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Heat shock attenuates VEGF expression in three-dimensional myoblast sheets deteriorating therapeutic efficacy in heart failure

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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Summary

Background: Myoblast sheet transplantation is a promising novel treatment for ischemic heart failure. The aim of this study was to test the hypothesis that heat shock (HS) pre-treatment affects the angiogenic properties of myoblast sheets *in vivo* and *in vitro*.

Material/Methods: We studied HS preconditioning of L6 myoblast sheets in relation to their apoptosis, proliferation, and vascular endothelial growth factor (VEGF)-associated responses under normoxia and under hypoxia *in vitro*. *In vivo* evaluation of their therapeutic effect was performed with 60 male Wistar rats divided into 3 groups (20 each): sole left anterior descending (LAD) ligation (control); LAD ligation and non-conditioned sheet transplantation (L6 No-Shock); and LAD ligation and L6-heat shock conditioned sheet transplantation (L6 Heat-Shock). Left ventricular function was evaluated by echocardiography after 3, 10, and 28 days.

Results: Expression of HSP70/72 was strongly induced 24 hours after HS, and thereafter it decreased notably during 72 hours in hypoxia. Under normal growth conditions, HSP70/72 expression remained stable. HS delayed apoptosis-associated caspase-3 expression during 24-hour hypoxia compared to non-treated controls. However, VEGF expression reduced significantly in the heat shock pre-treated sheets. Ejection fraction of the L6-myoblast HS pre-treatment group (L6 Heat-Shock) decreased gradually during follow-up, in the same pattern as the controls. However, these functional parameters improved in the L6-myoblast normal sheet group (L6 No-Shock) at the tenth day and remained significantly better.

Conclusions: HS protects myoblast sheets from hypoxia-associated apoptosis *in vitro*, but reduces VEGF expression of the sheet, leading to lower therapeutic effect in heart failure.

key words: heat shock • cell sheet therapy • hypoxia • angiogenesis

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Background

Cell therapy has emerged as a new treatment method for cardiac tissue repair. Skeletal myoblasts possess several optimal qualities for cellular transplantation, including easy harvest, autologous use, and resistance to hypoxia. Experimental and clinical studies have shown that skeletal myoblasts can engraft in postinfarction scars, differentiate into myotubes, and improve heart function [1,2]. The first, larger randomized clinical study, the MAGIC trial, showed no improvement in primary end-points, which brought basic monolayer myoblast cell therapy to an end [3]. This study forced us to set our sights on cell modifications and engineering.

Thermoplate-based cell sheet engineering is a technology in which sheets can be constructed in temperature-responsive, polymer-grafted cell-culture dishes, which release the confluent cells from the dish surface at less than 20°C [4,5]. Sheets can be placed topically on the myocardium (Figure 1). This delivery system resolves problems related to the intramyocardial injection of the cells [6]. This method maintains cell-cell interactions and is preferred over an artificial tissue-engineered scaffold because it does not raise material-related issues of biocompatibility, biodegradability, or cytotoxicity. The cell sheet structure preserves intercellular communication junctions, and the extracellular matrix deposited by the cells themselves is the adhesive agent [7]. Using culture surfaces covalently grafted with this temperature-responsive polymer poly (*N*-isopropylacrylamide), cell sheets can be harvested by a simple temperature change and can be layered upon each other to improve the desired effect [8].

Improvement of graft cell survival after skeletal myoblast transplantation is a major concern. When developing 3-dimensional cell constructs, it is of utmost importance to generate vasculature for survival of the graft. Heat shock proteins (HSPs) protect cells during various stress conditions [9], and their apoptosis-blocking properties have an important function in cyto-protection. HSP70/72, one of the most studied subtypes of these chaperones, is well known to be up-regulated by heat shock pretreatment of mammalian cells [10,11]. Heat shock (HS) has served as a pre-conditioning method prior to myoblast transplantation [12,13]. It has been suggested that by using HS as a preconditioning method, there is no need to modify the cells genetically before their transplantation. However, it is unknown how the HS affects the angiogenic capacity of the myoblast sheet.

Myoblast sheet implantation has been shown to improve cardiac function by attenuating cardiac remodeling and metabolic recovery in the impaired myocardium by paracrine effects [4,13]. In this study, we investigated the angiogenic effects of HS treatment in the myoblast sheet. We induced HS by incubating the sheets at 42°C for 45 minutes and noted its effect by the expression of heat shock protein 70/72 (HSP70/72). Subsequently, we tested the sheet *in vitro* and *in vivo* in a rat model of acute heart failure.

Material and Methods

Antibodies and reagents

Anti-HSP70/72 (Stressgen, Victoria, BC, Canada), anti-PCNA (Neo Markers, Fremont, CA), anti-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-caspase-3 specific for the active cleaved fragment (Cell Signaling Technology Inc, Danvers, MA), Alkaline phosphatase (AP)-conjugated secondary antibodies, anti-mouse IgG, and anti-rabbit IgG were from Promega (Madison, WI), and the AP-conjugated anti-mouse IgM was from Jackson ImmunoResearch Europe Ltd (Soham, UK).

Myoblast cell culture

The L6 rat myoblast cell line (CRL-1458, Manassas, VA) from the American Type Culture Collection was cultured in growth medium (GM): DMEM supplemented with 10% fetal calf serum (FCS) (Gibco, Paisley, Scotland), antibiotics (antibiotic-antimycotic: penicillin 100 µg/ml, streptomycin sulfate and 0.25 µg/ml amphotericin B as Fungizone, Gibco). Cells were used at passages 5 to 15.

