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Molecular Mechanisms underlying the potential of shock wave treatment for cardiac therapy

104 – Biomedizin Innovativ – patientInnenfokussierte, anwendungsorientierte sowie interdisziplinäre Forschung am Puls der Zeit

Abstract

Coronary heart disease constitutes the most deadly condition in the world. Globally, over 17.3 million patients pass away annually due to cardiovascular disease, and this number is predicted to increase to almost 24 million by the year 2030. Among various therapeutic approaches available to physicians, medication with nitroglycerin, β-blockers or nitrates, percutaneous coronary intervention or coronary artery bypass grafting are used. Unfortunately, they have some limitations: they are invasive, costly and require expertise. In the light of these facts, cardiac shock wave therapy may offer a non-invasive and safe therapeutic alternative, which could not only significantly improve the lives of patients who suffered from myocardial infarction, but also reduce the costs of hospitalization. In our study we investigate the effect of shock wave treatment on the differentiation of murine embryonic stem cells (mESCs) and murine cardiovascular progenitor cells (mCVPCs) into the cells of the cardiac lineage. Our experimental approach is based on the use of mESC-derived embryoid bodies or mCVPC-derived cardiac bodies, which are three-dimensional spherical aggregates of the respective cells. The spheroids are subjected to shock wave treatment of variable energy flux densities. Cardiac-specific gene expression or the activation of intracellular pathways over time have been investigated following treatment. On the protein level, a dose-dependent activation of ERK1/2 immediately after treatment has been observed, corroborating the previously reported effect of SWT in a three-dimensional model. When analyzing gene expression, a significant upregulation of the expression of early mesodermal marker Brachyury and the early cardiac marker Nkx2.5 was shown already 7 days after the formation of spheroids. Our studies aim to justify the use of shock wave treatment in the therapy of patients who suffered from myocardial infarction or other conditions associated with death of cardiomyocytes and other surrounding cells. Our results point to a putative beneficial effect of shock waves on the expression of genes specific for the cardiac lineage when differentiating mouse embryonic stem cells from an undifferentiated state. Taken together, we suggest that shock wave treatment of the post-ischemic myocardium contributes to its regeneration by improving the differentiation of cells.

Keywords:

Shock wave treatment, cardiac stem cells, mouse embryonic stem cells, embryoid bodies, cardiac shock wave therapy

Coronary heart disease, having surpassed all types of cancer taken together, currently constitutes the most deadly condition in the world. Globally, over 17.3 million patients pass away annually due to cardiovascular disease, and this number is predicted to increase to almost 24 million by the year 2030 (Mozaffarian et al., 2016). Coronary heart disease is a pathological condition of the heart, which results from an insufficient blood supply to the heart tissue. The most common symptom of the disease is myocardial infarction, but also unstable and stable angina, atherosclerotic coronary artery and sudden cardiac death (*Medical Dictionary for the Health Professions and Nursing*, 2012).

Currently, various therapeutic approaches are available to physicians. Among others, medication with nitroglycerin, β-blockers or nitrates, percutaneous coronary intervention or coronary artery bypass grafting are used (Libby & Theroux, 2005; Yang et al., 2013). Unfortunately, they have some limitations: due to the invasiveness of the latter two methods, many patients are excluded from the treatment as their condition is not stable enough. Another important factor is the poor economic situation and lack of technical expertise in the developing countries to perform such complicated procedures (Yang et al., 2013). In the light of these facts, cardiac shock wave therapy may offer a non-invasive and safe therapeutic alternative, which could not only significantly improve the lives of patients who suffered from myocardial infarction, but also reduce the costs of hospitalization.

Shock waves are non-periodical acoustic waves of high initial peak pressure and a high amplitude (see Fig. 1A), propagating in three dimensions (Frairia & Berta, 2011; Notarnicola & Moretti, 2012). The dose of shock waves can be expressed by “energy flux density” [mJ/mm^2], describing the maximal energy being transmitted through a 1 mm^2 area with each pulse (Ogden, Tóth-Kischkat, & Schultheiss, 2001). Clinically, shock waves were first used for the disintegration of kidney stones and are to date the golden standard for this procedure. However, multiple side effects of the treatment have soon been observed, including its beneficial role in wound healing and the therapy of musculoskeletal disorders (Wang, 2012; Weihs et al., 2014). For the latter condition, shock wave treatment remains a prospective therapy undergoing clinical trials. Inspired by those findings, researchers have investigated the effect of SWT on the cardiac tissue, the cells of the cardiac tissue or the whole heart *in vivo*. One such study focused on discovering the *in vitro* effects of SWT on human cardiac precursor cells isolated from healthy and diseased hearts. The results showed a beneficial effect of the said treatment both on the proliferation as well as differentiation of these cells (Nurzynska et al., 2008). The same authors have shown a similar activation of c-Kit$^+$ cells after the application of shock waves *in vivo*, in rat models (Di Meglio et al., 2012). This ability of shock waves to activate cells to proliferate or differentiate holds great promise for future regenerative therapies for post-ischemic hearts. Undeniably, the assessment of safety of cardiac shock wave therapy is a crucial aspect, due to the physical nature of the waves. The treatment generates mechanical force that could affect the action potential of the membranes, thus making cells of the myocardium particularly sensitive to it (Di Meglio et al., 2012). Several independent *in vivo* studies have been conducted and no obvious adverse effects (e.g. arrhythmia, myocardial necrosis, cardiac fibrosis) of shock wave treatment were observed (Di Meglio et al., 2012; Kikuchi et al., 2010; Nishida et al., 2004; Yang et al., 2013).

