Shockwave

INTERNATIONAL SOCIETY FOR MEDICAL SHOCKWAVE TREATMENT

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EDITORIAL

Shockwave Medicine
Past, Present and Future

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Shockwave Medicine. Two words that mean much more than just a therapy. The simple concept of enhancing tissue regeneration through mechanical controlled stimuli is a great step ahead in the management of many low healing diseases. The more complex effect of neo-angiogenesis and the generation of capillaries enhancing blood supply to avascular areas through mechanotransduction is just fascinating. The findings of gene expression changes, the stimulation of cell metabolism pathways, the secret language of cell migration and activation has shown us a rather unknown life line: mechanical forces. We have been able to translate part of this language, and create systems that can be used in reproducible parameters to obtain constant repeatable cell and tissue responses. This is Shockwave Medicine. Not an analgesic extracorporeal device, but a safe non-invasive tissue regeneration system.

Two decades of basic and clinical research are very encouraging. These years of research and development have shown solid scientific results. Many of the most relevant findings in medicine and therapeutics have started this way: following a biologically logical pathway, asking simple research questions and organizing the results in simple research answers. Many of our ISMST researchers started in Shockwave Medicine with the surgeon's incredulity for a funny therapeutic procedure coming from urologic lithotripsy. Once the results came up, the patients healed, the pain subsided, there were more questions than answers. Many turned their backs. Many continued against the wind with the clear commitment of finding why and how did this work.

Orthopedic sports medicine had a new tool. Shockwave Medicine gave the opportunity to researchers to understand the physiopathology of tendinopathies. Now we know the lack of regeneration of tenocytes in tennis elbow, the poor vascularity patterns of plantar fasceiitis and the limited capacity for healing of the proximal patellar tendon. We also know that using extracorporeal stimulation we can enhance all these conditions, control pain and recover function at no risk. Pain is an emotion, so it is very hard to measure. But after 20 years of shockwave medicine I only see good results everywhere. Some bad reports have been published, but most of them with a poor design, done by non-experts and with unknown protocols. If Shockwave Medicine was a bad thing it would have disappeared years ago. On the contrary, it grows day by day.

Bone healing is the most important challenge for the orthopedic surgeon. The transition from the full stability treatment protocols from the past century to the full biology treatment of our days is quite a change. The minimally invasive fracture fixations done today by my residents would have had me expelled from my hospital

in the 80`s. We know now that the vascularity and biological environment of the fracture is key to bone healing. Evidence was always our enemy, until researchers like Wang, Schaden, Caccio and Rompe have shown level one EBM data that proves equivalent results in bone healing using Shockwave Medicine or Surgery. The literature results, with all the possible variables in power and evidence, can only show one thing: we have at least the same results as surgery, with no complications and at a lower cost.

I always remember when Prof. Wolfgang Schaden came to my hospital`s orthopedic grand round a few years ago. He showed the evidence, the data, the cases. He brilliantly answered all questions in the most scientific manner. In the end, a young orthopedic surgeon said she will never recommend Shockwave Medicine in the hospital because of a conflict of interests with the providers. I must confess I was really angry with this answer. Wolfgang was smiling. I asked why. He said: When there is nothing else to argue in a medical procedure but a commercial issue, is because all the scientific answers are undisputable.

Plastic surgery faces a huge challenge with tissue regeneration: complex wound healing. The poor vascularity results in delayed coverage and increases infection rates. It is costly, and the surgical solutions are usually large and painful. Bad healing causes functional and cosmetic problems that require many other interventions. Shockwave Medicine has proved a solution in skin regeneration. Mechanotransduction has been studied thoroughly in skin fibroblasts with great results. The work of researchers like Stodjadinovic has shown amazing results in both in vitro and in vivo series. The clinical use of Shockwave Medicine for wound healing is now widespread, and patients with these difficult conditions are healing all over the world. The protocols for diabetic foot and burns are showing excellent results. I believe skin applications will soon be the most popular and well accepted protocol of Shockwave Medicine.

Research in myocardium tissue regeneration has also been very active in the past five years. The use of extracorporeal systems for angina has evolved to the next step: intra-corporeal Shockwave stimulation of ischemic heart during open coronary by-pass surgery. The logic is there: changing the heart`s pipeline sometimes is not enough. Some kind of tissue stimulation must be used. Many researchers have found the use of intra-cardiac injected growth factors can enhance muscle regeneration. The results are encouraging but variable. The use of intra-corporeal Shockwave stimulation has proved more reliable results both in animal and human trials. We are excited to see the results that will come up in the next years.

Many other applications of a non-invasive system that creates angiogenesis, tissue regeneration and pain control are being used and under investigation. The development of pressure and radial wave systems have proved a great therapeutic tool in superficial conditions such as insertional tendinopatias, trigger points and bursitis. These conditions are by far the most common consults at the Shockwave Medicine units all over the world. Providing a non-expensive, easy to use system that controls pain and regenerates local tissue is very attractive, and that is the reason for this growing field.

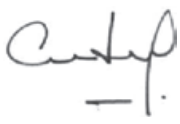
I am very encouraged to see the results in spasticity and rehabilitation of muscle complications of strokes and cerebral palsy in the near future. Some of our ISMST researchers are moving forward on this field. The local stimulation of bone turnover in osteoporotic patients may be the long seeked solution for prophylaxis in the contralateral hip in elder femoral neck fractures. The use of shockwaves in subchondral stimulation of articular cartilage lesions has proved some preliminary results. We hope to show some more numbers in the near future.

The main goal of a scientific society like the ISMST is to provide the direction of all efforts from our corner of science to improve health. We are the world experts in tissue regeneration by Shockwave Medicine, and we are responsible to keep the technology in the perfect balance between safety and efficacy. This is the place where scientists, clinicians, surgeons, researchers, industry, governments and insurance companies find a common ground to put all efforts together. We work on this goal day by day.

Every year we get together and tell each other what we are doing in Shockwave Medicine. Every year the ISMST gives the world an update of this fascinating and growing technology. This year we have a beautiful congress that brings together the world leaders to discuss in 14 keynote lectures, 51 scientific papers and one full-day Instructional Course the best knowledge update in Shockwave Medicine.

I have been privileged not only to direct the efforts of the ISMST in the past year, but also to organize the XV world congress in my country, Colombia. The city of Cartagena is ready to welcome all the ISMST family in the best Caribbean atmosphere. We will be able to discuss science and to meet our friends from all over the world. This is also the first congress of ONLAT, the Ibero-American Shockwave Medicine Society as a regional intercontinental federation. We expect to have the best congress ever, and we are committed to it.

There is a lot of past, present and future of Shockwave Medicine. It is the mission of the ISMST to keep it growing.



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EDITORIAL

RICHARD THIELE,MD

MEMBER OF THE ISMST
SUPERVISORY BOARD

Dear ISMST Colleagues and Friends around the world,

As you can see from our current 8th issue of the newsletter its look and layout have received a complete makeover. We have embarked on an update and hope the redesign meets with your approval. Alongside the annual ISMST Conference, we will, as usual, be publishing research works of interest on musculoskeletal shockwave therapy.

Acceptance of this therapy continues to gain ground. Ever more people the world over are gaining access to this exciting therapy. The Austrian National Institute of Health, for instance, has designated the treatment as the "therapy of choice" in the care of pseudarthrosis. Consequently, if a physician in Austria operates on a patient without first proposing shockwave therapy, this practitioner may become criminally liable.

It is encouraging to see that ESWT is increasingly being covered by insurances and medical plans. A survey on the current state of affairs regarding international acceptance, application and medical coverage will be presented at the end of the conference. Shockwave therapy is nowadays finding application in urology, orthopedics, surgery, dermatology, plastic surgery, dental and veterinarian medicine.

There are hundreds of valid studies worldwide, and all of which would not have been possible without basic research. Only through basic research work has it been possible to discover ESWT's multifaceted mechanisms of action. It was believed 30 years ago that shockwaves could effectively break up kidney stones, calcification, e.g. in the shoulder, and thereby generate a healing effect. Today we know that the mechanical impact is a mere secondary effect, that these waves can be healing and pain relieving. They have a positive impact on the metabolism of living cells and can moreover, as these studies have demonstrated, have a beneficial effect on mesenchymal stem cells.

This has led to the broadening of ESWT's spectrum of mechanism of action on bones, tendons, skin, nerves and the heart muscle.

Because basic research is so vitally important, also for the further development of this therapy, a gathering of many of the world's leading scientists in Innsbruck (Austria) was held a second time this January for the purpose of presenting and exchanging the latest findings in basic research. Meetings of this kind should continue to take place for the coordination of scientific research around the world and the exchange of knowledge at the highest levels.

So that these exciting discoveries can be made available to the public, we are publishing the presentations of the most important events in this issue of the newsletter. For this reason, we would like to express our heartfelt gratitude to the authors Prof. Wang (Taiwan), Prof. Frairia (Italy), Prof. Bloch (Germany), Dr. dÀgostini (Italy), Dr. Antonic (USA), Dr. Neumann (Germany) and Dr. van der Jagd (Netherlands).

Unfortunately a few of the presentations on research findings on the heart and skin are not yet available, but which we hope to publish later on.

I wish all of you great enjoyment in the reading of these important works and most of all lots of fun and new discoveries at the 15th International Congress of the ISMST in Cartagena led by our President Professor Carlos Leal.

Yours sincerely,
Dr. Richard Thiele

INSIDE THIS ISSUE

Early Angiogenic Response to Shock Waves in a Three–Dimensional Model of Human Microvascular Endothelial Cell (HMEC – 1)

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In the last few decades the field of Shock Waves (SW) has been characterized by some great progresses: their clinical applications have widened from the urological field (lithotripsy) to the treatment of both inflammatory and degenerative orthopaedic diseases (tendons, ligaments, soft tissues and bone pathology) (Valchanou and Michailov 1995, Schaden et al. 2001, Wang et al. 2005, C. d'Agostino et al. 2011).

Moreover, in recent years, they have been applied also in the field of wound care management and "scar pathologies", thank to their tissue trophic effect, generated by the dissipation of the high pressure focused acoustic waves into low energy unfocused shock waves (uSW) (Schaden et al. 2007, Romeo et al. 2011).

As a general concept: SW in orthopaedics are not used to disintegrate tissues, rather to induce neovascularization, improve blood supply, and tissue regeneration (Wang CJ, 2003). This is possible thanks to mechanotransduction, that is the possibility to convert a mechanical stimulation (SW) into a series of biological reactions at the tissue level; this mechanism is active in all cells that are responsive to physical stimulations.

Experimental data suggest that one of the main biological effects induced by mechanical stimulation of SW is the production of Nitric Oxide (NO) (Mariotto et al. 2005), which is well known to promote angiogenesis (formation of new blood vessels from pre-existing capillaries). Angiogenesis is itself one of the first step in tissue healing and it is induced by a variety of growth and angiogenic factors (Cooke and Losordo 2002).