Sheet formation and heat shock

The cell sheets were made as described earlier [4]. Briefly: 6 million L6 myoblasts were seeded in temperature-responsive culture dishes (Cell Seed Up Cell). After 24-hour incubation, the cell sheets were harvested by temperature changes from 37°C to 20°C. Prior to transplantation, L6 cell sheets

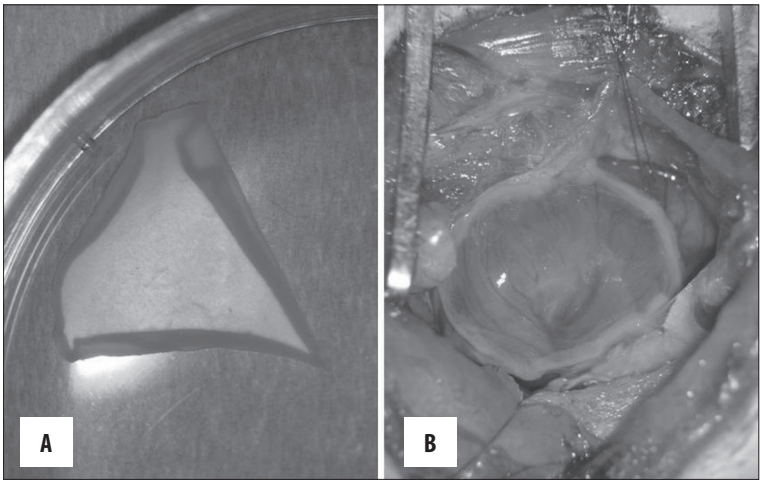

Figure 1. (**A**) Myoblast cell sheet after harvesting on a cell culture plate and (**B**) Graft implanted onto the infarcted area of a rat heart.

were subjected to hyperthermia (+42°C for 45 minutes) on plates in a water bath. Sheets without HS-treatment (No-Shock) served as controls *in vivo*.

Sheet resistance to oxidative stress, and proliferation

Sheet resistance to apoptosis was studied by exposing HS-conditioned and non-conditioned sheets (No-Shock) to pathological hypoxia (1% O_2). After harvesting and HS pretreatment, sheets (Heat-Shock and No-Shock) were immediately transferred to a hypoxia incubator (In Vivo2, Hypoxia Workstation 400; Ruskinn Life Sciences Ltd., Bridgend, UK). Western blot samples were collected after exposing cell sheets continuously to hypoxia for 24, 48, and 72 hours.

Immunoblotting

For immunoblotting, cell samples were lysed to SDS-PAGE sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.005% bromphenol blue) and boiled for 5 min. SDS-PAGE (gradient of polyacrylamide 5–15% with 4% stacking gel) was used to separate proteins, thereafter they were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was then blocked with 2.5% low-fat dried milk in Tris-buffered saline (20 mM Tris- HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5), and was incubated with primary antibodies, followed by incubation with an alkaline phosphatase-conjugated secondary antibody (Promega). The protein bands were visualized according to manufacturer's recommendation. Densitometry of images was performed with the ImageJ program (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, *http://rsb.info.nih.gov/ij/*, 1997–2008).

VEGF-A secretion of myoblast sheets

VEGF-A protein secretion from heat shock-conditioned and non-conditioned sheets was determined from growth medium after 24, 48, and 72 hours in hypoxia with a rat VEGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Animal population

This acute myocardial infarction rat study model used 60 male Wistar rats (326±11 g). The rats were randomized into 3 groups of 20 animals each: Control (LAD ligation only), L6 No-Shock (LAD ligation and non-conditioned sheet transplantation), and L6 Heat-Shock (LAD ligation and heat shock-conditioned sheet transplantation) before LAD ligation. Experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals from the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were evaluated and approved by the ethics committee of the Hospital District of Helsinki and Uusimaa, Meilahti Hospital, Department of Surgery.

Myocardial infarction

The LAD coronary artery was ligated as described [14]; the same operation included myoblast cell sheet transplantation. Briefly, rats were anesthetized with 0.05 mg/kg of medetomidine (Orion Pharma Inc, Turku, Finland) and 5 mg/kg i.p. ketamine (Parke-Davis, Barcelona, Spain). Animals were intubated and remained under mechanical ventilation during surgery. The heart was exposed through a left thoracotomy. LAD was ligated at the lower border of the left atrial appendage. Immediately after LAD ligation, 2 myoblast sheets (total 1.2 10^7 cells) were placed on the left ventricular anterior wall. Each animal received either conditioned or non-conditioned sheets. Cell recovery time after HS preconditioning prior to transplantation was 5–10 hours. Anesthesia was antagonized after the surgery (atipamezole hydrochloride 1.0 mg/kg s.c., Orion Pharma, Inc.), and the animals were allowed to recover. Buprenorphine hydrochloride (0.05 mg/kg s.c., Reckitt and Colman Ltd, Hull, UK) served as post-operative analgesia.

Echocardiography

All animals underwent echocardiography under medetomidine anesthesia (0.5 mg/kg) at 3 (baseline), 10, and 28 days after surgery with a 7.5 MHz transducer (MyLabR25, Esaote SpA, Genoa, Italy). Anterior wall thickness was measured during end-systole (AWTs) and end-diastole (AWTd), as was posterior wall thickness during end-systole (PWTs) and end-diastole (PWTd) phases. Left ventricular internal diameter at end-systole (LVDs) and end-diastole (LVDd) were measured similarly in the short-axis right parasternal projection just between the mitral valve and papillary muscles. LVDd and LVDs served to calculate fraction shortening (LVFS) and ejection fraction (LVEF):

$$LVFS\ (\%) = (LVDd\text{-}LVDs)/LVDd$$

$$LVEF\ (\%) = (LVDd3\text{-}LVDs^3)/LVDd^3.$$

Histology and Immunostaining

At post-operation day 28, the rats underwent echocardiography and were euthanized. The excised heart was then and cut into 4 equal transverse parts. The 2 middle parts (next apex and next basal) were fixed in 4% neutral-buffered formalin for 48 hours. The samples were then embedded in paraffin and cut into 4-µm-thick sections. Immunofluorescent staining allowed identification of vascular structures and cell proliferation. To demonstrate vascular density, endothelial cells were stained with an antibody against von Willebrand Factor (vWF, AB7356, Millipore, Billerica, MA). Cell proliferation was evaluated with anti-Ki67 antibody (RM-9106-S1, Labvision Inc., Fremont, CA). Picric acid stain served to analyze fibrosis. The immunolabeled sections were examined under an Olympus IX81 fluorescent microscope. The vWF- and Ki67-immunostained sections from each of the infarct and border areas were photographed with a microscope in 2 to 3 randomly selected microscopic fields at 20X magnification. Fibrosis was evaluated from scanned images of picric acid-stained sections. Percentage of fibrosis was calculated as picric acid-stained area divided by whole section area as evaluated by use of Image J / Photoshop 7.0 (Adobe Inc, San Jose, CA).

