Expression of atrial-fetal light chains in cultured human cardiomyocytes after chemical ischemia-reperfusion injury

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Abstract. Atrial light chains (ALC1) are naturally present in adult heart atria, while ventricular light chains (VLC1) are predominant in ventricles. Degradation of VLC1 and re-expression of ALC1 in heart ventricles are associated with heart disorders in response to pressure overload. The aim of the current study was to investigate changes in myosin light chain expression after simulated ischemia and simulated reperfusion (sI/sR). Human cardiomyocytes (HCM) isolated from adult heart ventricles were subjected to chemical ischemia. The control group was maintained under aerobic conditions. Myocyte injury was determined by testing lactate dehydrogenase (LDH) activity. The gene expression of ALC1, VLC1 and MMP-2 were assessed by reverse transcription-quatitive PCR. Additionally, protein synthesis was measured using ELISA kits and MMP-2 activity was measured by zymography. The results revealed that LDH activity was increased in sI/sR cell-conditioned medium (P=0.02), confirming the ischemic damage of HCM. ALC1 gene expression and content in HCM were also increased in the sI/sR group (P=0.03 and P<0.001, respectively), while VLC1 gene expression after sI/sR was decreased (P=0.008). Furthermore, MMP-2 gene expression and synthesis were lower in the sI/sR group when compared with the aerobic control group (P<0.001 and P=0.03, respectively). MMP-2 activity was also increased in sI/sR cell-conditioned medium (P=0.006). In conclusion, sI/sR treatment led to increased ALC1 and decreased VLC1 expression in ventricular cardiomyocytes, which may constitute an adaptive mechanism to altered conditions and contribute to the improvement of heart function.

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Introduction

Myosin is composed of two heavy chains (MHC) and two pairs of light chains: essential (non-phosphorylatable) light chain 1 (MLC1) and regulatory (phosphorylatable) light chain 2 (MLC2) (1-6). MLC2 are indirectly responsible for modulating adenosine triphosphatase activity of MHC (7-11), while the main function of MLC1 is to stabilize the MHC head region where actin and ATP binding sites are located (4,5,12,13). At least two types of alkali essential myosin light chains 1 are distinguished in an adult human heart. There are atrial and ventricular myosin light chains (ALC1 and VLC1, respectively) (12,14-19). In fetal stage, fetal light chains 1 (FLC1) are present in the human heart ventricles. This form is identical to ALC1, hence also called atrial-fetal light chain 1 (1,5,7,19). After birth, the expression of FLC1 rapidly decreases in the ventricles and VLC1 plays the main contractile role (13,20). There are several reports showing that the expression of ALC1 in ventricles increases with the occurrence of congenital heart diseases such as tetralogy of Fallot, valvular heart defects or hypertrophic cardiomyopathy (1,13,16-18,21). ALC1 may also appear in the heart ventricles of the adults suffering from heart failure and in response to pressure overload (16,22).

Direct contractile machinery damage and degradation of sarcomeres during oxidative stress leads to disturbance of the balance between synthesis and degradation of contractile proteins (19,23,24). It has also been documented that oxidative stress induces post-translational modifications: nitration, nitrosylation and phosphorylation of MLC1 and MLC2 increasing their affinity to the proteolytic enzymes (4,14,15,25-27). Among the main contributors to ischemia-reperfusion (I/R) injury of the heart are enhanced production of reactive oxygen species, expression of nitric oxide synthase and overproduction of nitric oxide, which subsequently lead to increased matrix metalloproteinase-2 (MMP-2) activity (28-32). Therefore, MLC become more susceptible to degradation by MMP-2 (4,18). As a consequence, expression of the atrial light chain 1 gene (*MYL4* gene), encoding ALC1 and subsequent synthesis of this protein was observed (13,33,34).

Data showed that the contractile apparatus with myosin containing ALC1 is characterized by a faster cross-bridge kinetics, an increase in maximal shortening velocity, rate of

tension development, isometric force generation, duty ratio and higher calcium sensitivity of force generation (1,3,6,12,14). This effect results from weaker binding of myosin and ALC1 to actin compared to myosin and VLC1 (21,35,36). ALC1 is shorter than VLC1, hence it imposes a weaker 'molecular load' on the cross-bridge (37). The amount of interactions between myosin containing ALC1 and actin may be reduced. It results in an increase in kinetics and force generation, which causes an increase in shortening speed and an improvement in the state of contractility (38,39).

Taking the above into account, the main aim of this research was to explore, whether the expression of ALC1 is increased in the simulated ischemia/reperfusion (sI/sR) injury model of cardiomyocytes isolated from adult heart ventricles in comparison to the cardiomyocytes subjected to aerobic conditions. The next important objective was to examine the MMP-2 changes in the above mentioned sI/sR model. We explored changes in gene expression and concentration of ALC1 and VLC1 in human cardiac myocytes (HCM). We also performed indirect immunofluorescence staining to visualize the differences between ALC1 and VLC1 cell expression. We measured lactate dehydrogenase (LDH) activity as a marker of cell injury and MMP-2 gene expression, content and activity as an indicator of oxidative stress. We proposed the simulated sI/sR injury model of cardiomyocytes isolated from adult heart ventricles as a basic model for observing changes at the cellular level caused by sI/sR. The current study may constitute the foundation for further research on more advanced models.

Materials and methods

Cell culture. The cells of HCM, (commercially available, ScienCell Research Laboratories, Ink. LOT 16787, >1x10^6 cells/ml, 1 ml/vial) isolated from adult heart ventricles were cultured in Dulbecco's Modified Eagle's Medium with the addition of Cardiac Myocytes Growth Supplement (ScienCell Research Laboratories, Ink.; LOT RNBD9247, LOT 16425, respectively), 5% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, Merck KGaA). The cell line was cultured at 37˚C in a water-saturated, 5% CO_2 atmosphere. Cells passages were performed at 80% confluence by harvesting with 0.25% trypsin-EDTA (Sigma-Aldrich, Merck KGaA; LOT MS00JR).

