



**4th ISMST Basic Research Meeting**  
**Friday, March 4, 2016**

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## 4th ISMST Basic Research Meeting Saturday, March 5, 2016

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## Shock wave treatment for *in vitro* engineering applications

Anna M. Weihs<sup>1,2</sup>, Christiane Fuchs<sup>1,2</sup>, Dorota Szwarc<sup>1,2</sup>, Dominik Hanetseder<sup>1,2</sup>, Xavier Monforte Vila<sup>1,2</sup>, Andreas H. Teuschl<sup>1,2</sup> and Dominik Rünzler<sup>1,2</sup>

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**Introduction:** Recently, the cellular effects of shock wave treatment have been thoroughly studied using *in vitro* approaches. We already described intracellular pathways involved in the shock wave treatment effect using the *in vitro* shock wave treatment (IVSWT) water bath set-up and various cell types. We suppose that the beneficial cellular effects of shock waves – such as increased proliferation or enhanced growth factor expression – could also be exploited in the emerging field of tissue engineering. The adaptation of *in vitro* shock wave treatment parameters for the application on 3D cell culture systems and cell/scaffold constructs would therefore provide a promising tool for tissue engineering purposes.

**Methods:** Several three dimensional cell culture systems such as spheroids of stem cells, various cell-loaded hydrogels and scaffolds, were subjected to *in vitro* shock wave treatment.

**Results:** Diverse protocols were developed and tested to establish optimal treatment set-ups and parameters, e.g. stiffness of the used scaffolds, medium density as well as optimal number of shock wave treatments were evaluated in order to find the optimal conditions for diverse 3D systems.

**Discussion:** The potential of shock wave treatment to trigger mechanosensitive pathways can be used to improve proliferation or differentiation in 3D systems *in vitro* in order to support scaffold maturation and ultimately enhance cell-scaffold performance. Thus, the experience on the application of shock waves on 3D cell culture systems and scaffolds will help to establish shock wave treatment as a potent tool in the field of tissue engineering and regenerative medicine.

**Device and producing company:** Dermagold 100 with OP155 applicator, MTS

**Setup:** IVSWT waterbath, cells and scaffolds treated in a 15 mL polypropylene tube

This work was supported by the FFG COIN Disease Tissue Project (FFG #845443)

# The beneficial effects of *in vitro* shock wave treatment on cardiomyogenesis are energy dependent

Christiane Fuchs<sup>1,2</sup>, Anna M. Weihs<sup>1,2</sup>, Dorota Swarc<sup>1,2</sup>, Philipp Heher<sup>2,3</sup>, Andreas H. Teuschl<sup>1,2</sup> and Dominik Rünzler<sup>1,2</sup>

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**Introduction:** In the recent years, more and more evidence for the beneficial effect of shock waves for the treatment of cardiac diseases is arising. Myocardial infarction (MI) and other cardiac diseases are still the number one reason for death as stated by the WHO. In previous experimental and clinical studies it was shown that extracorporeal shock wave treatment (ESWT) significantly improved systolic function, number of blood vessels, angina pectoris symptoms and myocardial blood flow.

Embryoid Bodies (EBs) are commonly used 3D *in vitro* systems to study early embryonic development. Additionally, when using mouse embryonic stem cells, they are used as a feasible tool to study cardiomyogenesis. As stated above, there is evidence that ESWT can augment e.g. regeneration after MI, however the underlying mechanisms are still not fully elucidated.

**Methods:** Therefore we investigated the effects of ESWT on cardiomyogenesis in EBs and analyzed several parameters such as percentage of beating EBs, expression levels of cardiac markers and signaling pathways involved in mechanotransduction, proliferation and differentiation.

**Results:** We could show a dose dependent effect of shock wave treatment on the percentage of beating EBs. Investigating different signaling pathways, we could demonstrate that the ERK signaling pathway was induced upon ESWT. Moreover, on the molecular level ESWT significantly up-regulated lineage specific and cardiac markers compared to untreated controls.

**Discussion:** In current experiments we investigate the signaling and regulatory pathways involved in the beneficial effects of ESWT on cardiomyogenesis. The ultimate goal is to provide *in vivo* researchers and clinicians with a solid base to smooth the way of ESWT into the clinics as an alternative or additive treatment method for various cardiac diseases.

**Device and producing company:** Dermagold® 100 with OP155 applicator, MTS Europe GmbH

**Setup:** cells in suspension or as Embryoid Bodies / Cardiac Bodies placed in a 15 ml polypropylene tube in a waterbath

This work was supported by the FFG COIN Disease Tissue Project (FFG #845443).

