



News letter

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Editorial · Editorial comments

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Chicago is hosting the 13th Annual International Society for Medical Shockwave Treatment (ISMST) Congress, June 24-26, 2010 at the Hyatt Regency Chicago. It is the first Congress to be held in the United States since 2002 and much has changed in the world (including shockwave) since that time. The World has gotten much smaller with the universal use of the Internet. Medicine feels those ramifications on a daily basis. Patients have immediate access to all of the medical information being disseminated from all over the globe. With this access, patients have become more savvy and aware of their medical conditions and treatment alternatives. As a result, physicians have to be more prepared to deliver the care patients desire. Shockwave has become a mainstay of patient desires.

As technology evolves and the public is more aware of these changes in medicine, there is a greater desire for non-invasive alternatives. With this public pressure, medicine has had to adapt. Many non-invasive or minimally invasive treatments have become popular and mainstream but many have not gone through the rigors of clinical studies supporting their use. Shockwave therapy has met that burden of proof. Shockwave therapy technologies have performed prospective placebo controlled studies like no other technology or treatment alternative for similar indications. Continuously, shockwave has proved itself in peer reviewed publications worldwide.

The ISMST Congress will gather the best and brightest researchers and clinicians with expertise in shockwave technology and treatment from around the World. Abstracts from Europe, Asia, South and North America will highlight the ever growing use and applications of medical shockwave. Musculoskeletal, cardiac, and wound healing applications are just some of the areas that abstracts have been accepted for the 13th Congress.

While ESWT is the focus of the Congress, other treatment alternatives for conditions that shockwave is treated will be presented. An exciting list of invited speakers on non-shockwave treatment alternatives will round out an exciting weekend of information.

The United States has always been slow to universally adopt shockwave and this Congress is going to accelerate the knowledge and understanding of shockwave in the U.S.

Chicago is the perfect host city for such an important Congress. Known for its architectural beauty on the Lake Michigan, Chicago is also one of the most culturally diverse cities in the world. There may be no more perfect month than June to visit Chicago with a wide array of cultural and social events planned. The Hyatt Regency Chicago is situated in a perfect location with access to world famous museums, top restaurants, incredible shopping and lake front activities.

The 13th Annual ISMST Congress is sure to be one of the best ever.

Richard Thiele, MD
Member of the ISMST Supervisory Board



ISMST concept newslett

Ladies and gentlemen, let me extend a warm welcome to all of you who traveled far and wide to attend the 13th ISMST conference in Chicago (USA).

At the same time, we are pleased to present the 6th edition of the ISMST newsletter at this conference. The contents of this publication are on fundamental research. All the articles found within are the results of presentations conducted at the first ISMST Basic Research Meeting in Vienna (Austria), January 2010. You will see that musculoskeletal shockwave therapy has become a highly scientifically researched method of therapy administered not only in urology and orthopedics, but cardiology, veterinarian medicine, dermatology, surgery and plastic surgery as well. Renowned scientists like Prof. C. J. Wang from Taiwan (Molecular reactions of extracorporeal shockwave technology); Prof. R. Frairia from Italy (Shockwave basic research and some prospects in malignant tumor treatment); Dr. C. d'Agostini, Italy (Osteogenesis and bone turnover); Prof. W. Bloch, Germany (ESWT and Mechanotransduction); Prof. M. Branes, Chile (ESWT and Angogenesis); Prof. T. Matula, USA (**Measurements of acoustic energy deflection in the presence of replica bone**); Dr J. Hohlfield, Austria (**How can in-vitro shockwave therapy be performed best? - Preliminary results from cardiac cells**) have kindly provided us their papers for publication.

We would like to thank all our speakers for their generous participation in the creation of this important newsletter and the same goes for our industrial partners for their consistent support.

As joyful as the many years of development of ESWT has been and as wonderful as the annual opportunity to meet the familiar faces of ESWT is, and this year especially the participation of scientists and colleagues who made shockwave presentations on well-known indications, this year's shockwave get-together was overshadowed by a sad event. As you might have already discovered in this newsletter, the obituary of Professor Heinz Kuderna, a long-time proponent and pioneer of ESWT, who passed away earlier this year. Dr Helmut Neuland attended all of the twelve ISMST conferences - his presence will be sorely missed.

I hope you will find the articles published in this newsletter immensely interesting and informative. Last but not least, I would like to thank the founder and publisher of the ISMST newsletter Paulo Roberto Dias dos Santos of Brazil for his steadfast and dedicated engagement in ISMST and for this newsletter which he so tirelessly put together.

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It is with great sadness that we announce **Dr. Helmut Neuland** passed away on Saturday, February 12, 2010. Dr. Neuland had been a member of the ISMST since its foundation; a true ISMST “family member”.

It was at our first ISMST congress in Izmir, Turkey in 1997 that most of us met Helmut for the first time. We remember fondly how he spoke with fervor about concepts which no one, except him, seemed to understand (as most were not addressed by the content of his slides) before he suddenly realized his slides were in the wrong order. He remedied the situation by rearranging the slides then telling the audience, “You can read the words on the screen yourselves“. He then proceeded to re-present his information without comment, changing slides so rapidly they were impossible for the audience to read. It was a presentation none of us will soon forget!

It soon became a declared goal of our society to dispense with arguing the pros and cons of shockwave treatment by quoting statistical data but rather to become a forum for the exchange of new (including, still unproven) ideas concerning the effects of shockwave treatment. After reviewing Dr. Neuland's research, it became evident to the society that he would present his ideas at the second ISMST congress in London in 1998 and in succeeding congresses each year. During the discussions that followed his presentations year after year, Helmut responded with patience and generosity even to questions of a provocative or antagonistic nature. All the while, we members of the ISMST were becoming more and more aware of what a man of genius was standing before us. He overflowed with new ideas, many of which stretched beyond the current limits of understanding regarding shockwaves. As his audience would begin to comprehend his ideas, Helmut would smile and say, “This is Neuland” (which, in German, means “virgin soil”) “for you.”

Dr. Neuland's keen interest in mechanotransduction may have been triggered by his personal experience in the field of manual therapy. In cooperation with the Institute for Physiology of the University of Frankfurt, he investigated changes of redox potentials in tissue through the influence of shockwaves. Also, in cooperation with the Institute for Pharmacology of the University of Hamburg, he studied the influence of shockwaves on the depolarization of muscle cells by the opening of ion channels. In early 2006 he also initiated a cooperative and highly fruitful endeavor with the German University of Sports in Cologne. The research investigated the influence of shockwaves on stem cells as well as the influence of signal transduction by radial and focused shockwaves on cell biology, specifically the relation between dosage and effect. He worked on signal transduction in cartilage, the wound healing effects of shockwaves, and the increase of stem cell transplantation effects by shockwaves.

Dr. Neuland was not only a researcher rich in ideas, but also an open-minded, charming, and warm-hearted man who enthralled us with his enthusiasm. We are stunned and deeply grieved by his sudden and premature passing. We extend our sincerest condolences to his relatives and especially to Mrs. Pokorny, his life companion. May they find some measure of comfort in knowing that Helmut will be sorely missed by his ISMST colleagues who will keep alive the memory of this unforgettable member of our shockwave family.

BRIEF COMMUNICATION

Bone Pathology and Extracorporeal Shock Wave Therapy: Morphological insights within clinical lessons



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Introduction

An international body of literature has emerged on the application of shock waves for specific bone pathology based on a robust clinical experience. A number of unanswered questions remain particularly with respect to differential response to therapy amongst various pathologies, the morphological changes occurring with shock wave stimulation and the morphological correlates of shock wave therapeutic failure, particularly for delayed union and non-unions. The aim of this brief communication is to share our 10-year experience studying the pathological effects of therapeutic shock waves and to discuss several illustrative clinical cases. We also emphasize an imperative in the delivery of shock waves for bone pathology: close collaboration between treating physician and bone pathologist.

Clinical Approaches

We have developed a shock wave treatment strategy which is centered on the nature and severity of disease. We apply 4,000 pulses at energy flux density of 0.3 mJ/mm² for all tendinopathies and, 4,000 to 12,000 pulses at the same energy flux density for delayed and non-unions. The final shock wave dose is determined on an individual patient basis based on the duration of the problem and the type of non-union. Clinical decision factors include time or natural history of the pathology, type of non-union (hypertrophic vs. oligotrophic vs. atrophic), presence of third bone fragment, associated soft tissue disruption, clinical evidence of impaired regional perfusion, co-morbidity (e.g. tobacco use), co-existing soft tissue or

bone infection, and failure of previous surgical treatment. We apply ESWT in a single session under anesthesia and with fluoroscopic guidance. Shock wave dose distribution is as follows: 50% total dose to the non-union; and 25% total dose each to proximal and distal bone ends with the intent to stimulate proximal and distal bone blood-supply, analogous to a bone grafting technique. In 2002 we began to collect bone and tendon specimens from shock wave-treated patients, and published our initial experience [1] with 40 patients treated with ESWT for delayed and non-unions of involving various anatomic sites with 73% successfully healing (**Figure 1**). Our subsequent experience with 55 patients' non-unions demonstrated similar success, which is consistent with healing rates published by others, albeit with different shock wave treatment methods and total dose delivered [2,3,24]. We hypothesize that this consistency of clinical response is explained partly by ESWT's mechanistic effect: that of a "biological inducer of normal ontological repair mechanisms" requiring minimal energy flux density and dose to be effective.

Failure of osseous healing response to ESWT defined by lack of bone consolidation 8-12 weeks following initial ESWT raises several possibilities: 1. bone fracture end instability; 2. a metabolic impairing bone repair mechanisms; and, 3. delayed (>12 weeks), but present healing response to shock wave stimulation. With respect to the latter issue, anecdotal reports of delayed or protracted bone healing after delivery of shock waves to a non-union are not uncommon. The case of a slow responder demonstrating initial

resolution of non-union related pain, and swift resumption of crutch walking and full weight bearing despite incomplete radiological consolidation within the 8-12 week period, which then becomes complete within 12-24 weeks is a familiar clinical course of the so-called slow responder. It is common for slow responders to undergo repeat (up to three) shock wave treatments, much like those with infected non-unions in an effort to augment bone consolidation at the non-union site.

We have also observed cases refractory to other interventional treatment approaches respond with complete bone healing after shock wave treatment of metaphyseal fibrous defects, bi-partite patellae, congenital tibial pseudoarthroses/NF1 [4]. In a case of bone-grafted radial agenesis we did not observe any histological reaction to shock waves, likely a reflection of a biological principle: normal innervation plays a role in pain/pro-pioception, bone remodeling as well as vascular regulation. An uncharacteristic case of a teenager with a distal fibular cyst who after treatment with 4,000 pulses (0.3mJ/mm²) demonstrated initial cortical defect on computed tomography (CT), and 13 months later resolved completely. These sort of bone lesions are generally unsuitable for shock wave treatment in large part due to the absence of "stromal tissue" inducible towards ossification.

Histological insights

Although the bone and bone-marrow are often regarded as separate systems, they actually exhibit marked functional interdependence. The red-marrow contains haematopoietic and

non-haematopoietic stem cells, all having concatenated roles, which explain well defined mechanisms related to bone: osseous modeling (growth), remodeling (continuous maintenance, Bone Remodeling Units (BRU) within "compartments") and fracture repair [5]. As shock waves are most often applied at a time when growth and modeling have been completed, actual histological insights from bone samples taken around the time of shock wave therapy reflect the process of bone remodeling.

Bone remodeling is comprised of four sequential biological events that can be examined morphologically: 1. activation (recruitment of mononuclear osteoclast precursors, cellular fusion to form multinucleated pre-osteoclasts and their subsequent maturation to active osteoclasts); 2. resorption (active osteoclasts secrete proteins and proteolytic enzymes; sealing zones); 3. reversal (osteoclast apoptosis, osteoblast progenitors appear with activation of pluripotent mesenchymal stem cells; mononuclear cells occupy resorption lacunae); and, 4. formation (osteoblasts synthesize organic matrix directing its mineralization and become "incarcerated" as transforming osteocytes; developing canaliculae)[6]. The process of bone remodeling is essentially the same for cortical and cancellous bone, occupying a timeframe of 4 to 12 months; >80% of bone surface is thought to be in a resting phase (**Figure 2**), whereas ~20% of bone surface consist of recruited osteoclasts [7]. This multifaceted biology is inherently complex. Recent reports indicate that osteal tissue macrophages are intercalated throughout human bone lining tissues having the capacity to regulate osteoblastic function [8]; this is demonstrated by minimal mineralization in an osteoblastic environment deployed of macrophages. Other macrophage roles are metabolic in nature, as has been

demonstrated that macrophages are in fact "richer in collagenases than the osteoclast." [9] These macrophages have been implicated in the microstructure of BRU, being part of a canopy of flat cells that cover the entire remodeling site with a supporting vascular framework (**Figure 2b**) [8,10]; however, in BRUs with anomalous canopies, bone formation appears deficient.

Interestingly, CD34+ mononuclear cells have shown regenerative potential for vasculogenesis and osteogenesis [11, 12]. These cells have the capacity to promote both processes throughout differentiation into Endothelial Progenitor Cells and dedifferentiation into osteoblast. We have found that shock wave stimulation enhances CD34+ endothelial cell marker expression in human rotator cuff tendons (associated with the neoangiogenesis/vasculogenesis process) [13]. Previous studies have also demonstrated shock wave induction of an "osteoblastic response" that encompasses bone marrow stromal cell activation with differentiation towards osteoprogenitor cells with increased TGF-1 expression [14], neo-vascularization induced at bone-tendon junctions[15], and stimulated healing at segmental bone defects with enhanced expression of bone morphogenetic proteins (BMPS) [16].

Human bone samples exposed to shock waves 8 to 10 weeks previously, demonstrate a characteristic "stromal reaction," consisting of a highly cellular mononuclear component and active neo-vascular component (**Figures 2d, 3, and 4**). However, similar "stromal reaction" can be appreciated in other non-traumatic bone pathologies such as spontaneous bone necrosis [17] or osteoarthritis [18], suggesting that a non-hematopoietic bone marrow stem cell reaction is a fundamental response (**Figure 5**). This fundamental process can be harnessed in a clinically meaningful

way, as shock wave-induced bone repair encompasses several stem cell-based mechanism such as plasticity (differentiation), dedifferentiation (trans-differentiation) and modulator activity for inflammatory and immune reactions [19,20]. A recently described mechanism, Epithelial-Mesenchymal Transition (EMT), is considered a critical process for embryonic development and is re-engaged during adult wound healing and tissue regeneration [21,22]. In the adult, EMT involves repair-associated mesenchymal cells, fibroblasts and myofibroblasts [21,22]. Specifically, Type-2 EMT, involves repair-associated events engendering fibroblasts and other related cells to reconstruct tissue defects after trauma or inflammatory injury; once the inflammatory response ceases, the entire process is attenuated. Recently, Agrawal et al. [23] reviewed epimorphic regeneration mechanisms, showing that degraded molecules from injured extracellular matrix promote the appearance of cells with the capacity to differentiate into ectodermal and mesodermal phenotypes. This has led us to hypothesize that perhaps all these biological events may be related to shock wave-induced effects because various treated different tissue types exhibit the same sequence of events: baseline chronic inflammatory response impairing healing, active neo-vascularization in shock wave-treated areas and subsequent focal reparative hyper-cellularity that resembles mesenchymal stem cells, recommenced healing activity that ends in healing with reduced scar formation.

In summary, the relationship between acoustic pressure stimulation and living host tissue response is intriguing and fascinating. Continued expansion of knowledge in molecular biology permits novel insights into the mechano-biological effects of therapeutic shock waves.