Protocol for in vitro chemical ischemia and reperfusion. HCM underwent in vitro chemical ischemia. After 15 min of aerobic stabilization, cells were subjected to 15 min of chemical ischemia and then 20 min of aerobic reperfusion at room temperature (RT) with an atmosphere of 5% CO_2. Cells were subjected to aerobic stabilization in the HEPES buffer (5.5 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 63.7 mmol/l $CaCl_2$, 5 mmol/l KCl, 2.1 mmol/l $MgCl_2$, 5.5 mmol/l glucose, 10 mmol/l taurine) supplemented with 55 μmol/l $CaCl_2$ and 0.75 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, Merck KGaA). After aerobic stabilization HEPES buffer was replaced (by centrifugation for 2 min at 1,500 x g at RT) with ischemia buffer (HEPES buffer containing 4.4 mmol/l 2-deoxyglucose and 4.0 mmol/l sodium cyanide (Sigma-Aldrich, Merck KGaA), which served as an inhibitor of electron transport chain). 15 min of chemical ischemia was established by measuring LDH activity (marker of cell damage) released from cells (data not showed). Next ischemia buffer was removed (by centrifugation for 2 min at 1,500 x g at RT) and the pellet was resuspended in fresh portion of HEPES buffer containing additional 55 μmol/l $CaCl_2$ and 0.75 mg/ml BSA at RT with an atmosphere of 5% CO_2 (reperfusion). Subsequently cells were centrifuged (for 5 min at 1,500 x g at RT) and cells pellet was homogenized and stored at -80˚C until further analysis. The aerobic control group was maintained in aerobic conditions for 50 min (Fig. 1). 'N' number per one group (Aero/sI/sR) was established as cells collected from one culture flask, from the same passage to keep the samples homogeneous, treated under aerobic or chemical ischemia conditions, respectively. The procedure was developed in our laboratory (40).

Cell homogenization. Cells pellets were suspended in the homogenization buffer (50 mmol/l Tris-HCl (pH 7.4) containing 3.1 mmol/l sucrose, 1 mmol/l dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 0.1% Triton X-100 (Sigma-Aldrich, Merck KGaA) on ice. Then, cells were degraded in three cycles of freezing in liquid nitrogen and thawing at 37˚C, and mechanically homogenized on ice (three times for 10 sec) using a hand-held homogenizer. Cell homogenates were centrifuged for 5 min (10 000 x g at 4˚C) to collect supernatant (cell-conditioned medium) and then stored at -80˚C until further analysis.

Determination of protein concentration. Bradford Protein Assay (Bio-Rad Laboratories, Inc.; LOT #SLBF1982V) was used to determine protein concentration in cells homogenates. BSA (heat shock fraction, ≥98%, Sigma-Aldrich Merck KGaA) served as a protein standard.

Assessment of LDH activity. Lactate dehydrogenase activity assay kit (Sigma-Aldrich,Merck KGaA; LOT #4J20K07260) was used to determine LDH activity in cell-conditioned medium from HCM collected after chemical ischemia, following the manufacturer's instructions. LDH catalyzed the interconversion of pyruvate and lactate with the reduction of nicotinamide adenine dinucleotide to reduced nicotinamide adenine dinucleotide. This reaction was detected by colorimetric assay (at 450 nm). LDH served as a marker of cell injury because of its cytoplasmic localization and immediate release into extracellular space during cell membrane damage/increased permeability.

MYL4, MYL3 and MMP-2 mRNA expression. Total RNA was isolated from HCM pellet using TRIZOL reagent (Thermo Fisher Scientific.; LOT 18132201) in accordance with the manufacturer's instructions. 2 μg of pure RNA was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.; LOT 64393525). The expression of following genes: *MYL4, MYL3* and *MMP-2* in relation to glucose-6-phosphate dehydrogenase (*G6PD*) was analyzed by RQ-PCR using CFX96 Touch (Bio-Rad Laboratories, Inc.). iTaq Universal Sybr Green Supermix (Bio-Rad Laboratories, Inc.; LOT 64326845), forward and reverse primers (final concentration 0.1 μm/l), Ultra Pure DEPC-Treated water (Thermo Fisher Scientific) and cDNA (100 ng) were used in a

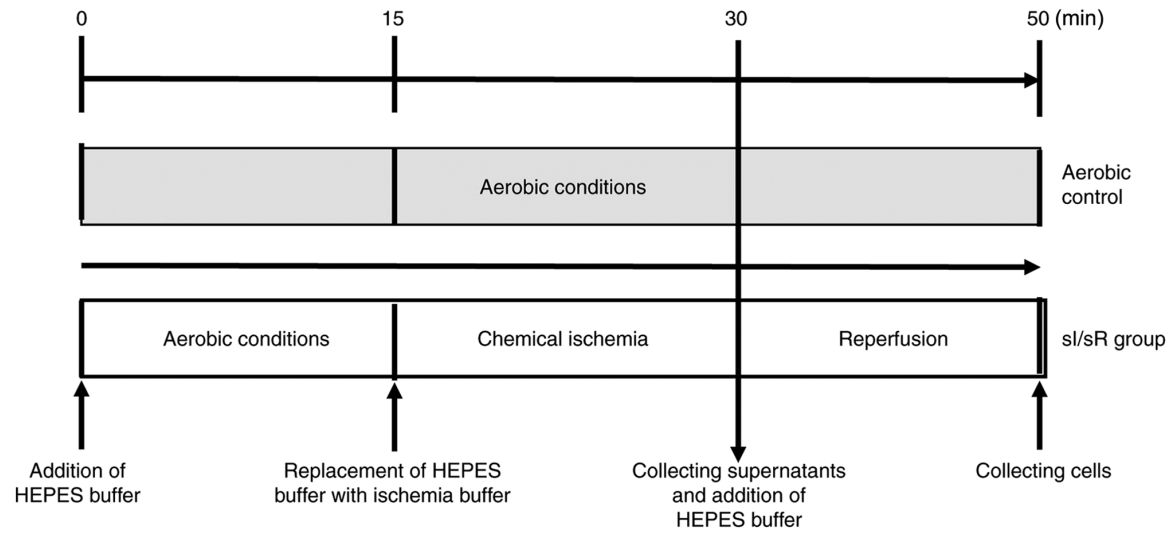

Figure 1. Experimental protocol for the sI/sR group and aerobic control. sI/sR, simulated ischemia and simulated reperfusion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

final volume of 30 μl. The 5'-3' sequences of primers were as follows: MYL4 F: AAGATCACCTACGGCCAGTG, MYL4 R: CCCTCCACGAAGTCCTCATA, MYL3 F: TACTTACAG CCCCCAATGGC, MYL3 R: TCTTGCCCAGCCATCAAC TT, MMP-2 F: ATCCAGACTTCCTCAGGCGG, MMP-2 R: CCTGGCAATCCCTTTGTATGTT. The amount of mRNAs in relation to *G6PD* was calculated as $2^{-\Delta Ct}$. The relative expression of respective genes were compared in cells exposed to aerobic conditions and cells subjected to sI/sR.