## Low-energy shock wave treatment induces angiogenesis in ischemic muscle by stimulation of Toll-like receptor 3 signalling

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**Background:** Low energy shock waves (SW) have been shown to induce angiogenesis in ischemic myocardium. The mechanism translating the physical stimulus to a biological signal is unknown. Toll-like receptor (TLR)-3 is activated by RNA binding. It plays a key role in inflammation and angiogenesis. We therefore hypothesized that SW cause cellular cavitation, thus liberating cytoplasmic mRNA that activates TLR-3 as does the specific agonist Poly I:C. Effects are suppressed in TLR-3 silenced cells and in TLR-3 knock out mice.

**Methods:** The effect of SW was tested in human umbilical vein endothelial cells (HUVECs): untreated (control) vs. SW treated (SW group) vs. treated with 200 µg/ml Poly I:C (agonist). TLR-3 gene silencing was done with siRNA. Hind limb ischemia was performed in wild type and TLR-3 knock-out mice. Expression of mRNA and proteins of the TLR-3 signaling pathway as well as typical angiogenic genes and proteins were measured. Laser Doppler perfusion imaging and necrosis score were assessed for clinical outcome evaluation (n=6).

**Results:** Shock wave treatment of HUVECs shows increase of mRNA expression (% of control) as does Poly I:C after 2 hours: TLR-3 (SW group  $123.8 \pm 8.0$  and agonist group  $237.7 \pm 14.1$ ,  $p < 0.0001$ ), Tie-2 (SW group  $154.3 \pm 20.0$  and agonist  $125.7 \pm 12.3$ ,  $p < 0.008$ ). TLR-3 gene silencing in SW treated HUVECs causes loss of response for TLR-3 mRNA ( $107.0 \pm 13.3$ ) as compared to SW group ( $378.3 \pm 14.2$ ) or agonist ( $1261 \pm 72.1$ ), both  $p < 0.0001$ .

SW treated TLR-3 knock-out mice showed no improvement of perfusion ratio 4 weeks after hind limb ischemia ( $0.52 \pm 0.07$  vs.  $0.53 \pm 0.02$  controls,  $p > 0.05$ ), whereas SW treated wild type animals improved significantly ( $0.78 \pm 0.03$  vs.  $0.48 \pm 0.08$  controls,  $p = 0.015$ ). Pro-angiogenic genes and proteins were up-regulated significantly. All known TLR-3 signaling pathways were involved as shown by significant increase of key proteins Trif, TRAF6 and IRF3.

**Conclusion:** Low energy shock waves induce angiogenesis in ischemic muscle by stimulation of Toll-like receptor 3 signaling in endothelial cells. Effects are suppressed in TLR-3 silenced cells and in TLR-3 knock-out mice.

## **Antimicrobial peptide LL37/ RNA complexes stimulate Toll-like receptor 3 upon shock wave therapy**

Graber M, Pölzl L, Primessnig U, Lobenwein D, Kirchmair E, Pechriggl EJ, Grimm M, Holfeld J, Tepeköylü Can

### **Background:**

Shock wave therapy (SWT) induces angiogenesis in ischemic heart disease. It is mediated via Toll-like receptor 3 (TLR3), an endosomal receptor of the innate immune system recognizing RNA. How TLR3 is activated upon SWT remains unknown. The antimicrobial peptide LL37 has been shown to be released after mechanical stress and to form complexes with RNA.

### **Purpose:**

We hypothesized that mechanical stimulation upon SWT leads to LL37 release, which forms complexes with RNA and leads to activation of endosomal TLR3.

### **Methods:**

Supernatant of treated human umbilical vein endothelial cells (HUVEC) was transferred onto TLR3 reporter cells and TLR3 activation was measured. To find out whether protein/RNA complexes play a role after SWT, supernatants were treated with RNase and proteinase. Treated HUVECs were analyzed for LL37 expression. To investigate the uptake of LL37/RNA complexes, premarked RNA was added to cells prior to treatment and uptake was tracked. C57BL/6 mice were subjected to acute myocardial infarction and subsequently treated with SWT. Echocardiography and pressure volume measurements were performed to evaluate cardiac function. Histological quantification of vessels and assessment of fibrosis was performed.

