Beneficial Effects of Mesenchymal Stem Cells on Adult Porcine Cardiomyocytes in Non-Contact Co-Culture

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Received March 21, 2018
Accepted September 12, 2018

Summary

Mesenchymal stem cells (MSCs) have been reported to improve survival of cardiomyocytes (CMCs) and overall regeneration of cardiac tissue. Despite promising preclinical results, interactions of MSCs and CMCs, both direct and indirect, remain unclear. In this study, porcine bone marrow MSCs and freshly isolated porcine primary adult CMCs were used for non-contact co-culture experiments. Morphology, viability and functional parameters of CMCs were measured over time and compared between CMCs cultured alone and CMCs co-cultured with MSCs. In non-contact co-culture, MSCs improved survival of CMCs. CMCs co-cultured with MSCs maintained CMCs morphology and viability in significantly higher percentage than CMCs cultured alone. In viable CMCs, mitochondrial respiration was preserved in both CMCs cultured alone and in CMCs co-cultured with MSCs. Comparison of cellular contractility and calcium handling, measured in single CMCs, revealed no significant differences between viable CMCs from co-culture and CMCs cultured alone. In conclusion, non-contact co-culture of porcine MSCs and CMCs improved survival of CMCs with a sufficient preservation of functional and mitochondrial parameters.

Key words

Mesenchymal stem cells • Cardiomyocytes • Co-culture • Regenerative medicine • Mitochondria

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Introduction

Cardiac diseases are a major cause of worldwide morbidity and mortality despite the progress in cardiovascular pharmacotherapy, cardiosurgery and interventional cardiology (Psaltis *et al.* 2014). Nowadays, stem cells-based therapies receive worldwide scientific interest as a promising treatment for variety of cardiovascular diseases (Mao *et al.* 2014, Kim *et al.* 2014). Hematopoietic stem cells (Nygren *et al.* 2004), mesenchymal stem cells (Li *et al.* 2007), embryonic stem cells (Dai and Kloner 2006) and fetal cardiac myocytes (Etzion *et al.* 2005) have been investigated, but mesenchymal stem cells (MSCs) received the highest attention as a promising cell type for cardiac repair.

It has been shown that only very small percentage of MSCs differentiate into cardiomyocytes (CMCs) (Noiseux *et al.* 2006, Siegel *et al.* 2012), but their paracrine and immunomodulatory effects are much more beneficial for the cardiac regeneration (Xiang *et al.* 2009, Bader *et al.* 2014). MSCs secrete a variety of

cytokines and chemokines that support survival of CMCs, as well as vasculogenesis and neoangiogenesis in myocardial muscle, which further improve survival and regeneration of heart tissue (Xiang *et al.* 2009, Narita and Suzuki 2015). Recent studies on non-cardiac tissues revealed that use of MSCs conditioned media containing bioactive factors released by cells has similar effects as transplantation of cells themselves (Linero and Chaparro 2014, Bortolotti *et al.* 2015).

Encouraged by these promising results, many clinical trials administering MSCs to patients with different heart diseases have been opened. Results showed MSCs application to be safe, but despite the described beneficial effect of MSCs on cardiac tissue, no consistent significant positive results were found in preclinical studies. Detailed analysis of clinical studies showed a significant association between the number of experimental discrepancies and the reported increment in left ventricular ejection fraction. Trials with no experimental discrepancies showed no or very mild effect of MSCs in patient hearts (Nowbar *et al.* 2014).

One of the reasons of failing translation may be that majority of the preclinical studies was performed in small animal models (Narita and Suzuki 2015). Large animal models with higher clinical relevance, such as pig, need to be implemented to obtain results relevant for human application (Crick *et al.* 1998, Reardon 2015). Therefore in our study, we focused on porcine cells and studied effects of MSCs on primary adult CMCs survival, functional and mitochondrial parameters in strictly controlled *in vitro* non-contact co-culture.

Material and methods

MSCs isolation

Porcine MSCs were obtained from bone marrow of adult pigs (*Sus scrofa*, n=12) in general anesthesia (anesthesia was induced with i.m. tiletamine, 2.2 mg/kg, zolazepam, 2.2 mg/kg, and xylazine, 2.2 mg/kg, together with i.v. propofol 2 %, 1-2 mg/kg, and maintained with continuous i.v. propofol, 1-4 mg/kg/h, and fentanyl, 5-10 µg/kg/h). The bone marrow was diluted with phosphate-buffered saline (PBS; Sigma-Aldrich, USA) in ratio 1:1 and layered on Ficoll-Paque^{TM} Plus (GE Healthcare, United Kingdom), 4 ml of Ficoll-Paque per 6 ml of diluted bone marrow were used. Centrifugation (435×g, 30 min, break 6, room temperature (RT)) followed. Opalescent layer of mononuclear cells was aspirated, placed into new tube and washed with PBS. Resulting pellet was resuspended in 5 ml of prewarmed complete cultivation media consisting of α-modified Eagle's medium (αMEM; Hyclone, GE Healthcare, United Kingdom) supplemented with 10 % fetal bovine serum (FBS; Biosera, France) and 100 IU/ml penicillin (Biosera, France) and 100 mg/ml streptomycin (Biosera, France). Cells were placed on 75 cm^2 flasks (TPP, Switzerland) and cultured in humidified incubator (37 °C and 5 % CO_2 atmosphere). After 48 h non-adherent cells were discarded together with old medium and fresh medium was added, medium was changed every 2^{nd}-3^{rd} day until the cells reached 80-90 % confluence. For all experiments MSCs from 2^{nd} to 6^{th} passage were used.