Analysis of MMP-2 concentration in cell homogenates. MMP-2 concentration in HCM homogenates was measured using quantitative Quantikine ELISA Assay for Total MMP-2 (R&D Systems, Inc.; LOT P256537) in accordance with the manufacturer's instruction. Total MMP-2 Quantikine ELISA assay recognized recombinant MMP-2, active and pro-MMP-2, as well as tissue inhibitors of matrix metalloproteinase complexed MMP-2. MMP-2 was immobilized with monoclonal antibody specific to this protein and detected using anti-Total-MMP-2 polyclonal antibody conjugated to Streptavidin-Horse Radish Peroxidase (HRP). Then 3,3',5,'-Tetramethylbenzidine (TMB) substrate solution was added to trigger the reaction. A minimum detectable dose was 0.033 ng/ml. MMP-2 concentration in cell homogenates was expressed as ng per μg of total protein.

Determination of MMP-2 activity. MMP-2 activity was examined by gelatin zymography with the modified protocol of Heussen and Dowdle (40,41). Protein concentrations in HCM and cell-conditioned medium collected after simulated ischemia were measured using Bradford Protein Assay. HCM homogenate and cell-conditioned medium from each sample were adjusted to contain the same protein concentration with distilled water, and mixed with 4x Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.) in a ratio of 4:1 (v:v). Samples containing 20 μg of protein were applied to 8% polyacrylamide gel copolymerized with gelatin (2 mg/ml) and 0.1% sodium dodecyl sulfate (SDS, denaturing, but not reducing conditions). After electrophoresis, gels were rinsed (three times for 20 min) in 2.5% Triton X-100 to remove SDS. Then, gels were placed in the incubation buffer (50 mol/l Tris-HCl pH 7.5, 5 mmol/l $CaCl_2$, 200 mmol/l NaCl, 0.05% NaN_3) for 18 h at 37˚C. After digestion of gelatin, gels were staining for 2 h (0.5% Coomassie Brilliant Blue R-250, 30% methanol, 10% acetic acid) and destaining (30% methanol, 10% acetic acid) until bands were clearly visible (Sigma-Aldrich, Merck KGaA). MMP-2 activity was visualized as dark bands on a bright background. Zymograms were scanned using GS-800 Calibrated Densitometer (model PowerLook 2100 XL-USB) and analyzed using Quantity One v. 4.6.9 software (Bio-Rad Laboratories, Inc.). The relative activity of MMP-2 was established and expressed in AU as an activity per μg of total protein.

Analysis of ALC1 and VLC1 concentration in cell homogenates. Atrial isoform of myosin light chain 1 (ALC1) concentration in HCM homogenates was determined using Human Myosin Light Chain 4 ELISA Kit (Bioassay Technology Laboratory; LOT 1812006). The concentration of ventricular isoform of myosin light chain 1 (VLC1) in HCM homogenates was determined using Human Myosin Light Chain 3 ELISA kit (Bioassay Technology Laboratory; LOT 201908009). Briefly, capture antibodies bound to ALC1/VLC1 from cell homogenates which were detected by biotinylated antibodies. Then, HRP was conjugated to the biotinylated ALC1/VLC1 antibody. TMB substrate solution was added to enable complex visualization. A minimum detectable concentration of ALC1 and VLC1 ELISA tests was 0.024 ng/ml and 12.93 ng/l, respectively. ALC1/VLC1 in cell homogenates were expressed as ng per μg of total protein.

Analysis of ALC1 and VLC1 by immunofluorescence microscopy. Indirect immunofluorescence method was used to determine the expression of ALC1 and VLC1 in cardiomyocytes after sI/sR. Cells were cultured at 24-well Cell Culture Plate (Greiner Bio-One, Cellstar) to reach 90% confluence, as previously described. Cells underwent in vitro chemical ischemia on culture plate according to the procedure described above (simultaneously aerobic control was

maintained in aerobic conditions). Next 4% paraformaldehyde (Sigma-Aldrich, Merck KGaA) was added to each well in order to fix the cells. After 15 min of incubation at RT, cells were washed with phosphate-buffered saline (PBS). Then blocking buffer (10% goat serum, 1% BSA, 0.3 m glycine, 0.1% Triton X-100 in PBS) was added to each well. Plate was incubated for 1 h at RT and then washed with PBS. Plate was incubated overnight at 4˚C with Rabbit Anti-MYL4 Antibody (Bioassay Technology Laboratory; LOT BT-AP05702) and Mouse Anti-MYL3 Antibody (Invitrogen; LOT UH2823151). After overnight incubation plate was rinsed with PBS. Then Donkey anti-Rabbit IgG conjugated to fluorochrome DyLight 488 (Abcam,; LOT GR177576-7) and Goat anti-Mouse IgG conjugated to fluorochrome DyLight 550 (Abcam; LOT GR155905-3) were added to the wells for 45 min in the dark to visualize the expression of ALC1 (green fluorescence) and VLC1 (red fluorescence), respectively. After wash, DAPI (4', 6-diamidino-2-phenylindole, Sigma-Aldrich, Merck KGaA ; LOT #034M4030V) was added to wells to stain cells' nuclei (blue fluorescence). After 15 min of incubation at RT in the dark and washed with PBS the emitted signal was read using Multifunction Reader Spark TK Biotech 2017 (Tecan Austria GmbH), Zoe Fluorescent Cell Imager (magnification x200, Bio-Rad Laboratories, Inc.) and Thunder Leica Imager (magnification x400, Leica Microsystems). The excitation and emission wavelength for DyLight 488 was 493 and 518 nm, respectively. The excitation and emission wavelength for DyLight 550 was 562 and 576 nm, respectively. The excitation and emission wavelength for DAPI was 358 and 461 nm, respectively. The cell expression of ALC1 and VLC1 in cardiomyocytes were expressed in arbitrary units (AU) as expression normalized to the total number of cells in each well determined by staining with DAPI.

