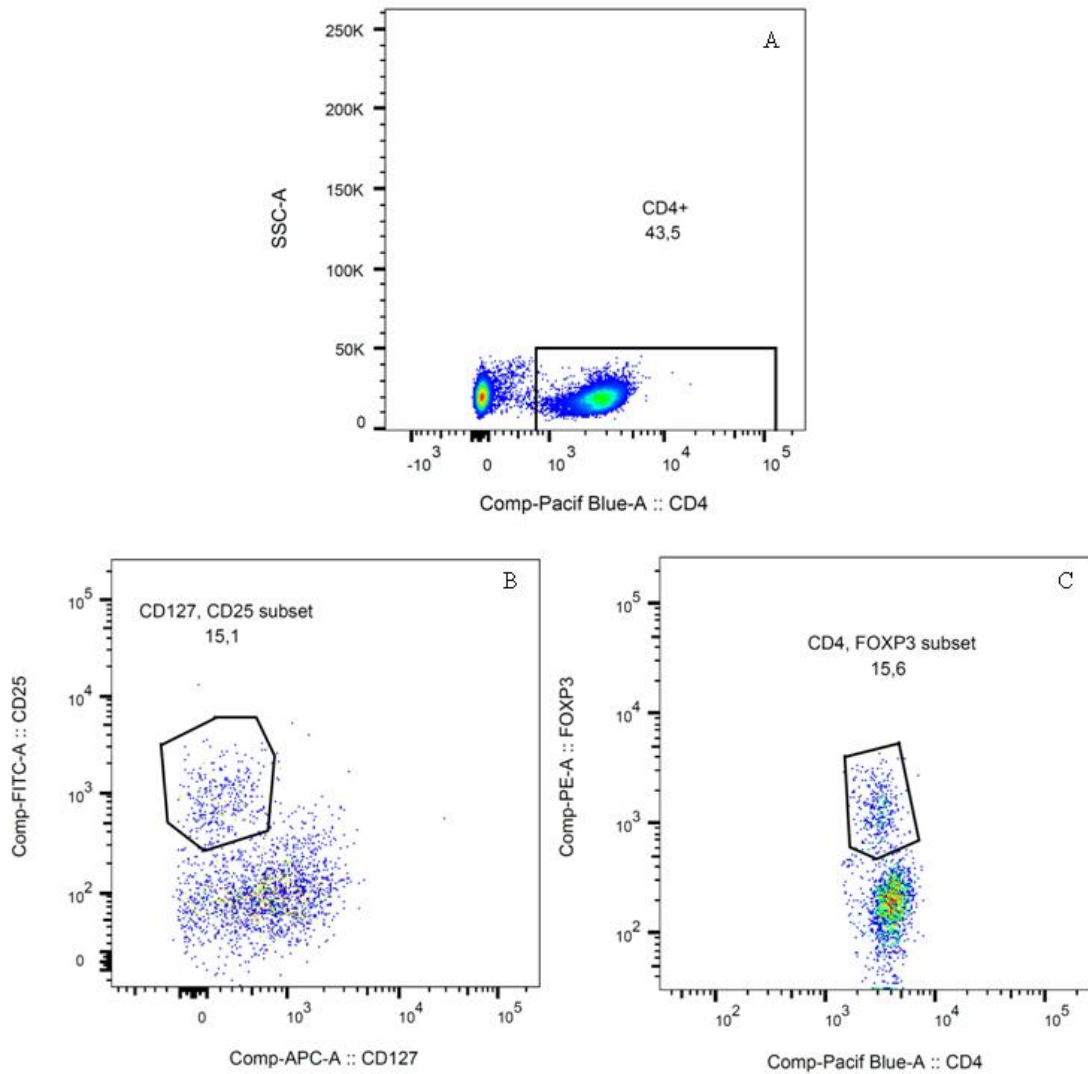
Figure 21 - Example of Tregs gating strategy in flow cytometry. Comparison of two different antibody sets (B,C) A – subpopulation of lymphocytes positive for CD4 (43,5 %). B – Tregs positive for CD25 and also CD127 (15,1 %). C – Tregs positive for CD4 and FOXP3 (15,6 %).
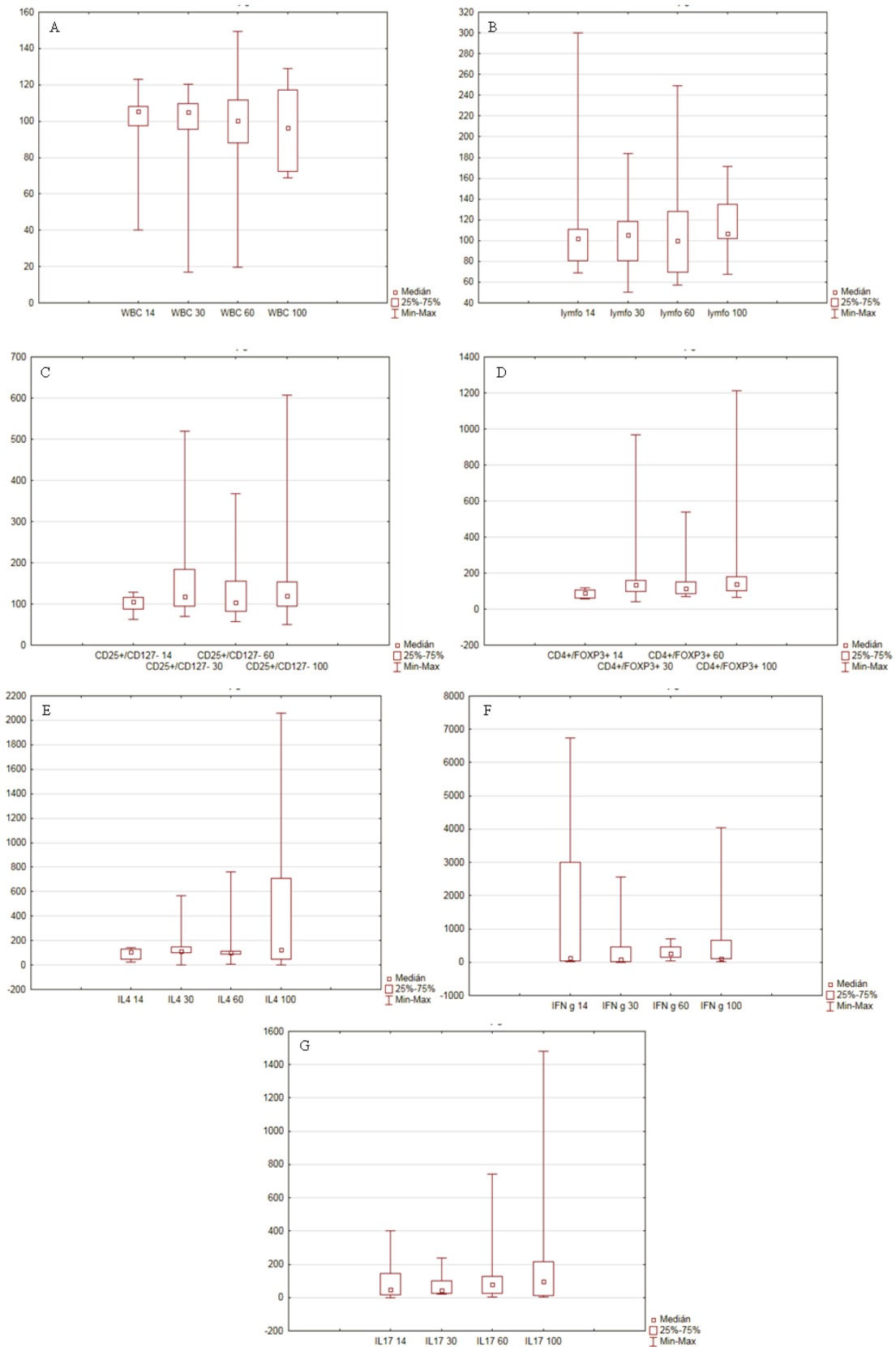
Figure 22 - Chronic GVHD after MSCs administration, changes of numbers of Tregs and helper T-lymphocytes in timepoints (14 days, 30 days, 60 days, 100 days). No significant differences were found. A – count of white blood cells (WBC), B – count of all lymphocytes, C – Tregs (CD25$^+$,CD127$^-$) count 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.

Figure 23 - Acute 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, G – levels of IL-17 in time.
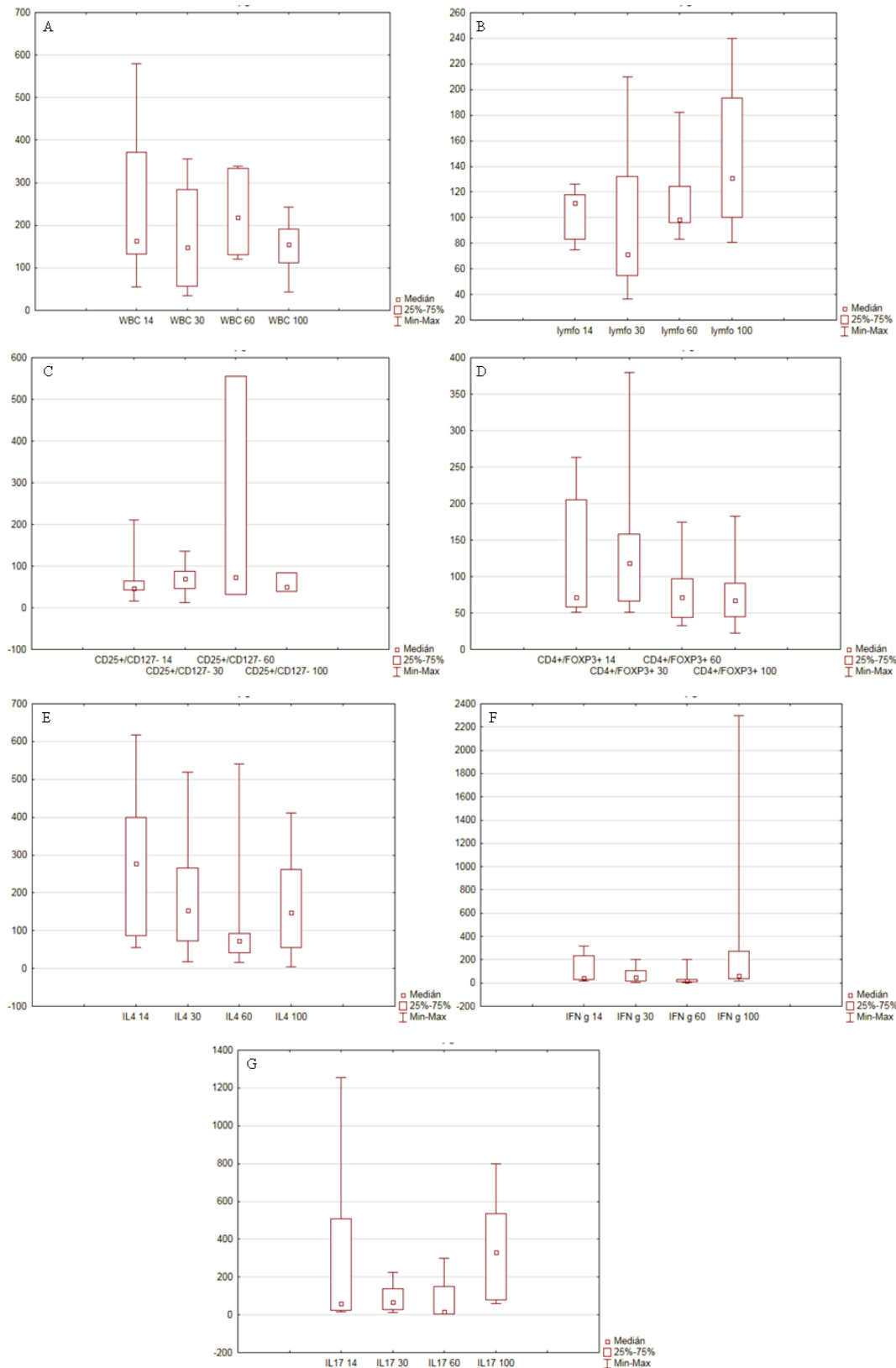
6 Disscussion

Mesenchymal stem cells are promising 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.

6.1 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.

6.2 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 promising 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.

6.3 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.

7 Conclusion

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

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

However, in preclinical and clinical settings of liver steatosis followed with liver lobe resection and GVHD, respectively, the MSCs administration into organism neither repair regenerated liver function nor improve GVHD progress significantly. Particulary, MSCs exhibited tendency to support connective tissue formation in liver, which may not be always beneficial for regenerating of the organ. MSCs immunomodulatory properties were visible in patients conditions improvement, but measured immune parameters were not significant.The discrepancies between in vitro and preclinical and clinical studies show the issue of proper validation criteria of MSCs influence. Requirement 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. Obtained results from large data sets will provide significant and meaningful information necessary for transfer of MSCs administration into clinical practice.

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9 List of attachments

ATTACHMENT I

PUBLICATIONS NOT RELATED TO THE TOPIC OF THE THESIS

ATTACHMENT II - V

PUBLICATION RELATED TO THE TOPIC OF THE THESIS:

ATTACHMENT II

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.**

ATTACHMENT III

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.**

ATTACHMENT IV

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.

ATTACHMENT V

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).

ATTACHMENT I

PUBLICATIONS NOT RELATED TO THE TOPIC OF THE THESIS:

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

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.

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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.

ATTACHMENT II

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.**

In vitro testing of immunosupressive effects of mesenchymal stromal cells on lymphocytes stimulated with alloantigens

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Aims. Mesenchymal stromal cells (MSC) derived from adult bone marrow or adipose tissue offer the potential to open a new frontier in medicine. MSC are involved in modulating immune response and tissue repair *in vitro* and *in vivo*. Experimental evidence and preliminary clinical studies have demonstrated that MSC exhibit an important immuno-modulatory function in patients with graft versus host disease (GVHD) following allogeneic hematopoietic stem cell transplantation. The immunosuppressive properties of MSC have already been exploited in the clinical setting. However the precise mechanisms are being still investigated.

Methods. We examined the immunosuppressive function of MSC by coculturing them with stimulated HLA incompatible allogeneic lymphocytes in a mixed lymphocyte culture test. The metabolic and proliferative activity of lymphocytes was determined by MTT test.

Results. After stimulation with alloantigens the presence of MSC caused significant decrease of absorbance levels by 62% ($P<0.01$), 26% ($P<0.01$) and 6% ($P=0.0437$) in comparison to positive control depending on the MSC/lymphocyte ratio (1:5, 1:50, 1:500). The mitogenic stimulation of lymphocytes with fMLP or PHA was also significantly reduced during MSC cocultivation. The absorbance was reduced by 42% ($P<0.001$) and 67% ($P<0.001$).

Conclusions. Allogeneic bone marrow is an ideal source of MSC for clinical application. The experiments confirmed the dose-dependent inhibitory effect of MSC on lymphocyte proliferation triggered by cellular or mitogenic stimulation. The mixed lymphocyte culture test offers a simple method for characterization and verification of the immunosuppressive potential of MSC, being prepared for clinical use.

Key words: mesenchymal stromal cells, allogeneic, immunosuppression, GVHD

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INTRODUCTION

Mesenchymal stromal cells (MSC) are multipotent non-hematopoietic progenitor cells of stromal origin that can be isolated from the bone marrow or other tissues (adipose tissue, cord blood). MSC can differentiate into cells of mesodermal origin (adipocytes, chondrocytes, osteoblasts) and likely into stromal fibroblasts, endothelial cells, neural cells and other cell types under certain condition[1]. MSC are not characterized by one specific marker. The absence of hematopoietic antigens such as CD45, CD34, CD14, CD19 and presence of surface antigens CD105, CD90, CD73 is typical for their immunopheno-type. The expression is dependent on the cell source and cultivation conditions[2].

Mesenchymal stromal cells are considered to be hy-poimmunogenic. They have low expression of HLA class I antigens and no expression of co-stimulatory molecules like CD80, CD86 and CD40. They do not induce a pro-liferative response in allogeneic lymphocytes and can be used and transplanted without respecting the usual transplant barrier represented by major histocompatibility complex[3,4]. MSC offer considerable immunomodulatory capacity. They are able to interact with the cells of innate and adaptive immunity and modulate some of the func-tions of the immune system. When cultivated with differ-ent immune system cell subpopulations, they can shift the cytokine profile of dendritic cells, T-lymphocytes and NK cells to the anti-inflammatory phenotype. MSC reduce the secretion of INF-γ in Th1 cells and increase the expres-sion of IL-4 in Th2 cells. Immature dendritic cells and Tregs increase in the presence of MSC expression of IL-10 whereas mature dendritic cells reduce the production of TNF-α and IL-12 (ref.[5,6]). Antigen-specific or mitogen-induced non-specific lymphocyte proliferation is signifi-cantly reduced in the presence of mesenchymal stromal cells. Reduced reactivity of T-lymphocytes is non-selective and concerns both CD4+ and CD8+ subpopulations[7,8]. The suppression is human leukocyte antigen (HLA) in-dependent and can be mediated through allogeneic and autologous MSC. Conversely, the degree of suppression is related to the dose of MSC (ref.[9]). T-lymphocyte pro-

liferation inhibition is mediated by arresting them in the G0/G1 phase of the cell cycle[10]. The biological relevance of these in vitro findings is not entirely clear and requires further studies.