MSCs phenotype confirmation

For confirmation of multidirectional differentiation ability of MSCs, cells were seeded on 12-well cultivation dishes (TPP, Switzerland) with seeding density 3.8×10^4 cells/well for adipogenic and chondrogenic differentiation and 1.9×10^4 cells/well for osteogenic differentiation respectively. After 24 h attachment period the media was discarded and replaced with 3 ml of specific differentiation medium; StemPro® Adipogenesis Differentiation Kit (Life technologies, USA) for adipogenic differentiation, StemPro® Chondrogenesis Differentiation Kit (Life technologies, USA) for chondrogenic differentiation, and StemPro® Osteogenesis Differentiation Kit (Life technologies, USA) for osteogenic differentiation. After differentiation period of 21 days, Oil Red O staining for lipid droplet visualization in adipogenesis, Alcian blue for glycoproteins visualization in chondrogenesis and Alizarin red S for calcium ions visualization in osteogenesis were performed.

The mesenchymal phenotype of MSCs was confirmed by flow cytometric detection of CD90, CD73 and CD44. About 500,000 MSCs were washed by PBS and stained by 5 µl of CD90-APC (Biolegend, USA), CD73-PE (Biolegend, USA), CD44-BV421 (Biolegend, USA), and CD45-FITC (Bio-Rad, USA) for 15 min at RT and dark. After staining, MSCs were washed once, resuspended in 300 µl of PBS and analyzed by BD FACS Aria Fusion and BD FACS Diva 8.0.1 software (both Becton Dickinson, USA).

Cardiomyocytes isolation

Porcine adult CMCs were isolated from left ventricles of young adult pigs (*Sus scrofa*, n=12).

After animal sacrifice (animals were euthanized

by anesthetic overdose and excision of the heart), 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; 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 apparatus and perfused with warm (37 °C) oxygenated solutions: 1, Tyrode solution without Ca^{2+} (5 min), 2, Tyrode solution with Ca^{2+} (0.5 μmol/l), collagenase A (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). CMCs were harvested from the wall of left ventricle after endocardial tissue removal and placed into Tyrode 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 to the resulting 0.2 mmol/l Ca^{2+} concentration.

Isolated CMCs were let to sediment, supernatant was removed and CMCs were resuspended in complete CMCs cultivation medium consisting of Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10 % FBS (Biosera, France), glucose (4,500 mg/l, Sigma-Aldrich, USA), L-glutamine (4 mmol/l, Biosera, France), and 100 IU/ml penicillin (Biosera, France) and 100 μg/ml streptomycin (Biosera, France). Resulting cell suspension was used for experiments.

Following each isolation, the evaluation of percentage of living CMCs in resulting cell suspension was performed by experienced observer using light microscopy (Nicon Eclipse Ti, Japan). All rod shaped cells with clear cross-striations were counted as living CMCs, the rest was considered to be dead or dying cells.

Co-cultivation experiments

MSCs in Complete MSCs medium were seeded to 3 wells of 6-well plate (TPP, Switzerland) in concentration 54,000 cells/well each and let to attach overnight in humidified incubator (37 °C, 5 % CO_2). After MSCs media removal, three membrane inserts Transwell® (Corning, Sigma-Aldrich, USA) with 3 microns membrane pores were placed into wells with MSCs and CMCs in complete CMCs medium were added to all 6 wells of 6-well plate. On day 1 and day 3 of co-cultivation morphological and functional properties of CMC were analyzed then.

Cardiomyocytes morphology

Morphology of CMCs cultured in both cultivation conditions was observed under light microscope (Nikon Eclipse Ti, Japan) and documented. On day 1 and day 3 of the cultivation, experienced observer quantified relative CMCs survival, all rod-shaped cells with clear cross-striations were considered to be living CMCs.

Cardiomyocyte viability – cell viability imaging kit

On day 1 and day 3 of the cultivation, ReadyProbes® Cell Viability Imaging Kit (Blue/Red) (Molecular Probes, Life Technologies, USA) was used. CMCs were transferred to clean cultivation plastic and culture medium was replaced with Live Cell Imaging Solution (Molecular Probes, Life Technologies, USA). Two drops of NucBlue® Live ReadyProbes® Reagent and propidium iodide were added per milliliter of medium. Cells were incubated in the dark for 15 min and then visualized and documented with a fluorescent microscope (Nikon Eclipse Ti, Japan). In magnification 10×, all living CMCs, rod-shaped cells with clear cross-striations and blue nucleus, were counted and counting of all other cells, round cells with violet nucleus (red + blue), in field of vision followed. Resulting percentages of living CMCs in both cultivation conditions were statistically evaluated.

Cardiomyocyte viability – alamarBlue assay

On day 1 and day 3 of the cultivation, CMCs were transferred into new 96-well plate in 100 μl of media and 10 μl of alamarBlue® (ThermoFisher Scientific, USA) was added to each well. In this stage of measurement, it was not possible to count the exact number of CMCs transferred into new 96-well plate, thus 12×100 μl of CMCs suspension from each well of 6-well plate was transferred. After 5 h of incubation at 37 °C, fluorescent signal (excitation 530/25, emission 590/35) was measured by plate reader Synergy HT (Biotek, USA). The signal from each 12 wells of 96-well plate was expressed as the mean and these means were compared within both CMCs groups.

Cardiomyocyte viability – mitochondria detection assay

For visualization of mitochondrial network as a marker of viable cells on day 1 and day 3 of co-cultivation experiments, mitochondrial dye MitoTracker® (579/599) (Molecular probes, Life Technologies, USA) was used. After 30 min incubation, the functional mitochondria showed red signal. Nuclei

were stained by NucBlue® (360/460) (Molecular probes, Life Technologies, USA).