Figures:

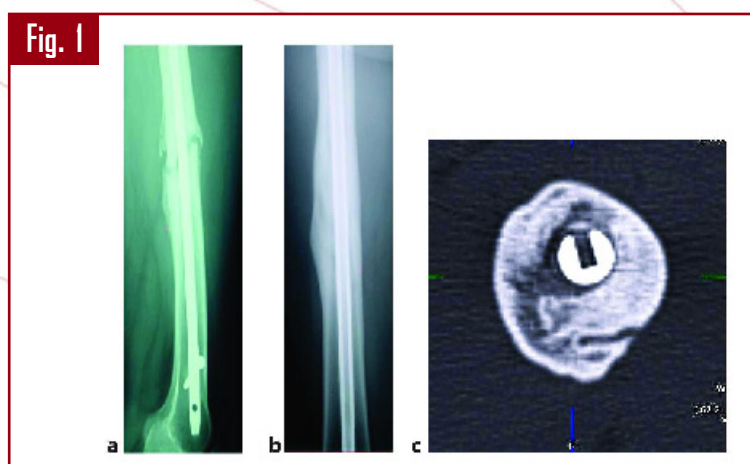


Fig.1-a. Delayed union (18 weeks after unstable ORIF). Fig.1-b, consolidation obtained after single SW-treatment. Fig.1-c, CT-Scan depicts bone repair quality with broad cortical bone (red dotted lines, images obtained 2 years post-SW).

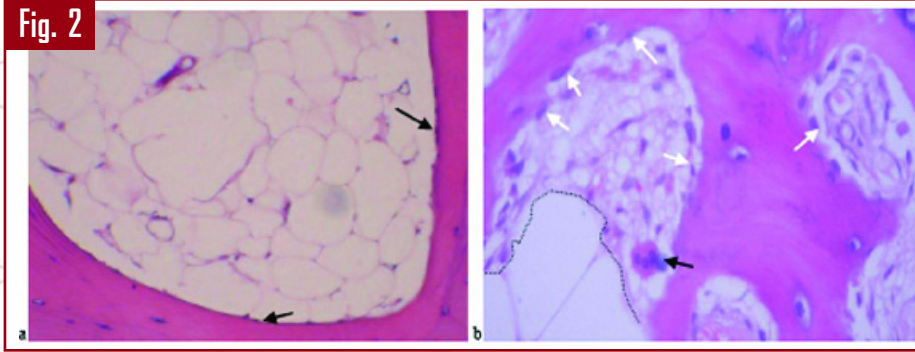
Fig. 2

Fig.2-a, section depicting endosteal quiescent area with some lining-cells (arrows) (H-E, 10x & zoom). **Fig 2-b**, trabecular bone area with BRU in activation phase with pro-osteoclast (black arrow), close to a bone area without lining-cells (white arrows); the entire area is closed by a canopy which contains some mononuclear cells (black dotted-line) (H-E, 40x).

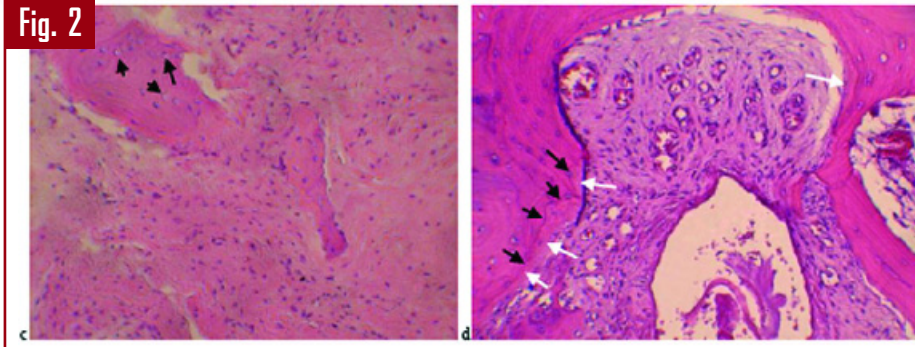
Fig. 2

Fig.2-c, trabecular bone (non-union, proximal tibia). Bone display some empty lacunae (black arrows) but fibroid tissue fill all spaces, featuring the scarcity of blood-vessels and chondral areas related to bone repair by callus formation. (H-E,10x). **Fig.2-d**, trabecular bone (shoulder rotator cuff, foot-print area, SW-treatment 10 weeks earlier). Stromal reaction with hyper-cellularity and important neo-vascular component, most of them actives. Cement lines on left (black arrows) helps to indicate the position of new osteoid-bone set downed (white arrows). (H-E, 10x & zoom).

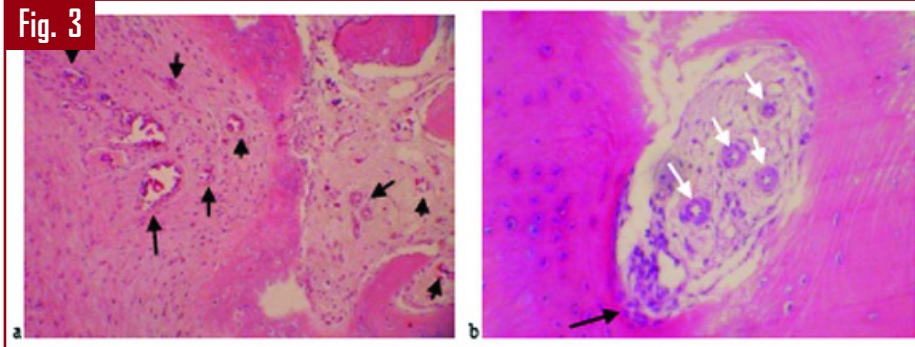
Fig. 3

Fig. 3-a, trabecular bone, SW-treatment 10 weeks earlier (foot-print area). Stromal reaction with significant number of active blood-vessels (black arrows) and evident hyper-cellularity in comparison to fig. 2 a-b-c. (H-E, 10x). We have not seen this kind of stromal reaction in bone material reviewed, including ectopic bone developed in the middle portion of Achilles tendon (Ectopic Ossification). **Fig. 3-b**, trabecular bone and SW-treatment 8 weeks earlier, depicting a BRU in reversal phase (black arrow); the only true difference with fig 2-b is the "hypermuskularized aspect" of blood-vessels integrated to BRU area (white arrows) (H-E, 10x & zoom).

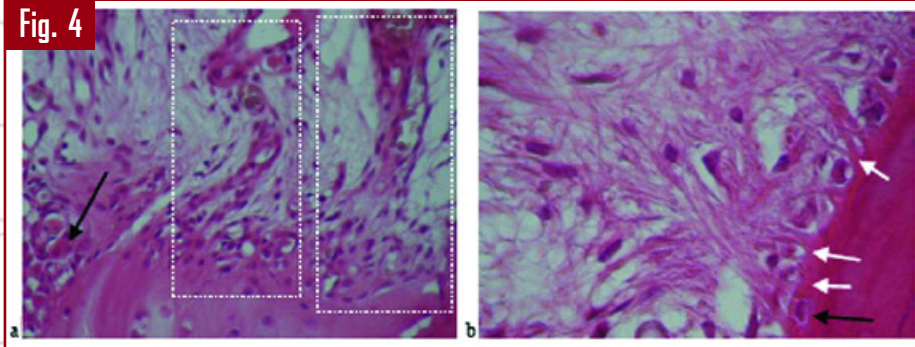
Fig. 4

Fig. 4-a, Trabecular bone, SW-treatment 22 weeks earlier, femoral neck area. This patient received SW because hip OA and subchondral focal necrosis; THR due to head collapse. The figure depicts less stromal reaction (perhaps due to time-span), with 2 defined active blood-vessels that are in relation to BRUs in reversal-formation phases (white dotted-rectangles). Another BRU in activation phase on left, where a pro-osteoclast is arriving to the scene (black arrow) (H-E, 40x). **Fig-4-b**, same patient, another section demonstrating an osteoblast in mineralizing activity (black arrow); new osteoid bone signaled by white arrows. (H-E, 100x).

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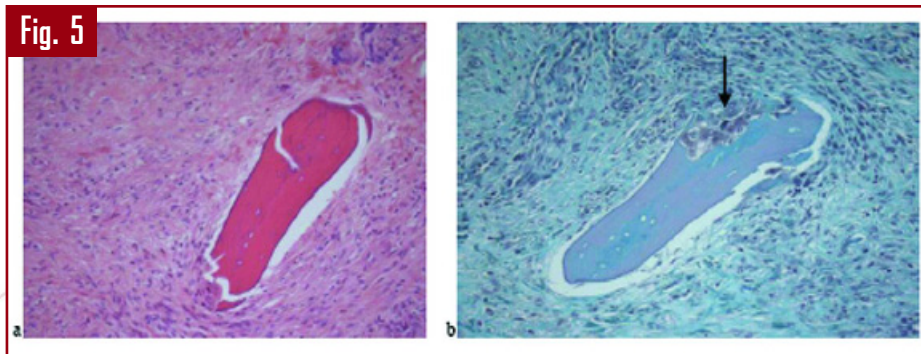


Fig. 5-a. Lunate necrosis (Kienbock disease). Impressive stromal cell reaction, developed in response to ischemia and bone necrosis, demonstrated by empty lacunae; noteworthy the absence of blood supply (H-E, 10x). **Fig. 5-b,** same patient showing focal bone resorption (black arrow), but it is not recognizable a normal BRU microanatomy (cells resemble macrophages activated not true osteoclasts, there is not identifiable canopy structure and blood-vessel normally associated (Alcian Blue, 10x & zoom).

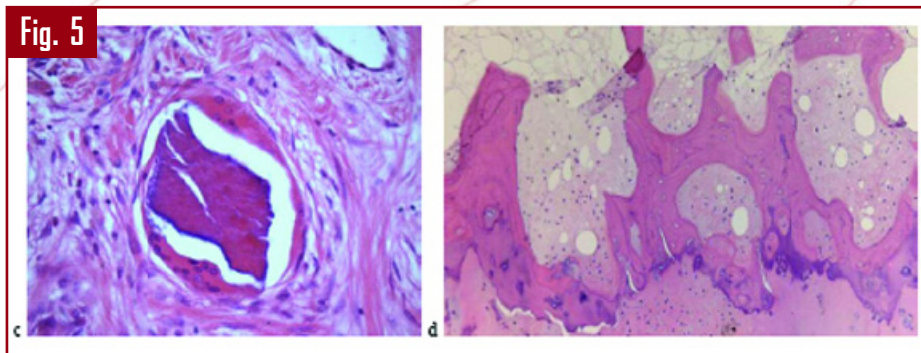


Fig.5-c, (Lunate necrosis)- this area is less ischemic (normal, single endothelial lined blood-vessel, top-right), 2 well-defined mature-macrophages encircling a necrotic bone fragment (H-E, 40x). **Fig. 5-d,** ankle traction-spur (distal tibiae). Another kind of stromal reaction, variable cellularity and fatty globules filling inter-trabecular spaces (H-E,10x); histological images for spurs developed in anterior shoulder acromion are quite similar.

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IVSWT - In Vitro Shock Wave Treatment



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Introduction

The number of in vitro experiments in shock wave science increases continuously. This fact reflects how important basic research findings on the cellular and sub-cellular level are for the future progress of shock wave treatment. In some very emerging fields better mechanistic understandings may be prerequisite for translation to clinical use or will at least support the application of already well established indications.

Today's knowledge of shock wave effects on cell cultures includes among others the increase of proliferation, alteration of cell membrane receptors, increase and acceleration of cell differentiation, release of several kinds of growth factors and chemoattractants

as well as increased cell migration (1-4).

Besides the cost-effectiveness and the reduction of animal experiments, the biggest advantage of IVSWT is the possibility of studying the specific behaviour of a certain cell type. In shock wave mediated tissue regeneration most likely all cells of the treated tissue are involved, even systemic effects are discussed. Nevertheless, each cell type plays a specific role in its tissue and has its own intrinsic function. These we are able to detect and better understand by doing IVSWT.

Some more applications of IVSWT are discussed. Maybe in future it could serve to improve tissue engineering or be used to enhance cell proliferation for (stem) cell treatment purposes.

Problem of in vitro models - distracting physical effects

Literature reveals very diverse methods of applying shock waves onto cell cultures. In general all existing models are focused on how to apply shock waves onto cells. It for example is done by direct coupling to a sample with ultrasound transmission gel, coupling through water tanks or even through pork skin (3-7).

However, this leads to the problem that it is highly difficult to compare results as physical mechanisms of cell stimulation are quite different between the models. As no standardized model for in vitro trials exists all the different models bring their respective advantage but are also associated with some distracting physical effects.

However, the question is not how to apply shock waves onto cells. This can easily be done with all the mentioned methods. But: What happens to the waves after passing the cell culture? The main problem is that the difference of the impedance between the cell culture medium and the ambient air is that high that nearly 98% of the shock waves get reflected (**Figure 1**).

Back running waves cause negative pressure onto the cells. Even if this negative pressure may not be harmful it interferes with the idea of mimicking in vivo shock wave effects in vitro. In vivo we would not find that high amount of negative pressure.

An even bigger problem occurs by back running waves disturbing the upcoming ones. This causes interference. Two types of interference are known. Constructive interference means that both waves are added thereby resulting in a doubled amplitude (**Figure 2A**). Destructive interference occurs if waves meet just oppositely. It causes abolishment of waves. (**Figure 2B**).

Therefore IVSWT needs a model that enables shock waves to propagate even after passing the cell culture. This can be done by putting cells into a water bath.

IVSWT water bath

Because of the above mentioned problems we asked a technician to build a water bath for us (**Figure 3A-3B**).

Basically this in vitro water bath exists of a plexiglass built container with a membrane to connect every kind of shock wave applicator. For coupling between this membrane and the applicator ultrasound transmission gel is used. The water bath is filled with degassed water to avoid cavitation that would occur if gas is soluted in the water. A heater at the bottom with a temperature sensor connected to a control unit enables to regulate temperature for imitation of in vivo conditions and to avoid cell cultures to

cool down. Temperature can be held stable at 37 degrees centigrade as it is done in an incubator. A holder for the cell samples allows to put in any kind of culture flasks or tubes. Of course samples need to be completely filled with culture medium as air bubbles would block shock waves. A wedge shaped absorber at the back wall destructs waves in order not to get reflected and run back. This is to avoid reflection and its distracting effects.

A further advantage compared to other IVSWT models is the possibility of varying the distance between the applicator and the culture flasks. Findings of our group and others who already use this model clearly show that every cell type reacts very specifically to different treatment parameters. Moreover, to define the distance between the waves source and the sample is crucial as it enables us to control the cells to be at a specific position to the focus of the applicator.

Besides our lab several other research groups already use this model and some of them presented their first results at the ISMST Basic Research Meeting in January 2010. All the feedbacks are very positive. Therefore this model seems to be a proper tool for IVSWT experiments and may to our today's knowledge avoid all kinds of physical limitations in our experiments. However, a continuous degassing system was discussed during the Basic Research Meeting and we are currently conducting a little experiment to find out whether the use of previously degassed water is sufficient or if we really need to continuously degas it.

Disclosure:

The author is not financially involved in distribution of the IVSWT water bath.

More information on the bath can be requested directly from the manufacturer by email: office@ing-hohenegger.at.

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Figures:

Figure 1. A shock wave applicator coupled directly onto a cell culture flask. When applied directly to the cell sample by e.g. ultrasound transmission gel shock waves get reflected after passing the cell culture due to the high difference of the impedance between culture medium and the ambient air. This causes negative pressure onto the cells and moreover leads to interference of upcoming and back running waves.

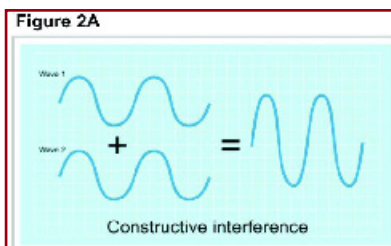
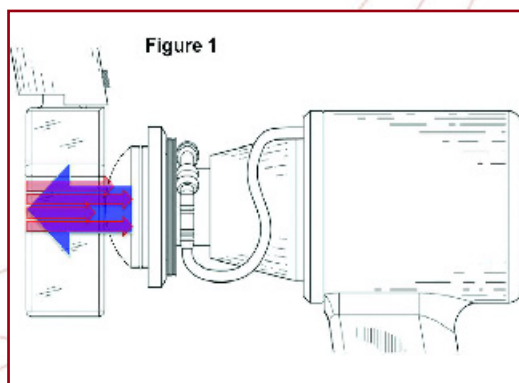


Figure 2A. Constructive interference occurs if upcoming and back running waves meet equally. They then are added thereby resulting in a doubled amplitude.