MSC can affect the outcome of the immune reaction and change the inflammatory environment to immune tolerant or even anti-inflammatory. The immunomodulative properties of MSC predetermine them for influencing the immune response in a number of diseases which originate in alloreactive immunity or autoimmunity. A number of studies target the issues of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation[11,12]. There is little information on the mechanism of GVHD treatment by mesenchymal stromal cells. MSC probably suppress donor T-cell responses to recipient alloantigens. The suppression is induced by several mechanisms, including induction of regulatory T-cells, production of soluble cytokines and repair of damaged target tissues[13].

In our study, we evaluated the inhibitory activity of MSC, which are being prepared as a part of a preclinical study of GVHD treatment. We used the one-way mixed lymphocyte culture (MLC) test and observed changes in metabolic activity of lymphocytes after stimulation with alloantigens (cocultivation of HLA incompatible lymphocytes) and after non-specific mitogenic stimulation, respectively. Our target was to create a simple in vitro model situation of GVHD and to confirm the functional activity and regulatory potential of MSC, which are essential for their clinical application in the treatment of severe GVHD.

METHODS

GVHD model

We used a one-way mixed lymphocyte culture for testing the immunomodulatory properties of MSC. Lymphocytes from healthy donors were stimulated with alloantigens (HLA incompatible inactivated lymphocytes), nonspecific mitogens and chemotaxis activators (PHA, fMLP) and cocultivated with MSC. The effect of the stimulation and immunomodulation was determined by the MTT test which corresponds to mitochondrial metabolic activity and cell viability. In total 32 tests were performed on lymphocytes obtained from 15 healthy donors. All donors provided written informed consent to the study.

Lymphocyte samples preparations

Lymphocytes' donors were not HLA compatible with the donors of MSC. The donors were typed at low resolution using commercial PCR-SSO kits (LIFECODES HLA-SSO Typing kits for use with Luminex®, Gen-Probe). PCR-SSO typing was performed for HLA-A*, HLA-B* and HLA-DRB1* loci. Lymphocytes were isolated by gradient centrifugation (Histopaque – 1077, Sigma, USA), washed and diluted with cultivation medium RPMI 1640 (Lonza, Belgium) to a final concentration 1×10^6 cells/mL. Half of the prepared lymphocytes were inactivated

by cultivation in 1% solution of paraformaldehyde in PBS for 5 min.

Mesenchymal stromal cell cultivation

Mesenchymal stromal cells were derived from iliac crest bone marrow aspirate performed under general anaesthesia. All donors provided written informed consent for MSC donation. MSC were isolated by gradient centrifugation using separation solution. Briefly, 10 - 20 mL of BM aspirates were diluted 1:1 with HBSS (PAA, Austria) and layered over LSM 1077 solution (PAA, Austria). After centrifugation at 1000 g for 15 min, the mononuclear cells were collected into a new tube and resuspended in 20 mL of PBS (PAA, Austria). Cell suspension was washed by centrifugation at 1000 g for 10 min, the supernatant was discarded and pellet was resuspended in 1 mL PBS. All the cells were put into 175 cm² flask (Corning, USA) containing 30 mL of Complete Culture Medium (α-MEM, PAA, Austria; 10% poled human platelet lysate, local source) and cultivated in humidified incubator at 37 °C and 5% CO_2. The non-adherent cells were removed after 48 h and the remaining cells were further cultivated (the medium was changed every 3-4 days). After reaching 80% confluence the cells were detached with TrypLE Select solution (Invitrogen, USA) and passaged in a concentration 1×10^4/175 cm² flask. The MSC from 2nd to 4th passage were used for the cocultivation experiments.

Mitochondrial activity testing (MTT)

This method is based on the reduction of soluble 3-[4,5-dimethylthiazol-2-yl]-2,5-difenyl tetrazolium bromide (MTT, Sigma, USA) to insoluble formazan. The reaction takes place on the mitochondrial membrane of living cells. Formazan dissolves after adding strong solvent (DMSO, Sigma USA) and detergent (SDS, Sigma, USA). The arising color is detected by spectrophotometry with a wavelength 540 nm (MRXII, Dynex, CZ). The level of absorbance indicates the number of living and metabolic active cells. 200 µL of the lymphocyte mixture with 50 µL of MSC were incubated for 6 h in humidified incubator at 37 °C and 5% CO_2 in 96 well plates. The mixture was prepared from 100 µL of living lymphocytes and 100 µL of inactivated lymphocytes, always from different, HLA-incompatible donors. MSC were added to the culture in three different concentrations: 4 x 10⁵ cells/ml (MSC/lymphocyte ratio 1:5), 4 x 10⁴ cells/mL (1:50) and 4 x 10³ cells/mL (1:500). Phytohemaglutinin (PHA) and N-formyl-Met-Leu-Phe (fMLP, both Sigma, USA) were used at concentrations of 0.05 mg/mL and 0.025 mg/mL. The MTT solution was added after 1 h incubation and the mixture was incubated in for 2 h. The spectrophotometric analysis was performed after this time.

Statistical methods

The Wilcoxon paired test was used for statistical evaluation. The analysis was performed in statistical software R project (The R Foundation for Statistical Computing). A P value equal to or lower than 0.05 was taken as statistically significant.

RESULTS

Thirty-two experiments were performed in our study. The lymphocytes from healthy donors were stimulated non-specifically with alloantigens or PHA and fMLP, respectively. Cocultivation with mesenchymal stromal cells enabled to analyze the immunosuppressive effect of MSC on the cultivated lymphocytes. The results were obtained through the determination of absorbance of formazan solution released from the cells in each test. The absorbance level represented the metabolic and proliferative activity of cocultivated lymphocytes.

The stimulation of lymphocytes with HLA incompatible inactivated lymphocytes (positive control) manifested as expressive increase in metabolic activity with an increment of absorbance by 0.24 ($P<0.001$) compared to the negative control (unstimulated lymphocytes). In contrast to this observation in cocultivation experiments in the presence of MSC, we observed an average decline of absorbance of 0.23 ($P<0.01$) in undiluted (MSC/lymphocyte ratio 1:5), by 0.10 ($P<0.01$) in 10 x diluted MSC (1:50) and by 0.02 in 100 x diluted MSC (1:500) ($P=0.0437$). The presence of mesenchymal stromal cells led to an absorbance decline in comparison with positive control in all tests. The absorbance levels decreased by 62%, 26% and 6%, respectively. The effect of mesenchymal stromal cells was dose-dependent. The higher amounts of mesenchymal stromal cells added to the lymphocytes at 1:5 or 1:50 ratios provided the best immunosuppressive response. Extreme dilution of MSC (1:500) weakened their effect on the lymphocytes. For details see Table 1. and Fig. 1.

Significant differences in absorbance were also found in tests with PHA or fMLP stimulated lymphocytes. The lymphocytes were cultivated without and with MSC (1:5 ratio). The presence of MSC decreased the lymphocyte activity. The absorbance was reduced by 0.17 ($P<0.001$) in samples stimulated with fMLP and by 0.31 ($P<0.001$) in samples treated with PHA, which corresponded to the decline by 42% and 67%. Details are provided in Table 1. and Fig. 2.

DISCUSSION

Currently, mesenchymal stromal cells (MSC) are a subject of a large number of studies in various fields of medicine. They represent a promising treatment method for severe chronic graft-versus-host disease refractory to corticosteroids in patients after allogeneic hematopoietic stem cell transplantation. Allogeneic bone marrow is an appropriate source of MSC for clinical application. The cells can be cryopreserved and cultivated on request in reasonable timeframe. MSC can avert rejection in recipients of solid organ transplants[14]. Another field of successful utilization of MSC is the treatment of autoimmune diseases such as Crohn's disease, multiple sclerosis and rheumatoid arthritis[15,16].

Mesenchymal stromal cells are able to interact with the immune system and have effective immunomodulative

Table 1. Lymphocyte cytotoxicity test (MTT absorbance values).

	mean	SD	P
negative control	0.1354	0.03	< 0.001
positive control	0.3746	0.06	
MSC 1:5	0.1427	0.02	< 0.01
MSC 1:50	0.2778	0.03	< 0.01
MSC 1:500	0.3505	0.05	0.0437
fMLP[a]	0.4136	0.05	< 0.001
fMLP/MSC	0.2405	0.08	
PHA[b]	0.4588	0.04	< 0.001
PHA/MSC	0.1506	0.04	

positive control – stimulation with inactivated lymphocytes; negative control – without stimulation; MSC 1:5, MSC 1:50 and MSC 1:500 – stimulation with inactivated HLA incompatible lymphocytes and MSC cocultivation at different MSC:lymphocyte ratios; [a]fMLP stimulation with and without MSC addition; [b]PHA stimulation with and without MSC addition; abbreviations: MSC – mesenchymal stromal cells, fMLP – N-formyl-Met-Leu-Phe, PHA – phytohemagglutinin
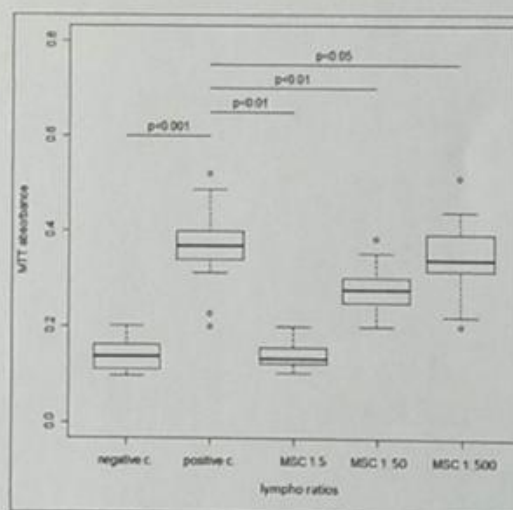

Fig. 1. Reduction of stimulated lymphocytes metabolic activity after MSC addition (stimulation with alloantigens). The cocultivation with MSC reduces the metabolic activity of stimulated lymphocytes in comparison to positive control (no MSC added). The MSC effect is dose dependent and higher MSC dilutions are less effective in lymphocyte suppression. See details in the text. MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max).
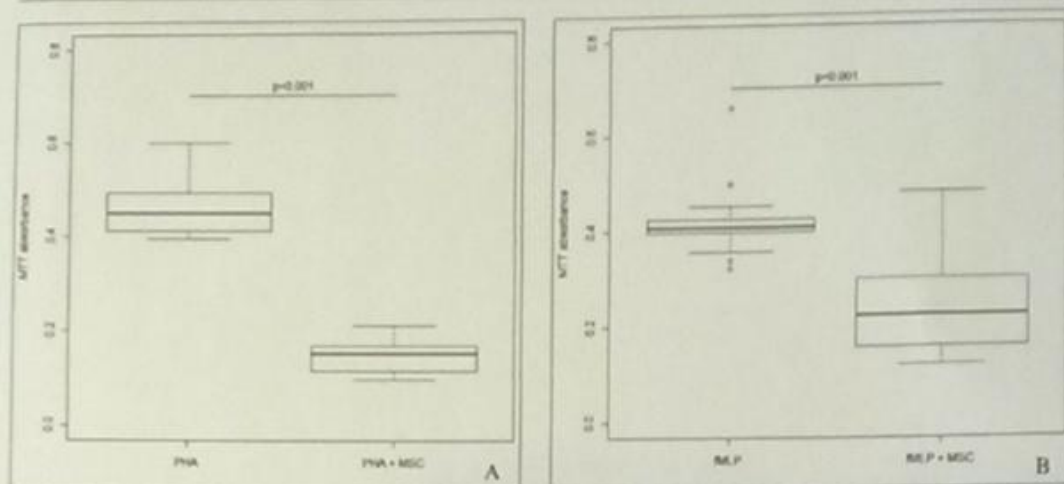
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Fig. 2. Reduction of stimulated lymphocytes metabolic activity after MSC addition (nonspecific stimulation). The effect of MSC addition to the culture of lymphocytes stimulated with PHA (A.) and fMLP (B.). Cocultivation with MSC reduces significantly the metabolic activity of lymphocytes ($P<0.001$). Abbreviations: fMLP – N-formyl-Met-Leu-Phe, PHA – phytohemagglutinin. MTT absorbance values showed, Wilcoxon pair test (median; box: 25%, 75% quantiles; non-outlier min, non-outlier max)

properties. They can affect the immune reaction by inhibition of inflammatory cytokines and by increase in expression of suppressive cytokines. Studies have confirmed that during the cocultivation, MSC can negatively influence T-lymphocytes proliferation[4,17,18]. Our results are consistent with these data. We observed that MSC possess the capacity to significantly affect the nonspecific activation of lymphocytes. When lymphocytes were cocultivated with MSC, there was a marked decline in absorbance, measured by MTT test of almost 60 percent in comparison with the positive control. The lower absorbance levels corresponded to reduction in the metabolic and proliferative activity of stimulated lymphocytes.

The immunosuppressive effect was present both during cocultivation of lymphocytes stimulated with alloantigens (inactivated incompatible lymphocytes) and in cultures with lymphocytes stimulated with PHA and fMLP. The laboratory analysis confirms that mesenchymal stromal cells could regulate the lymphocyte activation in vitro when stimulated with HLA incompatible lymphocytes. MSC can therefore be used for the treatment of severe forms of chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. This clinical application of MSC has begun to be studied in the first clinical trials with encouraging results. The immunomodulatory effect of MSC is dose-dependent[4]. An MSC/lymphocyte ratio of 1:5 provided the most noticeable suppression of lymphocyte proliferation in our analysis. The dose dependent nature of MSC inhibitory effects emerged from a similar study showing statistically significant suppression at MSC/lymphocyte ratios ranging from 1:1 to 1:10 (ref.[19]). It appears that the higher doses of MSC produce stronger effect. However, the exact dose

and frequency of application optimal for the GVHD treatment must be determined in larger clinical studies. Lower numbers of MSC are unable to inhibit mitogen-induced T-cell response. Some studies have reported stimulatory effects at very low concentrations of MSC (1:100 – 1:10000) on allogeneic lymphocytes provoking speculation that surface structures on MSC can act synergistically with HLA-DR antigens after mitogenic stimulation[4,20].

Our data confirm the inhibitory effect of MSC on lymphocyte proliferation triggered by cellular or mitogenic stimulation. The mixed lymphocyte culture offers a simple way of confirming the immunosuppressive potential of MSC and validating the cell therapy medicinal product intended for clinical use. Cocultivation experiments of lymphocytes and MSC offer a simple in vitro method for simulating the MSC effect on alloreactive interactions of donor and recipient lymphocytes similar to the processes during the post-transplant period and GVHD reaction.

ACKNOWLEDGEMENT

ATTACHMENT III

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.**

Original Article

The Quality Control of Mesenchymal Stromal Cells by *in Vitro* Testing of Their Immunomodulatory Effect on Allogeneic Lymphocytes

(mesenchymal stromal cells / allogeneic / immunosuppression / GVHD)

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Abstract. Mesenchymal stromal cells (MSC) represent a promising treatment of graft-versus-host disease (GVHD) in patients after allogeneic haematopoietic stem cell transplantation. We performed co-cultivation experiments with non-specifically stimulated lymphocytes to characterize the immunosuppressive activity of MSC. MSC influenced expression of some activation antigens. CD25 expression was lower with MSC and reached 55.2 % vs. 84.9 % (CD4^{+}, P = 0.0006) and 38.8 % vs. 86.6 % (CD8^{+}, P = 0.0003) on day +4. Conversely, CD69 antigen expression remained higher with MSC (73.3 % vs. 56.8 %, P = 0.0009; 59.5 % vs. 49.7 %, ns) and its down-regulation along with the culture time was less pronounced. MSC reduced proliferation of the stimulated lymphocytes. The cell percentages detected in daughter generations were decreased (32.82 % vs. 10.68 % in generation 4, P = 0.0004 and 29.85 % vs. 10.09 % in generation 5, P = 0.0008), resulting in a lower proliferation index with MSC (1.84 vs. 3.65, P < 0.0001). The addition of MSC affected expression of some cytokines. Production of pro-inflammatory cytokines was decreased: IL-6 (19.5 vs. 16.3 MFI; P < 0.0001 in CD3^{+}/CD4^{+} and 14.5 vs. 13.2 MFI; P = 0.0128 in CD3^{+}/CD8^{+}), IFN-γ (13.5 vs. 12.0 MFI; P = 0.0096 in CD3^{+}/CD4^{+}). Expression of anti-inflammatory IL-10 was only slightly increased after the addition of MSC (ns). The analysis confirmed the immunomodulatory activity of MSC. The functional tests have proved to be an important part of the quality control of the advanced therapy cellular product intended for GVHD treatment. Future research should more closely focus on the interaction between MSC and the patient immune environment.

Introduction

Human mesenchymal stromal cells (MSC) are a population of multilineage progenitor cells with the ability to differentiate into multiple mesenchymal lineages including chondrocytes, osteoblasts and adipocytes (Abdallah and Kassem, 2008). These non-haematopoietic stem cells can be found, isolated and expanded from many tissues including bone marrow, umbilical cord blood or adipose tissue. The immunophenotype of MSC is dependent on their source and cultivation conditions. MSC are distinguishable from haematopoietic cells by being negative for the surface leukocyte markers CD45, CD34, CD19, CD3, HLA-DR, but express CD105, CD73, CD90 (Dominici et al., 2006).

MSC do not express class II human histocompatibility antigens or costimulatory molecules (e.g. CD80, CD86). The effects of MSC on T cells are independent of HLA matching between MSC and lymphocytes; therefore, MSC can be administered repeatedly without

provoking an immunologic response in the HLA-incompatible recipient (Sundin et al., 2009).