### **Results:**

Supernatants of treated cells activated TLR3 reporter cells (CTR  $7.346 \pm 2,173$  vs. SWT  $146.005 \pm 12.508$ ;  $p < 0.0001$ ). Analysis of the supernatant revealed increased RNA levels (CTR  $21 \pm 2.444$  vs. SWT  $37 \pm 1.5$ ;  $p = 0.0174$ ). The effect could not be abolished by pre-treatment of the supernatant with RNase, but only by a sequential digestion with proteinase and RNase hinting strongly towards the involvement of protein/RNA complexes. Indeed, LL37 expression was significantly increased after SWT. Pre-marked RNA was added to HUVECs, followed by subsequent SWT. Cellular RNA uptake was significantly increased after SWT (CTR  $31.67 \pm 28,17$  vs. SWT  $19757 \pm 1054$ ,  $p < 0.0001$ ). Finally, SWT resulted in significantly higher numbers of capillaries (SWT 1262 vs. CTR 461,  $p = 0.001$ ) and arterioles (SWT 461 vs. CTR 160.5,  $p = 0.001$ ), decreased fibrosis (CTR  $\pm 2,76$  vs. SWT  $8,97 \pm 3,08$ ,  $p = 0.01$ ) and improved ejection fraction (CTR  $35.25 \pm 1.11$  vs. SWT  $46 \pm 2.83$ ,  $p = 0.01$ ) in treated hearts.

### **Conclusion:**

TLR3 activation upon SWT is mediated via the release of LL37. The antimicrobial peptide forms complexes with extracellular RNA and can thus stimulate endosomal TLR3. SWT subsequently induces angiogenesis in ischemic myocardium and might therefore develop a potent regenerative treatment alternative for ischemic heart disease.

## **Shock waves induce angiogenesis via exosome release**

Tepeköylü C, Pözl L, Lobenwein D, Graber M, Blumer M, Kirchmair E, Kirchmair R, Paulus P, Davidson S, Grimm M, Holfeld J.

### **Background:**

Shock wave therapy (SWT) is developing a promising approach for the regeneration of ischemic myocardium by induction of angiogenesis. However, the mechanism of action remains unknown. Exosomes are released by mechanical shear stress and have been shown to induce angiogenic effects. We hypothesized that SWT induces exosome release and thus exerts its angiogenic effects.

### **Methods:**

Human umbilical vein endothelial cells (HUVECs) were treated with SWT. Subsequently, exosomes were isolated from the supernatant and analyzed by transmission electron microscopy (TEM) and nanoparticle tracking analysis. In a next step, exosomes were characterized and analyzed for their angiogenic potential in vitro. Exosome content was evaluated via a sequencing array. Finally, isolated exosomes were injected into subcutaneously implanted matrigel plugs in nude mice. Perfusion of the plugs was measured via Laser Doppler perfusion imaging (LDPI). Arterioles and capillaries were quantified histologically. In vivo imaging was performed to analyze functionality of the vessels.

### **Results:**

SWT caused exosome release in HUVECs. Supernatants of treated cells showed significantly higher concentrations of exosomes. Exosomes showed a characteristic cup-shaped morphology in TEM analysis. Treatment of HUVECs with exosomes induced phosphorylation of Akt and ERK, caused increased tube formation (CTR  $19,5 \pm 7,79$  vs. SWT  $178,5 \pm 31,14$ ,  $p=0,004$ ) and endothelial cell proliferation (CTR  $0,59 \pm 0,02$  vs. SWT  $0,77 \pm 0,04$ ,  $p=0,011$ ). Pre-treatment with exosome-release inhibitor GW4869 abolished the angiogenic effects of SWT. Sequencing array showed the presence of angiogenic miRNAs in exosomes released after SWT. Injection of isolated exosomes into subcutaneously implanted matrigel plugs resulted in higher perfusion and increased number of capillaries (CTR  $0,53 \pm 0,19$  vs. SWT  $1,7 \pm 0,26$ ,  $p=0,0006$ ) and arterioles (CTR  $0,8 \pm 0,23$  vs. SWT  $4,5 \pm 0,54$ ,  $p<0,0001$ ). In vivo imaging of the matrigel plugs showed formation of functional vessels after exosome injection.

### **Conclusion:**

We show for the first time how the mechanical stimulus of SWT is translated into a biological response. SWT causes exosome release. Released exosomes show a very potent angiogenic effect. SWT might develop a potent therapeutic intervention for the treatment of ischemic heart disease.



## ***In vitro* Extracorporeal Shockwave Treatment has Beneficial Effects on Rat Schwann Cell Isolation and Culture**

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**Introduction:** As new approaches for peripheral nerve regeneration are sought, there is an increasing demand for native Schwann cells for *in vitro* testing and/or reimplantation. Extracorporeal shockwave treatment (ESWT) is an emergent technology in the field of regenerative medicine, which recently has also been shown to improve peripheral nerve regeneration. In this study we elucidate the effects of ESWT on Schwann cell isolation and culture.