Cardiomyocyte mitochondrial function measurement

For assessment of CMCs mitochondrial functions, high-resolution respirometry was used. Mitochondrial oxygen consumption in isolated CMCs from both cultivation conditions groups on day 1 and 3 of co-cultivation was measured by oxygraph Oroboros (Oroboros, Innsbruck, Austria) at 37 °C in chambers of 2 ml volume. Cultivation medium was placed into oxygraph chambers and let to equilibrate for 40 min. At the end of equilibration period, concentration of oxygen in chamber corresponded 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 non-permeabilised CMCs was measured.

In titration protocol, different substrates and disruptors of oxidation chain were added, following respiration statuses, routinely present in intact cells, were measured; ROUTINE (basic oxygen consumption necessary for physiologic control of substrate transport of intermedial metabolism and energetic switch), LEAK (status after inhibition of ATP synthesis 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 (electrontransport capacity of respiration system, which corresponds to maximal oxygen consumption when oxidation and phosphorylation are disrupted), and ROX (residual oxygen consumption after rotenone, inhibitor of Complex I).

After state ROUTINE measurement, 2.5 µmol/l of oligomycin (Sigma-Aldrich, USA) was added to chambers and measurement of state LEAK followed after oxygen consumption stabilization. By titration of protonofor carbonylcyanid-p-trifluoromethoxyphenylhyd-rason (FCCP; 0.05-0.40 µmol/l) (Sigma-Aldrich, USA) the ETS state was induced. Finally addition of 0.5 µmol/l of rotenone (Sigma-Aldrich, USA), inhibitor of Complex I, the ROX state was induced, measurement followed.

CMCs oxygen consumption was analyzed on-line by DatLab 7 software (Oroboros Instruments, Innsbruck, Austria) as negative time derivation of decrease in oxygen concentration in chamber, expressed in pmol $O_2/(s \cdot 10^6$ cells) and corrected to ROX.

Cardiomyocyte contractions and calcium transients

In order to evaluate functional properties of CMCs cellular contractility and calcium transients were analyzed. For the experiment CMCs were transferred from cultivation media to normal calcium (2 mmol/l) Tyrode solution. CMCs sarcomeric contractions and calcium transients were measured with Ionoptix HyperSwitch Myocyte Calcium and Contractility System (IonOptix LLC, Westwood, USA), with the Sarclen sarcomere length acquisition module. Cells were loaded with Fura-2 (Molecular Probes, Invitrogen, USA). For 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 min in normal calcium Tyrode solution with 2 µmol/l Fura-2-am and then repeatedly washed with normal calcium Tyrode solution. After 20 min of incubation, measurements followed. Measurements were performed in normal Tyrode solution at 37±0.5 °C. Cells were stimulated with field stimulator (MyoPacer Field Stimulator, IonOptix LLC, Westwood, USA) at 1 Hz.

For offline analysis of sarcomeric contractions and calcium transients the IonWizard 6.5 software (IonOptix LLC, Westwood, USA) was used.

Statistics

Results are presented as means ± SD. After testing for normality of distribution, the datasets were compared using two-way mixed-design ANOVA (repeated-measures factor for days, non-repeated factor for cultivation groups) followed by *post hoc* Tukey test. Statistical analysis was performed with OriginPro 2017 software (OriginLab Corporation, Northampton, MA, USA). Values of $p<0.05$ were considered significant.

Results

Mesenchymal stem cells

MSCs were successfully isolated with procedure described above. After non adherent cells wash, remaining cells showed MSCs properties. Light microscopy of cells confirmed MSCs morphology, the spindle shape cells with several spurs and large nucleus adhered to cultivation plastic (Fig. 1A, B). Cultivation of MSCs with differentiation media confirmed ability of cells to differentiate into three basic lines. Cells differentiated into adipocyte line produced fat vacuoles, stained in red color after Oil Red O addition. Cells cultured in media for osteogenic differentiation produced

calcium ions, stained in red by Alizarin red S. Cells in media for chondrogenic differentiation produced mucopolysaccharides, stained in blue by Alcian blue.