MSC are able to interact with the cells of innate and adaptive immunity and influence secretion of certain cytokines. They can move the cytokine profile of dendritic cells, naive and activated T lymphocytes and NK cells to the anti-inflammatory or tolerant phenotype. MSC reduce secretion of IFN-γ in Th1 cells and increase expression of IL-4 in Th2 cells when cultured with these lymphocyte subpopulations. Immature dendritic cells and Tregs increase expression of IL-10 in the presence of MSC, whereas mature dendritic cells reduce production of TNF-α and IL-12 (Aggarwal and Pittenger, 2005; Le Blanc and Pittenger, 2005).

The MSC-mediated immunosuppression acts mainly through secretion of soluble molecules that are produced or upregulated following interaction between the immune cells and MSC. MSC can inhibit proliferation of T lymphocytes with production of indoleamine 2,3-dioxygenase, which catalyses conversion of tryptophan to kynurenin and reduces the T-cell answer through depletion of tryptophan and accumulation of its toxic metabolites. MSC express the enzyme cyclooxygenase, which increases production of prostaglandin E2 and induces regulatory T lymphocytes (Aggarwal and Pittenger, 2005; Le Blanc and Ringdén, 2007). Activated MSC can also produce other molecules with the capability to reduce the activity of immunocompetent cells, such as IL-6, nitric oxide, TGF-β. Therefore, immune regulation mediated by MSC is the result of the cumulative action displayed by several molecules and cell types.

Human MSC suppress lymphocyte alloreactivity *in vitro* in mixed lymphocyte reaction assays. The antigen-specific or mitogen-induced non-specific lymphocyte proliferation is significantly reduced in the presence of MSC. The reduction of reactivity of T lymphocytes is non-selective and touches naive and memory T cells as well as CD4+ and CD8+ subpopulations (Tse et al., 2003; Ramasamy et al., 2008). The suppression is independent of the major histocompatibility complex (MHC) and can be mediated through allogeneic or autologous MSC (Le Blanc et al., 2003). The inhibition of T-lymphocyte proliferation is mediated by arresting them in G0/G1 phase of the cell cycle (Glennie et al., 2005). The expression of early activation markers of T cells, notably CD25 and CD69, can be altered by MSC (Le Blanc et al., 2004). The immunosuppressive effect of MSC arises as a consequence of both anti-proliferative activity and the ability to affect T-cell activation.

A number of studies indicate that MSC possess an immunosuppressive function both *in vitro* and *in vivo*. The immunomodulative properties of MSC predetermine them for affecting the immune response in many diseases that are associated with alloreactive immunity or autoimmunity. MSC are an attractive candidate as a potential cellular therapy for the treatment of severe graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. GVHD represents a significant cause of morbidity and mortality after stem cell transplantation. This cellular therapy could be of great clinical importance as it may ameliorate the symptoms of the GVHD refractory to the standard corticosteroid based immunosuppression (Le Blanc et al., 2008; Ringdén and Keating, 2011).

From a regulatory perspective, all MSC-based products in the European Union are classified as advanced therapy medicinal products (ATMP). The culture process corresponds to "substantial manipulation" and the derived cells are qualified as an active substance of a medicinal product. The cell characterization and the product release criteria cover a complex testing, including the potency analysis. The potency assay represents quantitative measurement of the biological activity based on the attribute of the product, which is linked to the relevant biological properties and expected clinical response.

In our study, we tested the immunomodulatory properties of MSC, which were prepared under a clinical study of GVHD treatment. The repeated co-cultivation experiments were used to observe the changes in the proliferation rate, activation and cytokine production in non-specifically stimulated allogeneic lymphocytes after the addition of MSC. The goal of the study was to assess the capacity of MSC to modulate activation and proliferation of T-cell subsets and to prove the functional activity of MSC, which is essential for their clinical application in the treatment of GVHD. We tried to validate this "functional testing" as a suitable method for the quality control of MSC products.

Material and Methods

MSC isolation and cultivation

MSC were isolated from bone marrow (BM) aspirates obtained from healthy voluntary donors of the Czech National Marrow Donor Registry. Donors were enrolled for the purpose of unrelated allogeneic stem cell transplantation. The BM grafts were collected from the posterior iliac crest under general anaesthesia. All donors provided written informed consent for MSC donation. About 10 to 20 ml of the BM aspirate was diluted 1 : 1 with HBSS (PAA, Linz, Austria) and layered over LSM 1077 solution (PAA). Mononuclear cells were isolated by gradient centrifugation, washed and resuspended in 1 ml of PBS (PAA). All cells were then placed into a 175 cm² culture flask (Corning, NY) containing 30 ml of pre-warmed complete culture medium – CCM (αMEM, PAA; heparin, Biochrom, Berlin, Germany; 10% pooled platelet lysate – pHPL, local source) and cultivated at 37 °C and 5% CO_2. After 48 h, the medium with non-adherent cells was removed. The remaining cells were washed with PBS and fed with fresh medium. The medium was changed every 3–4 days. After reaching 80 % confluence the cells were detached with TrypLE Select solution (Gibco, Grand Island, NY) and passaged at concentration 1 × 10⁶/175

cm² flask. The MSC from the 2ⁿᵈ to 4ᵗʰ passage were used for the co-cultivation experiments. The MSC were tested for viability and for characteristic expression of positive or negative markers (CD45 – Becton Dickinson, San Diego, CA; CD34, HLA-DR, CD13, CD14 – Immunotech, Boston, MA; CD73, CD90 – Biolegend, San Diego, CA; CD19, CD105 – Exbio, Vestec, Czech Republic). The purity of MSC exceeded 90 %. Expression of all positive antigens (CD73, CD90, CD105, CD103) was over 90 % and all negative antigens (HLA-DR, CD14, CD19, CD34, CD45) below 10 %.

The differentiation capacity of MSC was tested during the validation of the cultivation protocol. The cells (from 4ᵗʰ passage) were seeded in 6-well plates and cultivated for two days in standard medium. Then the differentiation media for adipogenesis, chondrogenesis and osteogenesis were applied (StemPro, Invitrogen, Carlsbad, CA) and cells were further cultivated for 14 days. Finally, staining with Oil Red O, Toluidine Blue O and Alizarin Red S was performed (Sigma, St. Louis, MO).

Preparation of lymphocyte samples

Peripheral blood mononuclear cells (PBMC) used for co-cultivation experiments were isolated from healthy donors. Donors were not HLA compatible with the donors of MSC. The donors were typed at low resolution using commercial PCR-SSO kits (LIFECODES HLA-SSO Typing kits (Immucor, Norcross, GA) for use with Luminex® (Gen-Probe, Stamford, CT)). PCR-SSO typing was performed for HLA-A*, HLA-B* and HLA-DRB1* loci. PBMC were isolated by gradient centrifugation (Histopaque – 1077, Sigma), washed with the cultivation medium (RPMI 1640, Lonza, Verviers, Belgium) and diluted to the final concentration of 1×10^6 cells/ml.

Lymphocyte activation and proliferation analysis

Proliferation of the stimulated PBMC and expression of activation markers on the lymphocytes were evaluated in 20 co-cultivation experiments. The mixed cultures were carried out in 5 ml tissue culture tubes (TPP, Trasadingen, Switzerland), the final volume of cell suspension was 2 ml. Proliferation of PBMC was measured by the use of CFSE (carboxyfluorescein succinimidyl ester) tracking. After staining with CFSE (Molecular Probes, Inc., Eugene, OR), 2×10^6 cells were stimulated with 10 µl of phytohemagglutinin (PHA; 1 µg/µl; PAA) and cultivated with or without MSC (MSC/lymphocyte ratio 1 : 2) at 37 °C and 5% CO_2. The parent and successive populations were measured after four days of cultivation and analysed with ModFit LT software (Verity Software House, Topsham, ME).

The level of lymphocyte activation antigen expression was tested on days 2 (48 h), 3 (72 h) and 4 (96 h). Every test consisted of three tubes: 2×10^6 of stimulated PBMC only (activation control); 2×10^6 of non-stimulated PBMC plus 1×10^6 MSC (1 : 2, control of stimulation via MSC) and stimulated PBMC plus MSC. PBMC were stimulated non-specifically with phytohaemagglutinin (10 µg/2 × 10⁶ lymphocytes). The cells were cultivated in RPMI 1640 medium (Lonza) supplemented with 10 % pHPL and cultivated at 37 °C and 5% CO_2 for four days. About 200 µl of the cell suspension was taken from the cultivation tube and mixed with the antibodies cocktail (CD45 – Becton Dickinson; CD19, HLA-DR – Immunotech; CD3, CD4, CD25, CD69 – Exbio). The cells were washed after the incubation and analysed immediately. All analyses were performed in a FACS Canto II flow cytometer (Becton Dickinson) and with FlowJo software (Tree Star, Ashland, OR).

Evaluation of cytokine production

We further evaluated production of certain cytokines in another set of 25 co-cultivation experiments. PBMC were diluted to a final concentration of 1×10^6 cells/ml, and 10 µl of antibody-based stimulation reagent (CytoStim, Miltenyi Biotec, Bergish Gladbach, Germany) and 4 µl of monensin (intracellular protein transport inhibitor, Cytodetect kit, IQ Product, Groningen, Netherlands) were added according to the manufacturer's instructions. Then, the cells were incubated with or without the addition of MSC (1 : 2 ratio) for 6 h at 37 °C and 5% CO_2. Fixation with 1% paraformaldehyde and staining for the surface markers CD45, CD4, CD8 followed. Finally, the cells were permeabilized with saponin (Cytodetect kit) and the intracellular cytokines were stained (IL-6, IL-10, IFN-γ; Becton Dickinson). Data were acquired in a FC 500 flow cytometer, with Kaluza software (Beckman Coulter, Brea, CA), and expressed as mean intensities of fluorescence (MFI).

Evaluation of phosphorylated proteins of the STAT family

The identical set of 25 co-cultivation experiments was used for detection of phosphorylated signal transducer and activator of transcription (STAT) proteins. PBMC at a concentration of 1×10^6 cells/ml were incubated with 10 µl of CytoStim (Miltenyi Biotec), again with or without MSC for 24 h at 37 °C and 5% CO_2. The staining was performed after cell membrane permeabilization and inhibition of phosphorylation enzymes. The cells were fixed with 1.5% paraformaldehyde for 10 min, centrifuged and resuspended in 100% cold methanol (Sigma-Aldrich, St. Louis, MO) and incubated for 30 min at 5 °C in the dark. Then the cell wash was repeated, cells were stained with monoclonal antibodies against antigens CD3, STAT1, STAT3, STAT4, STAT6 (Becton Dickinson) and incubated for 30 min in the dark. After one wash step the cells were analysed in a FC 500 flow cytometer, with Kaluza software (Beckman Coulter), and mean fluorescence intensities were acquired.

Statistical methods

The mean, median, SD, variations, minimum, maximum and other basic statistical measurements were

computed for all the parameters. The Wilcoxon paired test (non-parametric ANOVA) was used for comparison of daughter populations in the CFSE tracking assay, and for comparison of the expression of cytokines, STAT proteins and activation antigens on the cultured lymphocytes. The kinetics of activation antigens was analysed by Friedman ANOVA. A P value equal to or lower than 0.05 was taken as statistically significant. Software Statistica 98 Edition (StatSoft, Inc., Tulsa, OK) was used for the analysis.

Results

Activation of lymphocytes

Twenty experiments were performed in our study of activation markers. Non-specifically stimulated PBMC were co-cultivated with MSC for four days and the expression of activation antigens CD25, CD69 and HLA-DR on lymphocytes was observed on days +2 to +4.

The expression of CD25 increased during the observed period on both CD4+ and CD8+ stimulated lymphocytes (P = 0.00077 and P = 0.000001). Conversely, no significant changes of CD25 kinetics were observed in the tests with addition of MSC (ns). The CD25 expression was about 20–30 % lower with the MSC and reached 55.2 % vs. 84.9 % (CD4+ lymphocytes, P = 0.0006) and 38.8 % vs. 86.6 % (CD8+ lymphocytes, P = 0.0003) on day +4. The addition of MSC caused downregulation of CD25 in all tests, see Fig. 1A, C and Table 1.

The CD69 antigen expression showed a slightly different characteristic. The maximum expression was detected on day +2 followed by a decrease, irrespective of the MSC presence. The level of CD69 expression on CD4+ cells was similar in the tests with and without MSC on day +2 (81.9 % vs. 87.6 %, ns). Thereafter, the lymphocytes co-cultivated with MSC showed a slower decline of CD69 expression, resulting in higher percentages on day +4 (73.3 % vs. 56.8 %, P = 0.0009), Fig. 1B. A similar trend was also observed for CD8+ lympho-

Fig. 1. Comparison of CD25 and CD69 kinetics on CD3+/CD4+ and CD3+/CD8+ lymphocytes
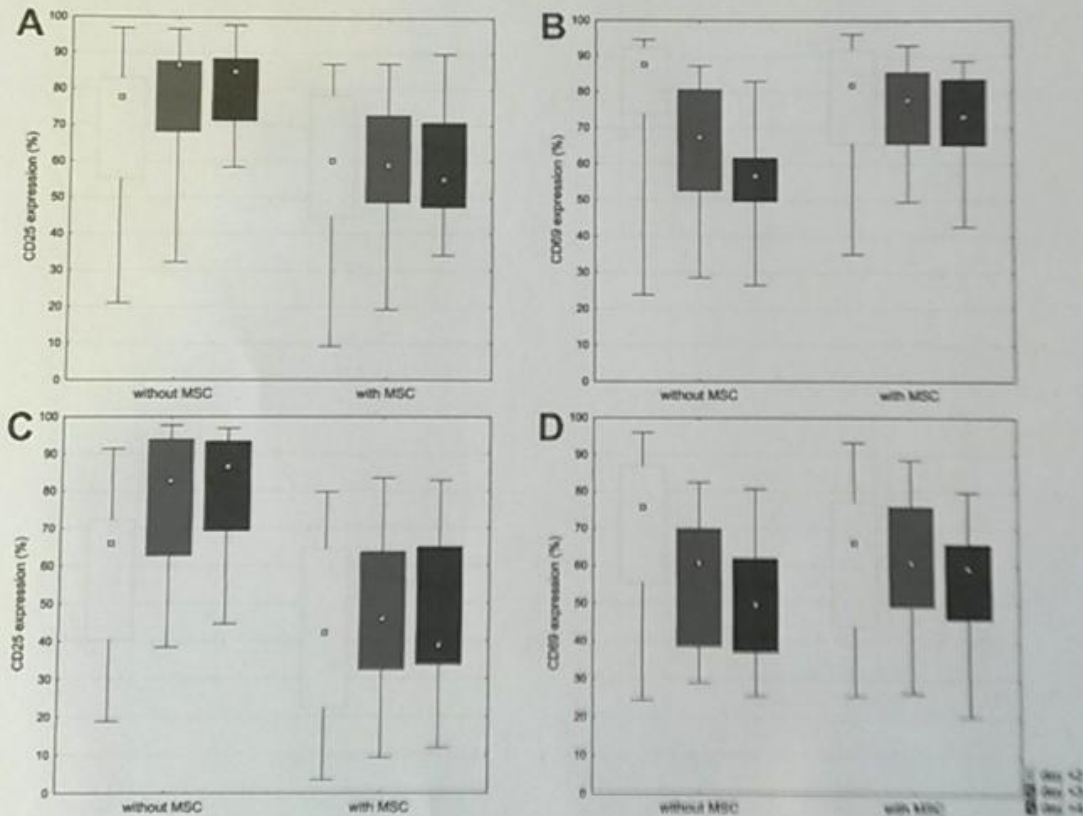
The lymphocytes stimulated non-specifically with phytohaemagglutinin were co-cultivated with MSC (MSC:lymphocyte ratio 1 : 2) for four days and the expression of activation antigens CD25, CD69 was observed on days +2 to +4. The addition of MSC to the culture resulted in different kinetics of activation antigen CD25 on both CD3+/CD4+ (A) and CD3+/CD8+ (C) lymphocytes as well as antigen CD69 (B for CD3+/4+ and D for CD3+/CD8+). See text for details (median, box 25 %, 75 % quantiles; non-outlier min, non-outlier max).

Table 1. Expression of activation antigens on T lymphocytes

% (median)		CD3⁺/CD8⁺			CD3⁺/CD4⁺		
		no MSC	with MSC	P	no MSC	with MSC	P
CD25	Day +2	65.9	42.2	0.0441	77.7	60.3	ns
	Day +3	82.8	45.8	0.0009	86.8	59.0	0.0036
	Day +4	86.6	38.8	0.0003	84.9	55.3	0.0006
CD69	Day +2	75.8	66.3	ns	87.6	81.9	ns
	Day +3	60.7	60.9	ns	67.4	77.9	ns
	Day +4	49.7	59.5	ns	56.8	73.3	0.0009
HLA-DR	Day +2	10.7	5.1	0.0166	6.4	2.6	0.0118
	Day +3	13.1	6.0	0.0008	8.7	3.3	0.0028
	Day +4	11.1	6.2	0.0750	9.8	3.6	0.0003

cytes cultivated with and without the addition of MSC on day +2 (66.3 % vs. 75.8 %, ns) and day +4 (59.5 % vs. 49.7 %, ns). The down-regulation of the CD69 antigen was less pronounced with MSC as its expression remained above the level observed without MSC, see Fig. 1D and Table 1. An example of how MSC influence CD25 and CD69 expression is given in Fig. 2.

HLA-DR was another activation marker evaluated in our experiment. No statistically significant changes of the kinetics were recorded between day +2 and day +4. HLA-DR levels remained low in both CD4⁺ and CD8⁺ lymphocytes. However, the co-cultivation with MSC resulted into the reduction of HLA-DR expression in comparison to tests without MSC addition.

Control tests demonstrated no significant induction of CD25, CD69 or HLA-DR antigens by MSC. The ex-pression was maintained between 0 % and 5 % (data not shown).