**Material and Methods:** Rat sciatic nerves were dissected, treated with ESWT and Schwann cells were isolated and cultured for 15 passages. Directly after shockwave treatment release of ATP and LDH were assessed. Cell yield and proliferation (BrdU assay) were evaluated. Cells were evaluated for expression of Schwann cell markers (Flow cytometry and Western Blot: S100b, P75, c-Jun, GFAP).

**Results and Discussion:** Single treatment of the whole nerve *ex vivo* led to significantly increased extracellular ATP as an immediate consequence, and subsequently a number of effects on the culture were observed, starting with a significantly increased Schwann cell yield after isolation. In the ESWT group quality of culture, reflected in consistently higher purity (S100b, morphology), proliferation rate (BrdU, population doublings per passage) and expression of regenerative phenotype-associated markers (P75, GFAP, c-Jun), was significantly improved. In contrast, the control group exhibited progressively senescent behaviour, reflected in a decrease of proliferation, loss of specific markers and increase in P16<sup>INK4A</sup> expression. Concluding, extracorporeal shockwave treatment has beneficial effects on Schwann cell isolation and culture.

**Device and producing company:** Dermagold 100, MTS

**Setup:** waterbath, whole nerve

## **ESWT affects Schwann cell phenotype *in vitro* and *in vivo* thereby accelerating nerve regeneration**

David Hercher<sup>\*1,2</sup>, Christina M.A.P Schuh<sup>\*1,2</sup>, Michaela Stainer<sup>1,2</sup>, Rudolf Hopf<sup>1,2</sup>, James Ferguson<sup>1,2</sup>, Dominik Rünzler<sup>2,3</sup>, Heinz Redl<sup>1,2</sup>, Antal Nógrádi<sup>1,2,4</sup>, Thomas Hausner<sup>1,2,5</sup>

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\*These Authors contributed equally

**Introduction:** Peripheral nerve injuries are common and a frequent cause of hospitalization displaying a major burden to patients and social health-care systems. ESWT has been shown to be one of very few treatment options which accelerates peripheral nerve regeneration. Despite recent advances in understanding the underlying mechanisms of ESWT, little is known of the effect on Schwann cells (SCs) and peripheral nerve regeneration. In this study we investigated these two aspects.

### **Methods**

*in vitro:* Schwann cells have been isolated from motor, sensory and mixed nerves, respectively. Dissected nerves have been treated with ESWT prior to isolation. Cultured SCs were evaluated using FACS analysis and western blot.

*in vivo:* A femoral nerve defect model was established in the rat. The effects of ESWT on motor fibers regenerating through a sensory environment have been evaluated using automated gait analysis, electrophysiology, histology and qPCR.

**Results:** In vitro data indicate a strong influence of ESWT on the activation status of SCs of different phenotype. Motor SCs differ from sensory SCs regarding proliferation and expression of myelination associated proteins. ESWT is able to induce proliferation of motor and sensory SCs.

In vivo data indicate inferior regeneration of motor axons through a sensory nerve graft compared to a phenotypically matched graft. ESWT can ameliorate this effect and accelerate nerve regeneration.

**Discussion:** This study indicates that ESWT is able to accelerate peripheral nerve regeneration in a model which reflects the clinical reality after autologous nerve transplantation. Thereby providing support for the use of ESWT after peripheral nerve injury.

**Device and producing company:** Dermagold 100, MTS;

**Setup:** *in vitro:* waterbath, whole nerve

*in vivo:* 1x transcutaneously after wound closure

## **Shock wave therapy enhances neuronal sprouting and improves neuronal survival**

An A\*, Nägele F\*, Irschick R, Lobenwein D, Venezia S, Stefanova N, Grimm M, Tepeköylü C, Holfeld J

\*authors contributed equally

### **Background:**

Shock wave therapy (SWT) has been shown to induce tissue regeneration and improve function in spinal cord ischemia via TLR3. Thereby, induction of angiogenesis and alteration of microglial response could be observed. However, it remains unknown whether SWT exerts a regenerative effect on neurons, too. We aimed to analyze whether (1) SWT improves neuronal survival and enhances neurite growth and (2) TLR3 signaling is involved.

### **Methods:**

Dorsal Root Ganglia (DRG) were isolated from Wild Type (WT) and TLR3<sup>-/-</sup> mice and subsequently treated with SWT (0.01mJ/mm<sup>2</sup>, 250 Impulses, 3Hz) or TLR3 agonist Poly(I:C). Control groups remained untreated. DRGs were analyzed via neuronal sprouting assay and survival was evaluated by TUNEL assay. Transmission electron microscopy (TEM) was used to evaluate the morphology of neurons and to assess vesicle release.