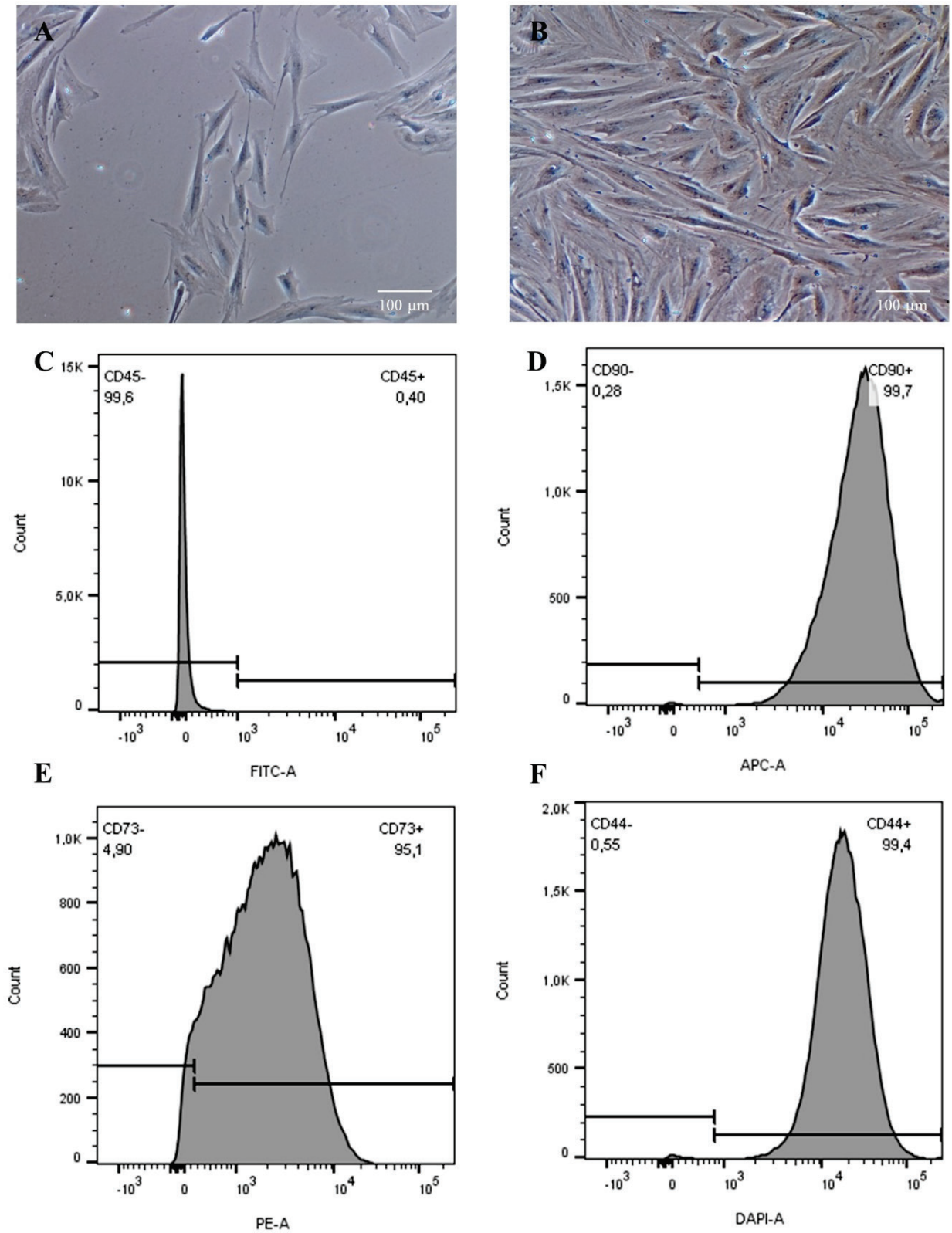

Fig. 1. MSCs morphology and representative histograms of evaluation of MSCs phenotype by flow cytometry. (**A**) MSCs in low density. (**B**) MSCs in confluency. (**C, D, E, F**) evaluation of MSCs phenotype by flow cytometry – MSCs showed to be negative in CD45 and positive in CD90, CD73, and CD44.

Flow cytometry proved the mesenchymal origin of MSCs. 99.7 % of cells expressed CD90, 99.4 % of cells expressed CD44 and 95.1 % of cells expressed CD73. 99.6 % of cells were negative for blood lineage markers CD45 (Fig. 1C-F).

Cardiomyocytes

Porcine primary adult CMCs were isolated by enzymatic dissociation and the percentage of rod-shaped CMCs with clear cross-striations was 20±10 %. Freshly isolated CMCs were rod-shaped with sharp edges and their cross-striations were clearly visible (Fig. 2A). In culture, the sharp edges of the cells started to round and cross-striations slowly disappeared. After three days of the culture, CMCs started to develop morphological pathologies (Fig. 2B-F).

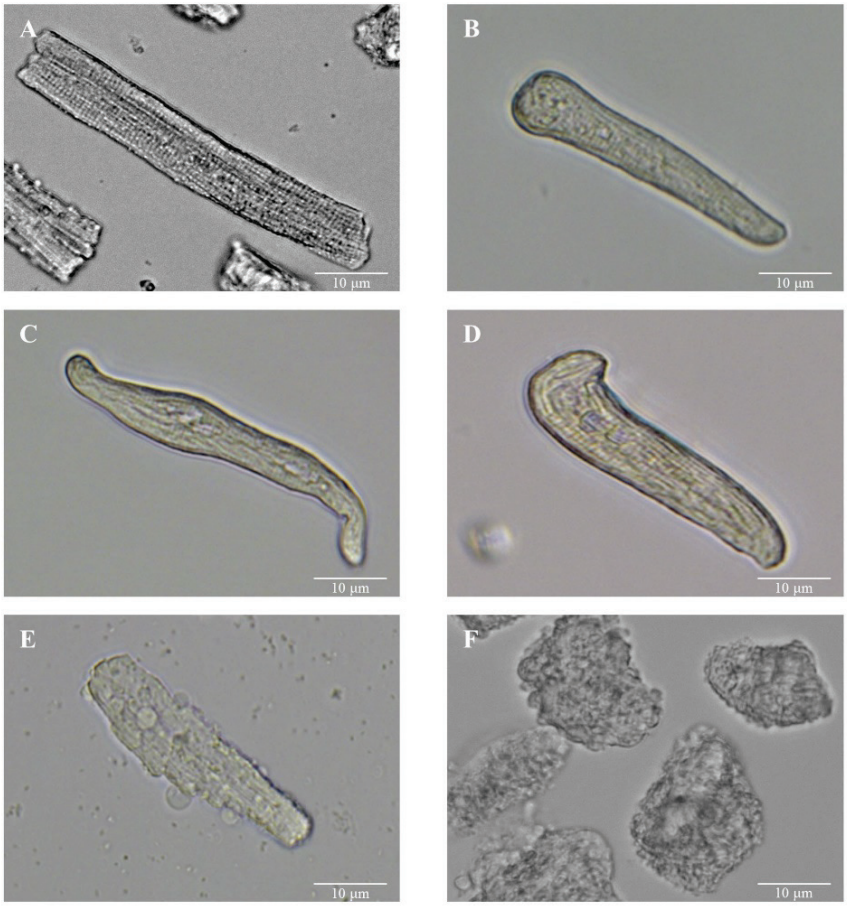

Fig. 2. Morphology of CMCs in *in vitro* culture over time. (**A**) freshly isolated rod-shaped CMCs with sharp edges and clear cross-striations. (**B, C, D**) morphological deterioration of CMCs in long term culture (3-10 days); CMCs loose cross-striations, become rounded and cell contracture develops. (**E**) dying cell. (**F**) dead cells and cellular debris.