Statistical analysis. The statistical analysis of the results was performed in GraphPad Prism v. 5. Shapiro-Wilk normality test or Kolmogorov-Smirnov test was used to estimate the normality of variances changes. Then results were analyzed by appropriate tests: unpaired t-test or Mann-Whitney U test. Correlations were assessed using Pearson's or Spearman's tests, as appropriate. The results were expressed as mean ± standard error of the mean. $P<0.05$ was regarded as a statistically significant difference. In selected tests we used up to 20 flasks per group (Aero or sI/sR, respectively)-it was based on the Power Analysis test (alpha =0.05, power =0.80), which was performed with the preliminary studies for each test. The difference in 'N' numbers between experiments also follows from the fact that some part of cells died, then the 'N' number had to be reduced. Part of results had to be removed from the analysis as well, as they were 'significant outliers' according to Grubbs test. Despite the different 'N' number, we obtained statistically significant differences in the presented experiments.

Results

LDH activity in cell-conditioned medium as a marker of cell injury. LDH activity in cell-conditioned medium of sI/sR group (as a marker of ischemic cells damage) was significantly increased in comparison to the aerobic (Aero) group (P=0.02, n=20) (Fig. 2). It confirmed the induction of sI/sR.

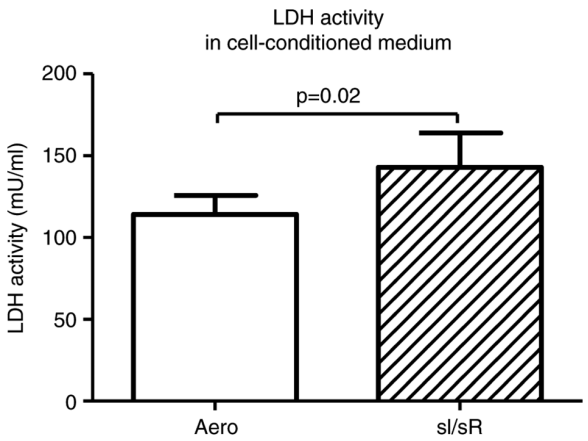

Figure 2. LDH activity in the cell-conditioned medium of cells subjected to sI/sR and aerobic conditions. Data are presented as the mean ± SEM (n=20). LDH, lactate dehydrogenase. sI/sR, simulated ischemia and simulated reperfusion; aero, aerobic control.

MMP-2 mRNA expression, synthesis and activity in myocytes subjected to sI/sR. The expression of *MMP-2* gene (P<0.001, n=7), as well as MMP-2 concentration (P=0.03, n=8) and activity (P=0.03, n=6) in HCM were decreased in sI/sR group in comparison to Aero group (Fig. 3A-C). However, MMP-2 activity measured in cell-conditioned medium collected after chemical ischemia and before reperfusion was significantly higher in sI/sR group (P=0.006, n=10) (Fig. 3D and E).

MYL4, MYL3 mRNA expression and ALC1, VLC1 concentrations in cell homogenates. *MYL4* gene (coding ALC1) expression level was significantly higher in sI/sR group in comparison to Aero group (P=0.03, n=14) (Fig. 4A). In contrary, the expression of *MYL3* gene (coding VLC1) was decreased in sI/sR group (P=0.008, n=14) (Fig. 4B). This was confirmed by negative correlation of *MYL4* and *MYL3* genes expressions (P=0.01, r=0.34) (Fig. 4C). Increased expression of *MYL4* mRNA negatively correlated with expression of *MMP-2* (P=0.04, r=0.36), while expression of *MYL3* mRNA correlated positively with *MMP-2* expression (P<0.001, r=0.67) (Fig. 4D).

The concentration of ALC1 in HCM was significantly higher in sI/sR group in comparison to Aero group (P<0.001, n=21). The difference in VLC1 content in HCM was not significant between Aero and sI/sR groups (P=0.18, n=14) (Fig. 5A and B). The negative correlation between ALC1 and MMP-2 concentration in cells was found (P=0.003, r=0.44) (Fig. 5C).

Cell expression of ALC1 and VLC1 in myocytes. Indirect immunofluorescence testing showed that the number of HCM after sI/sR was much lower in comparison to Aero group (P=0.003, n=12) (Fig. 6A). 4', 6-diamidyno-2-phenyloindole (DAPI) stained all nuclei in fixed cells-result of DAPI emission served as a marker of cells number. sI/sR group of myocytes showed higher cell expression of ALC1 (P=0.01, n=12) (Fig. 6B and E) and lower cell expression of VLC1 (P=0.04, n=12) (Fig. 6C and F) than the Aero group. This was confirmed by their negative correlation (P=0.02, r=0.46) (Fig. 6D).