Some other studies suggest that mechanical stimulation produced by SW can have also a direct effect on the ExtraCellular Matrix (ECM), which further triggers cytoplasmatic and nuclear reactions, varying according to the experimental model, the energy level, the number of impulses and the cell type (Speed 2004). As already demonstrated for some other specific biomechanical stimuli, SW could induce some biochemical reactions in responsive cells, thus affecting growth, development, differentiation, apoptosis regulation and gene expression via signal transduction pathways (Tarbell et al. 2005).

In particular, from the angiogenetic point of view, in vivo and in vitro models demonstrated the pro - angiogenic activity of extracorporeal SWs, via an up - regulation of mRNA levels for VEGF proliferation and differentiation of VEGFR-2 positive EC, and NO production (Wang CJ 2003, Mariotto S et al. 2009).

As already expressed, cell responsiveness to exogenous stimulation is correlated to a series of metabolic activities comparable, as a result, to the effects of Growth Factors (GF) on cellular transcription; in particular, endothelial cells (EC) are particular mechano - sensitivite cells.

It has been described that definite hemodynamic characteristics of the laminar flow induce, from 1 to 6 hours after stimulation, a regulatory effect on eNOS (endothelial NO – Synthase) and ICAM-1 (IntraCellular Adhesion Molecule) activation; both of them, in some way, can be considered as intracellular signalling events (Stolz et al. 2002, Davies et al. 1997).

Mechanical stimuli (as shear stresses) are described to induce hemodynamic responses of Endothelial Cells (EC), into different phases and times:

• EARLY RESPONSE (within some minutes /seconds, characterized by the activation of ion channels ad second messengers, as NO);

• INTERMEDIATE RESPONSE (within minutes/hours, characterized by endocytosis, cell replication, gene up/down - regulation);

• LATE RESPONSE (within hours /days, during which you can observe endothelial cells adaptation; structural and funtional adaptation are NO – mediated).

(Resnick et al. 2002).

More in details, after mechanical stimulation as shear stresses, the angiogenetic and antiapoptotic effects would interest some particular cellular compartments, engaged with selective sites of eNOS (Dimmeler S et al. 1999, Balligand et al. 2009).

FOR SUMMARIZING

• on UNSTIMULATED CELLS: eNOS is maintained in inactive state, through its association with Cav-1 (Caveoline);

• after MECHANICAL STIMULATION (shear stresses), it is possible to observe the following reactions (if there is an adeguate availability of substrate (Calmoduline) and Arginine Transporter CAT-1) (Traub O et al. 1998):

- eNOS - Cav1 dissociation
- eNOS activation

How to explain in detail angiogenesis and which effects on human endothelial cell lines, soon after uSW stimulation ?

On the basis of what above described, it is reasonable some questions arise: can SW, under definite experimental conditions, simulate the effects of shear stresses and stimulate endothelial cells? What does it happen in the earlier stages of EC stimulation ?

Aim of our study was to verify the capability of uSW to induce new vessels proliferation (neoangiogenesis) in vitro and, at the same time, to investigate the initial response of endothelial cells to this type of acoustic stimulation. For this purpose we employed an in vitro system, consisting of a matrix support, seeded with microvascular endothelial cells which resembles, as closely as possible, the structure of the natural tissues (Sansone V et al, 2012). By reproducing the architecture of a mechano-sensitive structure, such as the capillary network, one may provide a valid model for analyzing the behaviour of EC (HMEC - 1), when subjected to an acoustic signal comparable to shear stress.

HMEC-1 is the first immortalized human microvascular endothelial cell line, that retains the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells. When endothelial cells are plated on *BD Matrigel™*, they can form a three dimensional network of capillary tubes comparable to the final step of the angiogenic cascade (Ades EW et al. 1992).

In our experimental experience, cell cultures were stimulated with uSW, according to various protocols (different energies and number of shots).

For angiogenesis experiments, cells were grown in 24-well plates on *Matrigel* matrix and vessels-like structures were quantified by counting the capillary connections under an inverted microscope.

The most responsive group in terms of numbers of capillary connections underwent gene expression analysis using the Super Array kit-Signal Transduction Pathway Finder (SABiosciences, Qiagen), able to profile 84 key genes representative of 18 different signal transduction pathways.

After 12 hours, the treated cells showed a substantial increase in the number of new vessel-like structures if compared to untreated cells. This morphological differentiation was more remarkable in the samples that were exposed to low energies and limited numbers of shots **(fig. 1 and 2)**.
As reported by other authors (Steinbach et al. 1993), we observed a disaggregation of the Matrigel scaffold and a negative effect on the formation of capillary connections at higher energies (data not published).

In samples showing the most marked increase in number of capillary connections, we observed a decreased gene expression 3 hours after uSW treatment. Indeed, cells showed a strong down-regulation of genes involved in the apoptotic process (BAX, anti-apoptotic BCL2LI, GADD45A, PRKCA), also in the cell cycle (CDKN2C, CEBPB, HK2, IRF1, PRKCA), oncogenes (JUN, WNT1), cell adhesion (ICAM-1), and proteolytic systems (CTSD, KLK2, MMP10).

However, we did not observe any increased expression of receptors for angiogenic agents like endothelial nitric oxide synthase (eNOS) or vascular endothelial growth factor (VEGF).

• These preliminary results seem to indicate that endothelial cells in vitro quickly respond to SW, by proliferating and forming vessel-like structures, depending on the energy level employed and the number of shocks released.

In the first 3 hours after SW stimulation we did not observe either VEGF or eNOS modulation, whereas other authors were able to demonstrate the production of VEGF and the increased expression of the specific angiogenesis pathway after 6 hours (Stojadinovic A, et al. 2008, Wang FS et al. 2004).

• Nevertheless, the osberved downregulation of the genes involved in cell cycle and cell adhesion, could be interpreted as the preparatory signal correlated to an upcoming detachment of endothelial junctions, in other words: the "first reactive response" of the endothelial cells to the external stimuli and the prelude to the events characterizing the neo-angiogenic sequence.

COMMENTS

As already mentioned, Endothelial cells (ECs) are mechano-sensitive cells which physiologically react to flow shear stress. Particular regions of the cell membrane seem to be involved in the recognition of the different features of the laminar flow (Fleming and Busse 1999; Ziegler et al. 1998; Barakat et al. 2006) which are subsequently transferred

to the cytoskeleton (Corson et al. 1996; Davies et al. 1997). Those cellular compartments engaged with selective sites of eNOS are thought to mediate the angiogenic (Baum et al. 2004) and anti-apoptotic effect (Dimmeler et al. 1999) of the shear stress.

In clinical practice, several vascular pathologies are characterized by an intrinsic ECs dysfunction and a diminished production of growth factors (GF) (Madeddu 2005). Hence, new therapeutic options attempt to correct this sort of "biologic imbalance" by inducing neovascularisation, a process which can be achieved by supplementing the VEGF either via gene therapy or transplanting endothelial progenitor cells (Madeddu 2005; Chenggang et al. 2006).

On the other hand, SW stimulation represents an alternative, and innovative, therapeutic approach in those conditions where a strong angiogenic impulse is required – for example in severe skin wounds (Schaden et al. 2007) or in myocardial ischemic lesions (Fukumoto et al. 2006). Moreover, recent experimental studies suggest that the treatment of ischemic tissue with low energy SWs improves the recruitment of circulating endothelial progenitor cells (EPCs) due to enhanced expression of specific chemo-attractant factors (Aicher et al. 2006).

But, if the late neoangiogenic response has been documented adequately, much less attention has been given to the very early changes induced by mechanical stimulus. Our study was established to investigate the early effects of unfocused shock waves on HMEC-1 cultured in a three-dimensional Matrigel model, where cells were stimulated using a source of low energy unfocused shock waves with a pulsation frequency of 3 Hz per second. The experimental Matrigel model, as with any in vitro model, is obviously not a perfect replica of the biological tissues. However, by reproducing the architecture of a mechano-sensitive structure such as the capillary network, it is possible to provide a valid model for analyzing the behaviour of ECs when submitted to an acoustic signal comparable to shear stress, since with defocused waves the flow of the acoustic pulse is close to being laminar.

HMEC–1 are different from the lining EC of large vessels, and are thought to be involved in angiogenesis and in wound healing. We demonstrated that unfocused shock waves induced a quick morphological response (12 hours), characterized by a significant increase of vessel-like structures formation (Sansone V et al. 2012).

As described above, the pro-angiogenic effects of SW are most likely mediated by VEGF and NO. In our study, we did not observe either VEGF or eNOS modulation 3 hours after SW stimulation; we demonstrated a significant down-regulation of genes involved in the apoptotic process, in cell cycle and adhesion, and in proteolytic systems. However, this SW-induced modulation, which is more significant for the antiapoptotic genes and could represent the "early reactive response" of HMEC-1 to the physical impulse induced by the uSW. These observations compare favorably with Kim and Von Recum (2008) who remarked that mechanical stimulus induced by shear stress could improve the differentiation EPCs in ECs, regulating the expression of several genes involved in apoptosis and inducing EPCs to form capillary–like networks in 3D cultures.

In conclusion, our results seem to confirm that some aspects of the early gene response of ECs to uSW stimulation are comparable to those of the laminar shear stress flow, mainly characterized by an anti-apoptotic effect.

Further experimental studies are necessary to validate these hypotheses and to investigate if the biological response of EC to SW stimulation involves the same intercellular pathways and regulatory mechanisms that characterise other types of biophysical stimuli. At the same time, new research into the gene expression could shed light on the triggering of the angiogenic process when acoustic stimulation is applied.

FIGURES

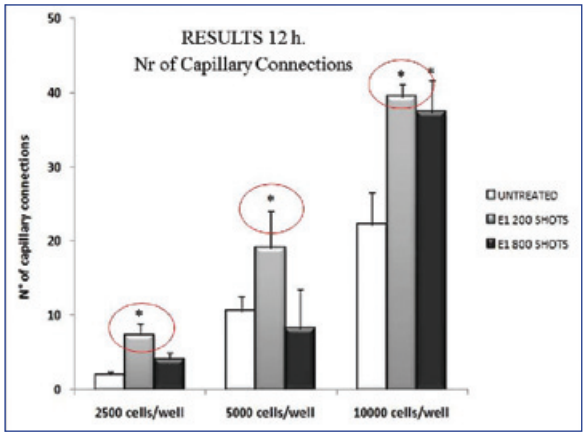

Fig. 1: Count of capillary connections (bifurcations) in HMEC-1 seeded at different cell concentrations, 12 hours after low energy uSW treatment (E1) with different number of shots (200 shots, light grey bars; 800 shots, dark grey bars). Untreated HMEC-1 are shown as negative control (white bars). Under E1 condition the behaviour of EC was related to the number of shots applied; indeed at all densities, cells receiving 200 shots showed a significant increase in the number of bifurcations in comparison to untreated cells. On the other hand, E1 uSW treatment with 800 shots was able to induce a significant increase in the number of bifurcations just in HMEC-1 cells plated at high density (10000 cells/well) in comparison to untreated cells (Sansone V et al, 2012).