Co-culture experiments – cardiomyocyte viability

Viability of CMCs based on morphological criteria (rod-shaped cells with clear cross-striations) showed the similar portion of living CMCs in both culture groups on day 1. On day 3, a significant decrease only occurred in CMCs cultured alone. We also observed a statistically significant decrease in percentage of living CMCs between day 1 and day 3 of experiment in CMCs cultured alone, but not in CMCs co-cultured with MSCs (Fig. 3A). Staining with Cell viability Imaging Kit showed similar results.

Analysis of alamarBlue® fluorescence revealed a significant decrease of cellular viability between day 1 and day 3 of experiment in both groups. A reduced level of cellular viability in CMCs cultured alone compared to CMCs co-cultured with MSCs was present already on day 1 and the significant difference between the groups persisted till day 3 of experiment (Fig. 3B).

Co-culture experiments – cardiomyocyte mitochondria

Fluorescent staining of mitochondria with MitoTracker showed sufficient mitochondrial membrane potentials in both CMCs groups (Fig. 4A-D) on both day 1 and day 3 of experiment. Similarly, no significant differences between CMCs groups on either day of experiment were found by high resolution oxygraphy. Oxygen consumption (Fig. 5A-C) of intact CMCs showed a tendency to decrease with cultivation time, but the trend did not reach statistical significance in either CMCs group. Basic respiration (ROUTINE state)

fluctuated from 249±62 pmol/(s·10^6 cells) in CMCs cultured for 1 day without MSCs to 126±42 pmol/(s·10^6 cells) in CMCs cultured for 3 days with MSCs (Fig. 5A). Similar trends were also found for the LEAK state (Fig. 5B). Maximal respiration induced by FCCP was nearly identical for both CMCs groups and did not decrease in time (ETS state in Fig. 5C).

Co-culture experiments – cardiomyocyte function

With time in cell culture, both sarcomeric contractions (Fig. 6A-D) and calcium transients (Fig. 6E-H) of CMCs cultured alone significantly decreased. Neither baseline sarcomeric length (Fig. 6A) nor the baseline Fura-2 fluorescence ratio (Fig. 6E) were affected by cultivation time. Besides the reduction of contraction and calcium transient amplitudes with time in culture the relaxations of both contraction (Fig. 6B, C) and calcium transient (Fig. 6F, G) were significantly prolonged. Co-cultivation of CMCs with MSCs did not prevent these changes. There were no significant differences in contractile and calcium parameters when CMC cultured alone and CMCs co-cultured with MSCc were compared.

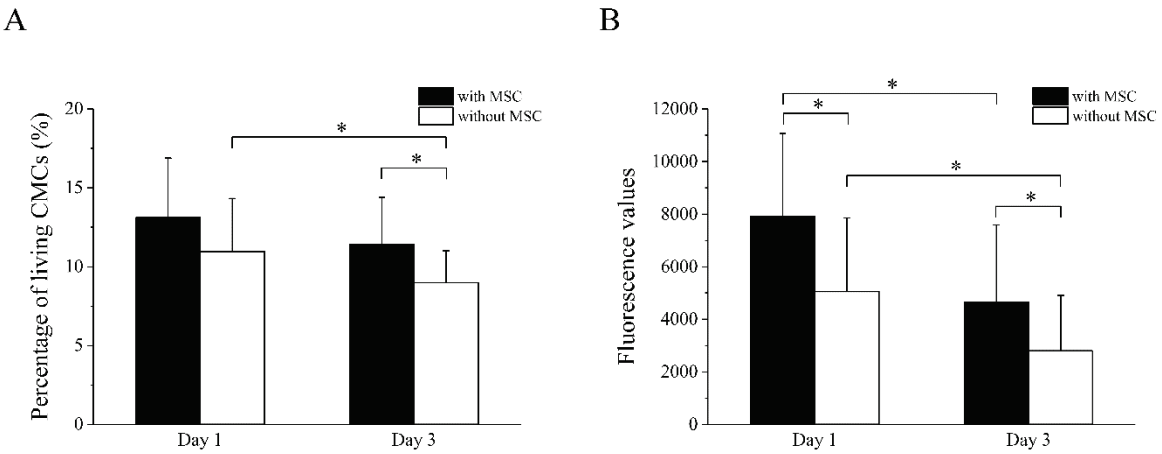

Fig. 3. Cardiomyocyte viability comparison of both CMCs cultivation groups (with and without MSCs). (**A**) percentage of rod shaped CMCs with clear cross-striations in field of vision co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**B**) CMCs viability measured by alamarBlue® assay in cocultivation with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. Values of * $p<0.05$ were considered significant.

Discussion

Although effects of MSCs on CMCs have been studied extensively (Silva *et al.* 2014, Psaltis *et al.* 2014, Narita and Suzuki 2015), precise mechanisms remain unclear and translation of these findings into clinical practice has not been satisfactory yet (Nowbar *et al.* 2014). More information from research in clinically more relevant animal and cellular models (Reardon 2015) need to be gathered first.

In this study, porcine cells were chosen because of the similarity of human and porcine cardiovascular physiology (Stankovicova *et al.* 2000). MSCs were isolated from porcine bone marrow, consequent evaluation of morphology and differentiation ability together with surface antigen verification by flow cytometry confirmed isolated cells as MSCs with 99 % purity. We successfully isolated primary adult porcine CMCs and described their phenotype with a number of morphological observations and functional tests. Primary adult CMCs, as fully differentiated cells, are unable to proliferate and their life span is short. In culture, fast deterioration and morphological transformation with a loss of typical phenotype occur within several days (Xu and Colecraft 2009, Banyasz *et al.* 2008, Mitcheson *et al.* 1998, Bugaisky and Zak 1989, Piper *et al.* 1988). In general, adult cardiomyocytes do not establish long-term cultures in a stable morphological state, in other than a surface attached spread cell form (Piper *et al.* 1988) and our experiments were in agreement with these findings.