CFSE evaluation

We used the CFSE cell tracking assay for analysis of proliferation of non-specifically stimulated PBMC cultured with and without the addition of MSC. After 4 days of cultivation, the CD3⁺/4⁺ lymphocytes divided into four to seven generations. In the tests without MSC, only 17.4 % of all lymphocytes remained undivided. When the PBMC were co-cultivated with MSC, their proliferation rate was reduced and about 50 % of all cells were retained within the parent generation (P < 0.0001). Figure 3 shows an example of CFSE analysis. Most of the lymphocytes only reached three to four divisions. The addition of MSC caused a significant reduc-

Fig. 2. Example of influence of MSC addition on the activation of antigen expression
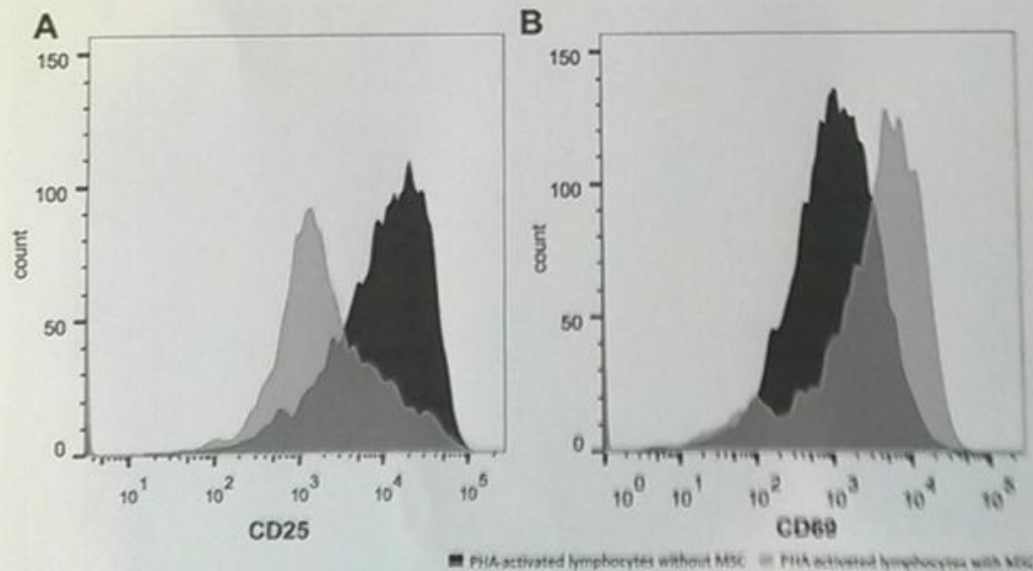
Co-cultivation with MSC changed the expression of CD25 and CD69 antigens on stimulated lymphocytes (an example after 72 h of cultivation); overlay histograms; Flow-Jo software; dark: without MSC, grey: with MSC, see Results for details.

Fig. 3. Example of CFSE proliferation analysis
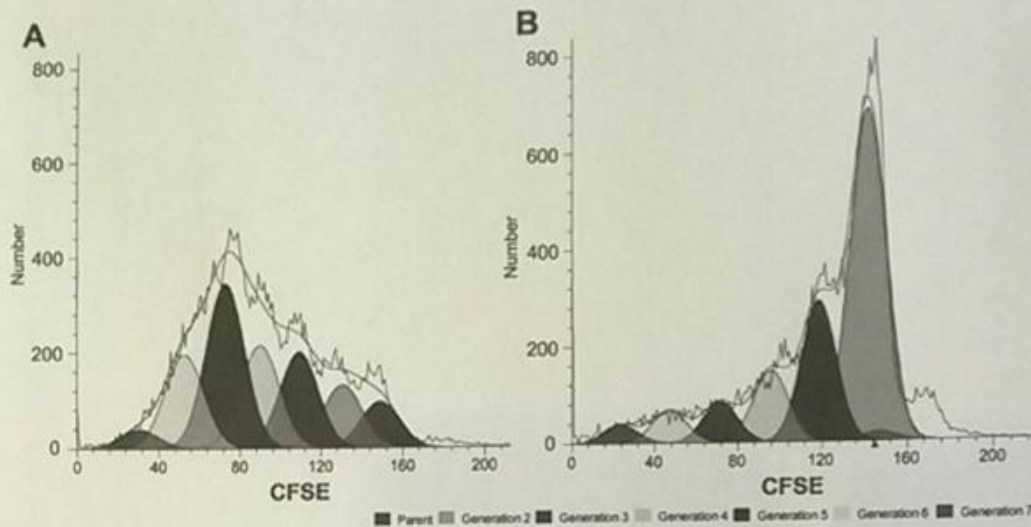
PHA-stimulated lymphocytes were cultivated for four days with or without MSC. The analysis of lymphocyte proliferation was performed with CFSE tracking and ModFit LT software. The addition of MSC reduced the numbers of lymphocytes dividing into daughter populations (**B**) in comparison to the culture without MSC (**A**); see Methods and Results for details; PARENT = parent generation, GEN2–8 = daughter generations.

Table 2. T-lymphocyte proliferation (CFSE tracking)

Generation*	no MSC	with MSC	P
Parental	17.41	50.95	< 0.0001
2nd	7.98	6.50	ns
3rd	12.92	13.01	ns
4th	32.82	10.68	0.0004
5th	29.85	10.09	0.0008
6th	12.52	10.05	ns
7th	4.58	8.90	0.0319

* percentage of cells in the generation (means)

tion of the percentage of cells detected in daughter generations (32.82 % vs. 10.68 % in generation 4, P = 0.0004 and 29.85 % vs. 10.09 % in generation 5, P = 0.0008). For details see Table 2 and Fig. 4A. Also the cumulative percentage of the lymphocyte population representation from parent to third generation was different (38.32 % without vs. 70.55 % with MSC, P < 0.0001) as shown in Fig. 4B.

The proliferation index, calculated as the sum of the cells in all generations divided by the number of parent cells, theoretically present at the start of the experiment, was decreased in the culture with MSC (1.84 vs. 3.65, P < 0.0001). The tests without MSC possessed a higher representation of the new daughter populations.

Cytokine and STAT protein expression analysis

The non-specific stimulation resulted in increased expression of all the tested cytokines (IL-6, IL-10 and IFN-γ). The addition of MSC lowered pro-inflammatory

IL-6 and IFN-γ expression on both CD3+/4+ (from 19.5 to 16.3 MFI; P < 0.0001; from 13.5 to 12.0 MFI; P = 0.0096) and CD3+/CD8+ lymphocytes (from 14.5 to 13.2 MFI; P = 0.0128; from 12.5 to 12.4 MFI; ns). The opposite effect was observed for production of interleukin-10. IL-10 expression was slightly increased in the presence of MSC; however, these changes were not statistically significant. Details are given in Table 3 and Figure 5.

Furthermore, we tested expression of four phosphorylated proteins of the STAT family – STAT1, STAT3, STAT4 and STAT6. After mitogenic stimulation, the STAT expression increased in all tests. The MSC presence was associated with a significant effect on the phosphorylated STAT protein expression. STAT proteins STAT1, 4 and 6 were silenced and their expression decreased significantly with MSC. In contrast, there was no effect on STAT3. For details see Table 4 and Figure 6.

Discussion

Human MSCs have generated considerable interest in the field of cellular therapy. Experimental evidence and preliminary clinical studies have demonstrated that MSC have an important immunomodulatory function. Several studies have been focused on their ability to treat acute or chronic graft-versus-host disease after allogeneic haematopoietic stem cell transplantation.

MSC-based products belong to advanced therapy medicinal products. ATMP reflect a complex and innovative class of biopharmaceuticals as these products are highly research-driven, characterized by innovative ma-

Fig. 4. Changes in stimulated lymphocyte proliferation due to MSC exposure
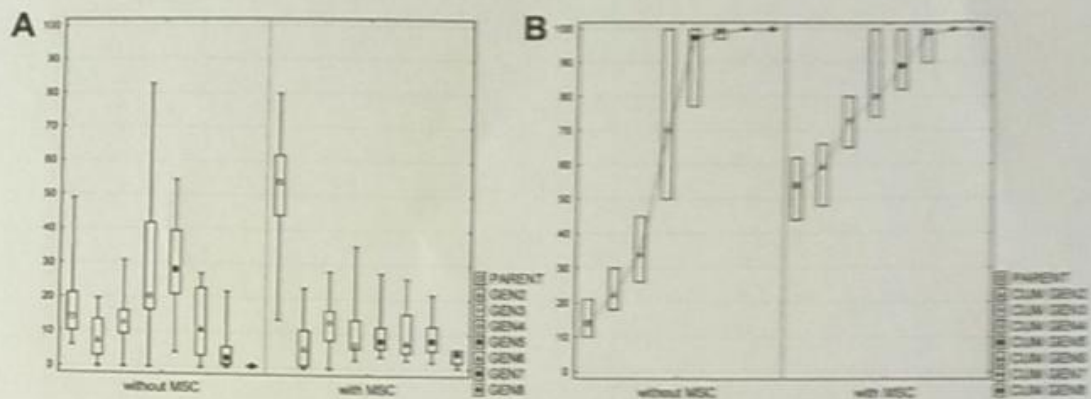
Lymphocytes labelled with CFSE were stimulated with PHA and cultivated with or without MSC (MSC/lymphocyte ratio 1 : 2). The presence of MSC reduced the proliferation rate of the stimulated lymphocytes, measured after four days of cultivation. **A. Parent and successive populations.** The number of cells retained within the parent generation was increased with MSC (50.95 % vs. 17.41 %, P < 0.001). **B. The cumulative percentage of lymphocyte populations.** Lymphocytes from parent to third generation accounted for 38.32 % (without MSC) vs. 70.55 % (with MSC) of all cells depending on the MSC addition to the culture (P < 0.0001). Median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PARENT = parent generation, GEN2-8 = daughter generations.

*Table 3.* Expression of cytokines on T lymphocytes

MFI	CD3+/CD4+					CD3+/CD8+				
	NK	PK	P[a]	MSC	P[b]	NK	PK	P[a]	MSC	P[b]
IL-6	3.5	19.5	< 0.0001	16.3	< 0.0001	1.0	14.5	< 0.0001	13.2	0.0128
IL-10	4.2	17.0	< 0.0001	17.4	ns	1.0	14.6	< 0.0001	15.2	ns
IF-γ	2.1	13.5	< 0.0001	12.0	0.0096	0.6	12.5	< 0.0001	12.4	ns

MFI – mean fluorescence intensity (median), NK – negative control (unstimulated lymphocytes), PK – positive control (stimulated lymphocytes without MSC), MSC (stimulated lymphocytes with MSC)
[a] negative vs. positive control
[b] positive control vs. lymphocytes co-cultured with MSC

Fig. 5. Comparison of cytokine expressions
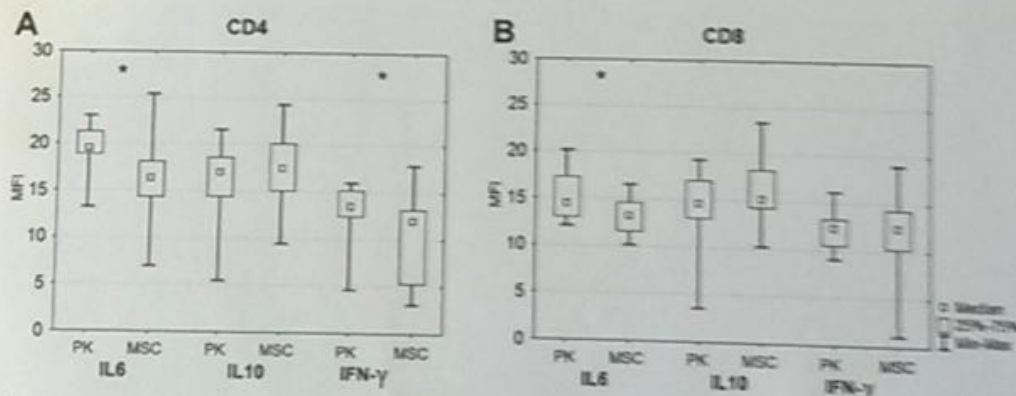
Lymphocytes were stimulated with Cytostim and monensin. The detection of intracellular cytokines was performed after 6 h of co-cultivation with or without MSC. The expression of pro-inflammatory IL-6 was reduced on both CD3+/CD4+ (19.5 to 16.3; P < 0.0001) and CD3+/CD8+ lymphocytes (14.5 to 13.2; P = 0.0128) in the cultures with MSC addition. IFN-γ expression was only significantly reduced on CD3+/4+ (13.5 to 12.0; P = 0.0096) lymphocytes. Wilcoxon test, median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PK = positive control.

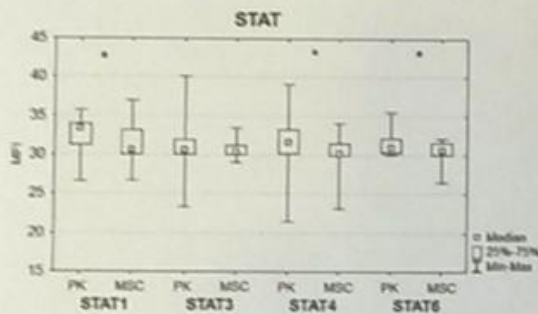*Table 4. Expression of phosphorylated STAT proteins on T lymphocytes*

MFI	NK	PK	P[a]	MSC	P[b]
STAT 1	0.5	33.4	< 0.0001	30.5	0.0335
STAT3	0.5	30.6	< 0.0001	30.5	ns
STAT4	0.4	31.6	< 0.0001	30.2	0.0058
STAT6	0.4	31.0	< 0.0001	30.5	0.0448

MFI – mean fluorescence intensity (median), NK – negative control (unstimulated lymphocytes), PK – positive control (stimulated lymphocytes without MSC), MSC (stimulated lymphocytes with MSC);

[a] negative vs. positive control

[b] positive control vs. lymphocytes co-cultured with MSC

Fig. 6. Comparison of expression of phosphorylated STAT proteins

Lymphocytes were stimulated with Cytostim and cultivated with or without MSC for 24 h. Staining with antibodies against phosphorylated STAT proteins was performed after cell membrane permeabilization and inhibition of phosphorylation enzymes. The expression of STAT1 (P = 0.0335) STAT4 (P = 0.0058) and STAT6 (P = 0.0448) was significantly reduced with MSC. Wilcoxon test, median; box: 25 %, 75 % quantiles; non-outlier min, non-outlier max; PK = positive control.

nufacturing processes and high complexity. The manufacture of ATMP cannot be controlled as precisely as the chemically synthetized small-molecule products. The analysis and quality control of these products are more complex in comparison to standard stem cell grafts as they comprise some functional potency assays.

We evaluated the immunomodulatory effects of MSC on PBMC when isolated from peripheral blood and activated by mitogens. The activation of lymphocytes is a complex cascade of events that results in the expression of several surface molecules, production and secretion of cytokines, lymphocyte proliferation, etc. There is still conflicting data in the literature regarding the mechanisms by which MSC modulate immune cells. Assessing the changes of immunophenotype and the functions of lymphocytes following immunotherapy provides information about the immune response mediated by MSC.

The resting lymphocytes from healthy donors show low or minimal expression of CD69, CD25 and HLA-DR.

The treatment with PHA leads to an increased time-dependent expression of these markers (Reddy et al., 2004). CD69's density grows from 3 to 12 h, reaches its maximum at between 16 and 24 h, and declines thereafter. The expression of CD25 and HLA-DR increases especially in the first 24 h after simulation and further grows until 72 h (Gibbons and Evans, 1996; Werfel et al., 1997; Arvá and Andersson, 1999). The peak is reached between days 4 and 8 (Caruso et al., 1997). Our analysis started 48 h after stimulation and monitored the period of decreasing expression after the presumed peak. A large number of studies have compared the activation marker expression within certain days of cultivation, while we focused on the entire kinetics of early (CD69) and late (CD25 and HLA-DR) antigens on the lymphocytes. We intended to take into account the dynamic nature of the activation process, where the regulation of surface protein expression accompany the transition to early, intermediate and later activated stages of the T-cell activation process.

CD25 antigen expression was down-regulated in our experiments on both CD4^{+} and CD8^{+} stimulated lymphocytes. This, similarly to the findings of Le Blanc et al. (2004), indicates a significantly lower expression of CD25 when MSC are present in the culture (Zheng et al., 2008). On the other hand, Maccario et al. (2005) reported increased numbers of CD4^{+}CD25^{high} lymphocytes in mixed lymphocyte reaction after the addition of MSC. The induction of "true" regulatory (FoxP3^{+} or CD127^{neg}) T cells, a lymphocyte subset with a presumed regulatory function, may be possibly mediated by different pathways in alloantigen- and mitogen-stimulated cultures. Moreover, alloantigen-reactive CD4^{+}CD25^{high} T cells may not be the principal mechanism responsible for the reduction of lymphocyte proliferation and cytolytic activity; probably a complex of other mechanisms, including release of multiple soluble factors, indoleamine 2,3-dioxygenase activity, etc., is involved.