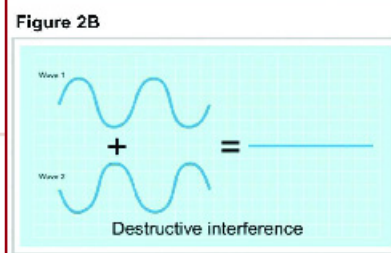


Figure 2B. Destructive interference occurs if waves meet just oppositely. It causes abolishment of waves.

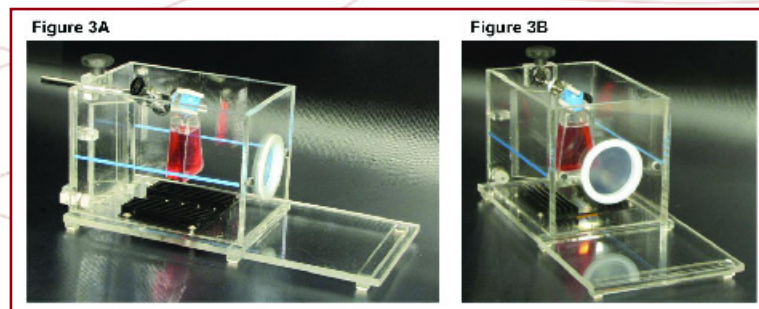
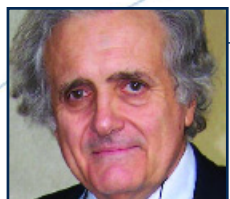


Figure 3A. The IVSWT water bath has a wedge-shaped absorber at its back wall to destruct waves in order not to get reflected and run back. A heater on the bottom controls temperature to be stable at 37 degrees centigrade. The universal clamp permits to put every kind of culture flasks or tubes into the bath.

Figure 3B. A membrane allows to connect every kind of shock wave applicator. Ultrasound transmission gel is used for coupling between the applicator and the membrane.

1st ISMST Basic Research Meeting

Shock Wave Basic Research and Some Prospects in Malignant Tumor Treatment



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The corner stone of cancer management is by far chemotherapy. Over the years, more and more powerful drugs have been developed with the purpose of increasing the rate of response to therapy. As molecular power of chemotherapeutic agents increased, unfortunately also toxicity and undesired side-effects increased as well. This is one of the reasons why so many efforts are being done to by-pass the toxicity and side-effects trouble of chemotherapy, without losing drug efficacy. Beside the identification of innovative methods to deliver drugs to cancer cells with the purpose to reduce the systemic dose but to increase the target cell concentration of the chemotherapeutic agent like new formulations in drug delivery, the search for new therapeutic strategies to be used in the management of cancer, like gene therapy, immunotherapy and the development of new pharmaceutical molecules, is one of the more promising strategies to reduce chemotherapy toxicity.

High Energy Shock Waves (HESW), that are widely used for the treatment of urolithiasis, have been reported to cause modifications of cell growth both *in vitro* and *in vivo* (Randazzo et al, 1988; Delius et al, 1990; Roessler et al, 1995; Miller and Tomas 1996, Huber and Debus, 2001). A substantial difficulty in comparing the various observations is due to the large heterogeneity of mechanical, biological and analytical variables in each experimental procedure. *In vitro* studies have shown that shock wave treatment elicits immediate reduction of cell viability and ability to form colonies (Randazzo et al, 1988). The impact of shock waves on cell survival varies not only with the cell type, but also between cell lines of the same type (van Dongen et al, 1989); it was hypothesized that cells in G2-M phase cell cycle can be more easily damaged by shock waves as compared to cells in G-0.

With regard to the mechanism by which the shock waves cause cellular damage, numerous hypotheses have been advanced. Based on the fact that cells did not show any damage when immobilized in agar, Brummer et al. (1989) suggested that the collision between cells could play a role in changing their viability; it was subsequently proven that viability was not influenced by varying cell concentration (van Dongen et al, 1989). It was also suggested a possible role of free radicals that are generated by shock waves in inducing cellular damage (Morgan et al, 1988). Currently, the best hypothesis on the mechanism of cell damage elicited by shock waves is that of cavitation and the generation of jet streams in the extracellular milieu.

Earlier changes include cell swelling and mitochondria fragmentation; other modifications include nuclear segmentation as well as changes in the morphology of the cell membrane (Randazzo et al, 1988). Moreover, shock waves are able to create lethal and sublethal damage to cancer cells *in vitro*. The cells that survive after shock wave exposure are still able to form tumors when inoculated in animal models, but these tumors are smaller than in controls (not treated with shock wave) since a significant percentage (40 to 60%) of surviving cells that have been inoculated show sublethal damages induced (Randazzo et al, 1988).

Numerous studies have shown that the combined treatment of tumor cells in suspension by some anticancer agents and Shock Waves elicits a significant enhancement of drug cytotoxic effect (Oosterhof et al, 1989; Gambihler and Delius, 1992; Kambi et al, 1997, Kato et al, 2000).

It was noted that the shock waves when applied to cells *in vitro*, are able (even at low energy) to determine a transient increase in permeability of cell membranes by opening pores, allowing

higher concentrations of drug within the cell (Delius and Adams, 1999, Kodama et al, 2000, 2002). Cells of human estrogen-dependent breast cancer (MCF-7) were sensitive to combined treatment with Shock Waves and paclitaxel, an antimicrotubule agent, active against a variety of solid tumors (Frairia et al, 2003). Recently, the suppression of cell proliferation induced by Shock Waves has been related to an apoptotic mechanism (Kato et al, 2000). Apoptosis, i.e. programmed cell death, is a cellular self-destruction mechanism, characterized by well defined morphological and molecular alterations, which plays a key role in the surveillance against tumors (Steller, 1995). Induction of apoptosis occurs in response to a variety of stress signals (Ashus et al, 2000) which may include Shock Waves (Kato et al, 2000).

Most recent studies have shown the cytotoxic action enhanced by Shock Waves in combination with some anticancer drugs *in vitro*: cell lines of human osteosarcoma (Palmero et al, 2006), human colorectal adenocarcinoma (Canaparo et al, 2006) and human anaplastic thyroid cancer (Catalano et al, 2008) were subjected to combined treatment (Shock Waves and anticancer drug). Moreover, a linked "sonodynamic/photodynamic" technique was adopted, based on ability of Shock Waves to activate and render cytotoxic a photosensitizing substance: the natural porphyrin precursor, (5-aminolevulinic acid, ALA), which is accumulated selectively by neoplastic cells.

In normal cells, protoporphyrin IX (PPIX), a substance with excellent photosensitizing properties, does not accumulate to a great extent because it is quickly transformed to heme by the action of ferrochelatase. In cancer cells, however, PPIX accumulates due to a defective heme biosynthesis, thought to be caused by abnormal levels of some of the enzymes involved in this pathway, since increased activity of porphobilinogen deaminase and/or decreased activity of ferrochelatase has been reported for a number of tumours. Exogenous application of ALA can lead to a pronounced accumulation of PPIX in tumour tissue and subsequent irradiation with light of wave lengths corresponding to the PPIX absorption bands can lead to specific destruction of tumour cells. Photodynamic therapy (PDT) has developed into an important new clinical cancer treatment modality in the past 25 years but the low penetration depth of light through the

skin and tissues, thus limiting PDT to the treatment of superficial, endoscopically reachable tumours.

High Energy Shock Waves induce acoustic cavitation, which results in a concentration of energy sufficient to generate a sonoluminescence emission, able to cause electronic excitation of porphyrins by energy transfer and initiate a photochemical process resulting in the formation of the cytotoxic singlet oxygen. "Sonodynamic therapy" is an analogous approach to PDT based on the synergistic effect of ultrasound and chemical compound referred to as "sonosensitizer" but the attractive feature of this modality for cancer treatment emerges from the ability to focus the ultrasound energy on malignancy sites placed deep in tissues. The Shock Waves source can be placed at direct contact with the body so that the maximum energy flow given to the inner part of the tumor can be precisely controlled.

This last technique proved to be effective even in vivo, in inducing necrosis and apoptosis of breast cancer and colon cancer implanted in laboratory animals (Canaparo et al, 2008; Frairia et al, 2009). There are therefore new possibilities in oncologic field: 1) Shock Waves are able to modify the permeability of cell membranes to allow a higher amount of antineoplastic drugs to enter into the cells; 2) a synergistic "sonodynamic/photodynamic" effect allows to treat malignancy sites placed deep in tissues. as the shock waves are able to activate and render cytotoxic chemical compounds referred to as "sonosensitizers" which are selectively accumulated within cancerous lesions.

Additional perspectives are provided by recent studies which show how the Shock Waves can support transfection, i.e. the transfer of therapeutic genes to target cells through a mechanism similar to electroporation: by temporarily increasing cell membrane permeability: DNA - or fragments, such as plasmids - can enter the cell (Tschoep et al, 2001, Song et al, 2002, Kodama et al, 2002; Miller and Song, 2002, Murata et al, 2007, Yamashita et al, 2009). A similar mechanism may be involved to "manipulate" stem cells, both as vectors for the expression of therapeutic genes (Berger et al, 2005), and as additional factors which can influence the proliferation and differentiation into specific cell lines (Nurzynska et al, 2008) The numerous data from the

literature allow to assume that Shock Waves - thanks to their ability to focus the energy in depth, to increase cell permeability (to antineoplastic drugs and/or genetic material) and, finally, to activate photosensitizing agents - can be considered for a further "multimodal" approach in antineoplastic therapy.

Concluding remarks: 1) Shock Waves act as an "*ultrasound-susceptibility modification agent*" since they may induce cell permeabilization, thus allowing better delivery of chemotherapeutic drugs into cytosol; 2) Shock Waves enhance both the cytotoxic activities of photosensitizers as well as the apoptotic signal transduction pathway: they can act as a further tool in "sonodynamic/ photodynamic" therapy; 3) Gene transfer can be induced by Shock Wave treatment in vivo, particularly with enhanced acoustic cavitation, which supports the concept that "Gene and Shock Wave therapy might be advantageously merged"; 4) Other treatment schedules are worth to be explored to evaluate the potential utility of Shock Waves in cancer therapy, especially in combination with other modalities.

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Extracorporeal shockwaves promote bone healing and systemic concentrations of nitric oxide (NO), TGF- β 1, VEGF and BMP-2 in long bone non-unions



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Summary

The systemic effects of extracorporeal shockwave technology (ESWT) in bone healing are not fully understood. This study investigated the effects of ESWT on bone healing and the changes in systemic concentrations of nitric oxide (NO), TGF- β 1, VEGF and BMP-2 in long bone non-unions.

Forty-two patients with 42 established non-unions of the femur and tibia were enrolled in this study (**Table 1**). Each long bone non-union was treated with 6000 impulses of shockwave at 28 Kv as a single session. Ten milliliters of peripheral blood were obtained for measurements of serum NO level and osteogenic growth factors including TGF- β 1, VEGF and BMP-2, serum levels of calcium, alkaline phosphatase, calcitonin and parathyroid hormone before treatment and at 1 day, 1, 3 and 6 months after treatment. The

evaluations for bone healing included clinical assessments and serial radiographic examinations.

At six months, clinical assessments revealed significant improvement in pain score, weight bearing, ability to work and overall function for activities of daily living (**Table 2**). Bony union was radiographically confirmed in 78.6%, and persistent non-union in 21.4% (**Table 3**). The serum NO level, TGF- β 1, VEGF and BMP-2 were significantly elevated at one month after ESWT (**Table 4**). Patients with bony union showed significantly higher serum levels of NO, TGF- β 1, VEGF and BMP-2 at one month after treatment as compared to patients with persistent non-union (**Table 5**).

Shockwave-promoted bone healing was associated with significant increases in serum NO level and osteogenic growth factors. The elevations of

systemic concentration of NO level and the osteogenic factors may reflect a local stimulation of shockwave in bone healing in long bone non-unions. It appears that shockwave-promoted bone healing in non-union of long bone was linked to NO modulation and activation of osteogenic growth factors including TGF- β 1, VEGF and BMP-2. The releases of systemic NO and osteogenic growth factors after a local application of ESWT to bone appears time dependent with peak levels at one month. Therefore, the systemic changes in NO level and osteogenic growth factors may represent a reflection of local stimulation with ESWT in long bone non-unions. It is reasonable to believe that shockwave treatment may provoke NO production, which in turn may activate the mitogenic, osteogenic and angiogenic responses within the bone microenvironment in a time fashion.

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Tables:

Table 1. Patient Demographic Characteristics.

| Number of patients / non-unions | 42 / 42 |
|---|---------------|
| Age (in years) | |
| Mean ± SD | 34.8 ± 13.6 |
| (Range) | (18~68) |
| Duration of fracture (in months) | |
| Mean ± SD | 15.02 ± 10.76 |
| (Range) | (6~48) |
| Gender | |
| Male | 22 |
| Female | 20 |
| Anatomical location | |
| Femoral | 28 |
| Tibia | 14 |
| Prior operation | |
| ORIF with IM nailing | 31 |
| ORIF with plating | 14 |
| ORIF with external fixation | 1 |
| Bone grafting | 10 |
| Type of non-union | |
| Atrophic | 7 |
| Hypertrophic | 35 |
| Length of Follow-up (in months) | |
| Mean ± SD | 15.24 ± 7.27 |
| (Range) | (6~24) |

Table 2. The Results of Clinical Assessment.

| Time | Pre-treatment | 1 month | 3 months | 6 months |
|---|---------------|-------------|-------------|-------------|
| Case number | 42 | 42 | 42 | 42 |
| VAS (Mean ± SD) | 3.19±1.55 | 1.17±1.08 | 0.45±0.71 | 0.19±0.4 |
| Range | 1~6 | 0~5 | 0~2 | 0~1 |
| P-value | | v<0.001 | <0.001 | <0.001 |
| Weight bearing (%) (Mean ± SD) | 35.35±18.43 | 47.62±20.81 | 64.76±22.55 | 80.0±17.81 |
| Range | 10~70 | 10~80 | 20~100 | 50~100 |
| P-value | | v0.003 | <0.001 | <0.001 |
| Ability to work (%) (Mean ± SD) | 25.58 ±14.19 | 34.63±18.32 | 52.2±21.27 | 76.19±19.87 |
| Range | 10~30 | 10~80 | 10~100 | 30~100 |
| P-value | | v0.007 | <0.001 | <0.001 |
| Improvement from last examination (%) (Mean ± SD) | | 30.26±13.47 | 55.00±22.22 | 79.76±18.93 |
| Range | | 10~50 | 20~100 | 30~100 |
| P-value | | | <0.001 | <0.001 |

VAS: Visual analogue scale from 0 to 10 with 0 for no pain and 10 for severe pain.