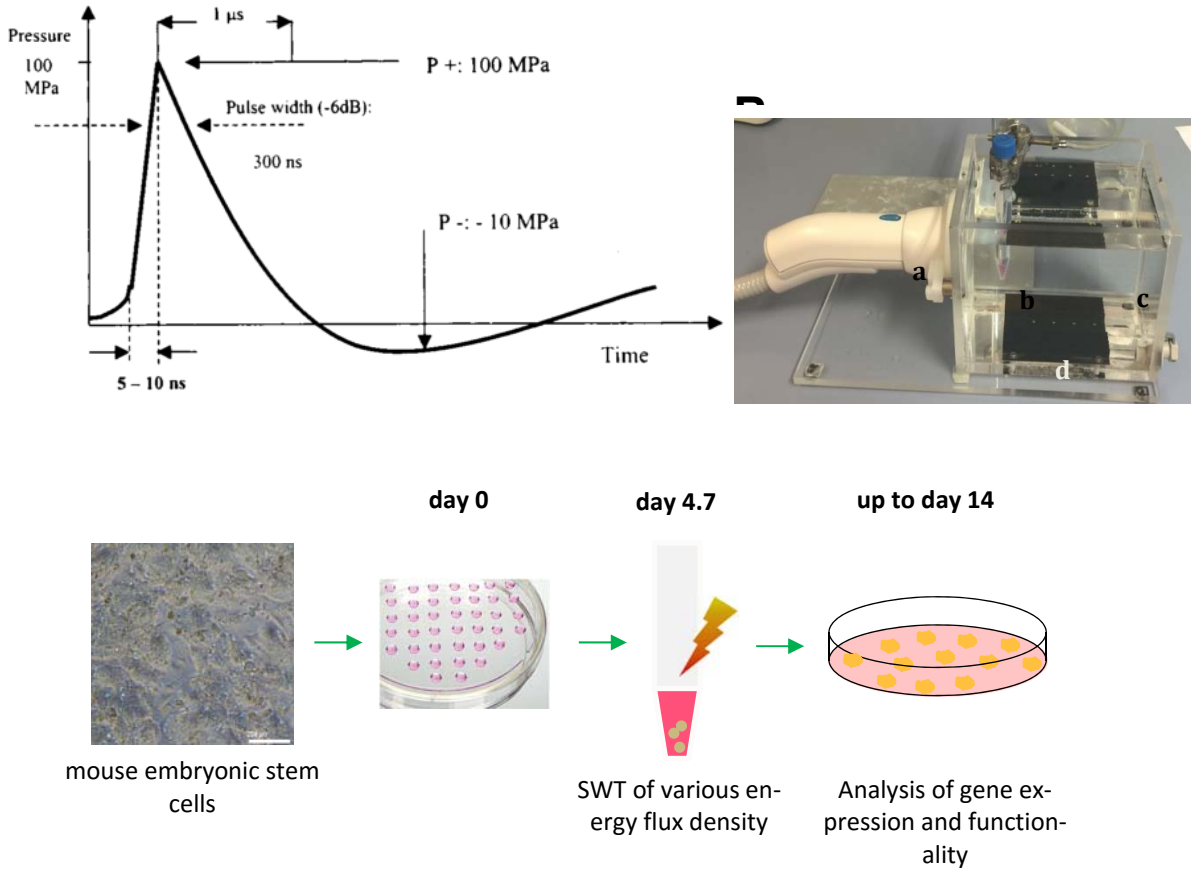

Figure 1. *Principle of shock waves and the experimental setup.* (A) SWT proved to activate ERK1/2 (A) Shock waves are characterized by a fast initial peak pressure reaching 10 MPa (Ogden et al., 2001) (B) Cells are subjected to treatment in 15ml polystyrene tubes (b) immersed in a water bath (c), which is equipped with a heating device (d) to maintain constant conditions; the shock waves are generated by the applicator (a) with a custom-made water bath adapter. (C) The experimental setup: mESCs are cultured in hanging drops for 4.7 days; after harvest, the cells undergo SWT and the subsequent "implantation" onto gelatinized cell culture vessels where they are cultured until analytical time points are reached.