We focused on potential paracrine effect of MSCs on CMCs exerted without direct contact of these cell types, therefore we compared CMCs cultured alone and CMCs cultured on transwell membranes with MSCs in non-contact co-culture. CMCs co-cultured with MSCs showed longer survival in culture: whereas CMCs without MSCs showed a significant decrease in percentage of living cells in culture between day 1 and day 3, difference

between day 1 and day 3 for CMCs in co-culture with MSCs was not significant. On day 3, comparisons of CMCs cultured with and without MSCs revealed a significantly better survival and viability of CMCs co-cultured with MSCs. This suggests protective role of MSCs conditioned media, which is in agreement with other studies performed in small experimental animals (Bollini *et al.* 2013, Dai 2005, Gnecchi *et al.* 2005). To the best of our knowledge, similar work on large animal cells has not been performed to date.

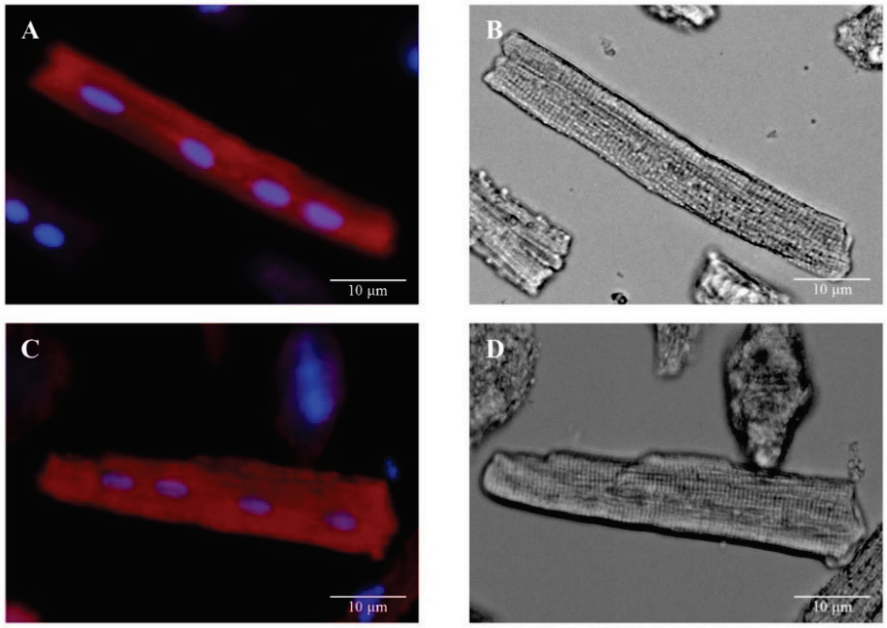

Fig. 4. Fluorescent microscopy of mitochondria in CMCs on day 1 and day 3 of cultivation (red color – functional mitochondria network, blue color – nuclei). Functional mitochondrial network was identified on day 1 and day 3 of the experiment in both CMCs groups. (**A**) Detail of single cardiomyocyte in fluorescence on day 1 of cultivation. (**B**) Detail of the same cell (A) in bright field. (**C**) Detail of single cardiomyocyte in fluorescence on day 3 of cultivation. (**D**) Detail of the same cell (C) in bright field.