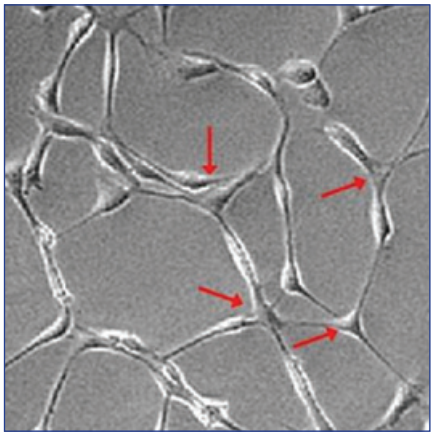

Fig. 2: Detail of strict capillary connections and cellular organization in HMEC-1 culture treated with uSW (E1, 200 shots) (Sansone V et al, 2012).

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Shock Waves Effects on Ca^{++} Deposition by Human Osteoblasts *in Vitro*

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BACKGROUND

In order to contribute to clarify the biological effects of Shock Waves (SW) treatment, we performed it on *in vitro* cultures of human osteoblasts. Here we evaluate the effects of SW on cell proliferation, Ca++ deposition and ALP and NOS activities.

METHODS

In vitro cultured human osteoblasts obtained from surgical fragments were submitted to three SW applications. Ca^{++} deposition, ALP and NOS activities and cell growth rate and differentiation were evaluated and data related with control group of cells.

RESULTS

SW treatment can inhibit both growth rate both Ca^{++} deposition. In the same time SW modifies the NOS activity in human osteoblasts, so presumably affecting ALP activity.

INTERPRETATION

The low rate of Ca^{++} deposition induced by SW treatment may be due to a decrease in enzymatic activity linked to bone mineralization, such as ALP activity, as well as to change in Ca^{++} intra and extracellular flow.

The use of Shock Wave (SW) in orthopaedic disease was reviewed with special regard to the clinical application (Haupt G,1997). Our interest was appointed on some diseases of bone and tendons, as abnormalities in intratendineous calcification, which are restored to normal conditions after SW treatment.

A SW is a transient pressure disturbance that propagates rapidly in three-dimensional space; it is associated with a sudden rise from ambient pressure to its maximum pressure and with a cavitation due to the negative phase of the wave propagation (Ogden JA et al, 2001). Various authors have studied the effects of SW on normal and pathological cells (Smits GA et al, 1991), and recently this physical stimulation has been shown to cause a transient increase in the permeability of the cell membrane. In fact it causes the formation of dimples on cell membrane and membrane potential hyperpolarization (Martini L et al. 2005).

In vivo and *in vitro* studies have shown that mechanical stimulation is associated with physiological changes in bone cells (Wang FS et al. 2003). The response to mechanical stimuli in bone cells is associated with an increase in ion-channel opening, an alteration in membrane potential, in cell proliferation and in the synthesis activity of proteins related to the regulation of bone formation and bone turnover (Harter LV et al. 1995).

As biological effects of these applications on Ca^{++} cellular metabolism are nevertheless quite unknown, our study was focused on the morphology, the proliferation and the enzymatic activities related to mineralization like Alkaline Phosphatase (ALP) of *in vitro* cultured human osteoblasts. *In vivo* conditions of SW treatment are obviously very different from our experimental system, as biological structures like muscle, articular and

tendon tissues and so on create a biological filter among the SW source and the cells that are the final objective of the treatment. This doesn't happen *in vitro*, where SW act directly on cell populations.

As vasodilation is probably important in SW *in vivo* effects, we decided to evaluate also NOS activity and NO production in our cultures after SW treatment.

Recently, it was demonstrated that extracorporeal SW, at a low energy density value, quickly increase neuronal nitric oxide synthase (nNOS) activity and basal nitric oxide (NO) production in the rat glioma cell line C6 (Ciampa A, 2005). Nitric oxide (NO) is a highly versatile signaling molecule which is produced in different cell types by at least three isoforms of NO synthase (NOS) through the conversion of L-arginine and oxygen into L-citrulline. Two enzymes, neuronal NOS (nNOS) and endothelial NOS (eNOS), are constitutively expressed and their enzymatic activity is Ca^{++}/calmodulin-dependent. These constitutive NOS (cNOS) are responsible for the production of physiological levels of NO involved in events such as vasodilation, angiogenesis, and neurotransmission (Ciampa A et al. 2005). The third enzyme is an inducible and Ca^{++}-independent isoform of NOS (iNOS), virtually expressed in all cell types and increasing after stimulation with different cytokines.

MATERIALS AND METHODS

Cell cultures

Surgical specimens of bone tissue were obtained from young patients (8 males 18-24 years range) under traumatic accident. They were washed with antibiotic-added physiological solution, submitted to microdissection both mechanically under the microscope both enzymatically by Collagenase (Collagenase IV-S8 Sigma, St. Louis, Missouri USA) for 40' at 37°C, and finally seeded in culture dishes and cultured in MEM α-medium with 10% FBS, L-glutammine 20mM, P/S 1%, Vitamin C 50µg/ml and fungyzone 0,2%. Cells were cultured in a humidified 95% air /5% CO2 incubator, at 37°C.

When cells migrated from the tissue microfragments into culture dishes after 3-4 weeks, we added 1-2ng/ml of basic FGF (FGF b, Sigma, St. Louis, Missouri USA) and Desamethasone 10^{-8} M and β-glycerol-phosphate 2mg/ml (Sigma, St. Louis, Missouri USA) to the medium to induce osteoblasts differentiation and bone matrix-like mineralization.

Treatment with shock waves

We performed the treatment with SW on our primary cultures of human osteoblasts as summarized in **tab. 1**; between every application there was a 48h break. The SW generator we utilized for our *in vitro* experiments is a electromagnetic device especially designed for clinical use in orthopaedics and traumatology. The lithotripter MODULITH SLK was kindly provided by STORZ Medical AG (Kreuzlingen, Switzerland). Cell cultured in 30 mm Petri dishes with 2 ml medium were treated with SW directly focusing the centre of the plate. The SW unit was kept in contact with the cell containing culture dish by means of a water-filled cushion. Common ultrasound gel was used as a contact medium between the cushion and the culture dish.

Tab. 1

Group	Impulses	mJ/mm²	N° pf applications
A	1000	0,1	3
B	1000	0,030	3
C	250	0,006	3
D	500	0,006	3
E	1000	0,006	3
Control	–	–	–

Cell proliferation

Cells were detached with 0,25% Trypsin in EDTA and cellular proliferation was evaluated with Neubauer haemocytometer at 2, 4 and 7 days of culture. Cell growth rate was studied during the treatment, i.e. between an application and the following one, and then at the end of the treatment.

Cytochemistry

For cytochemical analysis of Ca^{++} deposition, treated and untreated cells were plated on glass coverslips and stained with the Von Kossa method modified for cell cultures (Postiglione et al. 2003). Briefly, cells were fixed for 3 min with 3% formaldehyde in PBS, washed twice in PBS, covered with a 0.5% aqueous solution of silver nitrate and exposed to UV light (Philips, 30W) for 30 min at 25°C. Coverslips were then washed with distilled water and treated with 5% Na_2SO_4. After washing in tap water, coverslips were covered with 1% neutral red to stain nuclei and then submitted to the routinary passages in alcohol, xylene and mounting medium. The stain was performed 7 and 12 days after the treatment.

Enzymatic activity

ALP activity was observed after the staining by an enzymatic kit and quantified by an automatic analyser immediately after each application. We measured the eNOS and the iNOS activities using both DAF fluorescent staining both biochemical method as described, at the end of the treatment.

1) Alkaline-Phosphatase activity

According to the protocol suggested by Sigma Aldrich (Sigma Diagnostics, St. Louis, Missouri, USA) fixed cells were incubated at room temperature of 18-26°C in a solution containing Naphthol AS-BI-Phosphate and rieshly prepared Fast Red Violet LB salt or Fast Blue BB salt buffered at pH 9.5 with 2-Amino-2-methyl-1,3-Propanediol (AMPD). Sites of activity are either red or blue, depending upon the choice of Diazonium salts.

2) Alkaline phosphatase measurements

Alkaline phosphatase (ALP) was determined using p-nitrophenylphosphate as a substrate (Sigma). Cells were scraped into 500 µl ice-cold harvest buffer (10mM Tris HCl, pH 7,4, 0,2% NP-40 and 2mM phenylmethylsulfonyl fluoride, PMSF,) (Sigma). Enzimatic activity was measured by an automatic analyser (Hitachi 747, Boheringer Mannheim, Indianapolis, IN, USA). The results were expressed as UI/(enzyme activity)/10^4 cells.

3) nNOS assay

nNOS activity was estimated by measuring the conversion of L-2,3,4,5-[3H]arginine to L-2,3-[3H]citrulline, according to the method described by Colasanti M. et al. (1999).

The production of NO was assayed using the DAF-2DA detection system, as previously described (Mariotto S et al. 2005). Briefly, 10 lM 4,5-diamino.fluoresceindiacetate (DAF-2DA; Alexis-Corp., San Diego, CA, USA) was added to the cells cultured in serum free medium and incubated at 37° C for 10 min. After washings with PBS plus 1.2 mM CaCl2, the cells were fixed with 3% w/v paraformaldehyde plus 4% w/v sucrose, and cellular fluorescence was observed using a fluorescence microscope (Axioplan 2, LSM 510, Carl Zeiss, Gottingen, Germany) equipped for image acquisition.

Statistical analysis

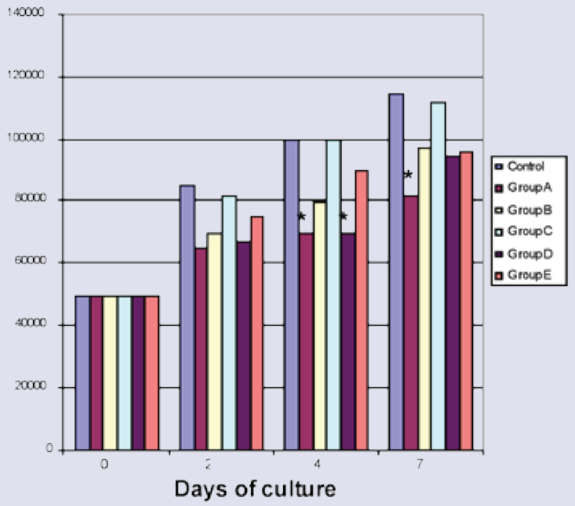
For statistical evaluation of our data on cell proliferation we used the t-Student test for unpaired data; probability value of $p \leq 0,05$ was accepted as statistically significant.