Table 3. The Results of Radiographic Evaluation.

| Time | Pre-treatment | 1 month | 3 months | 6 months |
|--|---------------|-------------|-------------|--------------|
| Case number | 42 | 42 | 42 | 42 |
| Maximal fracture gap (mm) (Mean ± SD) | 3.83±1.34 | 3.74±1.39 | 2.63±1.86 | 1.79±2.15 |
| Range | 1.5~7.33 | 1.2~ 6.27 | 0~6.27 | 0~5.4 |
| P-value | | -0.381 | <0.001 | <0.001 |
| Minimal fracture gap (mm) (Mean ± SD) | 1.98±0.62 | 1.92±0.68 | 1.46±0.99 | 0.9±1.13 |
| Range | 0.82~3.94 | 0.74 ~3.94 | 0~3.94 | 0~3.94 |
| P-value | | -0.318 | 0.002 | <0.001 |
| Callus at fracture gap (%) (Mean ± SD) | 29.88±17.72 | 57.38±26.14 | 80.83±24.49 | |
| Range | | 0~75 | 0~100 | 25~100 |
| P-value | | | <0.001 | <0.001 |
| Fracture healing by x-ray | 0% (0/42) | 12%(5/42) | 43%(18/42) | 78.6%(33/42) |
| P-value | | 0.03 | <0.001 | <0.001 |

Table 4. Serum NO Level, TGFβ1, VEGF and BMP2 at Different Time Intervals

| Unit | NO Umo/L | TGF-β1 Pg/mL | VEGF Pg/mL | BMP2 Pg/mL |
|---|--------------------|----------------------|----------------------|--------------------|
| Pre-treatment (N =42) Mean±SD | 62.2±42.2 | 49861±10838 | 334.6±214.5 | 71.2±16.6 |
| 1 day post-treatment (N = 42) Mean±SD P-value | 60.6±42.8 0.439 | 46394±14496 0.132 | 359.4±284.3 0.345 | 72.6±17.2 0.361 |
| 1 month post-treatment (N = 42) Mean±SD P-value | 92.1±49.2 0.003 | 59166±13547 0.002 | 476.7±306.9 0.028 | 82.1±26.9 0.018 |
| 3 months post-treatment (N= 42) Mean±SD P-value | 66.1±43.6 0.353 | 52918±15075 0.189 | 341.1±222.2 0.452 | 69.5±19.6 0.345 |
| 6 months post-treatment (N=42) Mean±SD P-value | 62.5±27.3 0.488 | 48116±13069 0.288 | 269.5±109.8 0.074 | 66.0±23.2 0.137 |

P-values: Comparison of pre-treatment data with the data at 1 day, 1, 3 and 6 months. Umo/L: Micromole/L. Pg/mL: Picogram/mL.

Table 5. Serum NO Level, TGF- β 1, VEGF and BMP-2 in Patients with Union and Patients with Non-union.

| NO and osteogenic markers | Union (N=33) | Non-union (N=9) | P-value² |
|----------------------------------|---------------------|------------------------|----------------------------|
| Pre-treatment | | | |
| NO (Umol/L) | 63.6 \pm 45.3 | 57.8 \pm 30.5 | 0.319 |
| TGF- β 1 (Pg/mL) | 50722 \pm 10807 | 46275 \pm 11175 | 0.179 |
| VEGF (Pg/mL) | 347.8 \pm 211 | 289.2 \pm 237.3 | 0.285 |
| BMP-2 (Pg/mL) | 72.9 \pm 18.0 | 66.6 \pm 6.7 | 0.074 |
| 1 day post-treatment | | | |
| NO | 64.7 \pm 47 | 56.9 \pm 19.1 | |
| P-value-1 | 0.416 | 0.494 | 0.264 |
| TGF- β 1 | 48175 \pm 15028 | 43937 \pm 9092 | |
| P-value-1 | 0.237 | 0.349 | 0.107 |
| VEGF | 367.6 \pm 281 | 332.7 \pm 313.1 | |
| P-value-1 | 0.389 | 0.383 | 0.391 |
| BMP-2 | 73.5 \pm 18.5 | 69.1 \pm 9.9 | |
| P-value-1 | 0.393 | 0.304 | 0.200 |
| 1 month post-treatment | | | |
| NO | 99.0 \pm 52.3 | 68.1 \pm 26.9 | |
| P-value-1 | 0.003 | 0.228 | 0.017 |
| TGF- β 1 | 60986 \pm 13661 | 49337 \pm 8132 | |
| P-value-1 | 0.002 | 0.306 | 0.034 |
| VEGF | 532.3 \pm 318.3 | 290.7 \pm 190.2 | |
| P-value-1 | 0.02 | 0.495 | 0.028 |
| BMP-2 | 86.4 \pm 29.5 | 68.8 \pm 5.5 | |
| P-value-1 | 0.035 | 0.266 | 0.003 |
| 3 months post-treatment | | | |
| NO | 67.7 \pm 43.9 | 61.5 \pm 44.7 | |
| P-value-1 | 0.361 | 0.408 | 0.361 |
| TGF- β 1 | 54250 \pm 15680 | 46791 \pm 11160 | |
| P-value-1 | 0.186 | 0.468 | 0.09 |
| VEGF | 353.3 \pm 194.1 | 276.2 \pm 351.2 | |
| P-value-1 | 0.462 | 0.47 | 0.311 |
| BMP-2 | 70.0 \pm 21.1 | 66.5 \pm 6.5 | |
| P-value-1 | 0.455 | 0.488 | 0.238 |
| 6 months post-treatment | | | |
| NO | 64.4 \pm 28.1 | 51.4 \pm 21.3 | |
| P-value-1 | 0.499 | 0.360 | 0.168 |
| TGF- β 1 | 48590 \pm 13457 | 46297 \pm 12437 | |
| P-value-1 | 0.275 | 0.498 | 0.309 |
| VEGF | 272.6 \pm 113.3 | 226.6 \pm 119.2 | |
| P-value-1 | 0.069 | 0.277 | 0.213 |
| BMP-2 | 66.5 \pm 24.4 | 63.5 \pm 17.5 | |
| P-value-1 | 0.161 | 0.347 | 0.362 |

P-value¹: Comparison of pre-treatment data with the data at 1 day, 1, 3 and 6 months after treatment.

P-value²: Comparison of patients with union with patients with non-union.

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Overview and General Aspects of the Subject

Bone is a highly specialized connective tissue, the unique for which damaged tissue is replaced without forming a scar. Osteogenesis is the process by which bone is formed, both in normal and pathological conditions, not only for bone replacement, but also in the growing phases of the skeleton.

Recent data from the literature described new insights into the cellular mechanisms involved in osteogenesis and bone turnover, thus introducing new perspectives in ESW treatments and indications (Issack et al. 2008, Secreto et al. 2009, Szczesny et al. 2002,).

Osteogenesis takes place mainly during: BONE GROWTH, HEALING and BONE TURNOVER (Modeling and Remodeling).

Bone Growth

In bone growth by endochondral ossification (osteogenesis preceded by a cartilaginous model), an important role has been recently described for Nitric Oxide (NO), especially in the regulation of cartilage development. It is known that not only chondroblasts and chondrocytes are involved in the sequence of cellular events of endochondral ossification, but also osteoblast, osteoclasts and endothelial cells. NO seems to have indirect effects in all these different cell types and distinct NO - pathways may be active at different stages of chondrocyte differentiation and maturation during endochondral bone formation (Teixeira et al. 2008).

Recently it has been described also a new role of angiogenesis as a crucial step for skeletal development (chondrocytes metabolism is regulated also by VEGF); the presence of osteoclasts and Matrix

Metallo Proteinase - 9 (MMP-9) produced by osteoclasts is essential for skeletal angiogenesis. In details, it would couple the invasion of blood vessels with hypertrophic cartilage remodeling during endochondral ossification (and in osteophytes formation for osteoarthritis as well) (Dai et al. 2007, Deckers et al. 2007, Gerber et al. 1999, Hasky - Negev et al. 2008, Mackie et al. 2008, Malemud et al. 2006,).

Bone Healing

Bone healing includes growth and differentiation of mesenchymal stem cells, regulation of inflammatory cytokines, synthesis and resorption of extracellular matrix, in well distinguished processes:

Inflammatory phase (Early healing) → *Reparative phase* (Well formed callus) → *Remodelling phase* (Remodelling of cortex).

Interestingly, some recent studies showed that gene expressions of some BMPs and BMP antagonists were significantly lower in non-unions, compared to standard healing fractures and down-regulation in expression of osteogenic BMPs may account for the non-unions of fracture (Wang CJ et al. 2009).

Bone Turnover

During life, bone is continuously self-renewed, both in childhood and in adult life: this turnover, other than for metabolic purposes, has the precise function to maintain skeletal integrity. On the basis of common cellular events, it is possible to distinguish: *Modeling e Remodeling*.

Modeling is the process whereby the skeleton is sculptured to achieve its definitive shape and size of adult life, by removing bone from one site and depositing bone at a different one. Once the skeleton has reached maturity, the periodic replacement of

old bone with new one occurs through a process of *Remodeling*, although the signals that regulate the transition from modeling to remodeling are still not well understood. First observations on this topic were proposed by Wolff (1836-1902) at the end of the 19th century (WOLFF's LAW), and then studied by HM Frost since 1960s, with the Mechanostat Theory (Frost 2003, Frost 2004). Briefly, bones adapt their mass and structure in response to the demands of mechanical loading: disuse/hypogravity induce bone loss (mainly due to an accelerated remodeling mechanism), while physical training (*overuse*) can increase bone mass, architecture and strength, in the presence of a healthy hormonal asset, synergistically with local factors controlling bone remodeling (Lee et al. 2002, Skerry et al. 2003, Turner et al. 1998, Turner et al. 2009).

Recently, it has been described an important role for osteocytes in mechanotransduction (other than in maintaining mineral homeostasis), as mechanosensors. They would be able to early respond to load *in vivo*, by rapid release of anabolic signals (NO, PG and other small molecules such as ATP). It is known that NO and prostaglandin E2 (PGE2) are molecules that regulate bone adaptation to mechanical loading. NO and prostaglandin E2 production are essential for the induction of new bone formation in response to mechanical loading *in vivo* and the constitutively expressed endothelial form of NOS (eNOS), is prominently expressed in osteocytes and up-regulated by mechanical loading (Bloomfield et al. 2001, Klein - Nulend et al. 2005, Liedert et al. 2006, Mullender et al. 2004, Papachristou et al. 2009, Rahnert

et al. 2008, Rubin et al. 2003, Santos et al. 2009).

According to some authors, there may be no single mechanoreceptors in osteocytes, but instead a combination of events that has to be triggered for mechanosensation and transduction of signal to occur: shear stresses along dendritic processes and/or the cell body; cell deformation in response to strain and the presence of primary cilia (Bacabac et al. 2006, Bacabac et al. 2008, Bonewald et al. 2006, McGarry et al. 2005, Nicoletta et al. 2002, Tan et al. 2008, Vatsa et al. 2007, Vezeridis et al. 2006).

The dynamic process of bone remodeling results from the coordinate action of bone resorption by osteoclasts (OC) and the formation of new bone by osteoblasts (OB) (Activation → Resorption → Formation, or *ARF Sequence*). Regulation of bone remodeling occurs through multiple mechanisms, that ultimately converge on the interaction of OC (or their precursors) with OB and bone marrow stromal cells. OCs are bone-resorbing cells derived from the myeloid lineage, that play a central role in bone remodeling and inflammatory bone erosion diseases; OBs regulate the production of osteoclasts by secreting M-CSF (Macrophage Colony Stimulating Factor) and displaying the Receptor Activator of Nuclear Factor K-B ligand (RANKL) on their cell surface, to induce cells of the monocytic/macrophage lineage to develop into Ocs. Osteoclast activation is a critical cellular process for pathological bone resorption, such as erosions in Rheumatoid Arthritis or generalized bone loss. In bone remodeling cycle, the OBs orchestrates this orderly process through activation signals from systemic and local factors: M-CSF and RANKL are the two major OB mediated factors, which regulate the recruitment and differentiation of the OCs. Osteoprotogerin (OPG) is also synthesized by OBs and serves as a soluble decoy receptor, blocking activation of RANK; inhibition or knockout of these signals from OB-OC results in reduction of bone resorption. Thus, in bone turnover, an important phenomenon is the so - called Osteoblasts - Osteoclasts Coupling (*OB - OC Coupling*): in normal conditions, bone resorption and formation are strictly related and new OBs assemble only at sites where OCs have recently completed resorption. It follows that pathological conditions can lead to a

loss of coupling between OCs and OBs, and the balance between RANKL and Osteoprotegerin (OPG) production determines how fast bone breaks down. Thus, (Cackowski et al. 2010, Matsuo et al 2008, Matsuo 2009).

Angiogenesis & Bone Remodeling

Recently, the existence of a microstructure in trabecular bone termed a Bone Remodeling Compartment (BRC), has been proposed. In this model, OCs and OBs, during bone remodeling, are enclosed in a dome of OB-like cells, that are in close association with a capillary. Most cells in bone have a regulatory role in angiogenesis, many through production of angiogenic factors and most osteoinductive factors, mainly derived from endothelial cells, are capable of inducing the production of VEGF by OBs (Cackowski et al. 2010). Like many other cell types, osteoblasts increase production of angiogenic factors in response to hypoxia (Wang Y et al. 2007).

Endothelial cells can stimulate also OCs formation by several mechanisms, including increased RANKL expression, and stimulate OC formation in co-cultures by a RANKL dependent mechanism. Endothelial cells may also regulate the recruitment of OC precursors to remodeling sites from the vascular compartment. MMP-9 was shown to be required for both blood vessel and OC entry into the primary ossification center (Cackowski et al. 2010).

Angiogenesis has also been shown to be crucial for the replacement of cartilage by bone during skeletal growth and regeneration. Vascular Endothelial Growth Factor- A (VEGF-A), produced by hypertrophic chondrocytes during endochondral bone formation, stimulates controlled invasion of chondroclasts into the cartilage. VEGF-A is also a chemoattractant for endothelial cells and regulates the growth plate vascularization of metaphyseal bone. Invasion of OCs into hypertrophic cartilage requires the presence of VEGF-A and VEGF-A may attract osteoclast precursor cells that are recruited from hematopoietic tissue (Daj et al. 2007, Deckers et al. 2002, Mackie et al. 2008).

Osteoclast & Angiogenesis & Bone Remodeling

As above described, bone remodeling is initiated by recruitment of OC precursor cells from bone

marrow or circulating monocytes: homing of these precursors through the vascular wall is required for successful bone turnover. Intimate contact of the bone remodeling site to adjacent vascular sinusoids indicates an interplay between the vascular endothelium and the bone cells. Thus, one of the key components of bone remodeling is constant development of vasculature and a novel role of OCs in skeletal angiogenesis was demonstrated. Not only angiogenesis is a crucial step for skeletal development, but vasculature is required for transport of nutrients and precursor cells, such as precursors of chondroclasts and osteoclasts, to the renewing bone tissue. The microanatomy of capillaries is characterized by their close association with bone cells. Signaling between the endothelial cells and the bone cells may have a role in recruitment of osteoclastic precursor cells; OCs are in close proximity to capillaries and are thus in a good position to signal to blood vessels (Cackowski et al. 2010).

Thus, it is possible to summarize as follow:

- Osteoclastogenesis and angiogenesis are correlated in bone, during physiological and pathological processes including development, fracture healing, bone metastases and inflammatory bone disease;
- **The Osteoclast Is an Angiogenic Cell in Bone:** it stimulate angiogenesis in the local microenvironment, by secreting factors, which directly or indirectly increase new vessels formation, although it is unclear if or how these two processes are linked;
- **MMP-9 Is Required for Osteoclast-Stimulated Angiogenesis:** it modulates OC - stimulated angiogenesis primarily by affecting OCs, most likely by migratory effects on them, especially during fracture healing and endochondral ossification
- OCs are rare cells in bone; induction of blood vessels and vascularization precedes the up-regulation of the transport machinery (Cackowski et al. 2010).

Nitric Oxide & Bone Cells

NO has long been implicated as one of the signals transducing mechanical stress in cells and has been implicated to be a critical effector to modulate OB

activities and bone remodeling, other than to have pleiotropic effects on bone cells *in vitro*. NO can be considered multifunctional signaling molecule and a key vasculoprotective and potential osteoprotective factor; it regulates normal bone remodeling and pathological bone loss, in part through affecting the recruitment, formation, and activity of bone resorbing osteoclasts. The source of NO production in bone cells is largely due to eNOS (endothelial NO - Synthase; it is prominently expressed in osteocytes and up-regulated by mechanical loading. Mechanical strain differentially regulates eNOS and RANKL expression from osteoprogenitor stromal cells in a magnitude-dependent fashion. The activation of eNOS seems to promote an anabolic picture in bone also during growth.