The lymphocytes co-cultured with MSC also displayed a different CD69 expression pattern compared to PHA-stimulated cells without MSC. The maximum activation was observed on day +2, and was further maintained up to day +4 with only a slow decline. There are conflicting reports in the literature about the effect of MSC on the expression of CD69 by activated lymphocytes. Some studies observed inhibition or no significant alteration of CD69 expression in the presence of MSC (Le Blanc et al., 2004; Groh et al., 2005; Ramasamy et al., 2008). In our analysis, the down-regulation of CD69 along with the culture time was less pronounced in the presence of MSC. The potential role of CD69 as a regulatory molecule has been reported by other authors, who even observed an increase in CD69 expression during co-cultivation experiments (Saldanha-Araujo et al., 2012). The receptor may modulate the inflammatory response by inducing TGF-β production, which is known to induce expression of the *Foxp3* gene and generation of regulatory T lymphocytes. This finding is consistent with CD69 being a marker of cells with a regulatory po-

tential, and a stable or late increase of CD69 expression could define cells with immunomodulatory properties.

MSC influenced the expression levels of lymphocyte activation markers, and this inhibitory effect of MSC was evident on both the CD4+ and CD8+ T-cell subsets. Although a direct comparison is not possible due to different culture conditions, cell types, nature of the stimulus or culture period, both CD4+ and CD8+ T cells were equally inhibited by MSC in other studies as well (Di Nicola et al., 2002; Le Blanc et al., 2004). Nevertheless, the inhibitory effect of MSC on the expression of the activation markers in response to PHA stimulation has not been documented in some similar studies.

Ramasamy et al. (2008) suggested that the immunosuppressive effect of MSC is exclusively a consequence of anti-proliferative activity since the expression of CD25 and CD69 were not significantly altered by MSC in their study. We confirmed a strong effect of MSC on the reduction of proliferation of the stimulated lymphocytes. The observation is in agreement with those described in the literature, demonstrating that MSC inhibit lymphocyte growth and decrease the mean number of new generations (Ramasamy et al., 2008; Najar et al., 2009). MSC inhibit T-cell proliferation in a concentration-dependent manner. We used the MSC/lymphocyte ratio 1 : 2, and in other studies the ratio 1 : 1 to 1 : 10 also allowed most significant inhibition (Di Nicola et al., 2002; Najar et al., 2009).

The activation with PHA induces both Th1 and Th2 cytokines. MSC cause a cytokine profile shift in the Th1/Th2 balance towards the anti-inflammatory Th2 phenotype. We observed significant changes in the expression of factors proposed to be involved in GVHD development on the one hand and the immunomodulatory activity of MSC on the other hand – IFN-γ and IL-6. The activation of lymphocytes and IFN-γ secretion in mixed lymphocyte reaction or the ability of antigen-specific T cells to secrete IFN-γ against cognate antigen re-challenge is reduced when co-cultured with MSC (Groh et al., 2005; Ramasamy et al., 2008). The elevated serum levels of IL-6 have been found in patients with acute GVHD, and IL6 gene polymorphism studies have shown an association with increased GVHD severity after allogeneic transplantation (Cavet et al., 2001; Morris and Hill, 2007). Co-cultures with MSC contain higher levels of IL-6, and neutralizing this interleukin-6 reverses their inhibitory effect (Melief et al., 2013). The inhibition of IL-6 in donor T cells in an experimental allogeneic bone marrow transplantation model led to a reduction in GVHD-induced mortality and prolonged survival (Tawara et al., 2011). There was a slight but statistically insignificant induction of IL-10 in the co-cultures of MSC and lymphocytes compared to lymphocytes alone. The results concerning production of IL-10 by stimulated lymphocytes under co-culture with MSC differ between studies. Some authors confirmed the increased IL-10 levels after MSC addition (Groh et al., 2005; Jui et al., 2012); however, others recognized this effect only in mixed lymphocyte culture experiments

without any difference in IL-10 when stimulating lymphocytes with PHA (Rasmusson et al., 2005). The recent work by Melief et al. (2013) revealed that MSC do not produce IL-10, but that IL-10 detected in cell-free supernatants is exclusively produced by monocytes in the culture.

The STAT proteins are critical mediators of cytokine and growth factor signalling. These proteins transmit signals from a receptor complex to the nucleus and activate transcription of their target genes in response to the cell stimuli. STAT proteins play an important role in many cellular processes involved in cell proliferation, apoptosis, immune cell development, etc. The transcriptional activity is regulated mainly by STAT serine phosphorylation. We detected decreased expression of phosphorylated STAT1, which targets genes to promote inflammation and antagonize proliferation (Schindler et al., 2007). The expression of STAT4 and STAT6 was also reduced under MSC co-cultivation. STAT4 is important in the differentiation of naive T cells into IL-2/IFN-γ-producing Th1 cells (Ross et al., 2007). Both STAT3 and STAT4 regulate Th17 differentiation and expansion (Schindler et al., 2007; Durant et al., 2010).

STAT6 is critical for a number of responses in T cells, including cell proliferation and development of Th2 cells. When we observed the slightly increased expression of IL-10, we expected STAT6 up-regulation in the context of the anticipated shift to the Th2 phenotype. In contrast to previous views equating STAT6 with Th2 differentiation, it appears that this process probably involves more complex interactions of STAT3, STAT5 and STAT6 with relevant target genes (O'Shea and Plenge, 2012). The STAT proteins interact with numerous transcriptional regulators. Most cytokines activate more than just one STAT. Our simple in vitro test cannot describe the complexity of the JAK/STAT pathways. Despite this fact we have demonstrated that MSC affect the immune cell activity on a transcriptional basis.

We used a relatively low MSC/lymphocyte ratio (1 : 2) in our in vitro experiments. The inhibitory effect of MSC is known to be dose dependent. Based on the concentration, some authors observed that MSC possess two distinctive activities. MSC support lymphocyte proliferation at high MSC/lymphocyte ratios (1 : 40 and 1 : 80). The stimulatory activity only happens at these low MSC concentrations and is mediated, in particular, by soluble factors (Najar et al., 2009). MSC become suppressive at lower ratios and acquire an inhibitory profile responsible for T-cell suppression. The dual ability of MSC to either stimulate or suppress T-cell proliferation according to the ratio of cells should be considered in the context of their clinical utilization.

ATMP are used in clinical settings, targeting many conditions with unmet medical needs. Numerous challenges arise from the derivation and nature of ATMP products. Functional assays such as analysis of MSC impact on stimulated lymphocyte activation and proliferation represent a useful tool for the verification of the potency of manufactured MSC-based products.

Significant progress has been made in the understanding of MSC immunomodulatory functions. Their activity was confirmed in a broad range of *in vitro* series and preliminary clinical studies, as has been the case for GVHD. Some of these studies produced conflicting evidence regarding the mechanisms of the MSC functions or their ability to promote or inhibit immune responses. In the present study, we confirmed the inhibitory effect of *ex vivo* expanded MSC on T-cell proliferation and the activation triggered by mitogenic stimuli. We suggest a combination of tests suitable for *in vitro* confirmation of the immunomodulatory activity of MSC that are produced for clinical application. Future research should more closely focus on the interaction between MSC and the local host immune environment and other factors such as infections or relapses. These aspects can appear during the GVHD treatment and could play an important role in a real clinical scenario.

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ATTACHMENT IV

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.

Vliv mesenchymálních kmenových buněk na *in vitro* kulturu dospělých prasečích kardiomyocytů

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1 Úvod

Kardiální insuficience široké etiologie je jednou z vedoucích příčin úmrtnosti v lidské populaci (1). Po prodělání koronární příhody se často stav pacientů ještě dále zhoršuje, mnoho z nich nakonec dospěje k fatálnímu srdečnímu selhání (2). Díky vývoji v odvětví farmakoterapie, kardiochirurgie a intervenční kardiologie jsou možnosti léčby koronární insuficience široké, přesto se stále objevují nové případy, kdy je postižení srdeční tkáně příliš rozsáhlé a terapie tudíž nedostatečná (3).

Bylo prokázáno, že mesenchymální kmenové buňky (MSC) mají příznivý efekt na hojení srdeční svaloviny (1,3,4). Tento efekt může být dvojího charakteru, MSC mohou diferencovat do buněk srdeční svaloviny, kardiomyocytů (CMC), a/nebo podporovat funkci CMC produkcí bohaté směsi regulačních a podpůrných molekul (např. interleukin 10 (IL-10), vaskulární endoteliální růstový faktor (VEGF), fibroblastový růstový faktor (FGF) a další), a podpořit tak regeneraci srdce jako celku (5–8). I přes značné množství *in vitro* (9–11) a *in vivo* (6,12,13) experimentů ukazujících pozitivní vliv MSC na regeneraci srdeční tkáně, jsou výsledky klinických studií, kdy jsou MSC aplikovány pacientům se širokým spektrem kardiálních poškození, stále nejednoznačné (3,14,15). Pro úspěšnou transformaci aplikace MSC do běžné klinické praxe je tedy nutné detailněji pochopit působení MSC na CMC a přesněji definovat podmínky případného pozitivního působení MSC.

Cílem této práce bylo připravit *in vitro* kulturu dospělých prasečích CMC a charakterizovat vliv prasečích MSC na přežívání CMC v *in vitro* kultuře. Pro tuto studii byly vybrány prasečí buňky, protože jejich charakteristiky jsou velmi blízké lidským (16). V rámci

studie byla provedena série *in vitro* experimentů od izolace a kultivace CMC až po jejich morfologické pozorování a kokultivační studie s MSC.

2 Materiál a metody

Veškeré zacházení s experimentálními zvířaty probíhalo v souladu se směrnicí Evropského parlamentu a Rady 2010/63/EU o ochraně zvířat používaných pro vědecké účely. Všechny experimenty byly schváleny Odbornou komisí pro práci s laboratorními zvířaty Lékařské fakulty v Plzni.

2.1 Izolace a kultivace mesenchymálních kmenových buněk

Pro experimenty byly využity mesenchymální kmenové buňky (MSC) izolované z kostní dřeně dospělých prasat (Sus Scrofa, n=5).

Kostní dřeň byla získána aspirací z tuberositas tibiae prasat v celkové anestezii. Aspirát byl zředěn přidáním fosfátového pufru (PBS) v poměru 1:1 a opatrně navrstven na Ficoll-Paque^{TM} Plus (GE Healthcare, Velká Británie) v poměru 6:4. Po centrifugaci (435x g, 30 min) následoval odběr opalescentní vrstvy mononukleárních buněk do čisté 15 ml zkumavky (Techno Plastic Products, TPP, Švýcarsko). Buňky byly promyty PBS a centrifugovány (1000x g, 8 min). Po odstranění supernatantu byla peleta resuspendována v kompletním MSC médiu, jež se skládalo z α-modified Eagle's media (GE Healthcare, Velká Británie) obohaceného o 10% fetální bovinní sérum (FBS) (Biosera, Francie), penicilin (100 IU/ml) a streptomycin (100 μg/ml) (Biosera, Francie). Buněčná suspenze byla nasazena na kultivační plast (TPP, Švýcarsko) a kultivována v inkubátoru za standardních podmínek (37 °C, 5% CO_2). Po 48 hod byly odmyty volně plovoucí buňky a přisedlé MSC zůstaly v kultuře. Výměna média probíhala každé 2-3 dny.

2.2 Analýza fenotypu mesenchymálních kmenových buněk

Pro ověření, zda se jedná o MSC, byla hodnocena morfologie buněk pod světelným mikroskopem, byl proveden test diferenciace, kde bylo sledováno, zda se buňky dokážou diferencovat do tří základních linií (adipocytární, chondrocytární a osteocytární) a průtokovým cytometrem byla ověřena exprese znaků typických pro MSC (CD44^{pos}, CD73^{pos}, CD90^{pos}, CD45^{neg}).

2.2 1 Světelná mikroskopie

Pomocí světelného mikroskopu (Nikon Eclipse Ti, Japonsko) byly identifikovány MSC jako přisedlé buňky s protaženým tělem, obsahujícím velké kulaté jádro, a s několika výběžky.

2.2.2 Test diferenciace MSC

Po oplachu neadherentních buněk (48h po izolaci) byla část MSC pasážována užitím trypsinu, TrypLE^{TM} Select (Gibco, Life Technologies, Dánsko) a nasazena na 3 jamky 6jamkové destičky (TPP, Švýcarsko), kdy do každé jamky bylo přidáno jiné diferenciační médium. Pro diferenciaci do osteocytární linie bylo k MSC přidáno komerčně dostupné médium StemPro® Osteogenesis Differentiation Kit (Life Technologies, USA), pro diferenciaci do chondrocytární linie médium StemPro® Chondrogenesis Differentiation Kit (Life Technologies, USA) a pro diferenciaci do adipocytární linie médium StemPro® Adipogenesis Differentiation Kit (Life Technologies, USA). Výměna médií probíhala každé 3 dny. Po 14 dnech kultivace v adipo- a chondro- diferenciačním médiu, respektive 21 dnech v osteo- diferenciačním médiu byly buňky opláchnuty PBS a obarveny. Adipocytární diferenciace byla potvrzena barvením Oil Red O (Sigma-Aldrich, USA), které červeně barví tukové částice uvnitř buněk. Chondrocytární diferenciace byla potvrzena barvením Alciánovou modří (Sigma-Aldrich, USA), která barví modře mukopolysacharidy přítomné v chrupavkách. A konečně, osteocytární diferenciace byla potvrzena barvením Alizarin Red S (Sigma-Aldrich, USA), které červeně barví kalceinové molekuly produkované diferencovanými buňkami.

2.2.3 Ověření markerů MSC průtokovu cytometrií

Část MSC (100 000 buněk) sklizená s využitím TrypLE^{TM} Select (Gibco, Life Technologies, Dánsko) byla použita k fenotypické charakterizaci MSC v průtokovém cytometru. Buňky byly promyty PBS, resuspendovány ve 100 µl PBS a smíchány se 3 µl protilátek; anti-CD44 DAPI (Biolegend, USA), anti-CD45 FITC (Bio-Rad, USA), anti-CD73 PE-A (RnD Systems, Kanada) a anti-CD90 APC (Biolegend, USA) a inkubovány 15 min ve tmě při pokojové teplotě. Následně byla suspenze promyta PBS a buňky byly resuspendovány v 300 µl PBS. Cytometrická analýza byla provedena přístrojem BD FACS Aria Fusion (Becton Dickinson, USA) a výsledky vyhodnoceny BD FACS Diva 8.0.1 softwarem. Jako MSC byly hodnoceny všechny buňky pozitivní na markery CD44, CD73 a CD90, které byly zároveň negativní pro CD45.

2. 3 Izolace a kultivace kardiomyocytů

Kardiomyocyty (CMC) byly izolovány z levých komor mladých dospělých prasat (Sus Scrofa). Ze zvířete (n=5) v celkové anestezii bylo vyňato srdce, které bylo ihned ponořeno do ledově chladného Tyrodova roztoku bez přídavku vápníkových iontů (složení v mmol/l: NaCl 137; KCl 4,5; $MgCl_2$ 1; $CaCl_2$ 2; glukóza 10; HEPES 5; pH bylo pomocí NaOH upraveno na hodnotu 7,4; vše Sigma Aldrich, USA). Po kanylaci přední sestupné větve levé věnčité tepny bylo srdce zavěšeno na Langerdorffův aparát a promýváno teplými (37°C) okysličenými roztoky. Nejdříve Tyrodovým roztokem bez vápníku (5 min), poté Tyrodovým roztokem s vápníkem (0,5 μM), kolagenázou (1 mg/ml; Roche Diagnostics, Německo) a hovězím sérovým albuminem (BSA, 0,5 mg/ml, Sigma-Aldrich, USA) po dalších 30 min. Na závěr bylo srdce promyto Tyrodovým roztokem bez vápníku (5 min). Po odstranění endokardu byly CMC získány z rozvolněné stěny levé komory, umístěny do Tyrodova roztoku (37°C) bez vápníku a opatrně přefiltrovány přes gázu. Koncentrace vápníku byla postupně navyšována v několika krocích (1; 5; 10; 100; 200 μmol/l) vždy po 10 minutách až na výsledných 0,2 mM Ca^{2+}.

Po izolaci byly CMC resuspendovány v kompletním CMC médiu Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, USA) obohaceném o 10% FBS (Biosera, Francie), 4 500 mg/l glukózy (Sigma-Aldrich, USA), 4 mmol/l L-glutaminu (Biosera, Francie) a 100 IU/ml penicilinu a 100 μg/ml streptomycinu (Biosera, Francie). Vzniklá suspenze buněk pak byla použita pro následující experimenty.

2. 4 Zhodnocení výnosu izolace

Po každé izolaci byl zkušeným pozorovatelem, s využitím světelné mikroskopie, zhodnocen procentuální poměr živých CMC ku mrtvým buňkám v buněčné suspenzi. Za živé CMC byly považovány všechny buňky s protáhlým tvarem, ostře ohraničenými konci a příčným pruhováním.

2.5 Zvýšení podílu živých kardiomyocytů v kultuře

2.5.1 Kultivační plast

Pro efektivní oddělení živých CMC od dalších buněk suspenze a optimalizaci kultivačních podmínek byly testovány čtyři rozdílné kultivační povrchy; 1. běžný kultivační plast bez speciální povrchové úpravy (TPP, Švýcarsko), 2. běžný kultivační plast (TPP, Švýcarsko) potažený myším lamininem o koncentraci 1,5mg/ml (Sigma-Aldrich, USA), 3.

běžný kultivační plast (TPP, Švýcarsko) potažený 0,5% želatinou (Sigma-Aldrich, ČR), 4. kultivační plast speciálně vytvořený pro zvýšení přilnavosti buněk (Corning, Sigma-Aldrich, USA).

Suspenze buněk byla resuspendována v kompletním CMC médiu, rozdělena do 4 skupin, nasazena na výše zmíněný kultivační plast, a kultivována v inkubátoru za standardních podmínek (37°C, 5% CO_2).