RESULTS

Cell proliferation

Cell proliferation at 2, 4 and 7 days after cell seeding and during the treatment is represented in **tab. 2**, were statistically significant steps are evidentiated. Cellular growth decreased in group A, which was submitted to the stronger treatment, while the decrease in the proliferation was lighter for lighter treatment, as growth rate in the group C indicates.

Tab. 2 Osteoblast proliferation during the treatment with SW

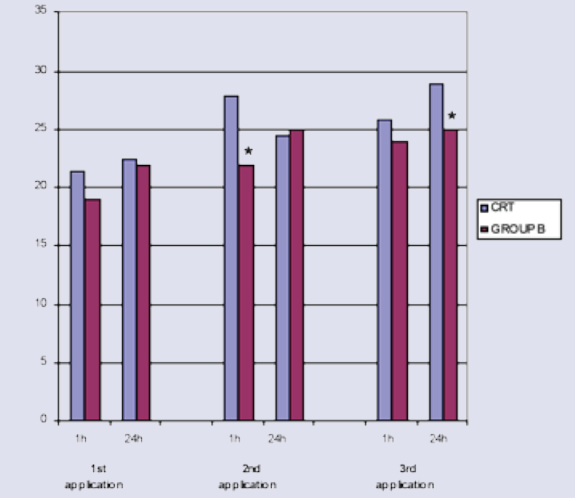


Enzymatic activity

1) Alkaline-Phosphatase activity

ALP reaction staining was evident after 4 days in vitro in our cultures. This stain was performed both in basal condition to confirm that our cells were differentiated osteoblasts both after the treatment. The enzymatic activity is well evident in all cell dishes when examined by the light microscope, but the morphology of cells, as well as their number, are really different. In particular, the treatment with SW is able to modify cell shape, which appears more flat, and slightly decreases the ALP staining in all treated groups **(Fig. 1)**.

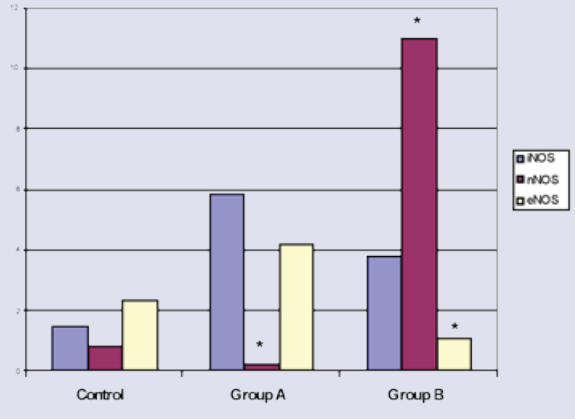
Tab. 3 ALP activity in human osteoblasts during SW treatment



2) Alkaline phosphatase measurements

ALP measured by an automatic analyser and expressed as UI/(enzyme activity)/10^4 cells demonstrated a decrease in ALP activity in SW treated cells beginning from the first application **(Tab.3)**. Enzymatic activity quantification is related to the number of cells, for control as well as for treated cells; so the decreased number of osteoblasts after SW cells is not responsible for the decreased ALP activity we measured. There is a restore in ALP levels in the 24h following the first and the second application of SW, but this does not happen after the third application.

Tab. 4 Effect of SW treatment on NOS activity



3) NOS assay

The SW induced variations in NOS activity are represented in **tab. 4**. There is a dramatic increase in nNOS activity in cell group B, when compared with control group, while the value of this type of NOS decreased in group A. The iNOS increases in apparent relation with the increasing power of the treatment. The endothelial form, on the

contrary, is less expressed as compared with control group for low treatment and more expressed after strong treatment. To verify whether enhancement of nNOS activity after SW resulted in an increase of the NO synthesis, the intracellular NO production was measured using the DAF-2DA detection system. When the cells were treated with SW, DAF-2T fluorescence was significantly enhanced above the background level (data not shown). As expected, this fluorescence response was prevented in cells treated with 1 mM N-nitro-L-arginine methyl ester (L-NAME) for 30 min before SW treatment, thus indicating that the increase of DAF-2T fluorescence was consequent to the activation of the L-arginine-NO pathway.

Cytochemistry

We used Ca^{++} deposition, showed by Ca^{++} brownish precipitates in the mineralized matrix, as an evidence of cell differentiation. The stain was performed after 7 and 12 days after the treatment with SW. The Ca^{++} brownish precipitates of mineralized matrix are always more early evident in control cells; mineralized ECM appeared in cell cultures treated with SW only after 7 days and also then the amount of Ca^{++} precipitates was less evident than in untreated cells.

The stain performed at 12 days after the treatment demonstrated strong evidence of mineralization for all cell groups, but confirmed the decreased level of Ca^{++} deposition in cells submitted to SW treatment **(Fig.2)**.

DISCUSSION

Our interest was appointed on some diseases of bone and tendons, as abnormalities in intratendineous calcification, which are usually restored to normal conditions after SW treatment. We investigated the biological effects of SW treatment by using a simply experimental model consisting of human osteoblasts cultured in vitro in a medium which is permissive for their differentiation and for mineralized matrix deposition. This method permits the evaluation of some morphological and physiological aspects of cells, such as growth rate, NOS activity and the expression of markers of bone differentiation like ALP activity and Ca^{++} precipitation.

Cell cultures were submitted to the same treatment used for patients, and our data indicate that cell proliferation decreases after SW treatment at all the used intensity while cell morphology is early enlarged, poligonal-shaped and "adhesive" in treated cells. The "adhesive" phenotype is usually associated with the decrease in cell proliferation in vitro and it is not surprising, but at the same time we expected an increase of cell differentiation and Ca^{++} deposition. On the contrary, Ca^{++} deposition is clearly more slow in SW treated cells when compared with control ones.

We investigated NOS activity in our experimental system because we think that our data are probably related to NO anti-inflammatory action after SW. It is well known that SW are implied in NO increase in treated tissue (Ciampa A et al. 2005). We observed that iNOS activity is increased by SW in a energy-dependent way. On the contrary, nNOS activity is dramatically increased by low levels of energy and inhibited by higher levels while eNOS is influenced in an opposite way. It is interesting that both their enzymatic activities are responsible for the production of physiological levels of NO involved in vasodilation and angiogenesis and are Ca++/calmodulin-dependent, and it is well accepted that SW influence ionic and Ca++ transmembrane currents. Recently it was demonstrated that SW lead to an increase in eNOS activity and NO formation in other experimental systems (Mariotto S et al. 2005), while the presence of nNOS in human osteoblasts is not yet commonly accepted (MacPherson H et al. 1999). In effect, the nNOS level is very low in human osteoblasts when cultured in vitro, but it rapidly increases al lower levels of energy in our experimental system. Our results confirm that SW rapidly increase NO production by enhancing catalytic activity of nNOS with the maximum value (about 10 folds over the control value) being reached at the medium level of energy densities.

On the other hand, the observed decrease in eNOS could contribute to inhibit the Alkaline Phosphatase activity and the Ca++ deposition in mineralizing matrix. Recently, it was demonstrated a marked retardation both in postnatal bone formation both in osteoblasts maturation in eNOS knockout mice, resulting in reduced bone volume (Aguirre J et al. 2001).

All together, these results contribute to clarify why SW treatment is a useful tool for decreasing Ca^{++} deposition, as they act retarding the mineralization of bone Extra Cellular Matrix in in vitro cultured osteoblasts as well as they do in athypical localizations of calcified tissue in vivo. As regards the different energy levels we used, our opinion is that medium-low levels are to prefer for studies on biological effects of SW; stronger levels (as group A) might cause cellular damage while too low applications (group C) might be virtually undistinguishable from control cultures. So we decided to show mainly our data on group B and group E, as indicate.

It is interesting that some evidences indicate that SW at higher energy levels induced increase in NO production and could exert a positive effect on the differentiation of mesenchimal cells toward osteoprogenitors (Wang FS et al. 2002). This is not inconsistent with our data, as it is well known that SW are useful in repairing processes as pseudoarthrosis, in vivo: they could act inducing differentiation of resident or circulating mesenchimal cells. On the contrary, we utilized lower energies and only well differentiated osteoblasts; the differences we observed in SW effects could indicate that we can modulate their effects by varying their energy level.

Our data indicate that cell proliferation is always decreased by SW but do not clarify if there is a proportional relation between SW energy level and growth rate. It could be useful to perform more experiments on the cell cycle profile of human osteoblasts to evaluate the influence of SW on the growth rate as well as on the production of various components of ECM. Other studies in progress will help to clarify the mechanism of action of SW on Ca^{++} transport and deposition.

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FIGURES

Fig. 1

ALP staining in in vitro cultured human osteoblasts. SW treatment modifies cell morphology and decreases ALP enzymatic activity (A) Control group (B) Group E Magnification 130X

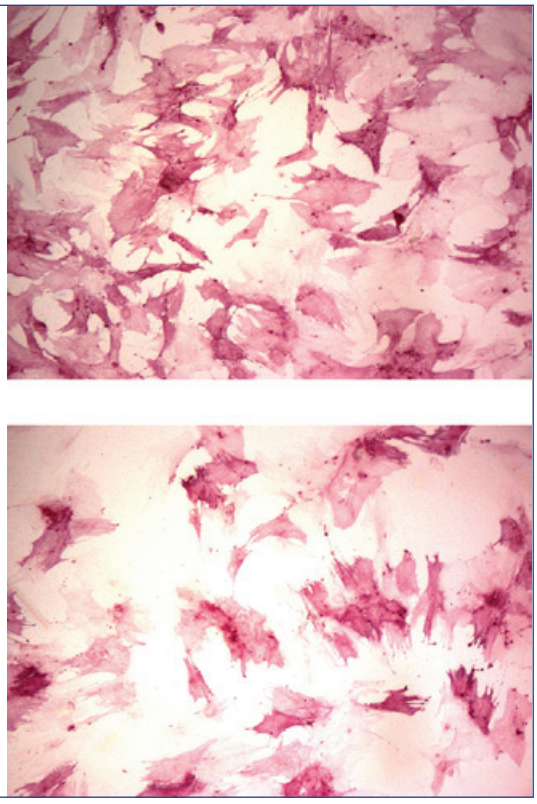
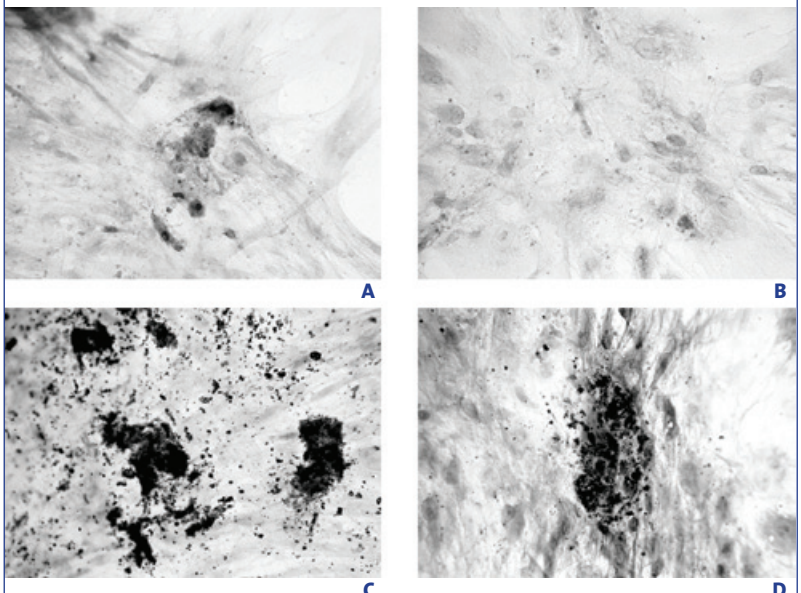