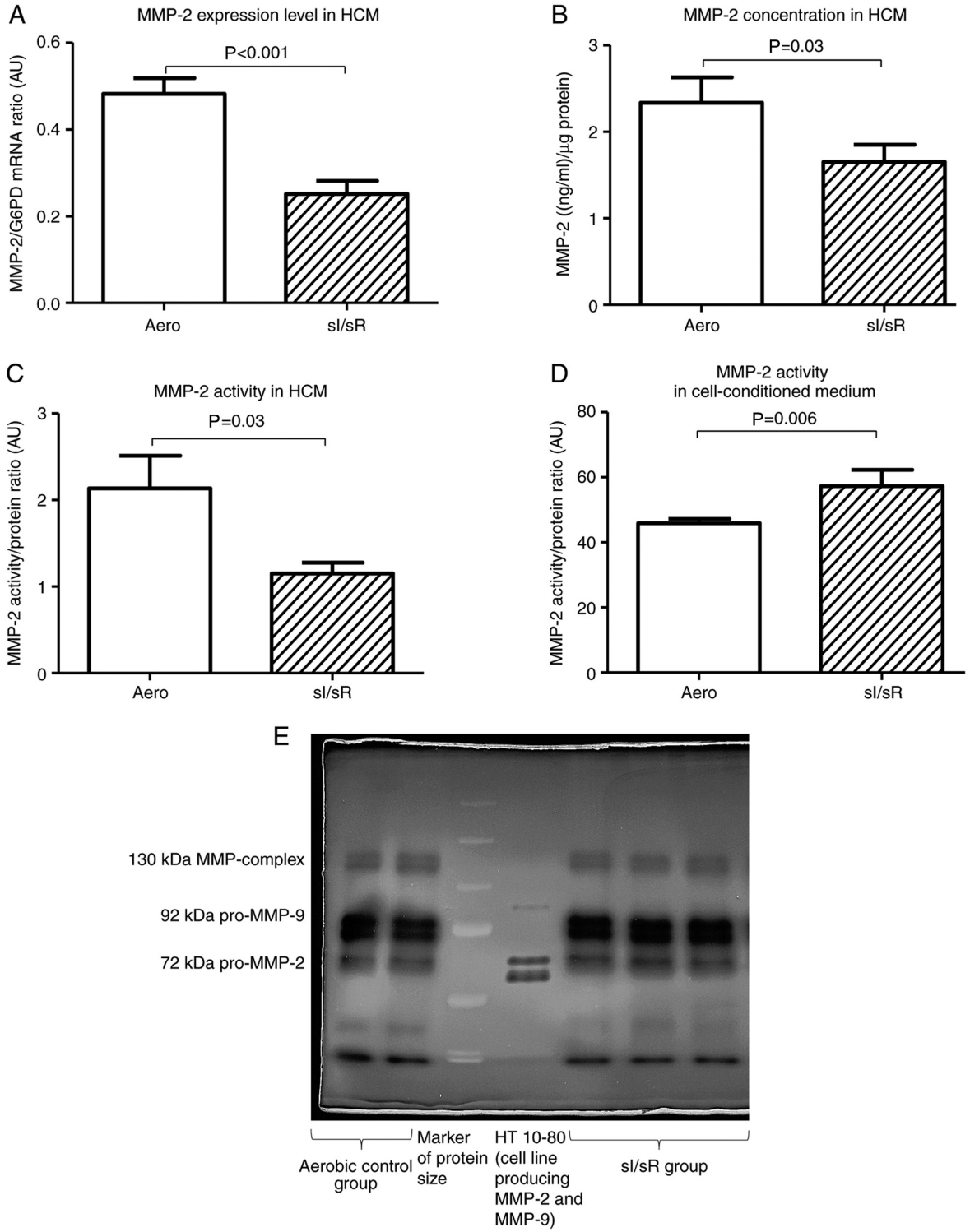

Figure 3. MMP-2 measurement. (A) *MMP-2* mRNA expression normalized to the *G6PD* gene (n=7). (B) MMP-2 concentration in cell homogenates normalized to the content of protein in each sample (n=8). (C) MMP-2 activity in HCM (n=6). (D) MMP-2 activity in the cell-conditioned medium of HCM (n=10). (E) Representative zymogram of MMP-2 activity in cell-cultured medium. Data are presented as the mean ± SEM. G6PD, glucose-6-phosphate dehydrogenase; HCM, human cardiomyocytes; sI/sR, simulated ischemia and simulated reperfusion; aero, aerobic control; AU, arbitrary units.

Discussion

Human myocardium contains a wide range of proteins isoforms responsible for the heart contractile function (38,42-45). During the development of cardiac pathologies, changes in the contractile apparatus of cardiomyocytes striving to maintain myocardial contractile activity occur (38,46). Transformation of atrial and ventricular types of myosin is one of the detectable alterations (47). Expression of MHC does not change substantially in adult hypertrophic ventricle, while ALC1 partially replaces the endogenous VLC1 isoform (9,22,35). As a result of the increased effort, cardiomyocytes undergo both hypertrophy and changes in the cell phenotype. Adult myocardial cells strive for restoring the original protein synthesis