Statistical analysis

Data are presented as mean ±SEM. Statistical significance was determined using ANOVA and student *T* test. Differences were considered statistically significant at $p<0.05$.

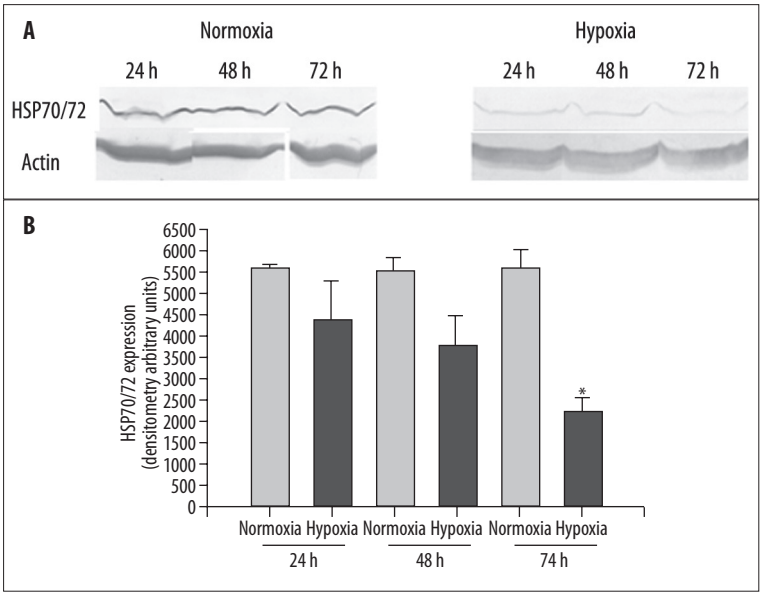

Figure 2. (**A**) Expression of HSP70/72 in L6 myoblast sheets at indicated time under normal growth conditions (Normoxia) and 1% O_2 (Hypoxia) after heat shock pre-treatment. Sheets were prepared as described and subjected to hyperthermia (+42°C for 45 minutes) on plates in a water bath and then transferred to hypoxia. Control sheets were cultured under normal growth conditions. Actin was used as a loading control. (**B**) Densitometry of HSP70/72 immunoblots of heat-shocked L6 myoblast sheets under normoxia and hypoxia from two to three independent experiments. Under normoxia, HSP70/72 expression remained stable, but under hypoxia expression significantly reduced within 72 hours. (* $p<0.05$ compared to normal growth condition cultured sheets).

Statistical analyses were performed with Graph Pad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

Results

HSP70/72 expression after heat shock preconditioning in normal and hypoxic growth conditions

The expression of HSP70/72 was used to evaluate the effectiveness of the heat shock protocol. After 24-hour incubation, the myoblast cell sheets were harvested by temperature changes from 37°C to 20°C and subjected to hyperthermia (+42°C for 45 minutes) in a water bath. Control cells were cultured under normal growth conditions. Under both growth conditions (normal and 1% O_2) HSP70/72 expression was strongly induced 24 hours after HS. Under normal growth conditions, HSP70/72 expression remained stable, but under hypoxia expression it significantly reduced within 72 hours ($p<0.05$ compared to controls, Figure 2A, B). Sheet formation itself induced no HSP70/72 expression (data not shown).

Sheet apoptosis, resistance to oxidative stress, and proliferation

We evaluated the expression of apoptosis and proliferation-associated proteins, caspase-3 and PCNA, in L6 myoblast sheets with and without HS pretreatment under normal and hypoxic growth conditions. Under normal growth conditions, there was no significant difference in caspase-3 expression between non-shocked and heat shocked cell sheets during 72 hours. However, in hypoxia the caspase-3 expression of heat shocked sheets was significantly lower ($p<0.01$) compared to No-Shock sheets after 24 hours, suggesting an anti-apoptotic effect of heat shock pretreatment. PCNA expression was higher ($p<0.01$) in heat shock pretreated cells under both growth conditions after 24 hours (Figure 3A, B).

VEGF-A secretion of myoblast sheets in hypoxia

To study the potential paracrine effect of heat shock-preconditioned myoblast sheets, we determined the secretion of VEGF under hypoxic conditions up to 72 hours. HS did not enhance L6 myoblast sheet VEGF secretion under hypoxic conditions. On the contrary, VEGF secretion was significantly lower ($p<0.05$) in heat shock-conditioned sheets than in non-conditioned (No-Shock) sheets during follow-up (No-Shock: 24 h 10 740±3 830 pg/ml, 48h: 12 390±1 242 pg/ml 72 h: 17 880±5 193 pg/ml and Heat-Shock: 24h: 6 660 ± 4 101 pg/ml, 48 h: 8 869±3 408 pg/ml, 72 h: 11 580±2 933 pg/ml) (Figure 4).

Echocardiography

Of the total 60 rats included in the study, 13 died (mortality 22%). Seven animals were excluded due to lack of myocardial infarct in the pretreatment echocardiography. One animal died during the surgical procedure, 7 died immediately after the surgery while recovering from anesthesia. During the follow-up period 3 of the control rats dies, as well as 1 rat in the non-conditioned group and 1 rat in the HS preconditioned sheet group. There was no difference in cardiac functions between the groups. LVEF and LVFS of the L6-myoblast HS pre-treatment group decreased gradually until the end of the study, with almost in the same pattern as in the control group (Heat-Shock: 0.295±0.015, 0.272±0.009, 0.237±0.011 and 0.145±0.008, 0.133±0.005, 0.116±0.005, respectively, Control: 0.297±0.019, 0.264±0.016, 0.234±0.017 and 0.147±0.01, 0.129±0.008, 0.114±0.009). However, these functional parameters improved in the L6-myoblast normal sheet group (No-Shock) (0.298±0.012 to 0.319±0.016 and 0.146±0.007 to 0.159±0.008) on the tenth day of the study, but then declined (0.266±0.011 and 0.13±0.005) until the 28th day. Anterior wall transverse diameter in the diastole phase (AWTd) was significantly higher (No-Shock: 0.785±0.048 *vs.* C: 0.615±0.042) on the 28th day of study verses the Control group (Table 1 and Figure 5).