Fig. 2



The deposition of Ca^{++} in vitro is decreased by SW treatment also in the presence of Dexamethasone and β-glycerophosphate. On the left are imagines from control groups, on the right from SW treated group B. Data are referred to 7 (A-B) and 12(C-D) days of culture after SW treatment.
Von Kossa, Magnification 450X

Mechanotransduction – Role in tissue adaptation

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INTRODUCTION

Biological tissues, such as tendons, cartilage, skeletal muscle, connective tissue, endothelium or epithelia possess the ability to sense a variety of different kinds of stress modes. 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 new aspects, such as **1)** is there a link between mechanical and redox signaling and **2)** is mechanotransduction linked to epigenetic regulation.

WHAT DOES MECHANOTRANSDUCTION MEAN?

Mechanotransduction describes the sensing and transmission of externally induced mechanical forces into a cellular system. In a recent review, 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 stimulations generate acute functional responses in the affected tissue/cells 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/cells in the way that the tissues'/cells' are longtime modulated leading to structural and functional adaptations of the tissues and organs.

The process of cellular mechanotransduction follows several steps including different phases finally resulting in cellular responses and adaptations. Wu *et al.* (2009) defined three phases of mechanotransduction. The first phase is called *signal transduction phase*. This phase is a highly complex phenomenon as diverse cellular mechanisms are switched on to lead to the second phase called *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. 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, including the regulation of calcium-dependent pathways, mitogen-activated protein kinases, second messenger systems, etc. The signal propagation phase will finally lead to the *cellular response phase*.

In the following part, we will focus on a possible link between mechanical force sensing and direct redox signaling as well as on implications of mechanotransduction in epigenetic regulations.

IS THERE A LINK BETWEEN MECHANOSENSING AND REDOX SIGNALING?

Redox signaling is significantly involved in cellular regulation and homeostasis, as almost all cellular substructures are regulated by redox systems (Bedard & Krause, 2007). Important protein complexes involved in these processes are NAD(P)H oxidase (Nox) complexes (Bedard & Krause, 2007). These Nox complexes crucially involve the translocation of $p67^{phox}$ subunits to the complex. Otherwise Nox complexes cannot exert their redox signaling potential, leading to different pathologies (Bedard & Krause, 2007). The translocation of $p67^{phox}$ to the Nox complex is mediated by its interaction with the small GTPase Rac1. Rac1 have been attributed significant roles in cytoskeletal arrangements and cellular migration regulation (Sepulveda & Wu, 2006). Therefore, it seems to be highly interesting to explore the possible link between Rac1 dependent mechanotransduction and redox signaling.

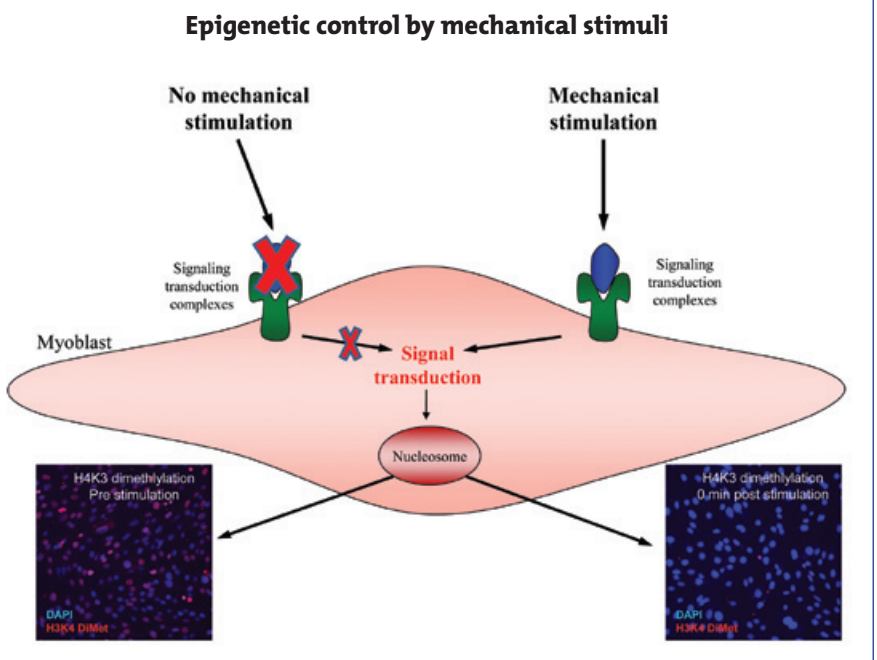
Mechanical impacts are sensed by extracellular matrix (ECM)/basement membrane (BM) structures that travel the mechanical impact downstream to intracellular structures via the connection to focal adhesions (FAs) (Legate *et al.*, 2006). A central player of FAs is the integrin-linked kinase (Ilk) that assembles different regulatory proteins to the FA site (Legate *et al.*, 2006; Lange *et al.*, 2009). β-parvin (Parvb) is one of these regulatory proteins interacting with Ilk at FA sites to transduce mechanical stimuli in cellular processes. Importantly, Parvb has been shown to interact with Rac1 and thereby stabilizing Rac1 at the plasma membrane (Sepulveda & Wu, 2006). We thus have investigated the role of Parvb in redox signaling events in more detail. We observed an important role of Parb for tissue adaptations towards physical training. This cardiac adaptation was associated with impaired redox signaling (Thievessen, Suhr et al., unpublished data), because physiological redox signaling has been shown for physiological tissue adaptations (Zhang *et al.*, 2010).

These preliminary data demonstrate that a direct between mechanosensing structures and physiological redox signaling exists at least at the plasma membrane. These findings are of very high significance as they demonstrate that physiological loading can result in maladaptive tissue adaptations due to disturbed interactions of mechanical and redox signaling components.

MECHANOTRANSDUCTION AND EPIGENETICS

Epigenetics is a growing field of research investigating gene manipulations by external factors rather than by classical inheritance processes. A diversity of external factors has been described in epigenetic gene control and regulation (McGee & Hargreaves, 2011). Among these factors, recent evidence arose attributing mechanical stress a central role in these epigenetic control mechanisms. Skeletal muscle tissue is a classically stimulated by mechanical impacts, either by external impacts or by internal forces or by a combination of both. It was demonstrated that exercising conditions result in transcriptional activation in skeletal muscle by increasing acetylations of histones 3 and 4 (McGee & Hargreaves, 2011). These data highlight the importance of mechanical stimulations on skeletal muscle plasticity related to epigenetic modifications and gene regulation. We used extracorporeal shock waves as a model of defined mechanical stress application. Murine immortalized myoblasts, C2C12, were subjected to extracorporeal shock waves to investigate the influence of mechanical forces on histone modifications, such as methylations, because methylations are known to have central roles in gene transcriptional suppression (McGee & Hargreaves, 2011). The application of focused extracorporeal shock waves resulted in time-dependent regulation of methylations of histone H3 at lysine residue 4 (K4) **(Fig.1)**. We have first evidences that methylation pattern of shock wave treated cells is decreased during the first hour post treatment and turned to an increase of methylation in the following hours(Willkomm, Suhr, Bloch, unpublished data). These data show for the first time the importance of mechanical stimulations as exerted by extracorporeal shock waves on the transcriptional regulation pathways, because histone structures are directly affected by mechanical impacts.

Fig. 1



CONCLUSION

Mechanical stimuli possess great potentials in regulating cellular responses and adaptation. A multitude of mechanosensitive structures and molecules are involved in transmission of the mechanical stimuli in biological response by different signal pathways. As highlighted in this review, new and important aspects of mechanotransduction pathways should be taken into account regarding mechanical regulation of tissue plasticity and disease manifestations. Interactions between mechanical and redox signaling components seem to be of very high importance. Furthermore, the emergence of genetic control by epigenetic pathways, such as direct histone modifications by mechanical impacts highlights the significance for defined investigations exploring the potential of mechanical impacts in disease development and gene transcriptional control. In the future, these two central aspects will be crucial for the understanding of cellular processes involved in disease development and manifestations. However, mechanical stimulations seem to offer a promising tool to successfully treat different diseases by inducing a myriad a signaling pathway positively influence cellular homeostasis.

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Anti-Inflammatory Effects of Extracorporeal Shockwave Therapy

1 

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ABSTRACT

Extracorporeal shockwave therapy has been in use for over two decades as treatment for disintegration of kidney stones and more recently for orthopaedic indications. Experimental observations that soft tissues enveloping surrounding bones of interest healed faster after application of ESWT, established a new therapeutic direction for ESWT, that of soft tissue pathology such as tendinopathies. With this finding and further scientific advances, the list of indications for ESWT was ones more expanded, this time to include difficult to heal and non-healing wounds. Even though these pioneering steps toward soft tissue applications are yet to be fully supported by experimental and well-designed prospective clinical trials with large cohorts of patients, preliminary data and studies thus far demonstrate efficacy and safety of this promising technology. Inflammation is a crucial process in wound healing that has an established role not only in killing pathogens and debridement of damaged tissue, but it continuation of the healing cascade and ultimately restoration of tissue structure and function. The specific aim of this review is to provide a brief overview of the current peer reviewed literature on the effects of ESWT on the inflammatory response and components of this important biological process.