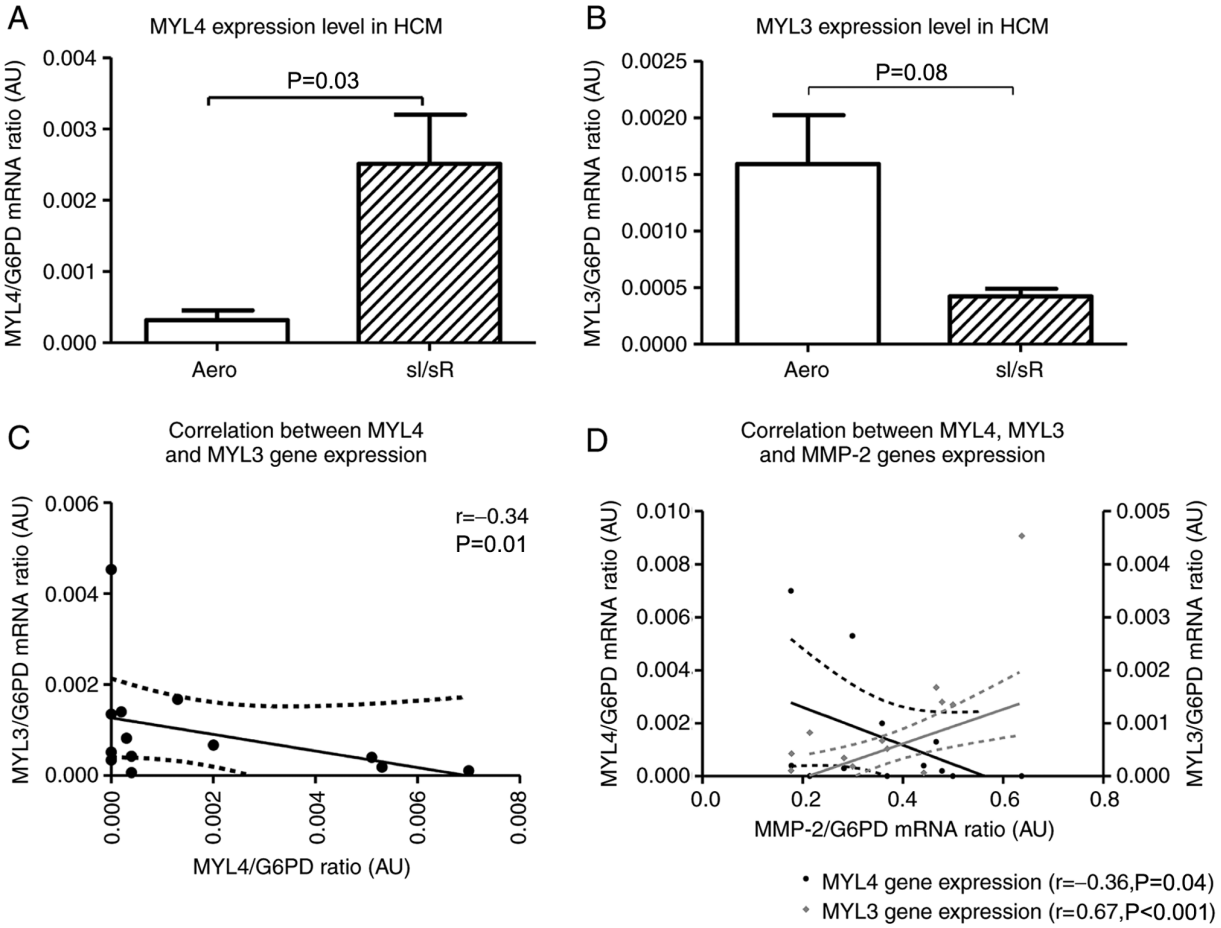

Figure 4. *MYL4 and MYL3* mRNA expression. (A) *MYL4* and (B) *MYL3* mRNA expression was normalized to the *G6PD* gene (n=14). Subsequently, the correlation between (C) *MYL4* and *MYL3* (n=13), and (D) *MYL4, MYL3* and *MMP-2* gene expression was assessed (n=12). Solid lines represent the residual plot. Dotted lines represent the 95% confidence interval. Data are presented as the mean ± SEM. MYL4, atrial light chain 1 gene; MYL3, myosin light chain 3; G6PD, glucose-6-phosphate dehydrogenase; HCM, human cardiomyocytes; sI/sR, simulated ischemia and simulated reperfusion; aero, aerobic control; AU, arbitrary units.

pattern previously observed in ontogenesis, to regain their original ability to perform proper function. The 'fetal gene program' is induced which results in reduction of the expression of adult isoforms of several heart proteins and increase in the expression of particular genes such as *MYL4*, which is known as a key gene responsible for atrial contractile, structural and electrical integrity (9,13,47). Fujimoto *et al* (1993) showed that ALC1 was strongly expressed in the overloaded adult ventricle as well as in the fetal ventricle (48). They indicated that proteins expressed in the fetal stage reappeared in an overloaded ventricle (48).

The main aim of this research was to explore, whether the expression of ALC1 is increased in the simulated ischemia/reperfusion injury of ventricular cardiomyocytes. We showed that sI/sR induced changes in *MYL4* and *MYL3* at the level of genes expression and protein synthesis. The expression of *MYL4* gene (encoding atrial form of MLC1) was significantly increased in cardiomyocytes subjected to sI/sR, while the expression of *MYL3* gene (encoding ventricular form of MLC1) was significantly reduced in comparison to cells maintained in aerobic conditions. In addition, a negative correlation between *MYL4* and *MYL3* was observed, which may confirm that the simultaneous change in the isoform expression occurred as an adaptation of myocyte contractile apparatus to sI/sR conditions and an activation of 'fetal gene program' (20).

Diffee *et al* (2003) presented the results of real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) for expression of *MYL4* and *MYL3* genes of the hearts of subjects undergoing exercise training, with respect to the control tissue from heart ventricles of healthy subjects (47). They observed an increase in mRNA expression and ALC1 production in those, who were forced through physical exercises. In the consequences, this was associated with enhanced cardiac contractile function through increased calcium sensitivity of tension and increased power output (47). However, in contrary to our results, the expression of *MYL3* did not decrease. It suggested that overexpression of *MYL4* in myocardium resulted in the inclusion of the recently synthesized ALC1 isoform in myofilament despite no change in VLC1 expression after exercise training (47). Ritter *et al* (1999) demonstrated that replacement of VLC1 with ALC1 in transgenic mice occurred at the level of protein synthesis, despite the changes being visible in gene expression in rats subjected to the endurance training (18). This means that the mechanism is not well understood and still requires further research.

In our research, changes of *MYL4* and *MYL3* mRNA expressions were accompanied with their synthesis in ventricle