Histology

Angiogenesis

Myocardial vessel density was evaluated at the end of the study. Paraffin-embedded myocardial transverse sections

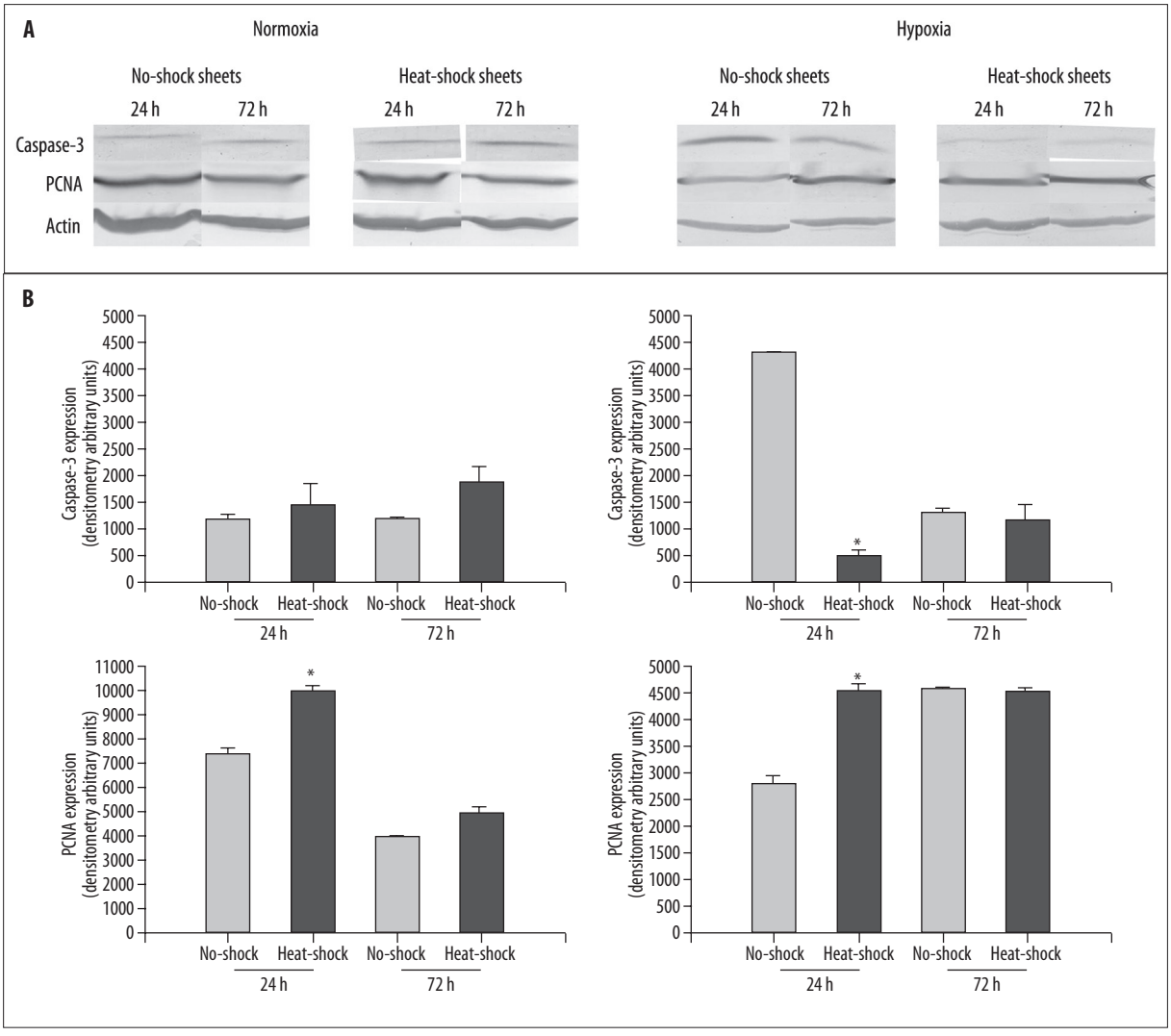

Figure 3. (**A**) Expression of apoptosis- and proliferation associated proteins, caspase-3 and PCNA, of L6 myoblast sheets with or without pre treatment. Sheets were heat shocked and thereafter immediately subjected to 1% O_2 hypoxia or let to recover in normal growth conditions. Western blot samples were prepared at indicated time points and actin was used as a loading control. (**B**) Densitometry of caspase-3 and PCNA immunoblots from two to three independent experiments of heat-shocked or non-shocked L6 myoblast sheets under normoxia (left panel) or hypoxia (right panel). * $p<0.01$ compared to No-Shock sheets.

were stained for expression of the endothelial antigen vWF as a marker of vascular density. There were no differences between groups in infarct area (Controls: 7.889±2.620, No-Shock: 6.417±1.197, Heat-Shock: 7.250±2.045). The higher vessel density of the No-Shock-group was evident ($p<0.05$ compared to the Heat-Shock group and Control group) in infarct border area, whereas Heat-Shock-group vessel densities remained at Control level (Controls: 16.41±0.24, No-Shock: 21.60±2.62, Heat-Shock: 16.20±1.01 (Figure 6A).

Proliferation

The number of proliferating cells was assessed in infarct and border areas by immunohistochemical staining of the proliferation-associated nuclear antigen Ki67 from paraffin-embedded myocardial sections. Ki67-positive cells were detectable only in the infarct border area. The number of proliferating cells was significantly lower in the Heat-Shock-group when compared to Controls ($p<0.05$, Controls: 1.000±0.1925, No-Shock: 0.7917±0.3688, Heat-Shock: 0.33330±0.1361, Figure 6B).