Hodnocení přilnavosti buněk ve zmíněných kultivačních podmínkách proběhlo na celkem deseti vzorcích z každé skupiny, 2 opakování pro každé zvíře. Hodnocení prováděl zkušený pozorovatel s využitím světelného mikroskopu. Hodnotou z číselné škály 1 – 5, kdy jednička značila, že většina (> 90 %) buněk plavala v médiu bez přichycení k povrchu a pětka značila situaci, kdy většina buněk přisedala k povrchu, byla označena každá jamka ve druhém dni kultivace.

Pro všechny čtyři sledované kultivační povrchy byly výsledky statisticky zpracovány a vyhodnoceny, běžný kultivační plast bez potahu sloužil jako referenční hodnota.

2.5.2 Magnetická separace

Pro získání většího podílu živých CMC v buněčné suspenzi byla testována metoda magnetické separace pomocí manuálního magnetického buněčného separátoru MACS™ (Miltenyi Biotec, Německo) s využitím Dead Cell Removal Kitu (Miltenyi Biotec, Německo), který obsahoval magnetické mikrokuličky schopné se navázat na mrtvé a umírající buňky a pufr usnadňující tento proces. Suspenze buněk byla centrifugována (300x g, 10 min), supernatant odstraněn a buněčná peleta resuspendována v pufru s magnetickými mikrokuličkami z kitu. Po 15 min inkubace byla celá suspenze přenesena do kolonek separátoru. Při průtoku suspenze kolonkou došlo k zachycení mrtvých buněk s navázanými magnetickými mikrokuličkami a do zkumavky pod kolonkou byly sbírány pouze buňky bez navázaných kuliček, tedy živé CMC.

Před magnetickou separací i po ní bylo zkušeným pozorovatelem s využitím světelného mikroskopu zhodnoceno procentuální zastoupení živých CMC v buněčné suspenzi. Výsledky byly srovnány.

2.6 Fluorescenční barvení

Pro znázornění mitochondriální sítě byla použita látka MitoTracker® (579/599) (Molecular probes, Life Technologies, USA), která se po přidání do buněčné kultury a 30 min

inkubaci dočasně navázala na živé mitochondrie buněk. Jádra byla nabarvena látkou NucBlue® (360/460) (Molecular probes, Life Technologies, USA).

2.7 Kokultivační experimenty

MSC byly nasazeny na 6jamkovou destičku (TPP, Švýcarsko) v nasazovací hustotě 54 000 buněk/jamka v kompletním MSC médiu a kultivovány do druhého dne. Po odsátí média byl do poloviny jamek vložen transwell, košíček s membránou s 3 μm póry, Transwell® (Corning, Sigma-Aldrich, USA) a do všech jamek bylo dáno kompletní kultivační CMC médium se suspenzí CMC. Jako kontrola byly použity CMC kultivované bez MSC (Obr. 1).
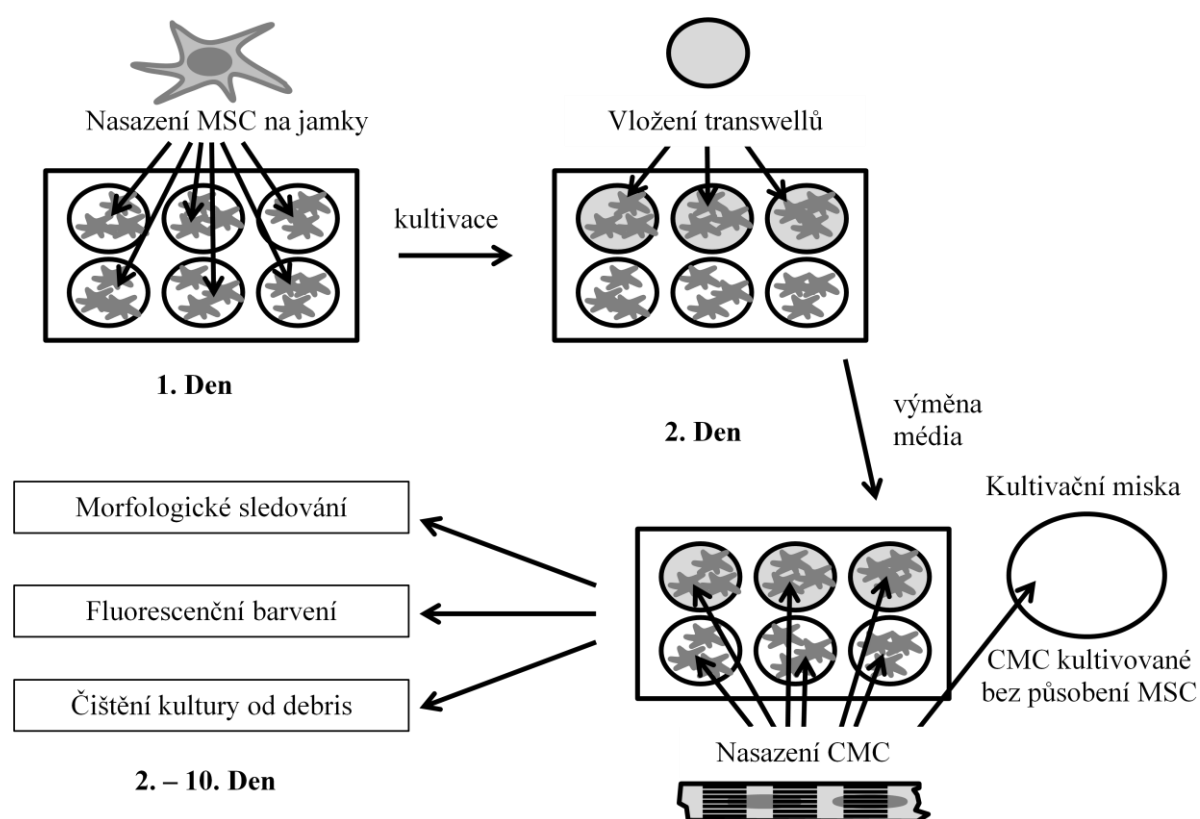
Obr. 1: Postup kokultivačního experimentu. V prvním dnu byly na kultivační plast nasazeny MSC. Další den byly do poloviny jamek vloženy transwelly. Čerstvé CMC pak byly rozděleny do tří skupin, část byla nasazena přímo k MSC, část k MSC přes transwelly a část byla nasazena na kultivační misky, pro kultivaci bez působení MSC.

2.8 Hodnocení přežívání buněk

Každý den až do 10. dne po izolaci byly CMC bez ovlivnění MSC, CMC v přímém kontaktu s MSC a CMC kokultivované s MSC přes transwelly sledovány pod světelným mikroskopem (Nikon Eclipse Ti, Japonsko).

V prvním a třetím dnu bylo prosté pozorování kvantifikováno. Na kultivačním plastu (n=20 pro každé zvíře) byly v zorném poli světelného mikroskopu, při zvětšení 10x spočítány

živé CMC (protáhlé buňky s příčným pruhováním a lomenými konci) a ostatní buňky. Výsledné procentuální zastoupení živých CMC v suspenzi ve všech opakováních u všech použitých způsobů kokultivace CMC s/bez MSC bylo statisticky vyhodnoceno.

3 Výsledky

3.1 Izolace, kultivace a fenotyp mesenchymálních kmenových buněk

Prasečí MSC lze izolovat dle uvedeného protokolu. Získané MSC vykazovaly charakteristický podlouhlý tvar s několika výběžky a přisedaly ke kultivačnímu platu.

Kultivace MSC s diferenciačními médii potvrdila schopnost buněk diferencovat do tří základních linií. Buňky diferencované do adipocytární linie obarvené Oil Red O vykazovaly červené zbarvení tukových vakuol. Buňky diferencované do chondrocytární linie produkovaly mukopolysacharidy, které se obarvením Alciánovou modří zabarvily modře. Buňky diferencované do osteocytární linie produkovaly kalceinové molekuly, které se po obarvení Alizarin Red S zbarvily do červena (Obr. 2).

Ověření povrchových markerů buněk průtokovým cytometrem prokázalo, že v průměru více jak 98 % buněk v kultuře bylo pozitivní pro markery typické pro MSC (CD44, CD73 a CD90) a zároveň negativní pro marker krevní řady (CD45) (Obr. 3).

3.2 Izolace a kultura kardiomyocytů

Primární prasečí CMC lze izolovat dle uvedeného protokolu. Získané buňky vykazovaly charakteristický fenotyp - protáhlý tvar, ostře lomené konce a příčné pruhování. Problémem byl vysoký podíl okolních mrtvých buněk a buněčné drti, výnos živých CMC byl pouze 20 % (± 10 %).

3.3 Zvýšení podílu živých kardiomyocytů v kultuře

CMC pěstované na běžném kultivačním plastu potaženém želatinou a také lamininem prokázaly statisticky významně (p = 0,00068 pro želatinu, p = 0,00011 pro laminin) vyšší přilnavost ve srovnání s CMC pěstovanými na běžném kultivačním plastu bez potahu či na výrobcem speciálně upraveném plastu pro zvýšenou přilnavost buněk (viz tabulka 1). Společně se zvýšenou přilnavostí CMC bylo pozorováno také zvýšené přisedání dalších buněk suspenze, prostá kultivace na různě upraveném kultivačním plastu tedy nepomohla ke zvýšení podílu živých CMC v kultuře.

TYP POVRCHU	POTAH	PŘILNAVOST CMC (průměr ± SD)	PŘILNAVOST OKOLÍ (průměr ± SD)
Běžný kultivační plast	-	1,8 ± 0,63	1,8 ± 0,63
Běžný kultivační plast	želatina	3,5 ± 1,08 *	3,5 ± 1,08 *
Běžný kultivační plast	laminin	3,8 ± 1,03 *	3,8 ± 1,03 *
Speciálně upravený povrch výrobcem	-	2,2 ± 0,92	2,2 ± 0,92

Tabulka 1: Srovnání přilnavosti buněk na jednotlivých typech užitého kultivačního plastu s různou povrchovou úpravou. Hodnoceno číselnou škálou zkušeným pozorovatelem, škála 1 – 5, kdy 1 značí většinu plovoucích buněk (> 90 %) a 5 většinu přisedlých.
* Statisticky významný rozdíl (t–test $p < 0{,}001$) v porovnání s běžným kultivačním plastem bez potahu.

Užití magnetické separace CMC z buněčné suspenze pomocí Dead Cell Removal Kitu bylo časově i finančně náročné, avšak umožnilo zvýšit podíl živých CMC v kultuře z iniciálních 20 % (± 10 %) na výsledných 75 % (± 15 %), s malou ztrátou CMC (15 % ± 5 %).

3.4 Morfologické změny kardiomyocytů v čase

Čerstvě izolované CMC mají dlouhý trabekulární tvar s jasně ohraničenými lomenými konci, jejich příčné pruhování je jasně viditelné (Obr. 4a). V průběhu kultivace se lomené konce pomalu zakulacují a příčné pruhování mizí (Obr. 4b). Po 3 dnech kultivace se u CMC začínají projevovat patologie ve fenotypu (Obr. 5), ale mnoho buněk si CMC fenotyp stále zachovává. Po 9. dni kultivace již byly morfologicky normální CMC pozorovány jen zřídka.

Užití MitoTrackeru pro fluorescenční mikroskopii ukázalo, že CMC obsahují mitochondrie s dostatečným mitochondriálním membránovým potenciálem pro efektivní oxidaci až do 3. dne po izolaci (Obr. 6), v dalších dnech pak byl pozorován prudký propad ve sledovaném fluorescenčním signálu.

3.5 Kokultivace kardiomyocytů s mesenchymálními kmenovými buňkami

CMC kultivované v přímém kontaktu s MSC ve zvýšené míře přisedaly ke kultivačnímu plastu a také k MSC, kdy společně vytvářely mnohovrstevnou kulturu. Tento způsob kultivace znemožnil hodnocení či použití buněk pro další experimenty, protože nebylo možné buňky od sebe vzájemně oddělit bez jejich poškození. Nicméně, dle pozorování lze říci, že při tomto způsobu kultivace buňky nejdéle přežívají a uchovávají si svůj tvar, pro ztíženou kvantifikaci bylo však toto pozorování nevyhodnotitelné.

CMC kultivované s MSC s využitím transwellů, bez možnosti přímého buněčného kontaktu, si uchovávaly své morfologické vlastnosti přibližně o den déle než CMC

kultivované samostatně. Kvantifikace s využitím zkušeného pozorovatele a světelné mikroskopie ukázala nerovnoměrné rozložení dat, proto byla použita neparametrická statistika, Wilcoxonův test. U CMC kultivovaných bez působení MSC byl přítomen významný rozdíl v počtu živých CMC mezi 1. a 3. dnem hodnocení (p = 0,0196). MSC kladně ovlivňovaly počet živých CMC ve vzorku, ve srovnání s CMC kultivovanými samostatně, ale v 1. dni nebyl rozdíl statisticky významný. Ve třetím dni kultivace již MSC statisticky významně (p = 0,0152) přispívaly k delšímu přežívání CMC, ve srovnání s CMC kultivovanými bez MSC (Graf 1.).
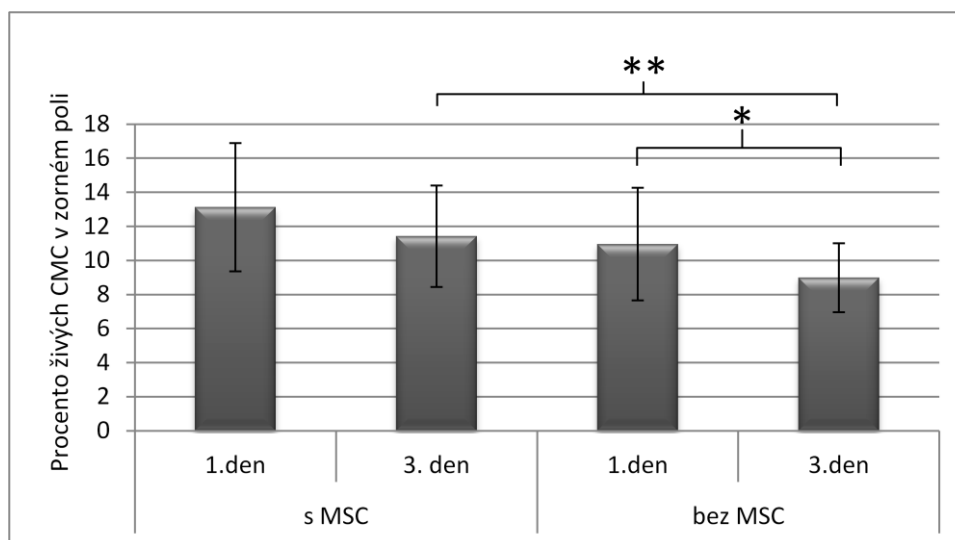
Graf 1. Srovnání procentuálního zastoupení živých CMC v kultuře s/bez MSC. Počet živých CMC v obou kulturách s časem klesá.
* Úbytek živých CMC kultivovaných bez působení MSC při srovnání 1. a 3. dne kultivace byl, dle Wilcoxonova testu, statisticky významný (p = 0,0196).
** Při srovnání počtu živých CMC v kultuře s/bez MSC byl ve třetím dni, dle Wilcoxonova testu, statisticky významný (p = 0,0152).

4 Diskuze a závěr

Vztahu a vzájemnému působení MSC na CMC se věnuje mnoho autorů (1-10). Většina prací je však provedena na malých zvířecích modelech a při přenosu získaných poznatků na člověka pak dochází k častým diskrepancím (14). Z důvodu co největší podobnosti s člověkem bylo pro naše experimenty zvoleno prase domácí, jehož kardiální charakteristiky jsou lidským velice blízké (16).

Izolace primárních prasečích CMC se ukázala být velmi obtížnou, proces vyžadoval mnoho optimalizačních pokusů, a přesto výsledek nebyl plně uspokojivý. Zaznamenali jsme pouze nízké procento živých CMC v suspenzi buněk ve srovnání s výsledky izolace CMC publikovaných u menších zvířat (17).

Z literatury vyplývá, že MSC jsou schopné přisedat ke kultivačnímu povrchu, čímž po výměně média dojde k vyčištění kultury a zvýšení procentuálního zastoupení CMC v kultuře (18,19). Vyzkoušeli jsme 4 různé kultivační povrchy, kdy CMC kultivované na běžném kultivačním plastu přisedaly jen minimálně, za to CMC kultivované na upraveném povrchu (laminin, želatina) přisedaly statisticky významně více. Oproti literatuře však v našich experimentech přisedaly také ostatní buňky suspenze (19) a to přímo úměrně CMC, tedy, čím více CMC přilnulo k povrchu, tím více buněk dalších buněk přilnulo také. Tímto způsobem tedy nebylo možné buněčnou kulturu CMC vyčistit.

Metoda magnetické separace zvýšila podíl živých CMC v suspenzi výrazně efektivněji, její širší použití bylo však pro časovou i finanční náročnost opuštěno.

Adultní CMC v kultuře běžně žijí 2-3 dny a poté dochází k dediferenciaci a postupnému zániku buněk (18,20). Naše experimenty tato zjištění potvrzují, kokultivace s MSC kardiomyocytům svědčí. Kultivace CMC společně s MSC, s umožněním buněčného kontaktu, se zdála být nejvíce efektivní pro přežívání CMC, avšak s buňkami již nebylo možné dále pracovat, protože došlo ke spojení s MSC, a nebylo možné je dále oddělit bez jejich poškození. Přijatelným kompromisem v podpoře přežívání CMC se ukázala kokultivace CMC s MSC prostřednictvím transwellů. Přímý buněčný kontakt byl sice znemožněn, avšak dá se předpokládat parakrinní působení MSC na CMC. Zatímco CMC kultivované bez MSC měly mezi 1. a 3. dnem po izolaci statisticky významný úbytek živých CMC, u CMC kultivovaných s MSC významný rozdíl pozorován nebyl. CMC kokultivované s MSC vykazovaly lepší přežívání než CMC kultivované bez jejich ovlivnění, ve třetím dni byl pak rozdíl statisticky významný.