INFLAMMATION

When describing inflammation it is imperative to distinguish between two very different types of inflammation: **acute** (a healthy response by the body to a harmful condition with the purpose of serving as the body's first line of defense); and, **chronic** (harmful, uncontrolled inflammatory response). The ultimate goal of acute inflammation is to neutralize/destroy injurious pathogens, create a barrier in order to limit the nature and extent of injury, to set in place cells and factors required for healing, and to alert the host to injury.

There are two major components of the inflammatory response: first, the vascular response, which includes all the changes of the vasculature within the inflamed area; and, second, the cellular response which represents changes of cells involved in the initiation, propagation and resolution of the inflammatory response. Because of the potential damaging effects of inflammation to healthy tissues, an active process of resolution of inflammation is required. Failure to resolution results in chronic inflammation, and concomitant cellular/tissue damage and destruction.

Effects of EWST on components of inflammatory response cascade.

This overview centers on research that at least partially explains the mechanism of the observed anti-inflammatory effects of ESWT. These studies were conducted *in vitro* and *in vivo*, e.g. ischemic flap animal model, ischemic muscle animal model, skin isograft mouse model, composite tissue allografts in rats, and animal model of severe burn wounds.

Vascular response to ESWT

Numerous studies have showed increased tissue perfusion and oxygenation after ESWT. We showed that not only tissue perfusion but also tissue vasculature permeability is affected by shockwaves. In a standard rat ischemic flap model, we showed an increase of topical perfusion in all zones and decreased edema formation (Mittermayr, Hartinger et al. 2011) following ESWT. In random patterns skin flaps (Yan, Zeng et al. 2008) and even in skin flaps with underlying co-morbidity (chemically caused diabetes in rats) a significant increase in blood perfusion was reported (Kuo, Wang et al. 2009). The induction of nitric oxide, a small ubiquitous molecule, is reproducibly correlated with ESWT, and its release from endothelial cells is one of the proposed mechanisms of the observed short term effects of ESWT on blood perfusion (Kuo, Wang et al. 2009).

Comparing pre-ischemic treatment vs. ischemia in the model of ischemic cremaster muscle, (Krokowicz 2008) showed a decrease in rolling and sticking of leukocytes as well as down-regulation of proteins involved in these two processes, specifically, ELAM-1, ICAM-1 and VCAM-1. An increased velocity of red blood cells was also observed in this model.

In 2011 the same group showed that shockwave pretreatment was associated with increases in red blood cell velocity in the arterioles of up to 40%, relative to ischemic controls ($p<0.05$). When shockwaves were applied after tissue injury, there was a quantifiable increase in functional capillaries of 21% ($p<0.05$), arteriole diameter of 33% ($p<0.001$), and red blood cell velocity in the arterioles of 65% ($p<0.001$) compared to ischemic controls.

Cellular response to ESWT

Several studies (Stojadinovic, Elster et al. 2008; Radu, Kiefer et al. 2011) have shown the effects of ESWT on tissue infiltration of inflammatory cells, notably a marked decrease in PMNs and macrophages (Davis, Stojadinovic et al. 2009), increase in fibroblast infiltration(Kuo, Wang et al. 2009) and their proliferation (Berta, Fazzari et al. 2009), recruitment of mesenchymal stem cells (Chen, Wurtz et al. 2004) and endothelial progenitor cells (Aicher, Heeschen et al. 2006). The observed changes in various cell populations most likely represents the combined effect of ESWT on vascular cell surface receptors (Krokowicz, Klimczak et al. 2012) and on gene expression of chemokines and cytokines in targeted tissue (Stojadinovic, Elster et al. 2008) of interst. Experiments utilizing ESWT in composite tissue allotransplantation, it was determined that rejection of the allogeneic hind limb was significantly delayed in ESWT compared to controls. Again, this was accompanied by significant reductions in inflammatory cell infiltrate. In severe, full-thickness, highly inflammatory burn wounds, Davis and colleagues (2008) evaluated the anti-inflammatory effects of ESWT. Analysis of chemokines, pro-inflammatory cytokines and matrix metalloproteinase (MMP) gene expression at burn wound margins of untreated and shock wave -treated BALB/c mice showed significant anti-inflammatory effects in the treated animals.

Shockwave therapy exerts its positive effects on the process of inflammation and healing by modifying the microenvironment within damaged tissue to a more favorable state through altered expression of genes crucial to the inflammatory process. In skin isografts (Stojadinovic, Elster et al. 2008) and hindlimb allografts (Radu, Kiefer et al. 2011) suppression of pro-inflammatory genes has been correlated with improved survival and healing after ESWT. In isografts, marked positive effects of treated animals when compared to sham controls was observed in the expression of chemokines (CXCL1, CXCL2, CXCL5, CCL2, CCL3, CCL4), cytokines (IL-1β, IL-6, G-CSF, VEGF-A), MMPs (MMP3, MMP9, MMP13), and macrophage derived factors [MIP-1a (CCL3), MIP-1b (CCL4), MMP-13].

Nitric oxide (NO) is one of the key molecules involved in angiogenesis and inflammation. Marked increases in blood vessels after ESWT was correlated to increased release and production of NO. Some authors propose that changes in NO levels are correlated with pathologies dependent on inflammation. In vitro studies on 2 cell lines showed that ESWT was able to counteract these changes and return NO to physiological levels by changes in gene expression of eNOS and consequent inhibition of NF-kB activation and other NF-KB-dependent inflammatory genes. Importantly, ESWT down-regulates NF-KB both before and after its activation. These findings provide further mechanistic insights to the clinically observed anti-inflammatory action of ESWT,

suggesting that it may be mediated by shockwave-induced increases in NO production. In vivo, pre-ischeminc treatment causes increases of iNOS, CXCL-5 and CCL-2 expression while post ischemic shock wave treatment decreases iNOS (Krokowicz, Klimczak et al. 2012)

Some authors have suggested that ESWT produces systemic effects. Kuo et al. 2009. showed that hydrogen peroxide (H_2O_2) levels in circulation were significantly decreased after ESWT, indicating potential systemic effect of ESWT on circulatory cells. Mittermayr et al. 2011. showed remote effects of ESWT in a transgenic mouse model for luciferase labeled VEGF receptor, finding increased expression of this protein in the contralateral untreated hind limb. Importantly, preconditioning of the tissues with shockwaves appears to have protective effects in ischemia/reperfusion injury; this occurs through the down-regulation of numerous pro-inflammatory proteins, which has been repeatedly shown to positively influence the healing process. Our team is currently working on evaluating ESWT pre-conditioning in two animal models: one of peripheral tissue trauma induced gastrointestinal dysmotility, another of postoperative inflammation and ileus. We are evaluating serum levels of various pro-inflammatory cytokines and chemokines after ESWT. Preliminary data indicates systemic effects of ESWT.

CONCLUSION

A growing body of peer reviewed literature supports ESWT effects on biological systems. Further laboratory studies and controlled clinical trials are indicated to better define the mechanism and role of ESWT, taking into account that this promising and seemingly effective therapy is currently approved for only limited a number of indications.

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Accumulated Total Energy Flux Density an Indicator to Compare Electrohydraulic and Piezoelectric Devices?

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INTRODUCTION

Piezoelectric and electrohydraulic devices are commonly used for shock-wave therapy. So far there is no way to compare the application parameters and energy flux densities of both devices. Generated shock-waves have different wave characteristics, which follow a slight curve in case of electrohydraulic and a cone shape in case of piezoelectric devices. Furthermore the load voltage of the generation principles varies whereby the emitted energy is different. The energy flux densities in the defined zones (-6 dB, 5 MPa, 5 mm) of a pressure-area curve of a single wave **(Figure Pressure zones)** can be measured and help to compare both machines.

MATERIALS AND METHODS

Normal human dermal fibroblasts were treated using the IVSWT Water Bath. Shock-wave machines used were Orthowave 180c CP-155, MTS Europe GmbH or PiezoWave F7G3, Richard Wolf GmbH. Seven Days after treatment proliferation results compared to control were measured.

Several distances between applicator and cells were tested, as well as different energy levels and number of pulses. With the theorem of intersecting lines the treated diameter was calculated **(Figure Treated diameter)**. Considering the number of applied pulses, the emitted energy in the observed zone and the treated area, an accumulated energy flux density for every zone was calculated.

RESULTS

First, the measured emitted energies, which imply the level of intensity, were plotted against the load voltage and compared in every energy zone **(Figure Regression curves)**. As a result it could be seen that only the 5 MPa zone was useful for analysis. The emitted energy of the piezoelectric device is in the -6 dB zone nearly stable for every level of intensity. This follows from an increase in energy flux density but decrease of the focus zone. In the 5 mm zone the area of the focus from the electrohydraulic device is not covered. Loss of effective energy compared to the piezoelectric device follows from that.

Proliferation results for both principles of shock wave generation compared to control were plotted against the accumulated energy flux density **(Figure Graphs proliferation and energy)**. Both devices show comparable results, which can be seen in the first graph. The curve, created from the results of the PiezoWave and dermagold, follows a skew distribution with steep increase to lower and slight decrease to higher accumulated energy flux densities. Statistically significant enhancement of proliferation is shown between an accumulated energy flux density of 1-4 mJ/mm^2 in both cases ($n \geq 3$; Mean±SD; *p<0,05; **p<0,01; ***p<0,001). Over 9 mm/mm^2 energy input a significant reduction of cell numbers due to cell loss is shown. That means that accumulated energy flux densities in the 5 MPa zone are comparable for electrohydraulic and piezoelectric principles of generation.

The second graph shows that the energy, which is necessary for a positive effect, cannot applied with a single pulse. Choosing energy flux densities over 0,01 mJ/mm^2 significant reduction of cell numbers is caused.

CONCLUSION

The effect of shock-wave treatment with both electrohydraulic and piezoelectric devices on the proliferation of fibroblasts can be compared for same accumulated energy flux density in the 5 MPa zone. Between 1-4mJ/mm^2 an equal enhancement of proliferation was proved in both cases.