Fibrosis

To study whether myoblast sheet therapy can reduce the amount of fibrotic tissue after acute MI, we assessed the percentages of fibrosis in histology sections. Fibrosis was evaluated from scanned images of picric acid-stained sections. Percentage of fibrosis was calculated as picric acid-stained divided by whole section area. No difference between groups was detectable. No decrease occurred in fibrosis (Controls 22.45±3.168%, No-Shock 19.76±1.622%, Heat-Shock 20.38±2.685%, Figure 6C, D).

Discussion

During recent years, cell-based transplantation research has expanded remarkably [15–17] Cell sheet technology shows

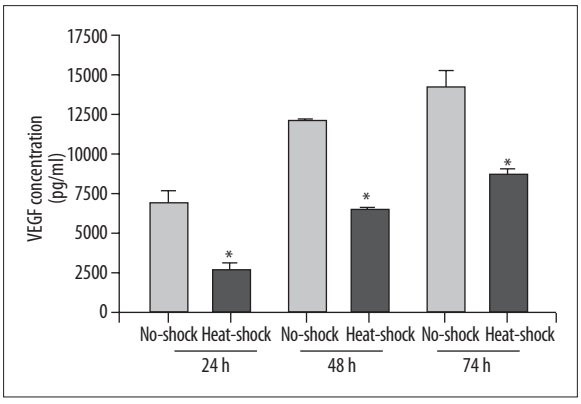

Figure 4. Secretion of VEGF by non-heat shocked and heat shocked L6 myoblast sheets under hypoxic conditions. Cell sheets were harvested as described in materials and methods and transferred to 1% O_2 hypoxia. Non shocked sheets served as a control. After 24-, 48-, and 72 hours continuous incubation in hypoxia, medium from non-shocked (No-Shock) and heat shock treated (Heat-Shock) cell sheets was subjected to a VEGF enzyme-linked immunosorbent (ELISA) assay.(* p<0.05 compared to No-Shock-group).

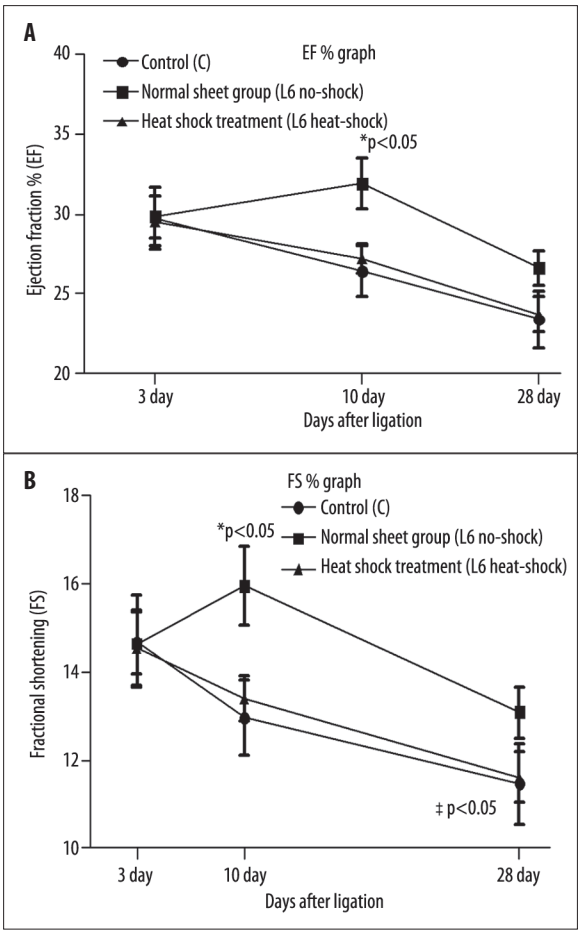

Figure 5. (**A**) Echocardiography data for left ventricle ejection fraction (EF), at three different time points after L6-myoblast sheet implantation without HS treatment (n=12, L6 No-Shock) and L6-myoblast sheet implantation which were pre-treated with heat shock (n=16, L6 Heat-Shock) in an acute myocardial infarction rat model (AMI). Control rats (n=12) underwent AMI without sheet transplantation. * P<0.05 as compared to the controls. (**B**) Echocardiography data for left ventricle fractional shortening (FS) at three different time points after L6-myoblast sheet implantation without heat HS (n=12, L6 No-Shock) and L6-myoblast sheet implantation with HS pre-treatment (n=16, L6 Heat-Shock) in an acute myocardial infarction rat model (AMI). Control rats (n=12) underwent AMI without sheet transplantation. * P<0.05 as compared to the controls; ‡ P<0.05 as compared to the L6 No-Shock group.

great promise in heart regeneration [18,19]. We studied the effect of HS preconditioning in myoblast cell sheets both *in vitro* and eventually in ischemic heart failure *in vivo*. The most valuable result contradicts the effect seen in monolayer cell HS preconditioning. In myoblast cell sheets, HS does not enhance their therapeutic effect in cell therapy. In fact, it may even limit the function of the cell sheet *in vivo*. We found decreased expression of VEGF in the HS pretreated sheets and the therapeutic effect of these sheets disappeared in the acute heart failure model with less vascular structures in the infarct border area. HS has a cell-protective effect in early stages after preconditioning, but hypoxic conditions also weaken HSP70/72 expression at 72 hours after HS, and eventually the HS-preconditioned sheets lack the myocardium-protective function seen in non-conditioned myoblast sheets.

The main effect of the myoblast cell sheet in ischemic cardiomyopathy is the reversal of left ventricular dilatation (ie, the remodeling process). This effect has been evident in several animal studies and also in right ventricular failure, where myoblast sheet therapy improved right ventricular diastolic dysfunction and suppressed fibrosis with increased capillary density [20,21].

The gene expression profile of the transplanted cells plays a key role in the paracrine effect, and also when sheet survival is assessed. A larger 3-dimensional structure requires blood supply in order to stay vital. The angiogenic properties of the myoblast sheet might play a significant role in its own blood vessel development and survival [22].