Nevertheless, nNOS (neronal) has been shown to play a role as a stimulator of bone turnover *in vivo* potentially through a neurogenic relay; a study of multiple human osteoblast cell lines suggested that eNOS but not nNOS was constitutively expressed and that inflammatory NOS was expressed only after cytokine stimulation. Inflammatory NOS and neuronal NOS (nNOS) are found during fetal development and fracture repair.

Really, NO has been described to have a regulatory effect on differentiation and proliferation of OB and OB-like cells; OBs can constitutively produce NO, following stimulation of inflammatory cytokines,

NO will be massively induced by osteoblasts. Constitutive NO can regulate OBs proliferation and differentiation; however, overproduced NO could also damage osteoblasts, mainly via an apoptotic pathway.

More interestingly, NO it has been shown to have an inhibitory effect on both OC formation and activation *in vivo* and *in vitro*. According to some of the authors, it could have a biphasic effects on osteoclastic bone resorption: low concentrations of NO have been shown to potentiate IL-1 induced bone resorption.

It was recently shown for the first time that RANKL induces iNOS expression and NO generation via an NF- κ B and IFN- γ mediated mechanism. This NO would function as an *autocrine negative feedback signal* to limit osteoclastogenesis concurrently stimulated by RANKL.

Specifically targeting this autoregulatory pathway may afford new approaches for therapeutic

interventions in bone diseases characterized by excessive OC formation and bone resorption.

Pharmacological NO - donors have been shown in fact to increase bone mass and these agents may also influence bone turnover in man. These data indicate that the L-arginine/NO pathway would represent a novel target for therapeutic intervention in the prevention and treatment of bone diseases.

Data from the literature indicate that eNOS and estrogen actions in bone are linked: NO is an ubiquitous estrogen-regulated signaling molecule, able to modulate responses to estrogen in the skeleton. Women treated with nitrates on a daily basis have greater hip and heel bone mineral density (BMD) (*Collin - Osdoby et al 2000, Collin - Osdoby et al 2002, Fox et al. 1998, Grassi et al 2006, Kiesel et al. 2007, MacIntyre et al. 1991, Rahnert et al. 2008, Tan et al. 2008, Van't Hof et al. 2001, Wimalawansa 2007, Zheng et al. 2006*).

What do we know About ESW Influencing the Subject?

The effectiveness of ESW in inducing bone healing can be related to direct stimulation of osteoblasts and periosteal cells, and to osteogenic differentiation of mesenchymal stem cells as well (*Martini et al. 2003, Tam et al. 2005, Wang FS et al. 2002*). These events has been described to correlate with enhanced expression of Extracellular signal - Regulated Kinase (ERK), Core Binding Factors (CBFs), Transforming Growth Factors (TGF - beta1) and Bone Morphogenetic Proteins (BMP-2). Moreover, early local production of pro - angiogenic molecules and growth factors seems to stimulate new vessel in-growth, thus improving blood supply and tissue regeneration (*Wang FS et al. 2003, Wang FS et al. 2004, Nishida et al. 2004, Meirer et al. 2007*).

Recently, it has been described that osteogenesis induced by ESW in non - unions, is associated with a meaningfully serum increase of angiogenesis-related growth factors, including eNOS, VEGF, TGF - beta1 and BMP2, as measured at the first month following the acoustic stimulation. (*Wang CJ et al. 2009*).

Cacchio et al. described that increased NO is related, in treated bones, to a mytogenic, osteogenic and angiogenic response (*Cacchio et al. 2009*).

A more recent study evidenced that ESW can influence osteoblastic differentiation and proliferation, via phosphorylation of ERK 1/2 signalling pathway, specific protein kinase of cells proliferation, as cyclin E2/ CDK2 complex, can induce an increase of RNA related transcription factors expression (RUNX2 RNA) and, interestingly, decrease the RANKL/OPG ratio, thus inducing inhibition of osteoclastogenesis (*Tamma et al. 2009*).

All these experimental findings suggest, and confirm, that the relationship between ESW and osteogenesis is a very complex pathway, in which many actors and molecules play different roles on stimulated cells and bone tissue. This complexity is partly related to the fact that exists a strictly interplay between cellular elements that form bone tissue both in normal and pathological conditions: first of all the above described OB - OC coupling and the correlation between OC angiogenesis and viceversa. Nevertheless, we can turn these findings as "the rationale" to employ ESW also for the treatment of vascular bone diseases, both due to an anatomical disfunction (as in Avascular Osteonecrosis or AON), both due to an altered bone turnover, as in Bone Marrow Edema Syndrome (BMES) and in Degenerative Osteoarticular Diseases (DOD) (*d'Agostino et al. 2005*).

In 2008 Wang et al., demonstrated that the regenerative effects of ESW on hip AON are related to angiogenesis, cell proliferation and bone remodelling as well (*Wang CJ et al. 2008*).

The positive influence of ESW on the vascular environment of bones is evident in relieving BMES, characterized by an "inflammatory" patterns in NMR imaging, without necrosis (ARCO stage I) (*d'Agostino et al. 2005*). Bone biopsy, in fact, did not evidence any features of avascular necrosis, while bone marrow abnormalities included accumulation of fluids, fat cells fragmentation, presence of fibrovascular tissue, and especially increased bone turn over with signs of active repair (*Disch et al. 2005, Hoffman et al. 2005*). BMES can be strictly related to the Regional Acceleratory Phenomenon (RAP), induced by different ischemic noxae. RAP has been described to be the loss of local bone homeostasis, characterized by accelerated bone turnover (with increased cell proliferation and activities), increased focal blood flow, but with the risk BME's

evolving in bony ischaemia, probably due to impaired venous outflow (Trevisan *et al.* 2002). Thus, it has been hypothesized that the positive effect of ESW on BME acts by "resetting" the mechanisms that control the bony response to local ischemia, probably by the vasoactive action of NO and some other factors (cytokines, chemokines, growth factors and neuropeptides) (d'Agostino *et al.* 2005).

One question arises: could this regulatory effect of ESW in bone vascular diseases be applied also in osteoarthritis and related osteochondropaties? Which could be the rationale for new applications of ESW in degenerative joint disease?

Consolidated hypotheses individualize in the cartilage damage one of the main causes of osteoarthritis. Nevertheless, more recent evidences suggest that subchondral bone alterations (like induced bone resorption and enhanced local vascularization, thus resulting in an altered bone turnover) are probably the main factors involved in both initiation and progression of the disease, due to some different causes (Anastassadies *et al.* 2005, Spector *et al.* 2005). Really, osteoarthritis features in NMR imaging are often characterized by BMES, notoriously considered a non-specific pattern of reaction, involving bone and its vascular supply, to some different disturbances, rather than an autonomous clinical entity (Hoffman *et al.* 1993, Papadopoulos *et al.* 2003). Moreover, it seems that subchondral bone marrow abnormalities can be considered predictor of radiographic progression of OA, while reduction in the extent of bone marrow abnormalities is related to a decrease in cartilage degradation. There is also an increasing interest in those drugs affecting bone turnover, which might positively interfere with the progression of OA (Garnero *et al.* 2005, Spector *et al.* 2005). In clinical practice, for example, antiresorptive treatments (nitrogen-containing bisphosphonates) can improve symptoms of OA, while reducing bone marrow abnormalities (Carbone *et al.* 2004).

In OA, angiogenesis and inflammation are closely related processes, which may affect disease progression and pain, while angiogenesis can promote also endochondral ossification (EO), responsible for osteophyte formation.

Thus, inhibition of angiogenesis and normalization of altered subchondral bone turnover may provide effective therapeutic strategies in OA (Bonnet *et al.* 2005, Ashraf *et al.* 2008). From this point of view the use of ESW in OA would seem to be contradictory, being well known their pro-angiogenetic effect. Nevertheless, it is to be considered their possible role in influencing the activity of those mediators that, as potential therapeutic targets, can slow or reverse the disease progression. First of all NO (one of the main mediators of ESW mechanism of action), whose regulatory role in vascular flow is well known, and a protective substance in acute inflammation and harmful in chronic inflammation (Mariotto *et al.* 2009, Moskowitz *et al.* 2004).

Another pathological condition determined by altered bone turnover is osteoporosis. Recent evidences from the literature seem to indicate that ESW could enhance local Bone Mineral Density (BMD), by inducing new bone formation in osteoporotic non-weight-bearing bone; more in details, shockwaves should enhance the osteogenesis of normal bone remodeling cycle through activation of osteogenic cells and it has been proposed that they may be an effective preventive approach to retard osteoporosis in osteoporotic fracture sensitive regions (Tam *et al.* 2009).

Another recent study seems to further confirm that bone microarchitecture can be positively affected by unfocused shock waves, by inducing biological responses without gross damaging effects. The authors described that unfocused ESW mainly affect the bone dynamics of existing surfaces and does not induce de novo bone formation (Van Der Jagt *et al.* 2009).

Outcome: Whose Would be the Most Interesting Steps in Future Research of this Subjects?

Recent data on the pathogenesis of bone vascular diseases, focalize the role of altered osteogenesis and bone turnover in the pathogenesis of osteoarthritis, osteoporosis and related pathologies.

Nevertheless, *in vitro* and *in vivo* experiments gave us growing evidences on the relationship between OC dysfunction, altered angiogenesis and all *bone diseases* (characterized by altered osteogenesis and local

tissue turnover), in which, other than cytokines, IL, growth factors and some other molecules, NO seems to have an important key - regulator role.

Thus, clinical and experimental trials are needed to confirm, in perspective, the possibility to employ ESW for controlling local bone turnover, thus providing a rationale for treatment of degenerative joints diseases and the polymorphical expressions of BMEs - AVN, and osteoporosis as well.

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Measurements of acoustic energy deflection in the presence of replica bone

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Abstract

The presence of musculoskeletal tissues can significantly distort incoming therapeutic shock waves. Instead of propagating undisturbed directly from the source to the focal region, the acoustic energy can be deflected and/or absorbed by intervening tissue, especially bone. The present study examines the deflection of acoustic energy, showing that energy deposition may not occur where expected. A model talus bone was manufactured and placed in a water-filled bath. Shock waves from an electrohydraulic lithotripter were focused on the talus. Cavitation bubbles generated by the negative pressure were imaged using a high speed camera. The bubbles mark the path of negative pressure. In parallel with the experimental work, we simulated the acoustic path under similar conditions using sophisticated numerical techniques. The results indicate that energy deposition can be significantly altered by the presence of bone tissue.

Keywords: energy deposition, cavitation, bone

Introduction

The propagation characteristics of shock waves used in clinical treatment are complicated by the physical mechanisms used to generate the energy. Sources can be focused, unfocused, de-focused, etc. Some devices don't generate actual shock waves at all, but instead, they generate 'pressure waves'. Furthermore, the incident energy can also be highly variable, for a given device, or among devices. Because there is such high variability, it can be difficult to compare the results of treatments between different machines. Often, physical comparisons are made by measuring the acoustic characteristics (peak positive and negative pressure, intensity, energy density, etc) from experiments in a water bath, and

extrapolating the results to soft tissue. This is often not a bad assumption, because soft tissue doesn't differ too radically from water. In soft tissue, the peak pressure appears slightly distal of the geometric focus and the negative pressure peaks proximal because of the nonlinear acoustic process self-refraction [1-3].

For these simple systems, models have been developed to accurately predict the acoustic propagation in water or even to some degree in (homogeneous) soft tissue [4, 5]. However, these models generally rely on axisymmetry, can predict only one direction of wave propagation (no reflections) and do not contain mode conversion or a method to account for the elastic properties of an object such as bone.

In both experiment and models, the presence of bone is often neglected. However, the presence of bone dramatically changes the classic hour-glass shaped focal zone that is often shown in the literature [6]. The presence of complicated bony structures may dramatically alter the acoustic path, cause refocusing at a new location, shield areas from shock wave exposure, and perhaps excite complex vibrations in the bone and in particular along the bone surface at tendon bone junctions.

As an example of how bones can deflect energy, we show a 2-Dimensional representation of an ankle, pressed against a water pillow in **Figure 1**.

Simulated acoustic energy is incident on the simulated ankle from a shock wave source on the left (not shown). The acoustic energy propagates into the soft tissue of the ankle, and then into the bones. Because of the difference in acoustic properties of bone relative to tissue, the acoustic energy, both positive and negative, can be deflected. In this example **Fig. 1(a)** shows the initial shock wave reaching the ankle from the spark discharge source. The arrival time is 160 μs after spark discharge. In **Fig. 1(b)**, the positive pressure has entered the ankle. Part of the positive pressure has entered the calcaneus and because the velocity is greater in bone, it has propagated further into the bone than in the surrounding tissue. At 190 μs , both the positive and negative pressure has worked its way through the bones and tissue of the ankle [7].

This coarse example shows only the instantaneous pressure at three different times. When the model is validated, it can be used to predict the highest concentration of positive or negative pressures, as a function of variables that are controlled by the clinician (orientation of the shock waves, intensity, etc.). The purpose of this paper is to examine shock wave propagation near and against musculoskeletal tissues in the ankle, specifically the talus, in order to better understand where and how the energy gets deposited, and to compare those results to a model. The long term goal

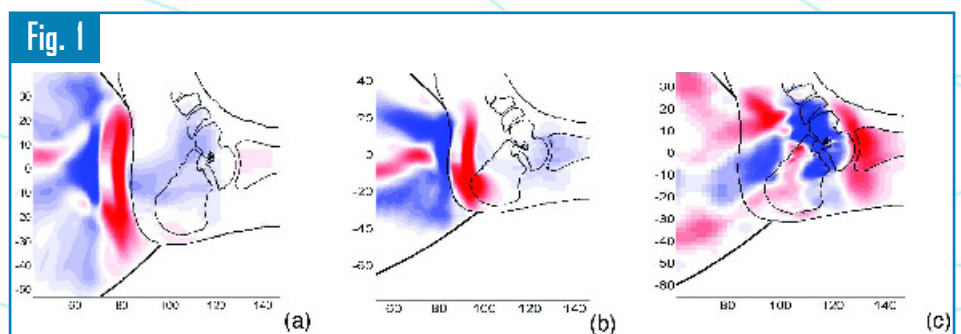


Figure 1: Snapshot in time of simulated shock wave propagating into an ankle. (a) $t=160 \mu\text{s}$, (b) $t=170 \mu\text{s}$, (c) $190 \mu\text{s}$ after spark discharge. The red color indicates positive stress, blue indicates negative stress.

of this research is to use actual patient MRI/CT scans in conjunction with simulations to provide patient-specific shock-wave treatment protocols of musculoskeletal injuries.

Materials and Methods

The mathematical model is relatively simple, using linear elastic, non-conservative equations to describe the musculoskeletal tissues, and solve the Riemann problem using a reconstructive evolve average algorithm based on a freely-available software package called CLAWPACK [7]. For purposes here, the most important aspect of the model is that it can handle 3-Dimensional objects, which is important for actual musculoskeletal tissues. The model is used below to compare with our physical replica experiments.

Physical replicas of the human anatomy, specifically the talus, were constructed from computed tomographic (CT) images with Rapid-Prototyping technology. The replicas were built from acrylic to provide transparency and because it is birefringent (designed for other experiments not described here) [8,9]. The CT images were scanned and segmented into a 3-Dimensional surface model that can be easily used to generate the physical replica and also used in computer simulations. An example of the virtual and physical model is shown in **Figure 2**.

The replica is placed in water. For hydrophone measurements, a needle hydrophone is used to record the relative pressure near the replica. For measurements of cavitation, a high-speed camera is used to image the bubbles using backlighting.