In our study we investigate the effect of shock wave treatment on the differentiation of murine embryonic stem cells (mESCs) and murine cardiovascular progenitor cells (mCVPCs) into the cells of the cardiac lineage. Cardiovascular progenitor cells, also termed cardiac stem cells, were first discovered in the early 2000s, contradicting the common theory of the vertebrate heart being a postmitotic organ, which maturation has completed several days after birth (Hoebaus et al., 2013). CVPCs have been identified as endogenous stem cells of adult heart. They were found to reside in the interstitial space, filling the space between the differentiated cardiomyocytes (Beltrami et al., 2003). These Lin^- $c\text{-}Kit^+$ cells are multipotent (able to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells) and capable of self-renewal (Taubenschmid & Weitzer, 2012). In an ischemic heart, CVPCs were able to differentiate into myocytes, as well as new blood-carrying vessels, thereby reconstituting the myocardium (Beltrami et al., 2003). In conclusion, the discovery of cardiovascular progenitor cells sheds new light on the regenerative potential of the adult heart, opening doors for novel therapies for heart regeneration after myocardial infarction.

Our experimental approach is based on the use of mESC-derived embryoid bodies (EBs) or mCVPC-derived cardiac bodies (CBs), which are three-dimensional spherical aggregates of the respective cells.

To create such aggregates, we use the hanging drop method. The cells are cultured in 20 µl drops of medium hanging from the inner side of the lid of a cell culture dish (see Figure 1C) and, due to the effect of gravitational forces, aggregate at the bottom of the drop forming spheroids. In order to expand embryonic stem cells *in vitro*, leukemic inhibitory factor is necessary to inhibit their differentiation. In our setup, the presence of this molecule is achieved by culturing the mESCs on LIF-expressing feeder layers, consisting of SNL76/7 murine fibroblasts. However, when the hanging drop culture is started, spontaneous differentiation of mESCs or mCVPCs is induced by the lack of LIF in the culture medium. Embryoid bodies undergo differentiation in a pattern resembling the first days of embryonic development *in vivo*, and have therefore become a commonly used model of early embryogenesis (Weitzer, 2006). Following this theory, we culture the cells in hanging drops for 4.7 days, at which point the aggregates are transferred onto gelatinized cell culture dishes to imitate the process of implantation (*in vivo*) in our *in vitro* setup (Weitzer, 2006). Before implantation, however, the spheroids are subjected to shock wave treatment of variable energy flux densities (0.04 mJ/mm^2, 0.07 mJ/mm^2, 0.13 mJ/mm^2 or 0.19 mJ/mm^2; non-treated samples are used as controls). Cardiac-specific gene expression analysis or the activation of intracellular pathways following treatment have been investigated.

On the protein level (Figure 2), a dose-dependent activation of ERK1/2 immediately after treatment has been observed, corroborating the previously reported effect of SWT (Weihs et al., 2014) also in a three-dimensional model. This ERK1/2-activating effect of SWT decreases over time, yet the dose-dependent trend seems to be preserved. Noticeably, there was no effect of SWT on the phosphorylation of neither AKT nor the ribosomal S6 protein (data not shown). These observations suggest that the overall effects of SWT are linked to the mechanical stimulus that the cells are subjected to, as ERK1/2 is a protein involved in mechanotransduction (Iqbal & Zaidi, 2005).