### **Results:**

SWT lead to enhanced neurite growth and an increase of branch points (CTR 1433 ± 76.61 vs. SWT 2061 ± 151.5, p<0.0001). Treated neurons showed improved survival rates. SWT effects were missing in neurons isolated from TLR3<sup>-/-</sup> animals. Poly(I:C) treatment mimicked SWT effects. TEM analysis revealed release of microvesicles in treated neurons.

### **Conclusion:**

SWT enhances neurite growth and improves neuronal survival via activation of TLR3. It could therefore develop as a potent therapeutic intervention for neuronal regeneration.

# **Shock wave treatment reduces neuronal degeneration upon spinal cord ischemia via a Toll-like receptor 3 dependent mechanism**

Lobenwein D, Tepeköylü C, Kozaryn R, Pechriggl E, Bitsche M, Fritsch H, Paulus P, Czerny M, Grimm M, Holfeld J

## **OBJECTIVES:**

Paraplegia following spinal cord ischemia represents the most severe complication of aortic surgery. Shock wave treatment (SWT) was shown to induce angiogenesis and regeneration in ischemic tissue. In pre-clinical as well as clinical studies SWT had a favorable effect on ischemic myocardium. We therefore hypothesized that SWT may have a beneficial effect on spinal cord ischemia as well.

## **METHODS:**

Aortic cross clamp was performed between left carotid and left subclavian artery in mice. Animals were randomly divided in a treatment group (SWT, 500 shock waves at 0.1mJ/mm<sup>2</sup>, 5Hz) and untreated controls (CTR), n=6 per group. RNA expression of angiogenic and inflammatory cytokines was measured after 24 and 48 hours. Immunofluorescence staining for degenerating neurons and macrophages was performed after 7 days. An ex-vivo spinal slice culture was performed for evaluation of Toll-like receptor (TLR) signalling. Spinal cords from wild type, TLR3 knockout and TLR4 knockout animals were cultured and set under hypoxia for 24 hours. Treatment groups (SWT) received shock wave treatment following hypoxia.

## **RESULTS:**

Real-time PCR analysis revealed higher gene expression of angiogenic factors VEGF-A after 24h (SWT 0.21±0.06 vs. CTR 0.07±0.01, p=0.028) and 48h (SWT 0.11±0.02 vs. CTR 0.07±0.01, p>0.05) as well as HIF-1a after 24h (SWT 0.11±0.04 vs. CTR 0.04±0.01, p>0.05) and 48h (SWT 0.09±0.02 vs. CTR 0.01±0, p=0.016). Early increase of inflammatory mRNA expression was observed after 24h by TNFα (SWT 0.03±0.003 vs. CTR 0.005±0.003, p=0.007) and TGFβ (SWT 0.57±0.05 vs. CTR 0.17±0.08, p=0.003). This resulted in a markedly decreased number of degenerating neurons in the treatment group 7 days after ischemia (SWT 74.50±8.14 vs. CTR 250.2±42.98, p=0.0025). Standardized coordination and motor tests performed at day 1, 3 and 7 postoperatively revealed a significantly better performance and outcome of the animals in the treatment group. In addition a Kaplan-Meier analysis revealed a survival benefit of SWT compared to normal animals. Effects of SWT were abolished in TLR3 knockout animals, whereas it was unchanged in TLR4 knockouts.

## **CONCLUSIONS:**

Shock wave treatment induces angiogenesis and modulates inflammation in spinal cord ischemia via the activation of TLR3. This results in a marked decrease of degenerating neurons and may therefore develop as an adjunct to the treatment armamentarium for paraplegia upon aortic cross clamp.

## **Molecular Changes after Extracorporeal Shockwave Therapy in Osteoarthritic Knee in Rats.**

Ching-Jen Wang, M.D.

Chang Gung University College of Medicine

Kaohsiung Chang Gung Memorial Hospital, Taiwan.