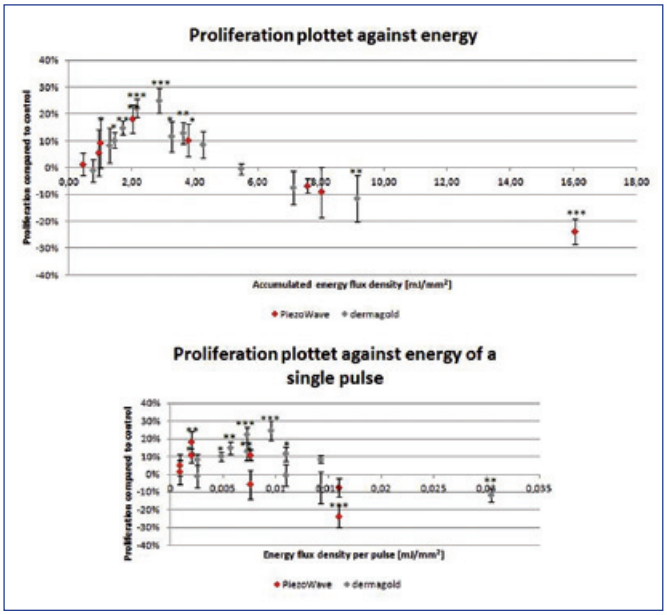
DISCUSSION

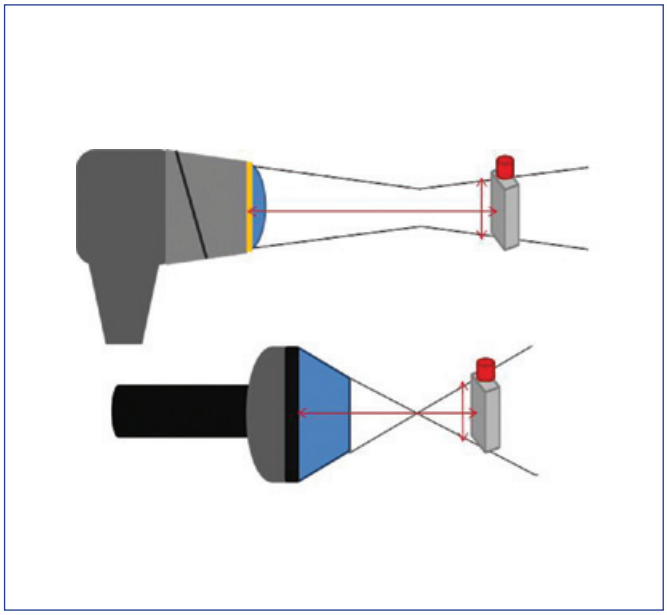
Presented data show results which are only measured for the proliferation of fibroblasts seven days after treatment. Some accumulated energy flux densities were not analysed because experiments were already finished before this way of analysis was developed. To compare both devices for clinical usage more experiments have to be carried out to verify these results.

ACKNOWLEDGMENT

We thank Mr. Christian Dorfmüller for his great idea to compare both devices in this way, his helpful explanations, interesting discussions and valuable support.

FIGURES





Extracorporeal Shock Waves modulate osteogenic differentiation of human mesenchymal stem cells

1 

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Human mesenchymal stem cells (hMSCs) are a promising candidate cell type for regenerative medicine and tissue engineering applications due to their capacity of self-renewal and multipotent differentiation. In particular, bone tissue engineering using hMSCs has the purpose to treat patients with trauma, spinal fusion and large bone defects. The adipose tissue holds MSCs which share characteristics with MSCs derived from bone marrow, such as high proliferative capacity, and the ability to differentiate into diverse mesenchymal cell lines, after the addition of peculiar growth factors (1). To date, little is known about the effects of physical stimulation on the differentiation of these cells.

Extracorporeal shock waves (ESWs) are acoustic waves that can induce a mechanical wave that passes through the cell compartment with cavitational effect; the cell response is proportional to the energy used. Bone and tendon regeneration enhanced by ESW treatment suggests that ESW may induce some signals for growth and maturation of the mesenchymal progenitors.

Aim of the present study was to evaluate the modulation of ESWs on the osteogenic differentiation of hMSCs induced by osteogenic medium.

METHODS

Human adipose-derived stem cells (LPA cells, provided by dr. Laura de Girolamo, IRCCS Galeazzi Orthopaedic Institute, Milan, Italy) - obtained from subcutaneous fat of healthy donors undergoing plastic surgery by elective lipoaspiration - were assessed for specific **mesenchimal stem cells markers** by cytofluorimetric (FACS) analysis. Cells were routinely maintained in control medium (DMEM/F12 plus FCS 10%). Control cells and cells to be submitted to ESW treatment (1 ml of cell suspension [1x106 cell/ml]) were placed into 20 mm polypropylene tubes completely filled with either control or osteogenic medium. Osteogenic medium consisted of control medium supplemented with 10 mM glicerol-2-phosphate, 10 nM dexamethasone, 150 µM l-ascorbic acid-2-phosphate and 10 nM cholecalciferol. Focused ESW treatment: 1000 shots, EFD: 0.32 mJ/mm^2 (Piezoson 100, Richard Wolf, Knittlingen, Germany).

After combined treatment with osteogenic medium and ESWs, we determined: cell viability by trypan blue exclusion; cell proliferation by WST-1 colorimetric assay; alkaline phosphatase (ALP), osteocalcin (BGLAP) and the two factors Runx2 and Ets-1 (transcription factor for the differentiation of MSCs to osteoprogenitors), Collagen type I and CD105 (co-receptor for Transforming Growth Factor-β1 [TGF-β1], involved in TGF pathway) by RT-real time PCR and, respectively, phenotypic profile by FACS analysis for cell markers: CD13, CD14, CD34, CD44, CD45, CD90, CD105. The nonspecific fluorescence was assessed by incubating cells with monoclonal anti-Human IgG. To detect and quantify the mineralization process, calcium deposits were shown up by staining the cells with Alizarin red S.

RESULTS

The combined treatment (osteogenic medium [OST] + ESW, 1000 shots) determined a higher increase of ALP

and Runx-2 expression (72 hours after treating cells) with respect to treatment with osteogenic medium alone, as shown in **Figure 1**.

In addition, ESW treatment determined a reduction of CD105 expression in cells maintained either in DMEM or in osteogenic medium (OST), both on day 5 and day 7 following treatment **(Figure 2)**.

CD105 has been shown to function as a regulator of TGF-β/TGF-β receptors signaling. In cells with lower CD105 expression, an enhanced human adipose-derived stromal cell osteogenesis has been reported (2).

Lastly, 21 days after maintaining cells in osteogenic medium (OST), histochemical analysis showed that calcium deposition was *more evident* in ESW treated cells than in control cells that did not receive ESWs **(Figure 3)**.

CONCLUSIONS

These data allow to regard ESW treatment as a new tool to accelerate osteogenic differentiation of human mesenchymal stem cells. Our previous observation on ESW induced activity of osteoblast-like cells in bioactive scaffolds (3) suggests to address future investigations to evaluate the role of dynamic cell seeding onto scaffolds. Seeding Mesenchymal Stem Cells exposed to ESW onto appropriate scaffolds will allow to verify the effect of ESW treatment on cell migration within the scaffold and on bone production.

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FIGURES

Figure 1

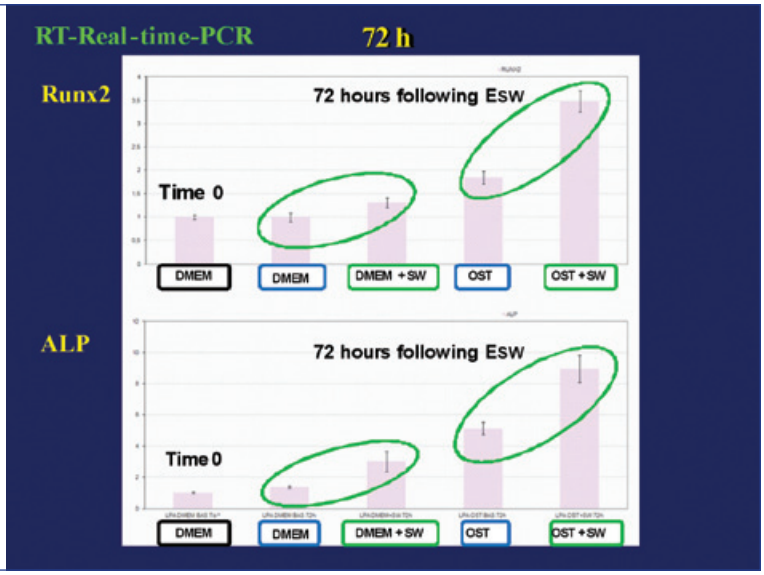


Figure 2

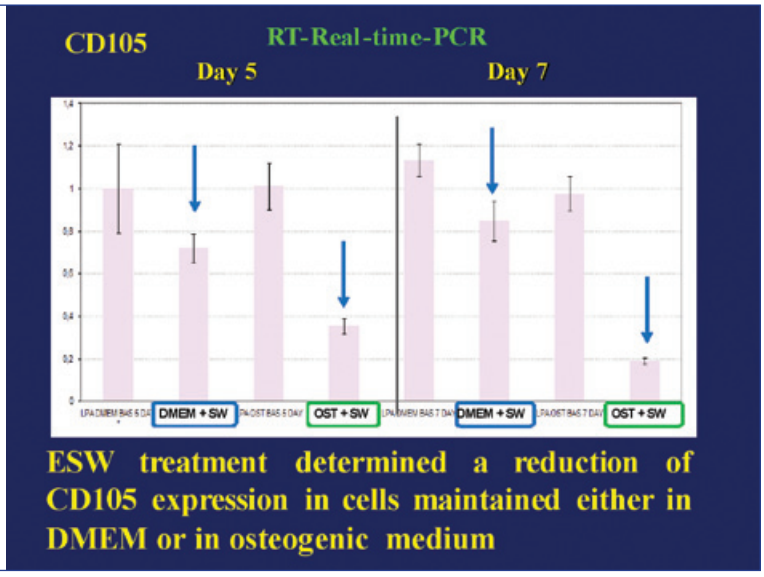
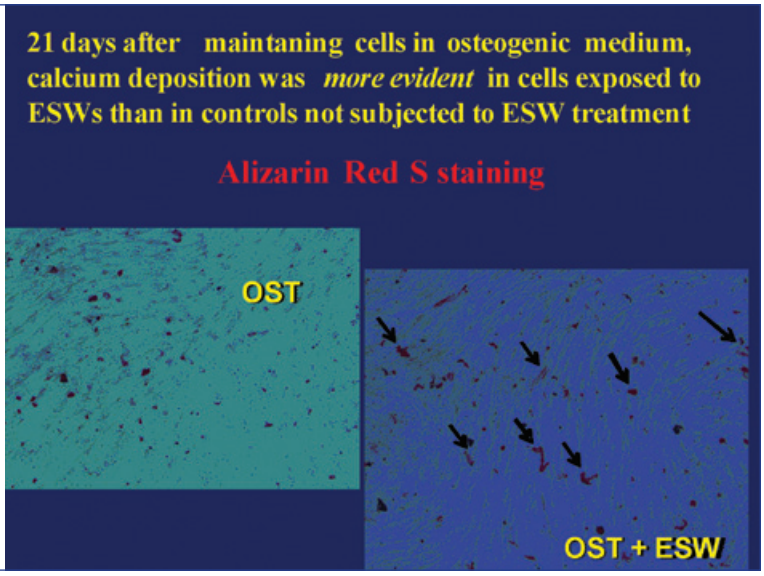


Figure 3



Unfocused Extracorporeal Shock Waves Induce Anabolic Responses in Osteoporotic Bone

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INTRODUCTION

Current therapy for osteoporosis aims at reducing further bone loss using bisphosphonates. It was previously shown that non-osteoporotic rats treated with unfocused extracorporeal shock waves (UESW) had higher cortical and cancellous bone volumes and improved mechanical properties (Van der Jagt et al. JBJS 2011;93:38-48) In the current study we examined the effects of unfocused ESW in osteoporotic rats. To explore the clinical value of ESW for patients that do or do not receive anti-resorptives, rats were treated with or without a bisphosphonate.