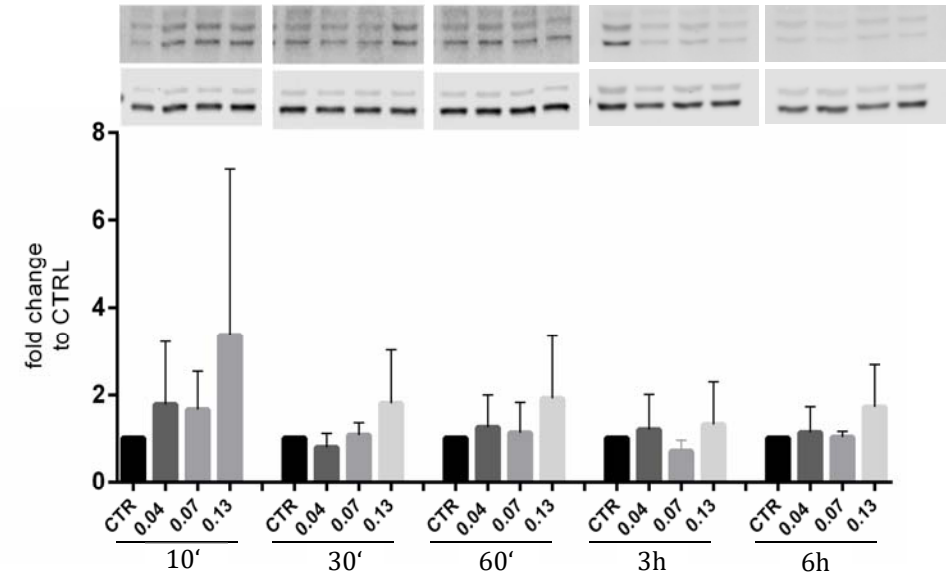

Figure 2. *SWT induces a dose-dependent activation of ERK1/2 immediately after treatment in mESC-derived embryoid bodies in the course of 6 hours.* SWT proved to activate ERK1/2 (A) in a dose-dependent manner immediately after treatment. This effect was maintained for up to 6 hours, with the phosphorylation levels decreasing with time. The bar graphs show the results of a densitometric analysis of the bands. The expression of phosphorylated protein was normalized to total protein level. The bars represent mean + SD, n≥4. Representative Western blots were shown

The expression profile of cardiac-specific genes is presented in Figure 3. A significant upregulation of the expression of early mesodermal marker *Brachyury* and the early cardiac marker *Nkx2.5* was shown already 7 days after the formation of spheroids. This effect was preserved over the following 7 days. On the other hand, the expression of *cardiac troponin T*, which is a marker of mature cardiomyocytes, only showed significant upregulation at day 14 after the induction of differentiation (data not shown). Finally, the cells also expressed the *smooth muscle protein 22-alpha*, which confirms that the cells constituting embryoid bodies can differentiate into different derivatives of the cardiac lineage (data not shown).

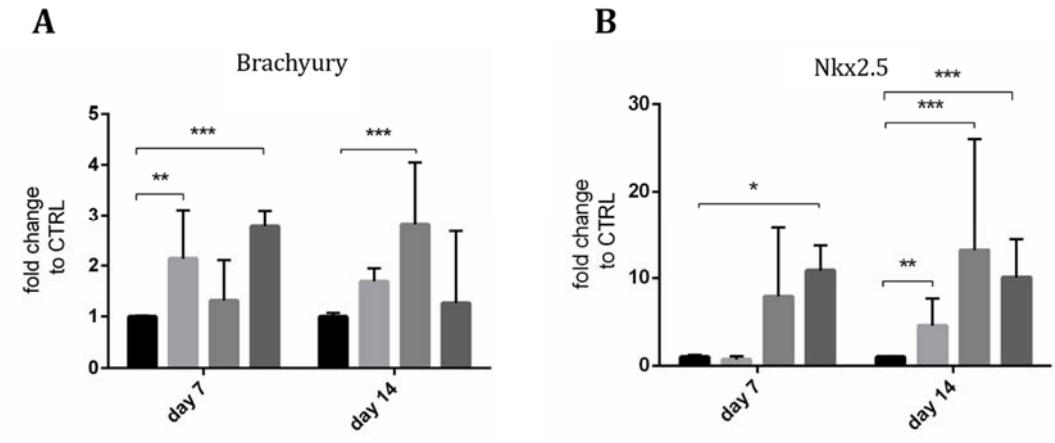

Figure 3. *The effect of shock wave treatment on the expression of cardiac markers in mESC-derived embryoid bodies over the course of 14 days.* The preliminary experiments show that SWT induced the expression of early mesodermal marker Brachyury (A) and early cardiac marker Nkx2.5 (B) in a dose-dependent manner both at 7 and 14 days after spheroid formation. The results are expressed as mean + SD, $n \geq 4$. Kruskal-Wallis and Dunn's/Holm-Sidak's/Dunnett's multiple comparison tests were performed, *p<0.05. ** p<0.01, ***p<0.001.

Our studies aim to justify the use of shock wave treatment in the therapy of patients who suffered from myocardial infarction or other conditions associated with death of cardiomyocytes and other surrounding cells. The reported ability to activate cardiac stem cells *in vivo* to regenerate the damaged myocardium holds great promise for future applications. Our results point to a putative beneficial effect of shock waves on the expression of genes specific for the cardiac lineage when differentiating mouse embryonic stem cells from an undifferentiated state. Similar results have been observed in cardiovascular progenitor cells under the same treatment conditions. Taken together, we suggest that shock wave treatment of the post-ischemic myocardium contributes to its regeneration by improving the differentiation of cells.

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