This study investigated the molecular changes of DKK-1, MMP13, Wnt-5a and  $\beta$ -catenin after extracorporeal shockwave therapy (ESWT) in anterior cruciate ligament transected (ACLT) osteoarthritic (OA) knee in rats. 27 male Sprague-Dawley rats were divided into three groups. Group I was the control one and received sham knee arthrotomy but no ACLT or ESWT. Group II underwent ACLT, but no ESWT. Group III underwent ACLT and received ESWT. The animals were killed at 12 weeks, and the harvested knee specimens were subjected to histopathological examination and immunohistochemical analysis. Radiographs of the knees were obtained at 0 and 12 weeks. At 12 weeks, radiographs of group II showed more arthritic changes with formation of osteochondral fragments, whereas very subtle arthritis was noted in groups I and III. In histopathological examination, group II showed a significant increase of Mankin score and a decrease of subchondral bone as compared to groups I and III. Group III showed a significant decrease of Mankin score and an increase of subchondral bone, with the data comparable to group I. In immunohistochemical analysis, group II showed significant increases of DKK-1 and MMP13 and decreases of Wnt-5a and  $\beta$ -catenin in articular cartilage and subchondral bone as compared to groups I and III. Group III showed significant decreases of DKK-1 and MMP13 and increases of Wnt-5a and  $\beta$ -catenin, with the data comparable to group I. In conclusion, the application of ESWT causes molecular changes that are consistent with the improvement in subchondral bone remodeling and chondroprotective effect in ACLT OA knees in rats.

## The effect of shock waves on *in vitro* cartilage development in silk scaffolds

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**Introduction:** Osteoarthritis (OA) is a degenerative condition causing joint pain and stiffness and, thereby, severely impairs everyday life of patients. It is predicted that by the year 2030, a quarter of the US population alone will be diagnosed with OA.

Silk is a biocompatible and biodegradable biomaterial, which has been implicated in a wide range of applications in biomedical engineering. Silk can be processed into very different forms, from hydrogels to solid bulk material, making it one of the most versatile biomaterials available. Furthermore, its outstanding mechanical properties permit the creation of constructs capable of withstanding physiological loads.

It has been shown that low-energy shock wave treatment (SWT), applied in combination with microfractures, resulted in increased production of cartilage-like tissue, affecting both chondrocytes and the surrounding blood vessels. Moreover, in another study, SWT was proven to slightly improve the differentiation potential of equine adipose tissue-derived mesenchymal stem cells *in vitro*.

**Methods:** We therefore studied the effect of SWT on articular chondrocytes *in vitro*. This included their expansion *in vitro*, resulting in their dedifferentiation, and finally their redifferentiation into functional cartilage in a silk scaffold upon SWT. We analyzed the distribution of cells within the scaffold, gene expression of cartilage-specific markers, as well as the activation of intracellular signaling pathways *in vitro*.

**Results:** Silk-based hydrogels and sponges were shown to be suitable scaffolds for cartilage engineering, providing the cells with a robust environment that preserves its architecture during long culture periods. The scaffold alone was shown to promote chondrogenic marker expression in cultured cells. This effect was further increased when SWT was applied.

**Discussion:** The use of shock wave treatment on chondrocyte-loaded silk scaffolds would provide a novel tool for tissue engineering in cartilage regeneration.

**Device and manufacturing company:** Dermagold<sup>®</sup> 100 with OP155 applicator, MTS Europe GmbH

**Setup:** cells in suspension or in a 3D silk scaffold placed in a 15 ml polypropylene tube in a water bath

This work was supported by the FFG COIN Disease Tissue Project (FFG #845443).

## **Functional Proteomic analysis of extracorporeal shockwave therapy in the potential for treatment of osteoporosis**

Shan-Ling Hsu, MD and Ching-Jen Wang, MD

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Taiwan

### **Abstract**

The Molecular mechanisms underlying extracorporeal shockwave therapy (ESWT) promoted tissue regeneration had been widely accepted. ESWT promoted proangiogenic factor eNOS, VEGF and bone morphogenic proteins that involving in tissue regeneration. But the responding molecules that modulated ESWT-regressed osteoporosis have not been investigated. Our preliminary proteomic data revealed abundant significant proteins that warrant further characterization. These proteins of interest were reported to participate in the cellular response to stress, calcium homeostasis, chemotaxis and lipid oxidative stress in several tissue types under pathological contexts. ESWT induced osteoblast activation and bone mineralization through upper-expression of protein-disulfide isomerase-associated 3 (Pdia3). We found the significant increase of bone mineralization density, bone formation markers including Matrix metalloproteinase-13 (MMP13), alkaline phosphatase (ALP), receptor activator of NF-kappaB ligand (RANKL) and osteoprotegerin (OPG) and extracellular signal-regulated kinases (ERKs), Pdia3 expression in the subchondral bone microenviornents actively responded to ESWT treatment in rats, which potentially regulate biological function of osteoblast in osteoporotic bone. The core unit monitored the standard protocol of shockwave therapy and facilitated subproject group to discover active molecules, thus provided a potential therapeutic regimen to alleviating osteoporosis. Also the results may help other subprojects to view newly molecular mechanisms widely.