Heat shock proteins, the best known endogenous factors, have an important function in cyto-protection with their apoptosis-blocking properties [9]. HSP70/72, the most studied subtype of these chaperones, is suggested to be the main factor of the endogenous pathways that limits the extent of myocardial damage in ischemia-reperfusion after cardiac surgery [23,24]. HS treatment protects cells from apoptosis *in vitro* [25,26]. Severe hypoxia has devastating effects on living organisms [27]. In our earlier work, heat-shocked myoblasts maintained their differentiation capacity under hypoxic conditions [28]. To evaluate the usefulness of HS pretreatment for myoblast sheet therapy *in vitro* and *in vivo*, HSP70/72 served as a marker for the efficacy of the pre-treatment protocol. Our HS protocol was sufficient to increase HSP70/72 expression for at least 72 hours under normal growth conditions *in vitro*.

Myoblast sheet HSP70/72 expression remained stable in normoxia, but in hypoxia, HSP70/72 expression showed a notable decrease during 72 hours. At early stages, the

Table 1. Echocardiography data at the 3-, 10-, and 28-days time points after LAD ligation and transplantation of an L6-myoblast sheet with heat shock pre-treatment (L6 Heat-Shock) and L6-myoblast sheets without heat shock pre treatment (L6 No-Shock), showing anterior and posterior wall thickness in diastolic (AWTd, PWTd) and systolic phases (AWTs, PWTs) and left ventricular diameter in diastolic (Dd) and systolic (Ds) phases. Dd and Ds served in calculating fraction shortening (LVFS) and ejection fraction (LVEF) percentages. Units are in mm.

3 days	N	AWTd	PWTd	Dd	AWTs	PWTs	Ds	FS	EF
Control	12	1.032±0.08	1.653±0.066	8.525±0.229	1.024±0.084	2.392±0.062	7.275±0.223	0.147±0.01	0.297±0.019
L6 No-Shock		1.086±0.049	1.751±0.049	8.305±0.189	1.049±0.055	2.484±0.049	7.085±0.157	0.146±0.007	0.298±0.012
L6 Heat-Shoch		1.162±0.07	1.643±0.045	8.359±0.176	1.135±0.086	2.392±0.056	7.172±0.165	0.145±0.008	0.295±0.015
10 days									
Control	12	0.874±0.039	1.837±0.066	9.384±0.217	0.863±0.05	2.604±0.049	8.176±0.234	0.129±0.008	0.264±0.016
L6 No-Shock		0.941±0.051	1.824±0.039	9.313±0.246	0.924±0.049	2.627±0.03	7.825±0.217	0.159±0.008*	0.319±0.016*
L6 Heat-Shoch		0.919±0.046	1.889±0.055	9.475±0.175	0.882±0.043	2.569±0.043	8.192±0.161	0.133±0.005‡	0.272±0.009
28 days									
Control	16	0.615±0.042	1.667±0.076	10.36±0.197	0.646±0.045	2.368±0.088	9.189±0.25	0.114±0.009	0.234±0.017
L6 No-Shock		0.785±0.048*	1.947±0.061*	9.827±0.261	0.78±0.054	2.708±0.046**	8.549±0.26	0.13±0.005	0.266±0.011
L6 Heat-Shoch		0.702±0.036	1.983±0.048**	10.26±0.136	0.697±0.031	2.639±0.046**	9.037±0.136	0.116±0.005	0.237±0.011

Values represent mean ± SEM. * $p<0.05$ as compared to control group; ** $p<0.05$ as compared to L6-No-Shock group.