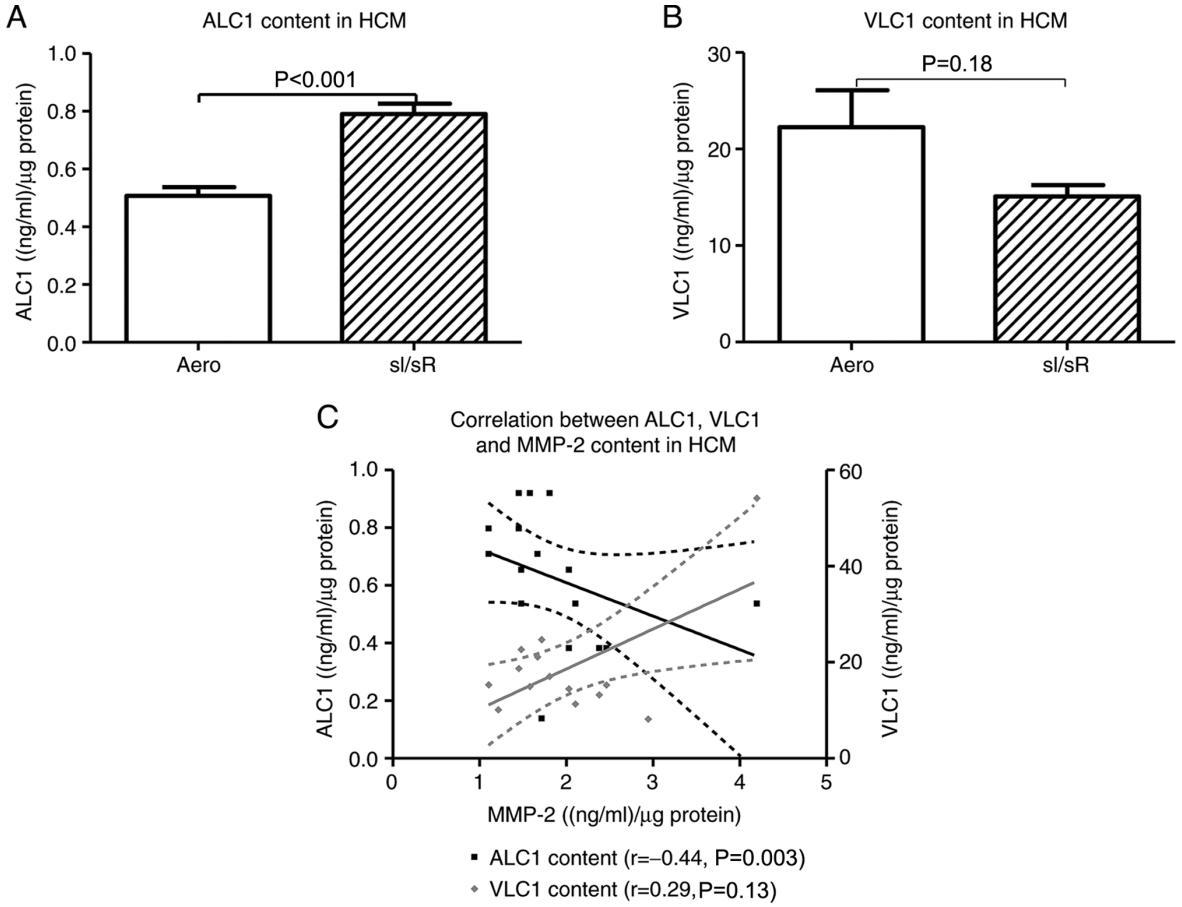

Figure 5. Synthesis of ALC1/VLC1 in myocytes. The concentration of (A) ALC1 and (B) VLC1 in HCM was measured using ELISA and normalized to the protein content of each sample (n=14-21). (C) The correlation between ALC1 and VLC1 content and MMP-2 concentration in cells was assessed (n=14-6). Data are presented as the mean ± SEM. ALC1, atrial light chain 1; VLC1, ventricular light chain 1; HCM, human cardiomyocytes; sI/sR, simulated ischemia and simulated reperfusion; aero, aerobic control.

myocytes. ALC1 concentration and tissue expression were significantly increased in cardiomyocytes subjected to oxidative stress. An immunofluorescence study confirmed negative correlation of ALC1 and VLC1 expression in cardiomyocytes. There are previous reports confirming compensatory mechanism observed in our study. Nakao *et al* (1992) showed that expression of ALC1 was increased in the heart ventricles with progressive heart failure and ventricular aneurysmectomy in patients with old myocardial infarction in their medical history. It was considered as a regional adaptation to the increased workload (19). Machackova *et al* (2006) observed an increased expression of ALC1 in the ventricles of patients with familial hypertrophic cardiomyopathy-ALC1 partially replaced VLC1, leading to increased cross-bridge cycling kinetics (16). Similarly, Morano *et al* (1996) observed, that increased expression of ALC1 modulated cross-bridge cycling kinetics by accelerating shortening velocity and isometric tension production (8). Diffee *et al* (2003) demonstrated that the level of changes in systolic properties significantly correlated with the increase of ALC1 expression (47). The replacement of VLC1 with ALC1 is a reversible phenomenon (8,12,14). This may indicate that changes in the expression of MLC1 isoforms are the basic molecular mechanism for adjustments in the systolic function during the pathological state (24,47,49).

Myocardial injury during I/R is a complex and multifactorial process in which reactive oxygen species activate MMP-2 (28-31,50), which in turn degrades the contractile proteins such as MLC1 (25,27,30,50-52). We previously showed that this process led to contractility disorders (53,54). In the current study, MMP-2 gene expression as well as its concentration in HCM homogenates and MMP-2 activity in HCM and cell-conditioned medium were examined. We showed that mRNA expression, synthesis and activity of MMP-2 in HCM were decreased in the sI/sR group, however, MMP-2 activity measured in cell-conditioned medium collected after ischemia was higher in sI/sR group. This confirmed that MMP-2 was released into the extracellular space due to cells damage.

Biały *et al* (2018) described similar relationship of MMP-2 and I/R (40). Reduced MMP-2 activity measured in HCM homogenates was observed, suggesting the release of MMP-2 into extracellular space (40). They also showed significantly reduced metabolic activity of myocytes, implying their inability to produce energy and, consequently, metabolic failure and death (40). Cheung *et al* (2000) also proved that in first minutes after reperfusion had started there was observed an acute enhancement in MMP-2 release into the coronary effluents from the hearts as shown by increase MMP-2 activity in coronary effluents (55), which was