Key words: shockwave therapy, osteoporosis, Pdia3

## **Shock wave therapy causes increased macrophage recruitment and enhances M2 polarization**

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### **Objective:**

Shock wave therapy (SWT) has been shown to induce angiogenesis in ischemic muscle. However, the mechanism of action remains unknown. Macrophages are crucial for angiogenic responses after ischemia. Proinflammatory M1 macrophages phagocytise necrotic tissue. M2 macrophages create a milieu of regeneration and enable angiogenesis. We hypothesized that the angiogenic effects of SWT are caused by enhanced macrophage recruitment.

### **Methods:**

C57BL/6 mice were subjected to unilateral hind limb ischemia with subsequent SWT (0,1mJ/mm<sup>2</sup>, 500 Impulses, 5 Hz) or sham treatment. Successful limb ischemia was confirmed via Laser Doppler perfusion imaging. Gastrocnemius muscle was harvested 72h and 28d after ischemia induction and further processed for immunofluorescence staining and RT-PCR analysis.

### **Results:**

Treated muscles show increased expression of the pivotal recruiting factor monocyte chemoattractant protein 1 (MCP-1) (217,9 ± 30,18 vs. 102,7 ± 14,08, p=0,0016). Indeed, an increase of the macrophage marker CD14 could be observed after SWT (118,1 ± 20,9 vs. 22,16 ± 2,874, p=0,0001). The higher numbers of macrophages could be confirmed in immunofluorescence stainings. The expression of the M2 polarization promoting chemokine IL-13 was significantly increased in the treatment group (517.7 ± 81,83 vs. 3087 ± 1043, p=0,0138). Increased levels of the M2 scavenger receptor CD163 could be found after SWT compared to untreated controls (172,4 ± 35,84 vs. 40,56 ± 6,266, p=0,0008). We found higher numbers of capillaries (CTR 8.18 ± 1.9 vs. SWT 16.25 ± 2.09, p=0.009) and arterioles ( CTR 1.11 ± 0.26 vs. SWT 3.78 ± 0.52, p=0.0003) after SWT. Treated animals showed significantly improved limb perfusion (CTR 0.45 ± 0.67 vs. SWT 0.76 ± 0.09, p=0.027).

### **Conclusion:**

SWT causes increased macrophage recruitment and enhanced polarization towards reparative M2 macrophages in ischemic muscle. It could therefore become a promising tool for the regeneration of ischemic myocardium.



## **Recommendation statement of the Conjoint Physics Working Group of ISMST and DIGEST on ESWT study design and publication**

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### **Introduction:**

Although many different applications of extracorporeal shockwave therapy are used for more than two decades, results are still not convincing or inconsistent. One big issue is that the results obtained with one device are difficult to transfer to a second device from another manufacturer. The reason may be that researches just refer to a small subset of the parameters defined in IEC 61846 to describe the applied shockwaves. The question is which other or even new shockwave parameters may be relevant to correlate with the clinical efficiency.

Furthermore we don't know yet the key effect of shockwaves on tissue – both, the biological response as well as the most effective “shape” (spatial and temporal pressure distribution) of the shock waves is not clear.

### **Methods:**

To overcome this problem the ISMST and DIGEST created a physics working group with delegates from *Dornier MedTech*, *MTS Medical*, *Richard Wolf*, *EMS*, *Nonvasiv Medical*, *Likamed*, *Jena MedTech* and *RP acoustics* to discuss about more suitable parameters for ESWT.

### **Results:**

As a result, a recommendation has been elaborated for researchers as well as for companies that should be considered in future clinical studies, in-vitro trials and especially in publication of the obtained data.

Furthermore, a proposal for a measurement standard for non-focused or weakly focused devices was discussed, which will be presented to the International Electrotechnical Committee (IEC) standardization organisation.

### **Discussion:**

These recommendations are just a first step on the long way to be able to find more suitable parameters. If researches and companies follow these recommendations, it may be possible to create new databases that can be used to determine the meaning and significance of different parameters with respect to the clinical outcome.

## **Biophysical signals from and stimuli to cells**

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### **Background/Aims**

Any fundamental features or traits in our cells are regulated in something of a rhythmic fashion. We play a part in the Universe's electromagnetic vibrations and sounds. For decades Scientists have used Chemistry to affect cell behavior.

We aimed at investigating whether exposure to physical energies, including electromagnetic fields and sound vibrations, may affect pluripotency expression and lineage commitment in human adult stem or somatic cells.