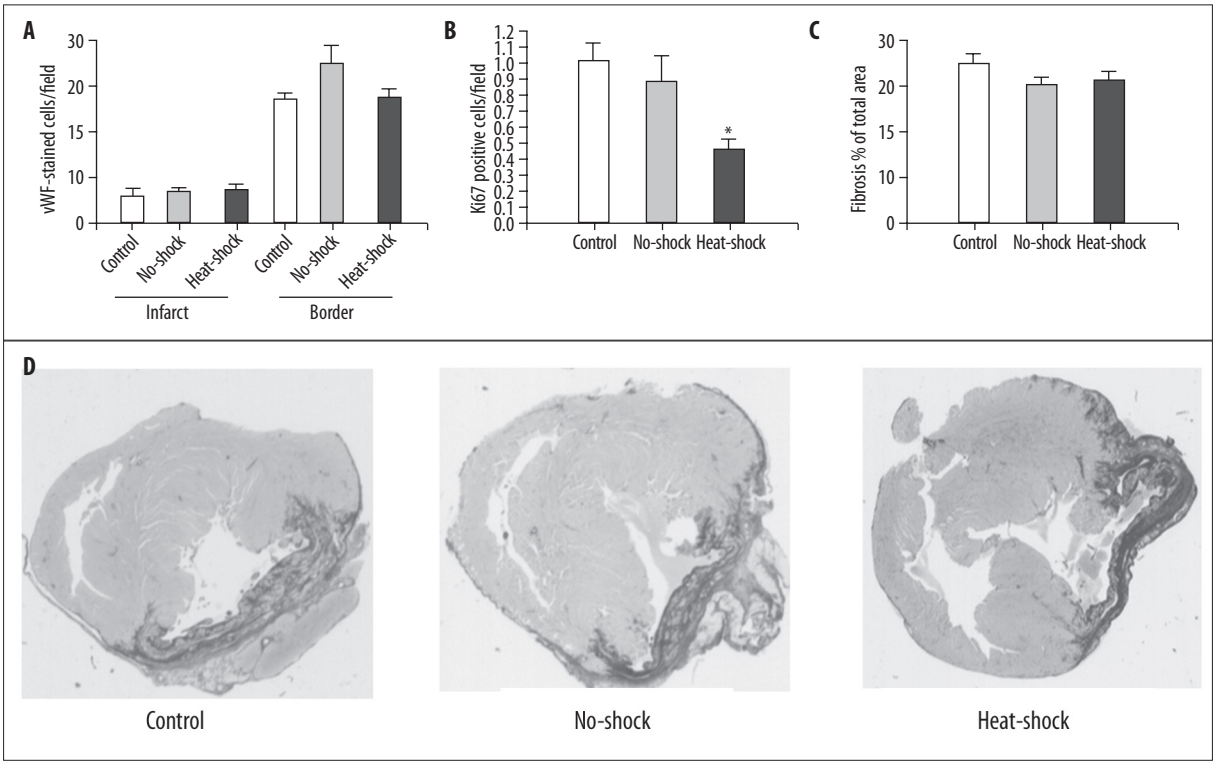

Figure 6. (**A**) Vascular densities of infarct area and border area (vWF positive capillaries) 28 days after infarction and sheet transplantation in control, non-shocked (No-Shock), and heat-shock group (Heat-Shock) animals. $P<0.05$, when compared to the non-conditioned sheets in border area. (**B**) Quantitative evaluation of proliferative cells (Ki67 positive cells) in infarct border area 28 days after the surgery in control, non shocked (No-Shock), and heat shock – group (Heat-Shock) animals. $P<0.05$, HS compared to control-group. (**C**) Quantitative evaluation of fibrosis by Picric acid staining of myocardial paraffin embedded sections from control, No-Shock, and Heat-Shock group animals. (**D**) Representative figures showing the amount of fibrosis from control, No-Shock and, Heat-Shock group animals.

HS-preconditioned sheets were better protected from apoptosis than were non-conditioned myoblast sheets *in vitro*.

When tested *in vivo* in rat ischemic heart failure, HS-conditioned myoblast cell sheet therapy failed to improve

cardiac performance, whereas non-conditioned myoblast cell sheets were able to significantly increase the cardiac ejection fraction at the tenth day of follow-up. These non-conditioned group results are in line with our previous findings of improved cardiac parameters at the tenth day of echocardiography recordings with wild-type myoblast cell sheets [29]. Behind the improved cardiac function are paracrine effects inhibiting fibrosis and stimulation of angiogenesis. Skeletal muscle cells engage in large-scale production of cytokines and growth factors involved in cell migration, adhesion and proliferation-like HGF (hepatocyte growth factor), IGF (insulin like growth factor, FGF (fibroblast growth factor) and VEGF (vascular endothelial growth factor), which is an important cytokine in the process of vascular endothelial cell differentiation and maturation [30–33]. Myoblast sheets have been shown to release more of these growth factors compared to injected cells [5].

Because of the potential of cell sheet therapy, several attempts have been made to modify the cell gene expression profile to further improve the effect of the myoblast sheets. After AMI, the Bcl-2-overexpressing sheets survived longer on the infarcted myocardium, and they significantly improved cardiac function. L6-Bcl2 sheet transplantation reduced myocardial fibrosis and enhanced vascular density in infarct and border areas in a Wistar rat acute infarction model [29].

However, our study's *in vitro* data showed a decrease in VEGF secretion in HS pre-conditioned myoblast sheets. This finding was supported by lower vascular density at the infarction border area. Furthermore, histological data showed no reduction in fibrosis, suggesting that the HS pre-treatment of the myoblast sheets provides no cardio-protective gain when compared to the non-conditioned sheet. In fact, the HS pretreated cell-sheet-group hearts behaved similar to the Controls.

HS has been shown to improve cell survival and engraftment when used in single-cell suspension [24]. In a study of Suzuki et al. the same L6 cell line was used. HS in single cell suspension demonstrated improved tolerance to hypoxia *in vitro* and significantly more HS-pretreated myoblasts were surviving when compared to the non-conditioned cells *in vivo* [34]. Myoblast sheets have different cell-cell communication status [5] and this might be the only reasonable explanation of the different response to HS in our study.

The sheet formation has been shown to protect cells *per se* and the sheet environment might change the cellular reaction to the HS. The lost therapeutic effect of the heat-shocked sheets might also affect the hypoxic environment during and after the sheet implantation procedure. This reduces the HS protein expression, and the pre-conditioning loses effect. However, this only partly explains the phenomenon, because the heat-shocked sheets work significantly differently from non-conditioned sheets. We believe that the reason why heat-shocked sheets lose their therapeutic effect might be that HS-preconditioning of myoblast sheets disturbs cell-cell contacts and the extracellular matrix required for the cell signaling and cell sheet functioning.

Myoblast cell sheets may also face the host immunoreactions attributed to culture serum and other additives in cell cultures, thus having an effect on myoblast cell sheet survival. Host immunoreaction even comes into play, despite the autologous setting. In our study we used L6-myoblast cells that are originally from Wistar rats, but we still expect some immunoreactivity to be exerted by L6 myoblast cells in recipient Wistar rats. In spite of immunoreactivity, many studies have shown the functional effects attributed by cytokine and growth factors expressed by L6-myoblasts, discussed by Memon et al. [14]. However, this cell line has been used without these types of concerns several times for cell transplantation therapy previously [25].

Heat shock treatment has been shown to improve graft cell survival of myoblasts when transplanted to injured tissue [25]. Combined with cell sheet transplantation, this could be a simple and safe method to achieve better survival of transplanted single cells. However, in this study, HS-preconditioning deteriorated the therapeutic effect of L6-myoblast sheet transplantation, with concomitant VEGF secretion and lower vascular density in the transplanted hearts. Since the pretreatment method evaluated in this study does not enhance the therapeutic potency of myoblast sheets, alternative methods need to be considered. Hypoxia preconditioning of mesenchymal stem cells have been shown to improve vascular and skeletal muscle fiber regeneration after ischemia [35] and also to up-regulate angiogenic cytokines in myoblasts [36]. In order to avoid the ethical and safety issues related to gene therapy, this could be the method of choice when developing myoblast sheet therapy.

Conclusions

Taken together, these results suggest that heat shock protects myoblast sheets from hypoxia-associated apoptosis *in vitro*, but reduces VEGF expression of the sheet, leading to lesser therapeutic effect.