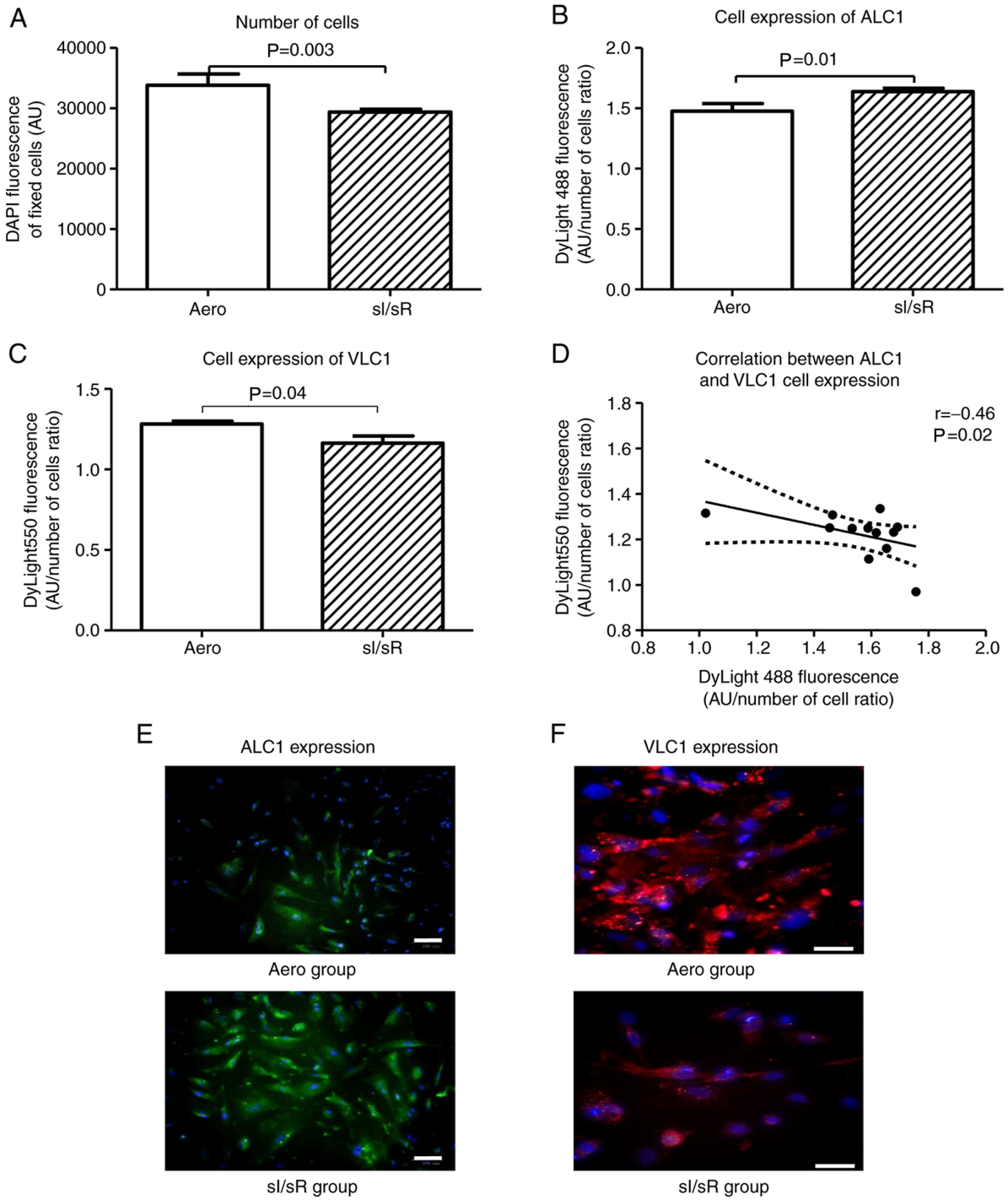

Figure 6. Fluorescence microscopy. (A) The number of cells fixed with paraformaldehyde was assessed by measuring the fluorescence of cell nuclei stained with DAPI (blue fluorescence) (n=12). (B) the HCM expression of ALC1 was assessed by measuring the green fluorescence of fixed cells stained with anti-ALC1 antibodies and DyLight 488 normalized to the total number of cells (blue fluorescence) (n=12). (C) The HCM expression of VLC1 was assessed by measuring the red fluorescence of fixed cells stained with anti-VLC1 antibodies and DyLight 550 normalized to the total number of cells (blue fluorescence) (n=12). (D) The correlation between ALC1 and VLC1 cell expression was established (n=12). (E) Immunofluorescence staining of fixed cells for ALC1 (green fluorescence) and DAPI for nuclei (blue fluorescence) was performed (magnification, x200; scale bar, 100 μm). (F) immunofluorescence staining of fixed cells for VLC1 (red fluorescence) and DAPI for nuclei (blue fluorescence) (magnification, x400; scale bar, 100 μm). Solid lines represent the residual plot. Dotted lines represent the 95% confidence interval. Data are presented as the mean ± SEM. HCM, human cardiomyocytes; ALC1, atrial light chain 1; VLC1, ventricular light chain 1; sI/sR, simulated ischemia and simulated reperfusion; aero, aerobic control; AU, arbitrary units.

consistent with our results. Ben-Yosef *et al* (2002) showed that MMP-2 activity changed from reduced after 6 h of hypoxia to increased after 24 h during remodelling of the extracellular matrix after I/R (56). Our study showed that both 15 min of chemical ischemia and 20 min of reperfusion led to the increased MMP-2 activity in cell-conditioned medium and decreased MMP-2 level in cells homogenates, confirming myocytes damage.

Bil-Lula *et al* (2018) also showed that increased synthesis of MMP-2 during I/R was associated with a decrease in

VLC1 concentration by about 50% in cardiomyocytes of ventricles (23). Importantly, the *MMP-2* gene expression in current study positively correlated with the expression of *MYL3* gene and negatively with the expression of *MYL4* gene. Moreover, a negative correlation between ALC1 concentration and MMP-2 content in cell homogenates may suggest that there is a relationship between ALC1 and MMP-2 activity, but this needs further investigation, as is only a speculation now.

In conclusion, the above data showed that the expression changes of ALC1 and VLC1 in an adult ventricle myocytes during simulated ischemia/reperfusion injury may be induced by the 'fetal genes expression program' as an attempt to adapt to sI/sR conditions. Described mechanism may lead to an improvement in heart function, but this needs further investigation. Moreover, data revealed that there is a relationship between ALC1 and MMP-2 activity, but this also needs further investigation. This preliminary study may provide the foundation for further studies on cardiac function, particularly cardiomyocyte contractility and actin - myosin interactions, on more advanced I/R models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MB and IBL conceived the current study. MB, AO and KH performed the experiments and stored data on external drives. MB, AKZ, AO, KH and IBL contributed to data analysis, investigation and methodology. MB and AO confirm the authenticity of all the raw data. AKZ and IBL coordinated funding to the project. MB and IBL contributed to project administration, supervision and construction of the final manuscript. MB contributed resources and conceived the original draft of the manuscript. IBL critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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