### **Methods**

Human adipose-derived stem cells (hADSCs), or human skin fibroblasts were exposed to asymmetrically conveyed radio electric fields (ACREF) by the aid of a Radio Electric Asymmetric Conveyer (REAC). Gene and protein expression were monitored by real-time RT-PCR and Western blot analysis, respectively. Confocal microscopy and immunofluorescence analysis were used to assess (stem) cell commitment.

### **Results**

Exposure to ACREF was able to transform hADSCs into cardiac myocytes, neurons, skeletal muscle, and endothelial cells. Moreover, ACREF acted as a sort of "time machine" even able to reprogram human adult non-stem somatic cells, like skin fibroblasts, into cell types in which these cells would never otherwise appear, including myocardial, neural, and skeletal muscle cells. Intriguingly, hADSC exposure to ACREF was found to counteract and reverse stem cell senescence *in vitro*, acting at the level of both telomerase-dependent and -independent pathways. Consonant with these observations is our discovery that cells can produce acoustic vibrations, and express "vibrational" signatures of their health and differentiating potential.

### **Conclusions**

The finding that (stem) cell fate can be remarkably modulated by physical energies prompts a deeper understanding of the interconnections between the physical universe and the living world in the attempt to further approach the information of Life.

## **A combination of physical and chemical stimuli on iPSCs increase the yield of cardiomyocyte differentiation**

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**Aims:** Stem cell therapy may become a valuable tool for heart regeneration. Within this context, induced pluripotent stem cells (iPSCs) are regarded as a new, ethically acceptable, potential source for cellular elements capable to rescue a damaged heart. In particular, iPSCs derived from urine exhibit high genetic stability and can be very easily obtained through a totally non-invasive procedure. Unfortunately, cardiogenic differentiation is an extremely low-yield process, and it's currently induced mainly through conditioned media.

**Methods and Results:** Here, we combined two different approaches for cardiogenic induction: a chemical treatment including Bmp4, recombinant Activin A, and the Wnt inhibitor IWR-1, and the exposure to physical energy, in the form of an electromagnetic field delivered through a radio electric asymmetric conveyor (REAC) a device that emit frequency waves around 2.4 GHz directly on cells in culture. Gene expression analysis revealed that our combinatorial strategy was able to induce the cardiogenic genes GATA4, and Nkx-2.5 and the cardiac specific transcripts MHC, TBX5, cTnT, MEF2C and ACT2. These data were also confirmed by confocal microscopy at the intact cell level. The cardiogenic induction was further supported by the remarkable increase in the number of iPS-derived beating clusters of myocardial cells at early day 8 and a persistence in culture until day 15.

**Conclusion:** On the whole, we have developed a new method based upon the combination of chemical and physical means to afford a high-throughput yield of complete cardiac differentiation from human urine-derived iPS. This method may pave the way to future developments in cardiovascular regenerative medicine.

## **Low level light therapy stimulates endothelial cells and promotes vasculogenesis**

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Low level light therapy (LLLT) with light-emitting diodes (LEDs) receives increasing interest in the fields of wound healing and angiogenesis. Endothelial cells play an important role in these processes. The aim of this study was to compare the effects of pulsing LED light of different wavelengths on endothelial cells and vasculogenic processes in vitro.

The effects of pulsed LED light on proliferation and migration of human umbilical vein endothelial cells (HUVEC) were investigated in several 2D and 3D cell culture models. Cells were treated with either blue (475 nm), green (516 nm) or red (635 nm) LED light. Control cells were not illuminated. 2D proliferation cells was determined at given time points by manual counting. 2D migration was assessed by scratch assays, 3D migration was evaluated by Cytodex bead assays. The vasculogenic potential of HUVEC in co-culture with adipose-derived stem cells (ASC) in response to LLLT was determined by analysing the network formation in a 3D fibrin matrix co-culture model after 4 and 7 days.

Stimulation with both red and green pulsed LED light significantly increased HUVEC proliferation. Moreover, HUVEC showed increased 2D migration potential with green light stimulation. The 3D migration was significantly enhanced by green and red light. In the 3D fibrin co-culture model, HUVEC elongation as precursor of vasculogenesis was enhanced by green and red light during the first 4 days while green light stimulation led to enhanced vasculogenesis after one week.

Both red and green light enhanced proliferation, migration and vasculogenesis processes while blue light was ineffective. Several parameters showed that green light was even more potent to stimulate regenerative processes than well-established red light therapy. Further studies have to focus on intracellular signalling induced by different wavelengths in order to optimize this promising, alternative application in tissue regeneration and wound therapy.

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