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References:

1. Hagège AA, Marolleau JP, Vilquin JT et al: Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. Circulation, 2006; 114(1 Suppl.): 108–13
2. Menasché P, Hagège AA, Scorsin M et al: Myoblast transplantation for heart failure. Lancet, 2001; 357(9252): 279–80
3. Menasché P, Alfieri O, Janssens S et al: The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation, 2008; 117(9):1189–200
4. Masuda S, Shimizu T, Yamato M, Okano T: Cell sheet engineering for heart tissue repair. Adv Drug Deliv Rev, 2008; 60(2): 277–85
5. Memon IA, Sawa Y, Fukushima N et al: Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. J Thorac Cardiovasc Surg, 2005; 130(5): 1333–41

6. Hata H, Matsumiya G, Miyagawa S et al: Grafted skeletal myoblast sheets attenuate myocardial remodeling in pacing-induced canine heart failure model. J Thorac Cardiovasc Surg, 2006; 132(4): 918–24
7. Shimizu T, Yamato M, Kikuchi A, Okano T: Cell sheet engineering for myocardial tissue reconstruction. Biomaterials, 2003; 24(13): 2309–16
8. Sekiya N, Matsumiya G, Miyagawa S et al: Layered implantation of myoblast sheets attenuates adverse cardiac remodeling of the infarcted heart. J Thorac Cardiovasc Surg, 2009; 138(4): 985–93
9. Benjamin IJ, McMillan DR: Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ Res, 1998; 83(2): 117–32
10. Sreedhar AS, Csermely P: Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. Pharmacol Ther, 2004; 101(3): 227–57
11. Beere HM: "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci, 2004; 117(Pt 13): 2641–51
12. Kojima A, Goto K, Morioka S, Naito T et al: Heat stress facilitates the regeneration of injured skeletal muscle in rats. J Orthop Sci, 2007; 12(1): 74–82
13. Bouchentouf M, Benabdallah BF, Tremblay JP: Myoblast survival enhancement and transplantation success improvement by heat-shock treatment in mdx mice. Transplantation, 2004; 77(9): 1349–56
14. Palojoki E, Saraste A, Eriksson A et al: Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. Am J Physiol Heart Circ Physiol, 2001; 280(6): H2726–31
15. Eve D, Fillmore RW, Borlongan PR et al: Stem cells have the potential to rejuvenate regenerative medicine research. Med Sci Monit, 2010; 1 6(10): RA197–217
16. Liu G-B, Cheng Y-X, Feng Y-K et al: Adipose-derived stem cells promote peripheral nerve repair, Arch Med Sci, 2011; 7(4): 592–96
17. Borsani P, Marazzi R, Passi A et al: Transplantation of bone marrow stromal mesenchymal cells in the treatment of acute myocardial infarction. Arch Med Sci, 2009; 5(2): 156–62
18. Sawa Y: Surgical regeneration therapy using myoblast sheets for severe heart failure Kyobu Geka. 2007; 60(5): 355–61
19. Kondoh H, Sawa Y, Miyagawa S et al: Longer preservation of cardiac performance by sheet-shaped myoblast implantation in dilated cardiomyopathic hamsters. Cardiovasc Res, 2006; 69(2): 466–75
20. Wu KH, Mo XM, Liu YL: Cell sheet engineering for the injured heart. Med Hypotheses, 2008; 71(5): 700–2
21. Hoashi T, Matsumiya G, Miyagawa S et al: Skeletal myoblast sheet transplantation improves the diastolic function of a pressure-overloaded right heart. J Thorac Cardiovasc Surg, 2009; 138(2): 460–67
22. Sasagawa T, Shimizu T, Sekiya S, et al: Design of prevascularized three-dimensional cell-dense tissues using a cell sheet stacking manipulation technology. Biomaterials, 2010; 31(7): 1646–54
23. Sreedhar AS, Csermely P: Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. Pharmacol Ther, 2004; 101(3): 227–57
24. de Jong PR, Schadenberg AW, Jansen NJ, Prakken BJ: Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. Cell Stress Chaperones, 2009; 14(2): 117–31
25. Riederer I, Negroni E, Bigot A et al: Heat shock treatment increases engraftment of transplanted human myoblasts into immunodeficient mice. Transplant Proc, 2008; 40(2): 624–30
26. Lepore DA, Knight KR, Anderson RL, Morrison WA: Role of priming stresses and Hsp70 in protection from ischemia-reperfusion injury in cardiac and skeletal muscle. Cell Stress Chaperones, 2001; 6(2): 93–96
27. Flisberg A, Kjellmer I, Bågenholm R et al: EEG and spectral edge frequency: analysis in posthypoxic newborn piglets. Neuro Endocrinol Lett, 2010; 31(2): 181–86
28. Kitabayashi K, Siltanen A, Pätilä T et al: Bcl-2 Expression Enhances Myoblast Sheet Transplantation Therapy for Acute Myocardial Infarction. Cell Transplant, 2010; 19(5): 573–88
29. Augustin M, Salmenperä P, Harjula A: Heat shock enhances troponin expression and decreases differentiation-associated caspase-3 dependence in myoblasts under hypoxia. J Surg Res, 2010; 161(1): 62–68
30. Miyagawa S, Saito A, Sakaguchi T et al: Impaired myocardium regeneration with skeletal cell sheets – a preclinical trial for tissue-engineered regeneration therapy. Transplantation, 2010; 90(4): 364–72
31. Czarkowska-Paczek B, Zendzian-Piotrowska M, Bartlomiejczyk I et al: Skeletal and heart muscle expression of PDGF-AA and VEGF-A after an acute bout of exercise and endurance training in rats. Med Sci Monit, 2010; 16(5): BR147–53
32. Baghdady Y, Hussein Y, Shehata M: Vascular endothelial growth factor in children with cyanotic and acyanotic and congenital heart disease. Arch Med Sci, 2010; 6(2): 221–25
33. Xie X-Y, Mo Z-H, Chen K et al: Glucagon-like Peptide-1 improves proliferation and differentiation of endothelial progenitor cells via upregulating VEGF generation. Med Sci Monit, 2011; 17(2): BR35–41
34. Suzuki K, Smolenski RT, Jayakumar J et al: Heat shock treatment enhances graft cell survival in skeletal myoblast transplantation to the heart. Circulation, 2000; 102(19 Suppl.3):III216–21
35. Leroux L, Descamps B, Tojais NF et al: Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway Mol Ther, 2010; 18(8): 1545–52
36. Egger M, Schgoer W, Beer AGE et al: Hypoxia up-regulates the angiogenic cytokine secretoneurin via an HIF-1_- and basic FGF-dependent pathway in muscle cells. FASEB J, 2007; 21: 2906–17