Two different shock wave sources were used for these experiments. An electrohydraulic spark discharge device, a replica of the Dornier HM-3 Lithotripter was used to image cavitation [2]. The device was operated at 22 kV, 2-Hz pulse repetition frequency. A Storz Masterpuls MP100 ballistic source was used for the hydrophone measurements. The ballistic source was pushed against a stretched tegaderm membrane with coupling gel to simulate application at the skin surface. The membrane forms a barrier to a tank full of water. A needle hydrophone is placed in the water to measure the relative acoustic signal. For both devices, the objective was to determine how the acoustic field would change if an object (in our case, a replica talus) is placed in the

path of the acoustic wave.

Results

Figure 3 illustrates how the replica talus changes the focal region of the ballistic source. They show a slice (orthogonal to the direction of propagation) of the pressure field in the presence and absence of the replica. In both cases, the hydrophone measurements were taken at the same location, but in (b) the talus was placed behind the hydrophone.

The talus modifies the pressure field, creating additional hot and cold spots (high and low pressure, respectively) that are otherwise absent from the field. In particular, at least two additional hot spots are observed to the left of the original focus. Also, the pressure amplitude at the focus in the absence of the talus is reduced when the talus is inserted.

The cavitation results are equally instructive. **Figure 4** illustrates how the cavitation field is deflected when the replica talus is brought closer and closer to the focal zone of the electrohydraulic source. In these series

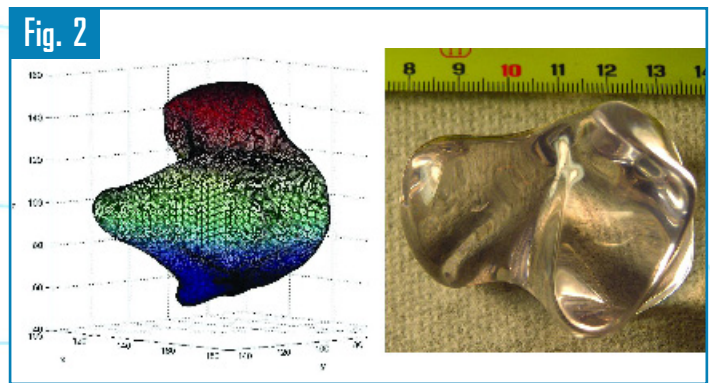


Figure 2: LEFT: Virtual model of a talus from a scanned CT image [10]. RIGHT: Physical model of a talus built from the virtual image using rapid-prototyping.

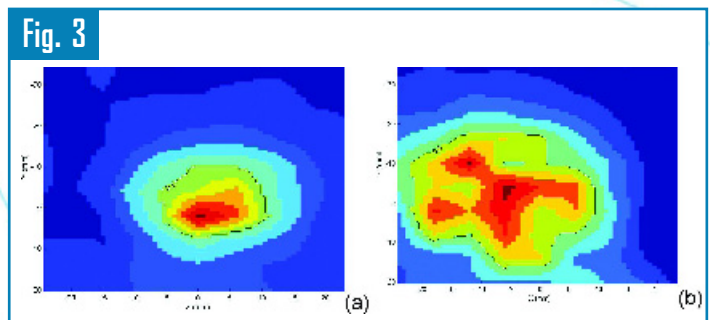


Figure 3: A Storz Masterpuls MP100 ballistic source is imaged by a needle hydrophone placed in the water. (a) Cross-sectional pressure field map in the free field, in the absence of any object. (b) Similar cross-sectional map with the talus replica placed behind the hydrophone. The talus modifies the pressure field, creating 'hot' spots near $[x,y] = [-17,3]$, $[-5,10]$, and $[12,-10]$ mm. A 'cold' spot occurs at $[0,0]$, the location of the original focus.

of photos, the high-speed camera catches the cavitation bubbles generated by the negative pressure of the shock wave (coming from the left). As the talus is moved closer and closer (labeled A-H) to the focal zone (defined by the bubble cloud), there is a real deflection of the bubble cloud. Since the bubbles are created by the negative focal pressure, the images of the bubbles also coincide with the negative pressure focal zone.

The numerical results are shown in **Figure 5**. Each image shows the deflection of the shock wave's tensile component with different bone

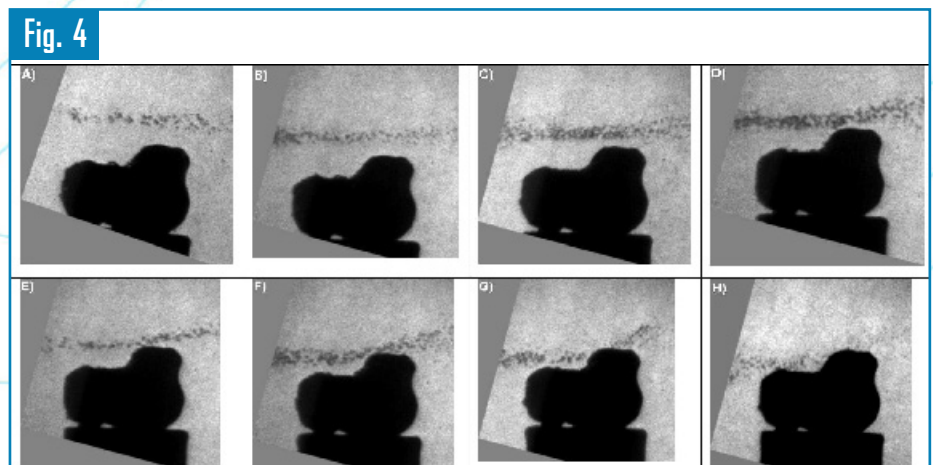


Figure 4: Laboratory experiment where a shock wave is incoming from the left and the tensile component of the wave creates a cavitation field. The interaction of the cavitation field with the talus is imaged with a high-speed camera. The bone height relative to the focal point changes from left to right where height corresponds to difference from the focus, in millimeters (A. -12, B. -9, C. -6, D. -3, E. 0, F. 3, G. 6, H. 9, I. 12).

Fig. 5

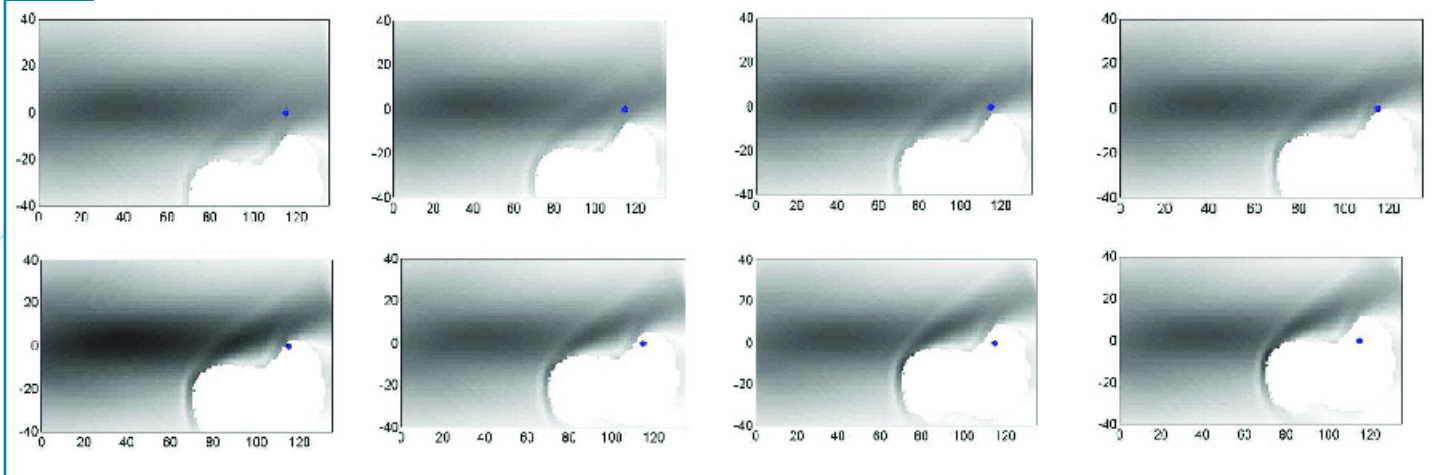


Figure 5: Maximum tensile component of the pressure wave (dark band) along a two-dimensional slice of the full 3D calculation. The dot is the location of the focus and the bone is shifted in the z-direction relative to the focus as in Figure 4.

locations. The regions of maximum tension (dark bands) indicate where cavitation would occur, thus, this is the basis for comparison. Due to the linear nature of the equations and computational limitations on the level of refinement, the pressure wave is more smeared out than in the laboratory experiment [10]. As a result, the pressure wave interacts more with the front of the bone than the more focused laboratory shock wave. The deflection of the wave path due to the bone geometry is different from the laboratory result, however, the general behavior is similar in that there is an upward deflection of the cavitation path.

Discussion

The experiments discussed above were performed with an electrohydraulic spark-gap device and a ballistic source. The acoustic output of these devices is very different. The ballistic source may not even generate an actual 'shock' wave. Nevertheless, the presence of bony structures will change the pressure field for any device. Acoustic energy will be absorbed, scattered, and re-focused.

The ballistic source hydrophone measurements show that when the talus replica is inserted into the field, the original 'focus' region changes dramatically. The focus becomes distributed over a larger region, with 'hot' and 'cold' spots. The 'hot' spot size increases from about 10mm to about 30 mm in size. The original focus shifts about 5 mm pre-focal. These results would change if the talus replica were

rotated, or if a different replica or real bone were inserted.

The cavitation measurements made with the Dornier HM-3 lithotripter clone reveal a similar phenomenon, but perhaps more visual. The line of bubbles generated by the negative pressure of the shock wave provides a good visual reference of the focal zone of the HM-3. In the free-field, the line of bubbles corresponds to the usual focal zone of a focused shock wave device - an hour glass. However, when the talus replica is brought closer to the focal zone, the shape of the focus actually changes, bending away from the replica. This bending suggests that when a shock wave is incident near bony structures, the acoustic energy is deflected to a different region. In this example, the focus shifts by about 15 mm or so (this is the difference in height between the original cavitation zone and the deflected zone in pictures A and H in Figure 4). As with the hydrophone measurements, the cavitation field deflection will depend on the orientation of the shock wave source and the bony structure.

Conclusion

The experiments discussed here provide just a small example of how the presence of bony structures can deflect acoustic energy. The deflected energy can be refocused to different locations, creating 'hot' and 'cold' spots, even centimeters from the expected focal region. These results suggest that the position and orientation of the shock wave source may have dramatic effects on the efficacy of shock wave therapy.

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Mechanotransduction – Mediators, Sensors, and Effects on Tissues and Stem Cells



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1. Introduction

Biological tissues possess the ability to sense a variety of different kinds of stress modes. These tissues include for instance tendons, cartilage, skeletal muscle, connective tissue, endothelium or epithelia. The mentioned biological structures are regulated, processed, and maintained by diverse stimuli that directly or indirectly fulfill their specific tasks in all kinds of biological structures. Among others the most important stimuli and the best characterized ones are hormonal stimuli, inflammatory stimuli, metabolic stimuli, and mechanical stimuli. In this review the focus will be put on the influences of mechanical stimuli on the described biological materials. Furthermore, the review will highlight some crucial aspects, such as 1) what mechanotransduction means and how it is sensed, 2) whether and how tissue plasticities can be influenced by mechanical impulses, and 3) in which way these kinds of stimuli can activate and regulate stem cells.

2. What does mechanotransduction mean?

Mechanotransduction in general means the sensing and transmission of externally induced mechanical forces into a cellular system. In a recent paper, Jaalouk and Lammerding (2009) define mechanotransduction as follows: “Mechanotransduction describes the cellular processes that translate mechanical stimuli into biochemical signals, thus enabling cells to adapt to their physical surroundings.”

According to this definition mechanical stimuli induce two rebuttals that have to be temporally discriminated. On the one hand, mechanical stress will generate acute functional responses in the affected cell types leading to rapid cellular shifts, like conformational changes of proteins or posttranslational modifications, including phosphorylation, acetylation or methylation. On the other hand, mechanical forces applied for a longer

period of time will remodel affected tissues in the way that the tissues' plasticity will be modulated. These fundamental modifications are associated with both structural and functional adaptations of the tissues (such as skeletal muscle, connective tissue, endothelium or epithelia).

The process of cellular mechanotransduction follows several steps including different phases finally resulting in cellular responses and adaptations. The first phase is a *signal transduction phase*. This phase is a highly complex phenomenon as diverse cellular mechanisms are switched on to lead to the *signal propagation phase*. The primary level of this complex cellular interplay is the force transmission into the tissue where it can be sensed mechanically. This mechanical signal has to be transduced within the cell into a biochemical signal to activate the downstream connected signal transmission. This event in turn will result in a molecular activation of a network of cellular signals. Important signals and signaling pathways activated by mechanical stimuli embrace calcium concentrations and calcium-dependent pathways, mitogen-activated protein kinases (MAP kinases) (e. g. extracellular-signal regulated kinases, protein kinase B [Akt], p38 kinase, JNKs, etc.), second messenger systems, like cyclic adenosine monophosphate etc. The sum of these signals downstream the initial mechanical stimuli direct to the already mentioned *signal propagation phase*. This phase is crucial in the way that specific transcription factors are activated to enter the cell nucleus to induce and to regulate specific gene transcriptions. Mechanically activated transcription factors comprise for instance hypoxia inducible factor-1 α (HIF-1 α) or NF- κ B. The events taking place in the *signal propagation phase* are essential for mechanotransduction, because these shortly described effects arise in a functional response or structural and functional adaptation and remodeling of the mechanically treated tissue, called

cellular response phase (Wu *et al.*, 2009).

To get an idea about the mechanisms available for a cellular system for sensing mechanical stimuli generated in the exterior periphery of a closed system, different kind of sensors and mediators of mechanical stress will be discussed briefly in the coming part. The sensors of mechanical stimuli have to be localized in the exterior periphery of the cellular system and in parallel as well as within the cell interior. This seems to be reasoned as the mechanical signal can be trapped in both the ECM and the cytoplasm induced by external stress and internal cellular movements and deformations.

Indirect sensors and mediators of mechanical forces. The best described and characterized indirect sensors and mediators of mechanical stress are growth factors (GFs) and hormones. To sense a certain stimulus in the first place these GFs and hormones have to be located extracellularly. In the extracellular matrix these molecules are attached covalently to heparan sulfate proteoglycans. This extracellular storage pool of GFs and hormones is critical for both rapid cellular responses and long-term adaptations, because the GFs and hormones are cleaved off from their respective storage anchor proteoglycan to be released into the extracellular space (Suhr *et al.*, 2009a). In a subsequent step these bioavailable proteins can bind to their receptors which are generally transmembrane receptors, such as receptor tyrosine kinases. The binding of GFs and hormones to their receptors elicits molecular responses in the cytoplasm. The essential mechanism is the activation of several signaling cascades (as mentioned above). These signaling cascades regulate a variety of rapid responses and/or permanent adaptations. For instance, by acting in the described manner GFs and hormones 1) regulate gene expression profiles and signals that have a major role in protein turnover; 2) signal transductions influence cellular

metabolism and mitochondrial biogenesis; 3) GF- and hormone-directed signaling pathways can lead to changes in cellular structures, protein translocation, protein bundles and assemblies, as well as protein conformations. Summarized, GFs and hormones influence in a critical way cellular functions as examples 1) to 3) will ultimately empty in a re-arrangement of cellular functions.