Závěrem lze říci, že izolace prasečích CMC je komplikovaná avšak reálná. Buněčná suspenze obsahuje mnoho elementů mimo živé CMC, které nelze příliš efektivně odstranit prostou kultivací, lze však využít i jiné metody, jako například magnetickou separaci buněk. Po celou dobu byl u CMC kultivovaných s MSC patrný podpůrný trend MSC, ve srovnání s CMC kultivovanými samostatně, ve třetím dni pak již byl rozdíl statisticky významný. Z našich výsledků vyplývá, že MSC podporují přežívání CMC.

**Dedikace**

Obrazová příloha:
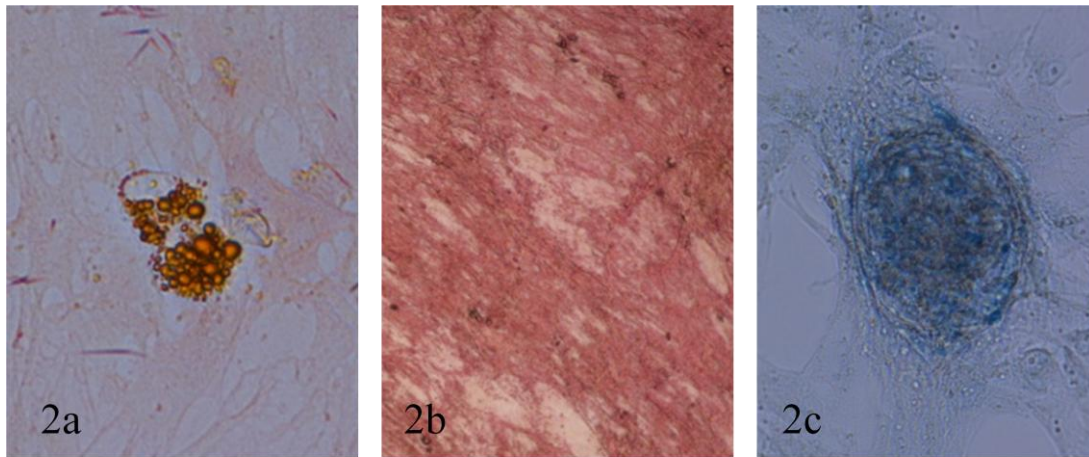
Obr. 2. Kontrola diferenciačního potenciálu MSC. 2a - MSC diferencované do adipocytární linie – oranžově zbarvené tukové vakuoly barvené Oil Red O. 2b – MSC diferencované do osteocytární linie – červeně zbarvené kalceinové struktury barvené Alizarin Red S. 2c – MSC diferencované do chondrocytární linie – modře zbarvené mukopolysacharidy barvené Alcian blue.
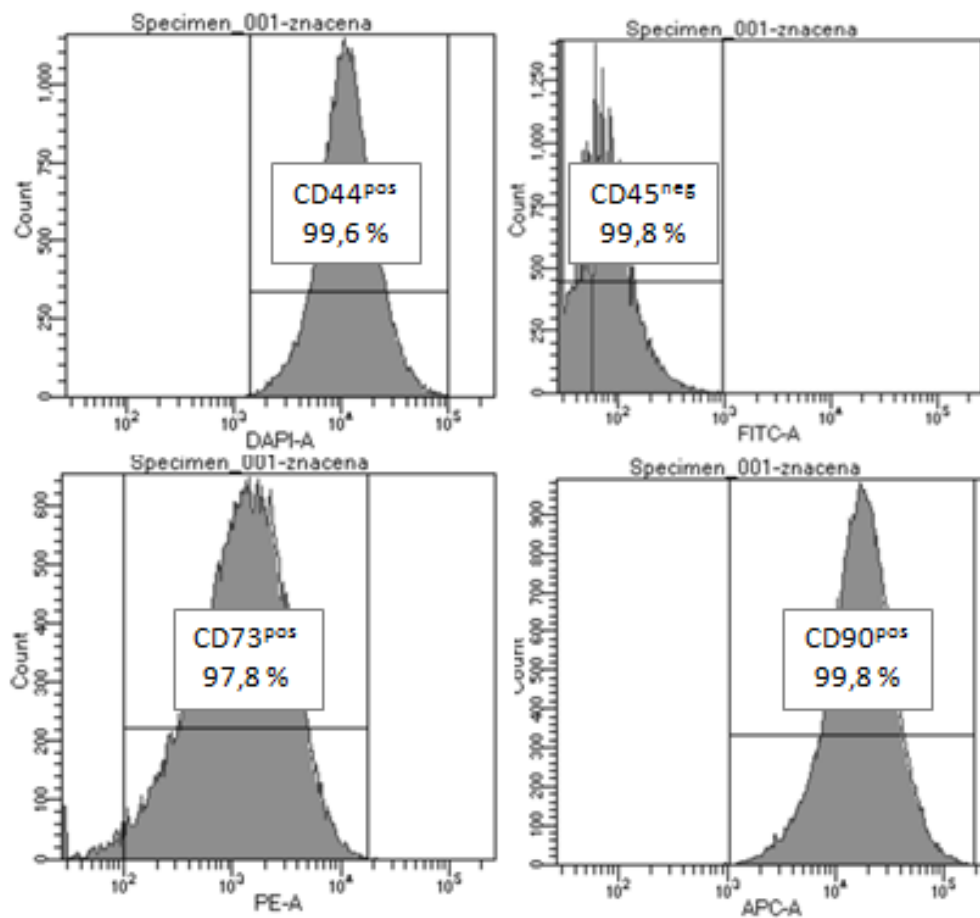

Obr. 3: Stanovení MSC markerů průtokovou cytometrií. Sledovaná suspenze buněk obsahovala průměrně 98 % MSC, tedy buněk pozitivních na CD44, CD73, CD90 a negativních na CD45.

Obr. 4: Morfologie normálních kardiomyocytů. 4a – čerstvě izolovaný kardiomyocyt s podélným tvarem, ostrými okraji a jasným příčným pruhováním. 4b –kardiomyocyt po 3 dnech v kultuře, kdy mají buňky stále ještě protáhlý tvar, ale již došlo k zaoblení konců a pomalu se vytrácí příčné pruhování. Zvětšení 40x.
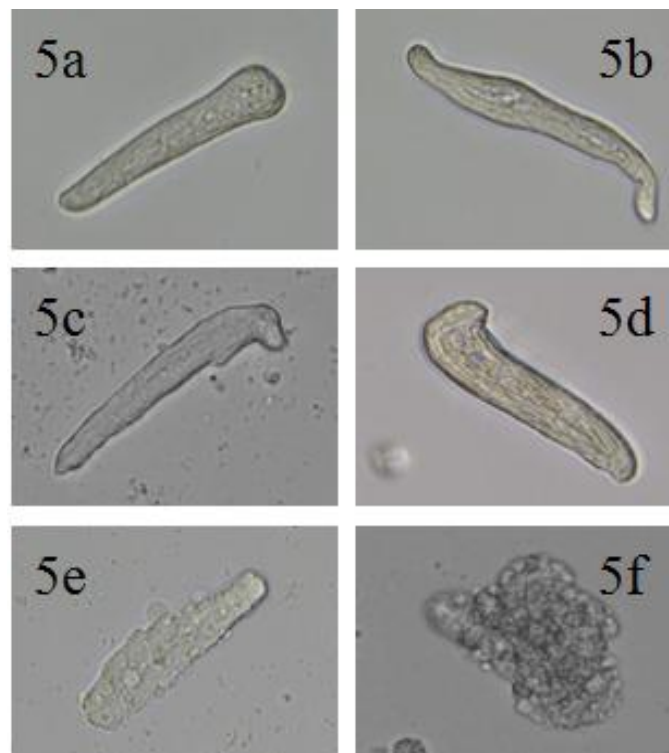
Obr. 5. Morfologické deformace CMC. Obrázky ukazují morfologickou deformaci CMC, která začíná po třetím dni kultivace (3 – 10 den). a – d, deformující se CMC, e – umírající CMC, f – mrtvý CMC. Zvětšení 40x.
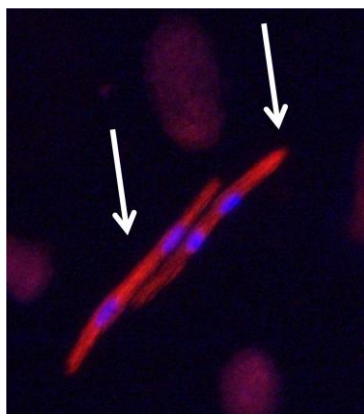
Obr. 6. Fluorescenční barvení mitochondrií CMC ve 3. dni kultivace. Červeně - funkční mitochondrie, modře - jádra buněk. Šipka označuje živé CMC. Zvětšení 40x.

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ATTACHMENT V

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).

Mesenchymal stem cells as the near future of cardiology medicine – truth or wish?

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Abstract

Cardiac damage is one of major cause of worldwide morbidity and mortality. Despite the development in pharmacotherapy, cardiosurgery and interventional cardiology, many patients remain at increased risk of developing adverse cardiac remodeling. An alternative treatment approach is the application of stem cells. Mesenchymal stem cells are among the most promising cell types usable for cardiac regeneration. Their homing to the damaged area, differentiation into cardiomyocytes, paracrine and/or immunomodulatory effect on cardiac tissue was investigated extensively. Despite promising preclinical reports, clinical trials on human patients are not convincing. Meta-analyses of these trials open many questions and show that routine clinical application of mesenchymal stem cells as a cardiac treatment may be not as helpful as expected.

This review summarizes contemporary knowledge about mesenchymal stem cells role in cardiac tissue repair and discusses the problems and perspectives of this experimental therapeutical approach.

1 Introduction

Cardiac diseases remain a major cause of worldwide morbidity and mortality [1]. In the United States of America every 34 seconds somebody suffers a coronary event [2]. Cardiac function of these patients is increasingly compromised with the progression of adverse cardiac remodeling and many patients eventually develop a fatal end-stage cardiac failure [3].

Progress in cardiovascular pharmacotherapy, cardiosurgery and interventional cardiology decreased mortality rate in cardiac diseases, but patients still remain at high risk of cardiac failure, especially when the damaged cardiac mass is large and the extensive cardiac cell loss is not compensated properly [4]. Only known effective treatment of cardiac diseases is heart transplantation, where donor shortage is a great problem [5]. New alternative approaches of endogenous repair have been investigated in adult mammalian hearts [6], consisting of mechanisms that involve mobilization of bone marrow and blood-derived progenitor cells [7], *in situ* turnover of regular cardiomyocytes [8], and the presence of resident cardiac stem cells having the ability to differentiate into vascular and mature cardiac cells [6]. However, all these mechanisms are not sufficient to prevent deleterious re-modeling of cardiac tissue.

Stem cells based therapies are now in worldwide interest as a promising treatment of various diseases [9], including cardiovascular diseases, where intensive research is performed [10]. Initially, the goal of stem cell based therapies was to provide a source of proliferating and functional cardiomyocytes, which will substitute cardiac cell loss and minimize damaged area. This aim has not been achieved to date. For clinical application various stem cell types are relevant, but their capability of creating mature cardiomyocytes *in vivo* is limited [11]. Therefore, stem cell based therapy aims have been expanded to more areas, including prevention of myocardial inflammatory and stress responses, improvement of myocardial perfusion via neovascularization, prevention of myocardial apoptosis and correction of metabolic and electromechanical disturbances [4].

Many cell types were investigated for cardiovascular repair properties and the most of attention was payed on stem cells exhibiting self-renewal, high replicative potential [12-18] (Table 1). Promising results were obtained in animal models by application of human embryonic stem cells [19] or cardiomyocytes derived from induced pluripotent stem cells [20]. However, it was not possible to verify these results in patients, because of ethical concerns and high oncogenic risks [21]. More easily available stem cell types for effective clinical application include hematopoietic stem cells, adipose

tissue derived cells or mesenchymal stem cells (MSCs), which undergone both preclinical and clinical testing successfully [22, 23]. Therefore, this review focuses on current knowledge, achievements and failures of MSCs application in cardiac tissue repair.

2 MSCs characteristics and sources

According to the minimal criteria of Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, MSCs are defined as adherent fibroblast-like cells expressing CD105, CD73 and CD90, not expressing CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR and with the ability to differentiate into adipocyte, osteoblast and chondrocyte cell types [24]. They can be found in different tissues including bone marrow [25], cord blood [26], placenta [27], fat [23], skin [28], muscle [29], tendon [30], synovium fluid [31] or teeth [32]. In opposite, MSCs are rarely detected in peripheral blood [33]. Further more, some studies indicate that the whole body MSC distribution can originate in a perivascular origin of MSCs. Perivascular CD146^{pos} cells isolated from many tissues (muscles, pancreas, fat, etc.) express MSC surface markers (CD73, CD90, CD105) and differentiate into osteo-, chondro- and adipolineage [34]. These findings suggest that distribution of MSCs in adult organism is related to their existence in the perivascular niche [33].

2.1 MSCs source variability

Although MSCs from different sources express the same set of surface markers and differentiate into three mesodermal lineages, various abilities are reported.

Bone marrow is a rich source of cells and the success rate of MSC isolation from it is nearly 100%. Bone marrow derived MSCs are able to proliferate *in vivo* and also *in vitro*, where their growth is reported to be arrested around 11-12 passage. According to colony forming unit-fibroblast assay (CFU-Fa), these MSCs form 16.5 ± 4.4 colonies in third passage [35]. In heart regeneration research, bone marrow MSCs improve heart regeneration after myocardial infarction in many species, reduction in scar size together with improved heart function was reported [14, 18, 20]. As they were discovered first [36], majority of MSCs characteristics was found through experiments with bone marrow derived MSCs. Those characteristics represent standards for comparison up to date.

Adipose tissue contains 500 times more stem cells in 1g of fat than in 1g of bone marrow. Many people undergo liposuction voluntarily, so it is easy to obtain material for adipose tissue MSCs isolation. Adipose tissue derived MSCs showed similar cardio protective potential as bone marrow MSCs when applied to doxorubicin treated diabetic rat model [37]. On the other hand it has been found that proliferation potential, growth rate and culture time of adipose tissue derived MSCs is lower. CFU-Fa showed only 6.4 ± 1.6 formed colonies in third passage, cell growth was arrested around passage 11 [35]. Evenmore, it has been shown that adipose derived MSCs possess different abilities according to the tissue of origin [38],[39]. Comparison of MSCs from abdominal fat, mesodermal origin, eyelid adipose tissue MSCs, and ectodermal origin, showed different phenotypes of cells together with variety in CD90 expression, suggesting higher abdominal fat MSCs response to angiogeneic factors [38]. Comparison of cardiac adipose tissue derived MSCs and abdominal fat MSCs, both of mesodermal origin, showed that cells were phenotypically identical, but cardiac MSCs constituted intristic properties toward myogenesis and vasculogenesis in significantly higher percentage and therefore have much better regenerative potential, especially for cardiac therapy [39].

Umbilical cord blood is a rich source of cells, whith MSCs also being present. MSCs isolation and cultivation from this source is complicated and the success rate of isolation is 63% [35]. If successfull, their culture lasts for long time periods, their proliferation is arrested at passage 14-16. CFU-Fa showed highest ability to form colonies (23.7 ± 5.8) [35] compared to others, but there are also evidence that in culture these MSCs display very low proliferation ability [39]. It was shown that MSCs from umbilical cord together with MSCs from amniotic membrane posses higher immunomodulatory capacity, based on gene expression profiling [40] than bone marrow MSCs.

MSCs from all three sources mostly used in research possess promising abilities for regenerative medicine, but, as it was mentioned before, they all have limits. Low-yielding isolation and complicated cultivation of umbilical cord MSCs makes them a not reliable source of cells. Easy isolation and cultivation of adipose tissue derived MSCs is very promising, but tissue specific effect

of MSCs from different adipose tissue sites makes them too variable. Human cardiac fat MSCs, showing the best qualities for cardiac regeneration, are hard to obtain and not convenient for detailed research. Therefore bone marrow-derived MSCs, a well documented type of MSCs, are in center of interest in cardiac regeneration research and are further discussed in more details.

2.2 Aging of MSCs

Important issue about therapeutical MSCs application is their aging. *In vitro* cultured MSCs obtained from older individuals, are larger, broader, flatten and show no spindle-formed morphology contrary to younger spindle shaped MSCs [41]. Aged MSCs contain more stress actin fibres, form small colonies and show telomerase deficiency [42]. Young MSCs are capable to reach 30-40 times maximal population doubling, aged MSCs have significant decline in replicative lifespan [43]. Aged MSCs also express different levels of various regulatory molecules. MSCs emission of pro-inflammatory interleukin 6 (IL6) increases with age [44], whereas production of anti-inflammatory and cell protective interleukin 11 (IL11) decreases with age [45]. Finally, aged MSCs have lower differentiation ability and proliferation potential [46, 47] and the age related loss of regenerative potential of MSCs is even dependent on the source of MSCs [47].

3 In vitro studies

Based on both *in vitro* and *in vivo* studies, three main mechanisms of action of MSCs implementation in cardiac tissue reparation process are suggested – differentiation into functional cardiomyocytes (CMCs), paracrine and immunomodulatory effect (Fig. 1).