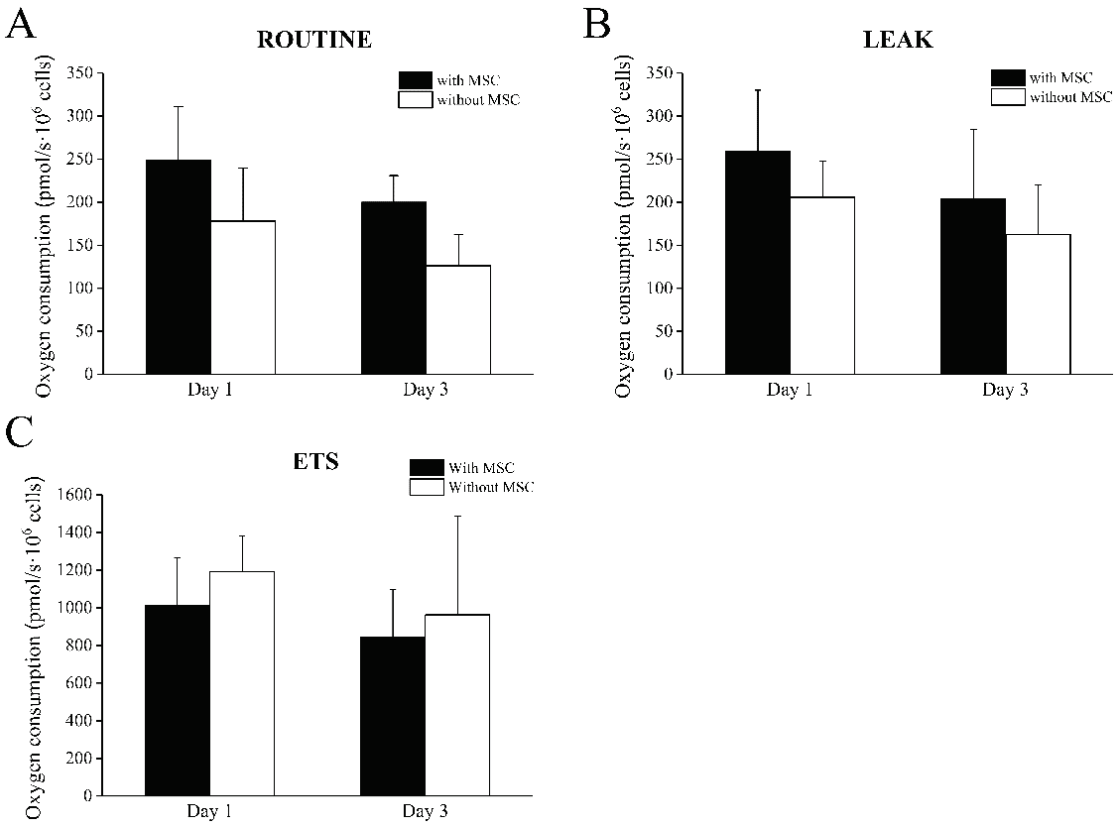

Fig. 5. Respirometry analysis. (**A, B, C**) oxygen consumption in states ROUTINE (A), LEAK (B) and ETS (C) in intact CMCs measured on day 1 and day 3 of cultivation with or without MSCs. Oxygen consumption was corrected for residual oxygen consumption and is showed in pmol per second per million cells.

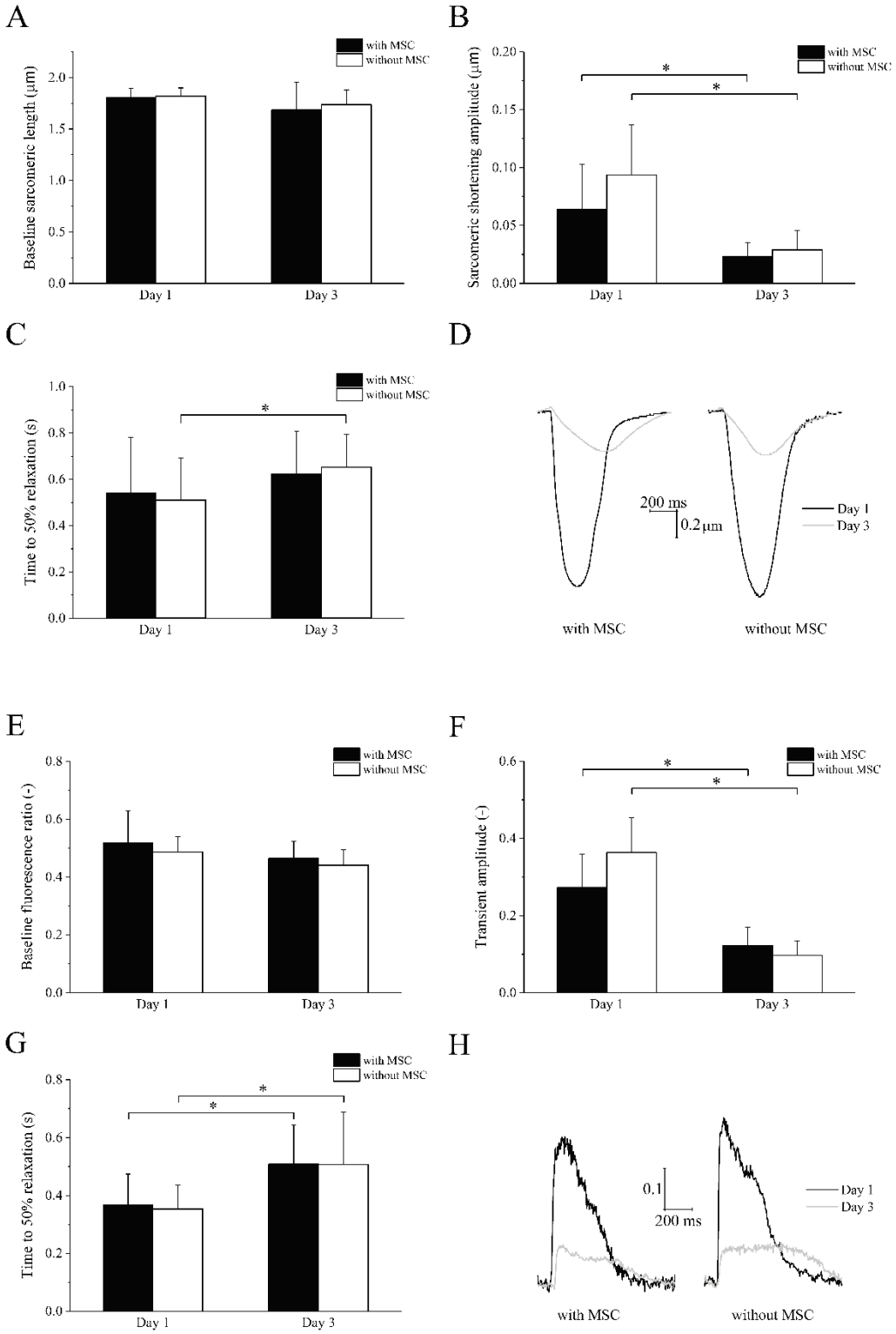

Fig. 6. Sarcomeric contraction and calcium transient. (**A**) baseline sarcomeric lengths in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**B**) sarcomeric contraction amplitudes in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**C**) times to 50 % relaxation in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**D**) representative examples of sarcomeric contraction in CMCs co-cultured with MSCs and CMCs cultured alone on day 1 (black line) and 3 (gray line) of culture. (**E**) Baseline Fura-2 fluorescence ratios in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**F**) Calcium transient amplitudes in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**G**) Times to 50 % relaxation in CMCs co-cultured with MSCs (black columns) and CMCs cultured alone (white columns) on day 1 and 3 of culture. (**H**) Representative examples of calcium transient in CMCs co-cultured with MSCs and CMCs cultured alone on day 1 (black line) and 3 (gray line) of culture. Values of * $p<0.05$ were considered significant.