Another class of interesting and recently in our lab focused indirect mediators are different families of proteases, especially cysteine (cathepsins L)- and metalloproteases (MMP-2 and MMP-9). It could clearly be demonstrated that mechanical forces in human tissues lead to the activation of these proteases resulting in the cleavage of basement membrane located collagens and proteoglycans (Suhr *et al.*, 2009c; Suhr *et al.*, 2007). The liberation of cleavage fragments from basement membrane constituents, such as endostatin, “transport” the mechanical stimulus to certain appropriate receptor systems localized on the cell membrane. The receptor systems include the receptor tyrosine kinases, integrins, syndecans, or glypicans (Suhr *et al.*, 2009a). Thus, on this note by cleaving and processing different kinds of ECM constituents following a mechanical trigger moment, proteases possess crucial functions as indirect mechanical mediators and sensors beside GFs and hormones (**Fig. 1**).

Direct sensors and mediators of mechanical forces. It is reviewed in an excellent manner elsewhere (Ingber, 2006), what kind of direct mechanical sensors and mediators are available in a cellular system. Here, the most critical ones will be shortly described and discussed.

Cell-cell contacts, including cadherins and gap junctions, are critical players in the translation of mechanical forces into physiological signals. Cadherins are calcium-dependent transmembrane glycoproteins that secure and stabilize cell-cell adhesions. By mediating signals which are critical for cellular differentiation the importance of this protein class can be estimated. Cadherins operate their signal transductions in the intracellular space by α - and β -catenin subunits, which are connected to the actin cytoskeleton of eukaryotic cells. Gap junctions display cell-cell channels composed of connexons. Each

connexon in turn consists of six connexins. Gap junctions therefore are clusters of proteins that have functions in channel-forming processes. These cell-cell channels have crucial roles in direct mediation of signals as a variety of molecules can pass through these channels to get from cell 1 to cell 2. Gap junctions transport inter alia nucleotides, which are important for gene expression regulation, amino acids for protein synthesis, ATP for energy supply, glucose, all kinds of ions, water, etc.

Cytoskeleton proteins encompass three major protein classes. Microfilaments are clusters of proteins that possess central roles in the movement of cells, in the mechanical stabilization of cells as well as in signal propagations. The basic microfilament protein is actin. Microtubules are a second class of the cytoskeleton and they are composed as tube-like structures. Comparable to microfilaments the microtubules are involved in cellular movements, stabilizations, and signaling. A further central challenge of microtubules is the transportation of vesicles and cell organelles, as they are associated with motor proteins like dynein and kinesin. The third class of mentionable cytoskeleton proteins are the intermediate filaments which are located between the microfilaments and the microtubules providing additional structural and mechanical support for cells during deformations and movements. All the mentioned systems can sense and can react on mechanical stress. In response to those stresses, externally or internally signals will be generated. Additional important intracellular mechanical sensors include z-disc proteins, like cardiac ankyrin repeat protein (CARP) and muscle ankyrin repeat protein (MARP) (Kemp *et al.*, 2000; Jeyaseelan *et al.*, 1997).

ECM proteins include a large number of huge and highly glycosylated proteins. Major protein classes are fibronectins, collagens, proteoglycans, and basement membrane proteins, such as laminins. These protein classes function in a crucial manner as tension sensors in the ECM. That means that these proteins are responsible for a sensitive registration of mechanical forces mediated by the ECM towards the cellular plasma membrane. Fibronectins, collagens, proteoglycans, laminins etc. show high affinity and strong binding properties to

components of the focal adhesion complex, like integrins or other surface receptors, such as proteoglycans. Due to their tight connection to transmembrane proteins that are linked to the cytoskeleton these extracellular tensile elements own a great significance as mediators and converters of mechanical stress into biochemical signals. Downstream of this complex protein network machinery a multitude of signaling molecules can be activated or inhibited to amplify or suppress cellular responses and adaptations (**Fig.1**).

Cell-ECM adhesions comprise a cluster of proteins known as focal adhesions. Focal adhesions can comprise up to 50 molecules and are one of the most critical sensors and mediators of mechanical stimuli as these protein bundles are directly associated with the cytoplasmic actin cytoskeleton. A central protein class of all focal adhesions are integrins, which consist as ?? heterodimers. Furthermore, focal adhesions include signaling molecules in the intracellular space, such as focal adhesion kinase (FAK), integrin-linked kinase (ILK), s-SRC and additional tyrosine kinases (Jaalouk & Lammerding, 2009). Geiger and colleagues (2009) outline the importance of integrin-mediated adhesions in the transformation of mechanical forces into biochemical signals: The integrin focal adhesions sites consists of about 160 components. Furthermore, an analysis of molecular interactions of the involved 160 components point out that almost 700 links exist between the elaborated constituents of the entire network (Geiger *et al.*, 2009).

By summarizing the term ‘mechanotransduction’ it should be highlighted that this mode of translating exterior impacts into cellular responses and adaptations is of an outstanding importance for all cellular systems. A force applied in the ECM environment is sensed by ECM proteins connected to focal adhesion complexes, especially to integrins. Integrins are connected to the cytoskeleton consisting of F-actin, vinculin, talin etc. The F-actin molecules in turn are associated by Nesprin 1 and Nesprin 2 to the inner nuclear envelope localized protein SUN1 (Dreger *et al.*, 2001). This connection is important as SUN proteins are indirectly attached to the chromatin and DNA within the nucleus (Haque *et al.*, 2006). By this connection network, mechanical

signals can be propagated into the nucleus within milliseconds and therefore execute for example alterations in gene expression extremely fast (included transcription factors: NF- κ B, GATA4, NFAT, STATs, etc.).

Thus, the cell is allowed to respond directly to the applied mechanical forces and can rapidly re-arrange the cellular setup, like protein synthesis, protein localization, protein degradation, posttranslational modifications of proteins and genes, etc. This fact is in strong contrast to indirect mediators and sensors, like GFs. GFs-mediated (mechanical) signals take up to several seconds until they reach their final destination, the nucleus.

As concluding remark it must be underlined that indirect and direct mediators and sensors of mechanical stimuli do not act and function separately. Both systems show tight connections and interplays, which make the molecular analysis and understanding of these network and interdependences excessively complicated.

3. What effects has mechanotransduction on tissue plasticity?

The tissue plasticities as well as structural and functional properties are highly dependent on the described indirect and direct mediators and sensors of mechanical stress. In this part, some recent key findings of tissue plasticity in response to mechanical stress in cartilage, tendon, skeletal muscle and vascular systems will be highlighted.

Tendon/Cartilage. Tendons and cartilage consist mainly of fibroblasts and chondrocytes. These tissues are typically highly stressed by mechanical impacts. Therefore, the study of these cell types *in vivo* permits insights into the effects of mechanical stimuli on tissue plasticity. Of course, these cell types contain the discussed mediators and sensors to translate the stimulus into biochemical signals. Once, the stimulus is translated kinases and transcription factors (MAP kinases, NF- κ B, PKC) are activated to initiate the transcription of different genes (e. g. collagen- or proteoglycan-coding genes) (Kjaer, 2004). In tendons, it could be demonstrated that mechanical stimuli induced by physical exercise lead to increased stiffness of this tissue (Arampatzis *et al.*, 2007). This increase might be related to changed cross-linker proteins of collagens and changes in the

fibril morphology of the stimulated ligaments (Hansen *et al.*, 2010; Kongsgaard *et al.*, 2010). Our group could recently demonstrate in cartilage that mechanical stimuli have clear impacts on central signaling molecules. Cartilage of porcine patellofemoral joint cartilage was used to assess the effects of dynamic and static loads on protein kinase B (Akt) in a dose- and time-dependent manner. It was clearly demonstrated that both types of mechanical loadings have significant influences on Akt phosphorylation in cartilage. In comparison to control tissue, phospho-Akt was downregulated 300 sec after the mechanical stress application (Niehoff *et al.*, 2008). These findings are highly interesting as the demonstrated temporal changes in Akt activation may have important roles in cellular signaling pathways, responses and time-delayed adaptations.

Skeletal muscle. Myocytes and myoblasts are the main cell types in skeletal muscle tissue. These cell types produce skeletal muscle specific proteins, such as actins, myosins, etc. Comparable to tendon and cartilage tissue, also skeletal muscle cells are exposed to mechanical loadings. The skeletal muscle tissue has developed different strategies to react and adapt to these impacts on variable levels. Mechanical stresses activate different signaling cascades in skeletal muscle cells, including Akt, IGF-dependent signals, PI3K, mTOR; p70S6K. These signaling molecules possess central abilities to regulate and re-arrange the plasticity of skeletal muscle tissues (Tidball, 2005). Gibala and colleagues (2006) showed that intensive cycling exercise has a strong impact on targets of the respiratory chain in skeletal muscle mitochondria. Mechanical stimuli also exert effects on satellite cells and thus regulating skeletal muscle regeneration after injuries, as demonstrated in both *in vitro* and *in vivo* (Tatsumi *et al.*, 2001; Mackey *et al.*, 2007). Our own data investigating phospho-Akt in the musculus masseter show interesting results as the muscle tissue seems to command a defined threshold regulating the activity of phospho-Akt (Korkmaz *et al.*, unpublished data). Further recent results from our lab indicate that physical exercise combined with vibration training resulting in highly intensive mechanical stimuli in the working skeletal muscle tissue induces rapid remodeling processes in the ECM of skeletal muscle tissue (Suhr *et al.*, 2007). Endostatin, the C-terminal

cleavage fragment of collagen XVIII, and MMP-2 and MMP-9 were increased after this kind of mechanical application a time-frame up to 4 hours post exercise. This finding indicates a strong potential of mechanical forces in the rapid remodeling of skeletal muscle tissue. In a current study (Suhr & Bloch, unpublished data) we can demonstrate that severe eccentric running exercise leads to the downregulation of several ECM- and connective tissue-related genes in rat skeletal muscle. For instance, mRNAs of integrins α 5 and α 7, collagens 1 and 3, as well as prolyl 4-hydroxylases α 1 and α 2 are significantly downregulated compared to sedentary controls. This study indicates that severe eccentric stimuli seem to influence skeletal muscle gene activation.

Vascular system. Endothelial cells of the vascular system are permanently exposed to a certain form of mechanical stress, namely shear stress induced by blood flow profiles. It was demonstrated by several investigations that shear stress activates signaling molecules, such as MAP kinases in endothelial cells (Michel & Feron, 1997). Side to the permanent exposure to mechanical influences the vascular system is a highly sensitive compartment susceptible for mechanical stress. The mechanisms underlying these phenomena are comparable to those observed in skeletal muscle. Mechanical stress activates indirect (e. g. MMPs and GFs) and direct (e. g. integrins, collagens, and proteoglycans) mediators and sensors that activate different signaling cascades in endothelial cells to regulate cellular responses (Suhr *et al.*, 2009a), such as proliferation and migration of endothelial cells or nitric oxide synthesis in endothelial cells and red blood cells (Kleinbongard *et al.*, 2006). Increased shear stress profiles induced by physical exercise were lately shown to exert the ability to increase and activate the endothelial nitric oxide synthase in human red blood cells and thus contribute to the microvascular environment in a prominent manner. This regulatory pathway seems to be PI3K- and Akt-dependent, but further research is needed to verify this hypothesis (Suhr *et al.*, 2009b). Interestingly, the former as anti-angiogenic characterized peptide endostatin, derived from collagen XVIII of basement membranes of endothelial cells (O'Reilly *et al.* 1997, PMID: 9008168) was demonstrated to function as a modulator as this molecule induces vasorelaxation by activating the nitric

oxide synthase pathway in the vascular periphery (Wenzel *et al.*, 2006).

4. What role has mechanotransduction on stem cell activation?

Regeneration, repair and growth of tissues are dependent on addition of new differentiated cells, these cells can mainly be delivered from undifferentiated cells which have the potential to proliferate and differentiate. These cells are called stem cells or progenitor cells (Beausejour, 2007). The stem and progenitor cells can be found in nearly all organs, where they can be activated by different stimuli. The stem and progenitor cells must be activated and mobilized before they can help to regenerate, repair or expand tissue. Stem and progenitor cells can be activated and mobilized by different stimuli and mechanisms (Rabbany *et al.*, 2003; Schmidt *et al.*, 2006). Mechanical stimuli are one of the central mechanisms which can activate stem and progenitor cells.

A major source of stem cells in the adult organism is the bone marrow which contains hematopoietic and mesenchymal stem cells (MSC). Especially for MSC recent findings demonstrate the activation by mechanical stimuli. It could be shown that the mode of mechanical load influence signal transduction of MSC, which can lead e.g. to a specific lineage commitment, alteration of proliferation and matrix remodeling (Kurpinski *et al.*, 2006) and by this way to tissue regeneration, repair and growth.

Beside of effect on growth and differentiation of stem cells, the migration and endothelial transmigration of stem cells can be influenced by mechanical stimuli which can be performed by focused ultrasound-mediated microbubble stimulation (hf-UMS) or by shock wave treatment. We could recently show that a pretreatment of MSC by hf-UMS leads to an improved trans-endothelial migration of MSC in the myocardium (Ghanem *et al.*, 2009) and unpublished data show that shock wave can influence migration of MSC in a dose dependent manner. Beside of them our investigations give further evidence that shock waves influence also proliferation and apoptosis in a dose dependent manner. This offers new therapeutic options for the use of shock waves as inductors of mechanical stimuli for activation of

MSC and possible also other stem and progenitor cells.

It can be concluded that stem cell and Progenitor cells are important targets of mechanotransduction also induced by shock wave.

5. Conclusion

Mechanical stimuli possess a great potential in regulating cellular responses and adaptation during cell development, tissue repair, regeneration and growth as well as in functional regulation of organs, tissue and cells. A multitude of mechanosensitive structures and molecules are involved in transmission of the mechanical stimuli in biological response by different signal pathways. The complexity of mechanotransduction indicates a strong dependency of biological effects by the mode of mechanical stimuli e.g. kind, direction, frequency, duration and intensity. Beside of the differentiated cells and tissue, stem and progenitor cells are an important target for mechanotransduction. Methods which can produce defined and localization directed mechanical stimuli, such as shock wave, can help to use the potency of mechanical stimuli for regulation of functional and structural adaptations of organs, tissues and cells.

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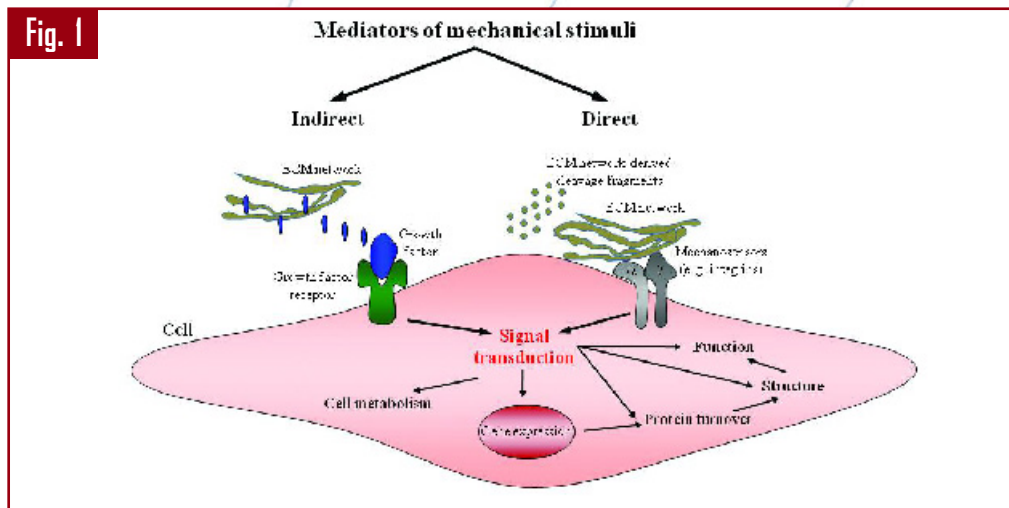
7. Figure Captions

Figure 1: Mediators of mechanotransduction. Mechanical stimuli can be sensed and mediated by either indirect or direct sensors. Indirect mediators and sensors are growth factors which will be released from the ECM and bind to their specific receptors. ECM constituents (like collagens, proteoglycans etc.) directly sense mechanical tensions mediating these to mechanosensors, such as integrins. These molecules thus serve as direct mediators and sensors. Both modes of force transmission lead to the activation or inhibition of a complex signaling pathway network in the cytoplasm (for detail see text). The activated or inhibited signaling pathways play crucial regulatory roles in cell metabolism, gene expression, protein turnover, structure, function, etc. of cells.