3.1 Differentiation of MSCs to CMCs

MSCs show ability to differentiate into CMCs *in vitro* [48]. This differentiation can be induced by addition of 5-azacytidine, retinoic acid and dimethyl sulfoxide (DMSO) into cultivation media [49, 50]. When MSCs are treated by 5-azacytidine they start to be positive for desmin and α-sarcomeric actin. Later, they show presence of sarcoplasmic reticulum, T-tubules and intercalated disc-like structures [51]. When MSCs are stimulated to CMC diffrentiation the expression of nesprin-1 protein is higher, which suggests it plays an important role in mediating MSCs differentiation [50]. MSCs can differentiate into CMCs also without 5-azacytidine, but the presence of insulin-like growth factor 1 (IGF-1), fibroblast growth factor 4 (FGF-4), hepatocyte growth factor (HGF), transforming growth factor β (TGF-β1) and bone morphogenic protein 2 (BMP-2) is required [52]. Even only cell-to-cell contacts of MSCs and isolated CMCs are able to support MSC differentiation into new CMCs [53]. It is further documented that N-cadherin (CD325) negative fraction of MSCs has lower CMCs differentiation potential than N-cadherin positive fraction of MSCs, which expresses significantly elevated mRNA levels of cardiomyogenic progenitor-specific transcription factors, including Nkx2.5, Hand1, and GATA4 [54].

3.2 Paracrine and immunomodulatory effect of MSCs on CMCs

Bioactive molecules released by MSCs can positively modulate the functions of CMCs by paracrine and trophic mode. Cytokines from interleukin 6 (IL6) family secreted by MSCs bind to receptor glycoprotein 130 (gp130) and activate the JAK-STAT3 signaling pathway which results in increased expression of STAT3 targets hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) [55]. Conditioned media from MSCs can also protect CMCs from apoptosis when it inhibits caspase-3 activation and the release of cytochrome C from the mitochondria. These findings suggest that MSCs paracrine signalling helps to protect CMCs by interfering with mitochondria-mediated apoptotic pathway [56]. Factors released by MSCs can also protect CMCs from ischemia, when MSCs conditioned media decreases the numbers of apoptotic cells, the numbers of dead cells and improves CMCs metabolic activity. These improvements are regulated via Akt, ERK1/2 and STAT3 signaling pathways [57].

4 Animal studies

The large number of studies investigating MSCs influence on variety of heart diseases on animal models has been done. Small animal models as rat, mouse and rabbit were used mostly and approximately 56% of studies were performed on rat heart disease models. Large animal models as swine or sheep were used in 29% of all preclinical studies [5].

Majority of studies investigate the effect of MSCs on heart function or explore the role of MSCs in the repair of acute as well as chronic heart failure such as myocardial infarction [58], dilatation cardiomyopathy [59] or Chagas disease [1].

4.1 Differentiation of MSCs to CMCs

As both cell types, MSCs and CMCs are of mesenchymal origin, it can be easily expected that MSCs retain the ability of differentiation into CMCs. This is evidenced in many studies with various experimental designs. In the rat model of myocardial infarction (MI) MSCs are infected via tail vein and at four days MSCs engraft MI injured area. Engrafted MSCs expresses cardiac troponin T, endothelial CD31 and smooth muscle major histocompatibility complex (sm-MHC) suggesting MSCs differentiation into all major cells of cardiovascular lineage. Moreover, hearts treated by MSCs show improved cardiac features such as left ventricular ejection, end diastolic and end systolic volume or left ventricular myo-mass [60]. Another study, where green fluorescent protein (GFP) labeled MSCs (GFP-MSCs) are injected into mouse model of MI shows that over 60% of GFP-MSCs co-expressed collagen type IV and troponin T or myosin heavy chain, characteristic for MSCs and cardiomyocytes, respectively, and were CD45^{neg}. This study further demonstrates that MSCs can differentiate into CMCs in various extent when nearly 25% of GFP-MSCs express one of two cardiomyocyte markers in the absence of MSCs characteristics. Despite the lower differentiation properties of GFP-MSCs, myocardium treated by these cells shows improved left-ventricular and end-diastolic pressure [61]. In the study employing porcine model of MI, MSCs overexpressing integrin-linked kinase (ILK-MSCs) improve ventricular remodelling and cardiac function by increased CMCs proliferation, cardiac angiogenesis and reduced apoptosis [10].

In contrary, some studies indicate that the differentiation of MSCs into functional CMCs hardly or even not at all occurs. MSCs overexpressing Akt (Akt-MSCs) injected into mouse model of infarcted myocardium engraft the infarcted area at higher extent than MSCs, but only rare differentiation of both types of MSCs into functional CMCs is observed. Despite this, Akt-MSCs restore early cardiac function and decrease infarct size indicating another mechanism of MSCs facilitated tissue repair [62]. In another study is demonstrated that MSCs injected into mouse infarcted myocardium migrate into site of injury and survive there for 14 days, but no significant differentiation into functional CMCs or improvement of cardiac function is detected. Evenmore, same MSCs treated by pro-cardiomyogenic agents or by co-culture with beating CMCs do not differentiate into new CMCs [63].

4.2 Paracrine and immunomodulatory effect of MSCs on cardiac tissue

Despite the inconsistent results in the ability of MSCs to differentiate into CMCs, some studies show that MSC transplantation improves cardiac functions. In these cases, the paracrine and immunomodulatory effect of MSCs on cardiac repair is more likely.

In many animal experiments, the application of MSCs after MI had a possitive effect on cardiac functions in comparison with controls. Improvement in left ventricular ejection fraction, reduction in infarct scar size and inhibition of left ventricle remodeling was observed together with decrease in end-systolic and end-diastolic volumes [64-66]. Anyway, the particular cellular mechanisms and regulating molecules or signalig pathways responsible for the cardiac function improvement remain undetailed. The decrease in CMCs apoptosis rate, decrease in inflammation and scar formation and increase in CMCs proliferation and cardiac tissue neovascularization are described as the most probable cellular mechanisms [67]. Particulary, diabetic rats treated by anti-cancer drug doxorubicin (DOX) possesing cardiotoxicity were co-treated by MSCs. MSCs prevented DOX-induced myocardial damage and significantly induced angiogenesis and reduced immune cell infiltration and collagen deposition [37]. In MI rat model, injected MSCs increased levels of angiogenic factors FGF-2, VEGF

and stem cell homing factor (SDF-1α) in infarcted hearts. This was followed by declined CMCs apoptosis, increased capillary density and improved left ventricular contractility [68]. In another study, mice suffered from insuline resistence and MI and treated by MSCs showed improved cardiac function connected with enhanced glucose uptake by peripheral tissues and mitochondrial oxidative phosphorylation efficiency. Moreover, MSCs improved insulin signaling via Akt phosphorylation and maintaining of glucose transporter type 4 [69]. Some investigators stimulated paracrine function of therapeutically applied MSCs by over-expression of VEGF [70] or by over-expression of miRNA-126 [71], which led to improved cardiac function after MI. The Akt molecule was identified responsible for the protective role of MSCs in cardiar repair function [71],[72].

4.3 Homing of MSC into damaged cardiac tissue

The ability of MSCs to home into damaged cardiac tissue is documented in some studies [60, 61], but it is still a very limited factor of MSCs cardiac therapy. Recent study demonstrated that up to 70% of MSCs applied into rat peripheral blood stream was trapped in lungs and some cells were detected in heart, kidney, spleen and bladder. The fraction of MSCs homed to the ischemic heart was only around 6 % [73]. Even the ability of MSCs to circulate in blood stream is limited [74]. Therefore, the extensive investigation is performed to describe MSCs homing mechanisms and to improve this process.

As in the homing of other cell types, the homing of MSCs is based on the process of chemotaxis. Ischemic myocardium is rich in many chemokines and adhesion molecules including chemokine (CC motif) ligands (CCL) 2, 6, 7, 9, chemokine (CXC motif) ligands (CXCL) 1, 2, SDF-1, IL-6, TGF-β, VEGF, intercellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM) or fibronectin [75], thus the expression of particular receptors on MSCs' surface should govern the process of homing.

Frequently investigated ligand/receptor pair is SDF-1/CXC chemokine receptor 4 (CXCR4). The level of surface CXCR4 in MSCs is low and unstable [76] and their expression needs to be stimulated to facilitate cardiac function repair [77, 78]. Also over-expression of CC chemokine receptor 1 (CCR1) promoted migration of MSCs and their homing to injured heart [79]. Another studies detected integrin β1 [80], hyaluronic acid/CD44 [76], N-formyl peptide receptor (FPR) and the formyl peptide receptor-like-1 (FPRL1) [76] or platelet-derived growth factor-AB (PDGF-AB)/PDGF receptor alpha and beta and insulin-like growth factor 1 (IGF-1)/IGF receptor [76] as crucial ligands and receptors for MSCs homing into injured cardiac tissue.

4.4 The importance of cell delivery routes

Current routes for MSCs delivery in heart treatment include intravenous injection (IV), where the MSCs are applied into the peripheral blood stream, intramyocardial injection (IM), where the MSCs are applicated by surgeons directly to heart and intracoronary injection (IC), where percutaneous catheter delivers MSCs into coronary arteries.

Systemic IV injection is used because of low invasiveness, low cost and reported MSCs homing ability [81]. In the case of heart damage, local and systemic chemo-attractants are upregulated, including various interleukins, stromal cell-delivery factors and adhesion molecules [4]. However, this homing signal seems to be not sufficient. It has been demonstrated that after IV injection of MSCs, only few of them were accumulated in infarcted myocardium of mice, majority of the cells was found in lungs [82].

Purpose of IM injection is to deliver MSCs directly to the damaged heart area via epicardial, endocardial or transvascular application. Advantages of this method are that it is similar to routine cardiac surgery, for surgeons it is easy to perform, and there is no risk of coronary embolism like in other application forms [5]. Also there is no need to rely on up-regulation of homing signal particles, because of MSCs delivery directly to the site of damage [4]. However, there is also a disadvantage, MSCs have tendency to form islet-like clusters consisting of donor cells and host inflammatory cells generating electrical and biological heterogenity in the host myocardium, which potentially results in arrhythmia occurrence [83].

IC injection method achieves higher first-pass delivery of MSCs into the heart and more homogenous cell distribution in target area with less inflammatory response [4]. Unfortunately donor

MSCs engraftment is similarly poor as after IM injection. It has been demonstrated that initial retention of applied cells is 15%, but after one hour only 5% of donor MSCs have been detected in damaged heart area [84]. Also it has been reported that IC applied MSCs are relatively large which may result in microvascular obstruction and ischemia [85]. Elevation of cardiac infarct markers and changes on electrocardiogram after IC injection of MSCs has been reported [86].

Despite extensive research, MSCs engraftment in damaged cardiac tissue is still poor and several explanations have been suggested. First, the injection of MSCs by thin needle can cause damage to MSCs, which could lead to their apoptosis or death [87]. Second, MSCs harvested for application by trypsin can lose their surface proteins and reduce cell-cell affinity, which can cause quick flush out of MSCs [88]. Third, MSCs in late passages can lose their surface expressions of chemokine receptors, which can disrupt their chemotactic ability [76]. Despite low MSCs retention in the damaged heart site, the majority of experiments show improvement in cardiac function and damaged area size after MSCs treatment.

5 Clinical studies

Till today, 13 clinical trials are registered at clinicaltrials.gov when searching for the keywords "mesenchymal stem cells" AND heart, which have been completed. From those, results were published from 8 trials and are summarized in Table 2 and the following text.

Patients with left ventricle disfunction, ischemic cardiomyopathy, acute or chronic myocardial infarction, idiopathic dilated cardiomyopathy or ischemic heart failure were included into clinical trials. At 5 trials out of 8, autologous MSCs isolated from bone marrow were applied by intramyocardially, intracoronary or transendocardially. The general effect of MSCs application was the improvement of left ventricule ejection fraction (LVEF) and reduction of infarcted tissue area (Table 2).

Hare et al. [89] performed a series of clinical trials focused on evaluation of safety and efficacy of MSCs application into patients with ischemic cardiomyopathy. POSEIDON, one of the first clinical trials in this field, compared the effect of autologous and allogeneic MSCs applicated transendocardially into 30 patients with ischemic cardiomyopathy. After 1year follow-up it was shown that this application is safe and beneficial for patients. Application of both auto- and allo- MSCs had low rate of serious adverse events (SAE), reduction in infarcted size area was observed, but no improvement in LVEF was shown. Only autologous MSCs application led to significant improvement in a 6 minutes walking test. However, lack of placebo control prevented additional comparisons [89]. In the following TAC-HFT clinical trial, autologous bone marrow MSCs application was compared with bone marrow mononuclear cells application and placebo group, also focused on safety and efficacy of cell application. In this trial, 65 patients with ischemic cardiomyopathy were enrolled. Transendocardial injection in 10 left ventricle sites showed that application is safe, with no SAE, but only MSCs improved myocardial functions, including contractility. No change in LVEF was observed [90].

In PROMETHEUS, the clinical trial where autologous bone marrow MSCs were injected into infarcted site of myocardium of 6 patients not eligible for bypass surgery, it was shown that MSCs reduce scar mass size for 48% compared to baseline. Also improvement in contractility and perfusion in these patients was shown together with improved LVEF [91].

Effect of clinical application of MSCs was also tested on accute myocardial infarction (AMI) patients a few days and up to a month after AMI. Lee et al. [92] applied bone marrow MSCs into infarcted site of myocardium of 80 patients and followed them for 6 months. 58 patients completed the trial and it was shown that application of MSCs is safe and even effective when performed month after AMI. LVEF, measured by SPECT, was improved for 6% in the 6th month of follow-up, in comparison to control group, receiving regular treatment only [92]. As a possible treatment for accute myocardial infarction also Wharton jelly MSCs (WJ-MSCs) application was tested. Intracoronary application of WJ-MSCs into 116 patients in 5-7 days after reperfusion treatment showed increased myocardial viability, measured by PET, and improved heart perfusion in 4 months. In the end of study, after 18 months of follow-up, LVEF was significantly improved (7%) in comparison to controls [93].

Autologous bone marrow MSCs were shown to be beneficial also for patients with ischemic heart failure where no more therapeutic options are available. 40 patients, out of 60 involved in study

called MSC-HF, received intramyocardial injection of MSCs, follow-up for 6 months was performed. In the end of the clinical trial, LVEF of patients who received MSCs was improved for 6% and their left ventricle end-systolic volume was reduced for 7%, in comparison to placebo control, suggesting improved myocardial function [94].

Contrary, in order to make MSCs application as less invasive for patients as it is possible, intravenous application was tested. In clinical trial STEMPEUCEL bone marrow derived MSCs were injected into antecubital vein of 10 patients with AMI two days after coronary intervention. After a 2 year follow-up and in comparison with placebo control it was shown that this application does not cause SAE and is safe for patients, but no beneficial effect was observed and no significant differences between MSCs and placebo group has been found in any tested parameter [95].

Metaanalyses of performed clinical studies showed confusing correlation between discrepancies and positive results. More methodical discrepancies have been found in research, where better results were reported. According to metaanalyses, studies with no discrepancies showed negative results [96], which is disturbing.

6 Problems and perspectives

MSCs and their influence on heart have been studied intensively. It has been reported that MSCs possess ability to home into site of cardiac damage and support damaged myocardium by differentiation into CMC, by paracrine signaling and immunomodulation properties. MSCs differentiation into CMC was shown to be not significant *in vivo*, but all other properties were confirmed *in vitro* and also *in vivo*.

In preclinical studies MSCs showed to be a safe and promising treatment for variety of cardiac tissue damages. Some clinical trials performed on patients also showed positive effect of MSCs application, but no all of them. All performed clinical studies agreed that the application of MSCs is safe for patients. However, new evidence is questioning the effect of MSCs in patients with cardiac diseases and therefore implementation of MSCs treatment as a regular therapy in the clinic might be further away than expected/hoped for. It has been also shown that application of MSCs may not be beneficial enough to use it as a standart treatment. This could depend on several factors including age and source of MSCs, their manipulation after isolation and the route of application.

As discussed earlier, age of MSCs is a very important factor. Usually, patients are older and therefore autologous transplantation of MSCs might not be efficient enough. In respect to therapy efficiency, use of MSCs from young and healthy donors, more active and capable of regenerative potential, should be considered. Especially after repeated prove, that allogeneic and autologous transplantation are both safe and have a similar effect.

Another consideration should be the source of MSCs. Bone marrow MSCs are the best investigated ones known to improve heart functions, but also cardiac adipose tissue derived MSCs, which are rarer and harder to harvest, show promising and even better cardio specific abilities.

Any MSCs chosen for application need to be cultured in order to achieve satisfactory numbers for application. Cultivation conditions as well as cell harvesting are well described, but there is room for improvement. MSCs have documented homing ability into site of injury, but a large number of researchers reported minimal homing to the cardiac damaged sites in human. The final harvesting procedure before application may destroy surface receptors of the MSCs, so they are unable to find the cardiac site of damage, instead they are trapped elsewhere.

In consideration of previous discussed challenges, the chosen form of application is crucial. Peripheral application is the cheapest and the most comfortable for patients and medical personal, but the risk that MSCs will be trapped outside of the heart is big. Intracoronary or transmyocardial application is more reliable, but possesses risk of microembolism. All these facts need to be taken into consideration together with application speed, application number and number of application doses.

The most important question for the future of MSCs therapeutical application is what should be considered as a positive result of application. Should it be any positive effect which is statistically significant or is it better to agree on a general evaluation protocol?

Many facts are well known, but many more questions need to be answered, before the MSCs application will become a real treatment option for cardiac patients.

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10   Acknowledgement