High-resolution oxygraphy of cell suspension of intact non-permeabilized CMCs did not show any significant decrease in mitochondrial respiration of CMCs during the cultivation time in any of the two groups. According to that, co-cultivation of CMCs with MSCs was not significantly harmful or beneficial for mitochondrial function. In literature, significant impairment of CMCs mitochondrial functions in culture has been reported as an off-target effect of addition of myosin II ATPase inhibitors blebbistatin and 2,3-butaneodine monoxime used for prolongation of culture time of viable CMCs (Hall and Hausenloy 2016), but in general, mitochondrial functions of CMCs in culture over time without any intervention have not been studied extensively.

Functional parameters of CMCs calcium transients and contractility showed a significant deterioration in time, which is in line with other studies (Banyasz *et al.* 2008, Mitcheson *et al.* 1998). Both calcium transient and sarcomeric contraction amplitudes were significantly reduced and the relaxation was slowed down. Co-cultivation of CMCs with MSCs did not prevent these undesirable changes. Therefore, it seems that co-cultivation of the CMCs with MSCs was not significantly beneficial for calcium handling and contractility of the isolated CMCs.

In brief, co-culture of CMCs with MSCs resulted in better viability and survival of CMCs without significantly affecting physiological and mitochondrial functions of myocytes. This lack of functional effects may be related to a selection bias, since only cells with normal CMCs phenotype in both cultures were addressed by the experimental methods. The high-resolution oxygraphy was performed in intact non-permeabilized CMCs and CMCs oxygen consumption as a negative time derivation of decrease in oxygen concentration in chamber was measured and expressed in pmol $O_2/(s \cdot 10^6$ cells). The functional parameters of sarcomeric contraction and of calcium transient were measured in individual CMCs and only cells in good condition that responded to electrical stimulation were selected for the measurement in both groups. It is possible that with a true representative selection of cellular populations significant differences in functional parameters would be found.

What is the mechanism of the beneficial effect of co-cultivation with MSCs? Since the direct cell-to-cell contact between CMCs and MSCs was prevented by the transwell membranes, a paracrine secretion of bioactive molecules by MSCs to cultivation media represents the most likely mechanism. MSCs were reported to secrete a variety of growth factors, anti-apoptotic, anti-inflammatory, and trophic molecules, including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), stromal cell-delivery factor (SDF), transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF), and matrix metalloproteinases (MMP) (Xiang *et al.* 2009, Guo *et al.* 2007, Siegel *et al.* 2012, Nauta and Fibbe 2007, Tang *et al.* 2005). All these molecules protect tissues and improve regeneration, e.g. in damaged myocardium it helps by reducing pathological fibrosis, by attenuation of cardiomyocyte apoptosis and hypertrophy and also by increasing neovascular formation and stimulation of endogenous stem/progenitor cells for myocardial infarction repair (Guo *et al.* 2007, Orlic *et al.* 2001, Xiang *et al.* 2009). MSCs released interleukin-6 family helps to activate signaling pathways resulting in an increased expression of the HGF and VEGF, both beneficial for cell survival (Shabbir *et al.* 2010). MSCs conditioned media can prevent CMCs apoptosis through inhibition of caspase-3 activation and release of cytochrome C from mitochondria, suggesting that MSCs protect CMCs by interfering with mitochondria-mediated apoptotic pathway (Xiang *et al.* 2009). However, with regard to the complexity of MSC secretome and very broad range of its possible effects (e.g. decrease in CMCs apoptosis rate, decrease in inflammation and scar formation, increase in CMCs proliferation, cardiac tissue neovascularization), it will be extremely difficult to dissect the most efficient signaling pathways and to tune the optimal therapeutic effects (Ranganath *et al.* 2012). Similar beneficial effects were observed also with use of MSCs conditioned media only, this media supported local neovascularization, showed ability to inhibit cell death and suppressed immune response of organism (Bollini *et al.* 2013). In another work, angiogenesis after MSCs conditioned media application was described and blood flow recovery was documented (Rehman 2004). Repeated application of MSCs conditioned media in comparison with repeated MSCs application showed similar therapeutic effects, such as neovascularization, decrease in necrosis and inflammation and stimulation of tissue regeneration (Bortolotti *et al.* 2015). Proteomic analysis of MSCs secretome revealed its sensitivity to culture conditions, for example in hypoxic conditions, MSCs produced significantly higher amount of VEGF, FGF-2, HGF, IGF-1 than MSCs cultivated in standard conditions. This hypoxic conditioned media were then much more

effective in treatment of myocardial infarction in rats, in which a significant reduction of lesion size was found together with improvement of ventricular function (Gnecchi 2006).

In conclusion, non-contact co-culture of large animal CMCs with MSCs improved survival and viability of CMCs without affecting main mitochondrial and contractile functional parameters of CMCs. The data indicate that paracrine effects of MSCs may significantly contribute to their beneficial actions and further analysis of MSCs secretome is warranted.

Limitations of study

The cellular mechanisms and signaling pathways responsible for the improved survival and viability of CMCs co-cultured with MSC were not addressed. With regard to the vast complexity and diversity of MSC secretome high-throughput cell biology methods will have to be employed to unravel the most likely mechanisms and signaling pathways involved and consequently to optimize the therapeutic profile of MSC secretome.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Charles University Grant Agency (Project 2090214) and by the Project 15-32801A of the Ministry of Health of the Czech Republic and by the National Sustainability Program I (Project LO1503) provided by the Ministry of Education Youth and Sports of the Czech Republic, and by the program for the Development of Scientific Fields of Charles University (Progres Q39).

Ethical statement

All animal experiments were performed in accordance to the guidelines of European parliament and European Council 2010/63/EU about protection of animals used in scientific experiments. All experiments were approved by Ethical committee for work with laboratory animals of Faculty of Medicine in Pilsen, Charles University.

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