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Short communication:

Influence of Shock Wave Treatment on Connexin Expression of Cardiac Cells In Vitro

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Introduction

Recently it is well known that in vitro shock wave treatment (IVSWT) has a high impact on cardiac cells. Previously we described that IVSWT causes changes in the expression of several kinds of proteins, induces

proliferation of all cardiac cells and even decreases the duplication time of cultured cardiomyocytes significantly. As Connexins (Cx) are of high importance for cell communication our goal was to analyze Cx40, Cx43 and Cx45 that are expressed between

cardiomyocytes. Cx are forming the main part of gap junctions [1,2]. Gap junctions allow direct exchange between neighbouring cells of small hydrophilic molecules and ions less than 1-2 kDa in size including metabolites and messengers such as sodium, potassium, calcium, cAMP/cGMP, ADP/ATP and inositol 1,4,5-triphosphate resulting in the metabolic and electric coupling of cardiac cells [3].

Materials & Methods

H9C2-cardiomyocytes (American Type Culture Collection) were used. A thermostatically controlled water bath was designed to avoid distracting physical effects. Adherent cells in common cell culture flasks fully filled with culture medium were dunked into the water bath. Unfocused SWT at an energy flux density of 0.15 mJ/mm²

were applied with a frequency of 5 Hz. Non-treated cells were used as controls. RT-PCR was performed for analysis of Cx40, Cx43 and Cx45.

Preliminary Results and Discussion

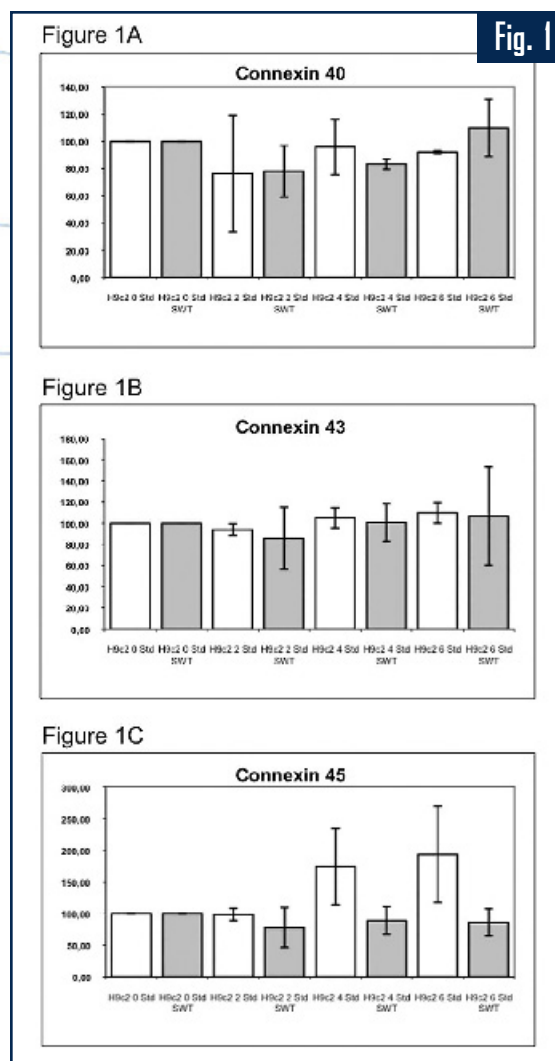
Expression of connexin 40 slightly increases 6 hours after treatment whereas connexin 43 remains nearly unaltered [Figures 1A-1B]. It is well known that Cx 43 transcription is up-regulated in different kinds of heart pathology, e.g. by a chronic mechanical load due to hypertension [4]. Connexin 45 shows a markable decrease compared to untreated cells starting 2 hours after treatment, remaining clearly down-regulated between 4 and 6 hours [Figure 1C]. Several analysis of this experiment are pending.

Physical stimulus of shock waves leading to numerous endogenous biological effects may on one side be directly mediated by mechanotransduction through activation of cytosolic components as well as the nucleus. However, mechanical forces also cause changes of the extracellular matrix thereby leading to the release of several kinds of growth factors. Nevertheless, the induction of tissue regenerative effects needs a functional and maybe even

increased cell communication. Therefore knowledge about connexin expression seems to be one important topic for better understanding of shock wave mediated effects. A lot of further research in this field remains necessary.

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Figures 1A-C: Expression of Connexines 40, 43 and 45 in H9c2 cardiomyocytes. White bars=controls, grey bars=treated cells.

Short communication:

Influence of extracorporeal shock waves on in vitro keratinocytes - Preliminary results

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Introduction

There are wide and diverse fields of application of extracorporeal shock waves in general, the scope constantly expanding. Lately, shock wave application has been put to use in the therapy of chronic dermal lesions such as ulcers or in the treatment of patients with II°-burns. Our trial is intended for the establishment of an *in vitro* treatment for keratinocytes to stimulate proliferation. A refined

methodology for the cultivation of cell cultures, and keratinocyte sheets in particular, could ultimately promote wound closure and wound treatment. The experimental setup was developed with close reference to the works of Holfeld et al. ["The importance of a standardized model for shock wave in-vitro trials - a proposal plus preliminary results of cardiac cells"; Abstracts ISMST 11th International ISMST Congress Juan les Pins 2008].

Materials & Methods

Primary keratinocytes harvested from human biopsies were cultured in modified serum-containing medium (DMEM) with different nutrients. Unfocused shock waves were generated with the Ortho-Wave 180C® by MTS Europe GmbH, Konstanz, Germany using reflector type CP 155. They were applied as single shock wave treatment to subcultures via a thermostatically controlled water bath using the IVSWT-Water Bath by Johann Hohenegger, Katzelsdorf, Austria. Frequencies (1, 3 and 5 Hz), numbers of pulses (25, 50 and 100) and the distance between applicator and cell culture flasks (5, 6 and 7 cm) were modified while keeping a constant total energy flux density of 0,1 mJ/mm². Untreated cells were used as a control group. Cell counting was primarily conducted by a haemocytometer and was eventually verified via LDH concentrations. For

Cytotoxicity we measured LDH concentrations after 15min, 4h, 72h, 120h and 168h in the respective supernatants. Metabolic activity was quantified through glucose/lactate consumption after 72h, 120h and 168h. Microscopical control for morphological cell alteration was ensured at all times.

Preliminary Results

The data for a distance of 7 cm shows no verifiable positive effect of shock wave treatment on keratinocyte cultures. (Figure 1a). Neither different frequencies nor impulse rates resulted in an increase in cell numbers or viability compared to the control group. The reduction in distance of 1cm to 6cm resulted in a measurable albeit minimal increase in cell numbers and cell viability: cell counts were closer to

the amount of viable cells in the control group [Figure 1b], expressing a slightly elevated/equal level of glucose consumption of most treated samples in comparison to the control. Currently, the best results with regard to a positive impact on cell viability can be demonstrated at a distance of 5 cm. In particular at 100 impulses, the total amount of viable cells exceeds the control group, reaching cell numbers as determined by LDH up to an additional 25% of the control [Figure 1c]. Within the given experimental parameters, it is indicated that increasing numbers of impulses lead to an increased number of cells with a concurring rise in glucose consumption. Microscopical analysis at all distances yielded a common result: After shock wave application, perforations of the cell layer could be

observed [Figure 2], which progressed to full closure after 7 days post shock wave application. The size of the disruption of the cell layer thereby varied in accordance with the total number of impulses. Additionally, cell layers of treated samples presented at the edge as clearly visible wavy arrangements of keratinocytes which could be observed after 72h post treatment while the morphology of the individual cells showed no direct visible change.

Conclusion

The preliminary data indicates that application of extracorporeal shock waves using specific distances, impulse rates and frequencies has a demonstrable impact on proliferation and cell viability in keratinocytes cultures.

Figures:

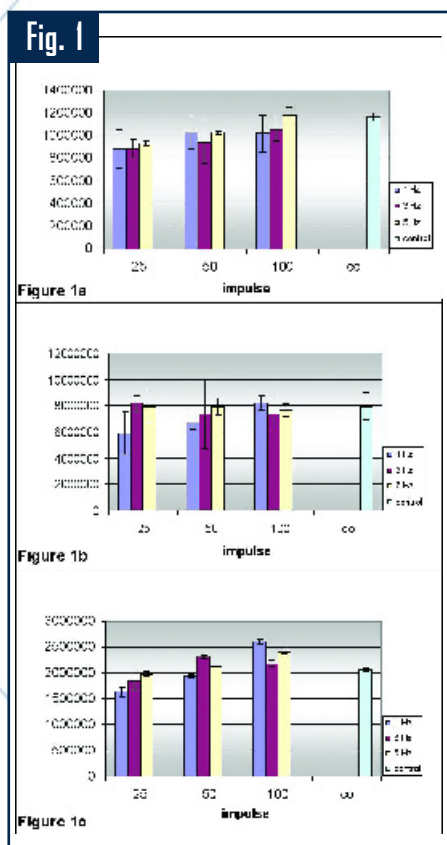


Figure 1: Number of viable keratinocytes via LDH concentration 7 days after shockwave application at different distances between cell culture flask and aperture. Fig. 1a: 7 cm distance Fig. 1b: 6 cm distance Fig. 1c: 5 cm distance

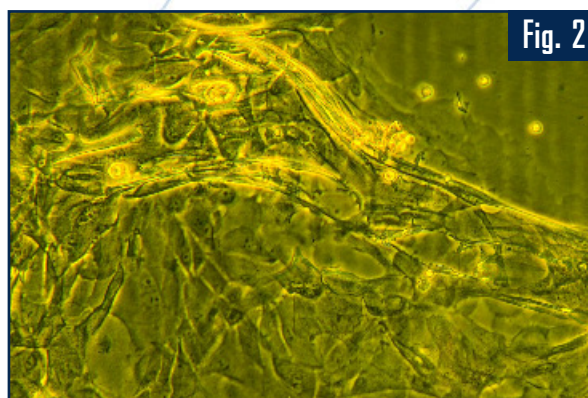


Figure 2: Microscopical analysis. Perforations of the cell layer after shockwave application

Short communication:

Evaluation of Shock Wave Treatment Parameters in an In Vitro Model

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Introduction

In vitro applications of shock waves are performed in various ways [1,2] and no standardized set-up is currently available. In particular, different set-ups using various methods of shock wave generation and different energy settings have resulted in hardly comparable findings. *In vitro* models have to be reproducible, feasible and they have to consider the technical properties of shock waves,

especially the distracting physical effects that might occur during application. The recently introduced IVSWT waterbath [3] holds a strong promise concerning these criteria, as it allows the reproducible shock wave treatment for various cell types in different culture containers as well as for different shock wave devices.

Our study aimed to identify the most effective settings on the electrohydraulic shock wave device DermaGold® (Tissue Regeneration Technologies LLC, Woodstock, USA, manufactured by MTS Europe GmbH, Konstanz, Germany) comprising energy flux density, number of pulses and frequency, used in combination with the IVSWT waterbath. As a model system the human leukemic monocyte lymphoma cell line U937 was chosen. The working hypothesis was based on the proposed transient membrane perturbation triggered by ESWT [4,5]. Based on studies regarding uptake [6] as well as release [7,4] of molecules, we used polar fluorescently labeled molecules for uptake experiments and lactate dehydrogenase (LDH) as an example for release of intracellular molecules.

Materials & Methods

Monocyte cells were cultured in suspension in RPMI 1640 medium, supplemented with 10% FCS, 1% L-glutamine, 1% Penstrep, 25 mM HEPES (PAA, Austria). To determine the effective energy settings for treatment, uptake rates of 3 polar membrane-impermeable fluorescently labeled molecules of different sizes - calcein (666 Da), dextran fluorescein

3 kDa and 70 kDa, respectively - were analysed. For shock wave treatment, aliquotes of 1 mL cell suspension at a concentration of 10^6 were prepared in sterile sample tubes. Directly before treatment, membrane-impermeable molecules were added, tubes were positioned in the waterbath and exposed to various shock wave settings. Immediately after treatment uptake of fluorescently labeled molecules was assessed by fluorescence microscopy and subsequently quantified using flow cytometry. Viability of cells was evaluated using propidium iodide by flow cytometry and additionally by a proliferation assay based on mitochondrial activity (EZ4U, Biomedica).

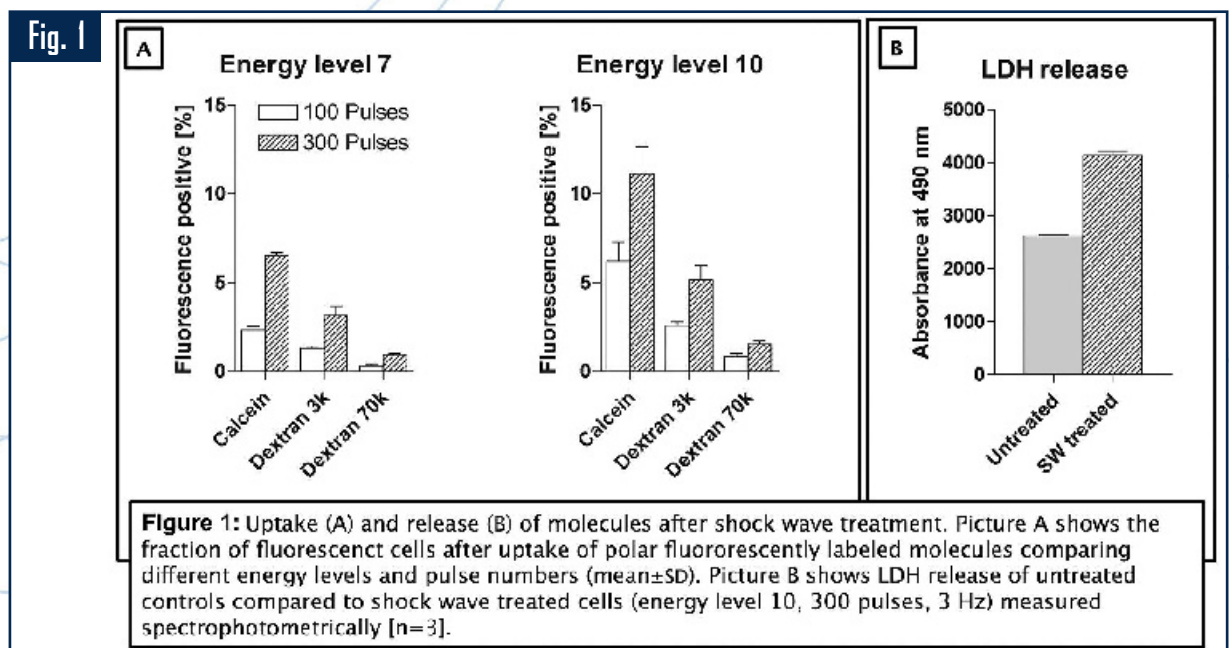
Results and Discussion

The uptake of polar molecules triggered by shock waves could be clearly demonstrated and quantified. As expected uptake rates of polar fluorescently labeled molecules increased with higher energy levels and decreased with increasing molecule size. The highest molecule uptake rates (up to 13% in the case of calcein, the smallest molecule) could be achieved using 300 shock wave pulses at energy level 10 (corresponding to an energy flux density of 0.3 mJ/mm^2) and a frequency of 3 Hz, as shown in **Figure 1A**. The release of LDH into supernatants of shock wave treated cell suspensions also indicates the release of macromolecules at the chosen settings [**Figure 1B**]. Obtained data demonstrate that the use of the

IVSWT waterbath allows reproducible application of shock waves in vitro to elucidate shock wave triggered effects on a cellular basis.

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