METHODS

Female Wistar rats received an ovariectomy (OVX). Two weeks after OVX one group received saline (n=9) and another group received alendronate (n=9). At 0 weeks 1000 ESW were applied to one hind leg, the other was not treated and served as control. At 0,2,4, and 10 weeks after ESW in vivo microCT-scans were made. Cancellous and cortical bone changes were analyzed. Furthermore mechanical testing and histological analysis were performed. Paired t-tests were used for statistical analyses.

RESULTS

In saline treated rats ESW resulted in higher cancellous bone volume at 2 weeks (p=0.003), but not at 4 and 10 weeks **(Fig.1a)**. ESW resulted in higher cortical volume at 2, 4 and 10 weeks with respectively 3.2, 5.5, 5.5 % more than the untreated control side **(Fig.2a)**.

In rats receiving alendronate ESW resulted in higher cancellous bone volume at 2, 4 and 10 weeks (p=0.002; p=0.001; p=0.001, respectively) **(Fig.1b)**. ESW resulted in higher cortical volume at 2,4 and 10 weeks with respectively 7, 10.5, 12 % more than the untreated control side **(Fig.2b)**.

In both groups ESW treated legs showed significant higher maximal force at failure. Large areas of direct bone formation were observed a the cortex and around de novo bone niches in the marrow of ESW treated legs. Intramedullary soft tissue damage, but no periosteal or bone micro damage was observed.

CONCLUSIONS

Unfocused ESW drastically increase cancellous and cortical bone volume and improve biomechanical properties. When shock wave treatment is combined with an anti-resorptive treatment these beneficial effects are enhanced and retained. This study shows promising results for the use of UESW in the treatment of osteoporosis, but more research is needed to further investigate the biological responses and the safety for human therapy.

FIGURES

Fig. 1 Cancellous bone volume.

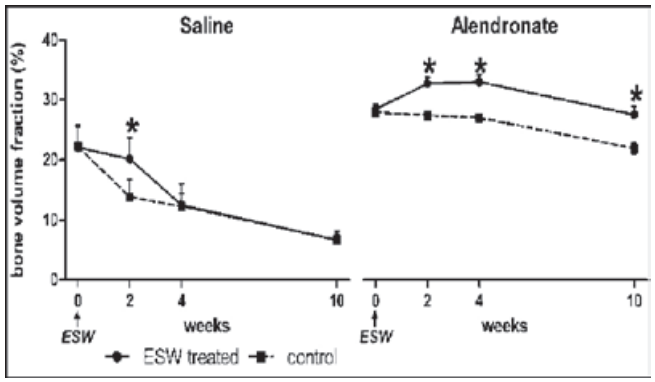
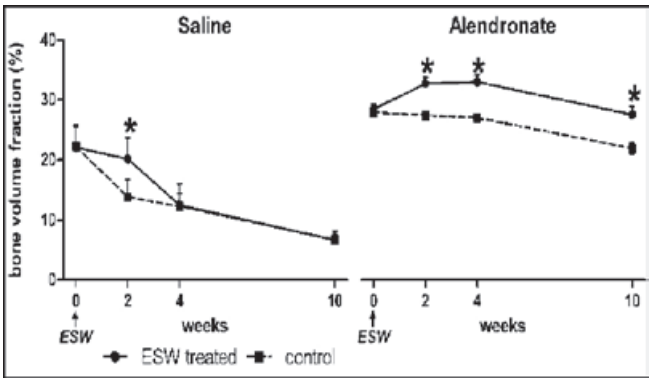


Fig. 2 Cortical bone volume



Extracorporeal Shockwave Therapy and Gene Expressions

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ABSTRACT

The recent advance in microarray technology has made it possible to investigate the tissue specific patterns of gene expression and their relationship with the specific

tissue lineages. Conservatively expressed genes or gene sets define common functions in a tissue group and are related to tissue specific disease. Differentially expressed genes contribute to the functional divergence of tissues.

The quantification of gene expression by real-time polymerase chain reaction (PCR) has revolutionized the field of gene expression analysis. Due to its sensitivity and flexibility, it is becoming the method of choice for many investigators.

The commonly utilized gene expressions in shockwave research include angiogenesis (vWF, eNOS, VEGF and CD31). Osteogenesis (BMP-2.osteocalcin, alkaline phosphatase, RUNX-2, DKK1 and Wnt /ß catenin), proliferation (PCNA, EGF,TGF-ß,BRDU and MMP13) and inflammatory (IL1-ß, IL 1& 6,TNF-α and TUNEL). The presentations illustrate the changes of gene expressions in chronic diabetic foot ulcers (DFU) and osteoarthritis of the knee.

In DFU, the hypothesis suggests that the effects of ESWT in DFU are linked to the improvement in blood flow perfusion and tissue regeneration. The blood flood perfusion scan confirmed the improved blood flood perfusion rate after ESWT that correlated with the healing of DFU. The analyses of gene expressions including vWF, eNOS, VEGF, PCNA, EGF and TUNEL confirmed the initial hypothesis.

In osteoarthritis (OA) of the knee, the initial hypothesis speculates that ESWT may improve subchondral bone remodeling that in turn prevents the initiation of OA with damage to the articular cartilage. X-rays, BMD and histomorphology (Mankin score and Safranin O stain) showed more advanced OA changes after anterior cruciate ligament transection (ACLT), while the ESWT-treated knees showed very subtle degenerative changes of the knee. The analyses of gene expressions including DKK-1, Wnt-5a, β-catenin and MMP13 confirmed the findings and concluded that ESWT shows chondroprotective effects in
the initiation of ACLT OA of the knee In rats.

In conclusion, the sensitive, convenient and flexible microarray technology has revolutionized in the investigation of the tissue specific patterns of gene expression and their relationship with tissue specific disease. With proper selection of the specific gene expressions for the specific disease condition, the researchers are able to accomplish the results more accurately and efficiently before full-blown disease manifestation.

Instructions for Authors

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We encourage authors to submit manuscripts via e-mail. When submitting by e-mail, print mail address and telephone and fax numbers also should be included.

MANUSCRIPT CATEGORIES

All articles should be well-written in plain English, whereby jargon, acronyms, abbreviations and complicated data should be avoided.

Scientific research

Theoretical or experimental (basic or applied) scientific research or the practical application of this research. The article should consist of an abstract, key words, introduction, methods, results, discussion, and conclusion.

Length: The manuscript should be no longer than 2,500 words, including title page, abstract, references, legends and tables.

Review articles

Review articles on topics of general interest are welcomed. Reviews should include the specific question or issue that is addressed and its importance for the shockwave therapy community, and provide an evidence-based, balanced review on this topic. The article should include a description of how the relevant evidence was identified, assessed for quality, and selected for inclusion; synthesis of the available evidence such that the best-quality evidence should receive the greatest emphasis; and discussion of controversial aspects and unresolved issues. Meta-analyses also will be considered as reviews. Authors interested in submitting a review manuscript should contact the editorial office prior to manuscript preparation and submission.

Length: Approximately 2,000 to 2,500 words and no more than 40 references.

Case reports

Authors are encouraged to submit articles with interesting case reports with relevant information regarding diagnosis and therapy, unique for shockwave therapy. The articles should be short, accurate and easy to understand, and should consist of the following:

- A summary with the clinical relevance;
- An introduction explaining the clinical problem;
- A short report of the cases, consisting of history, physical examination, further investigation, treatment and follow-up.
- A discussion, whereby the clinical consequences are described and the most interesting aspects of the case report.

Length: Approximately 750 to 1,200 words and a maximum of 15 references.

Clinical lesson

Authors are invited to give a description and background information of developments in the field of further diagnostics and clinical tests and methods that are relevant to all aspects of shockwave therapy, training and rehabilitation. It is not necessary to include examples of patients, as in case reports. The articles should be up-to-date, short, accurate, and easy to understand and should contain the following:

- A summary with the clinical relevance (max. 150 words)
- And introduction with the theme of the article
- A description of the used test method or diagnostic
- A conclusion with the practical relevance and practical tips.

Length: Approximately 750 to 1,200 words and a maximum of 5 references.

National organisation communications

National organisations are invited to describe any aspect of medical care or science in their country, e.g. the function of their medical committee, medical care of their players, research that is being conducted etc.

Approximately 300 to 500 words.

Letters to the editor

Letters discussing an article that has been published in Journal of Extracorporeal Shockwave Therapy have the greatest chance of acceptance if they are sent in with 2 months of publication. Letters that are approved will be forwarded to the author, who will have 6 weeks to respond. The original letter and the reply will be published simultaneously.

Length: Such letters should not exceed 400 words of text and 5 references. Research Letters reporting original research also are welcome and should not exceed 600 words of text and 6 references and may include a table or figure.

Review of the Literature

Authors are invited to submit summaries of published article of particular interest for the shockwave therapy community. The opinion of the author should be stated following each summary.

Length: Such a review should be approximately 500 to 700 words. A review of three articles simultaneously should be no longer than 1,000 words.

Conference reports and Abstracts

Authors are invited to submit reports of conferences they have attended, and to include one to three photographs taken at the meetings. Please include the names and highest titles of the persons that can be identified in the photographs. Summaries of work presented at the conference may be submitted for publication as well.

Length: 300 to 500 words per report or abstract.

MANUSCRIPT PREPARATION

Manuscripts should be prepared in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (Vancouver Style). http://www.nlm.nih.gov/bsd/uniform_requirements.html

- If submitting by e-mail, text, tables, and figures should be included in the same file. Do not submit duplicate copies by mail or fax.
- Articles should be in Microsoft Word format.
- Double-space throughout, including title page, abstract, text, acknowledgements, references, figure legends, and tables.
- Do not use abbreviations in the title or abstract and limit their use in the text.
- Please use Times New Roman, size 12.
- On the title page include the full names, highest academic degrees, and affiliations of all authors. If an author's affiliation has changed since the work was done, list the new affiliation as well.
- Figures, summary tables and diagrams should be numbered consecutively throughout the paper. Photographs should be clearly labelled.
- References. Number references in the order they appear in the text; do not alphabetise. In text, tables, and legends, identify references with superscript Arabic numerals. When listing references, follow AMA style and abbreviate names of journals according to Index Medicus. List all authors and/or editors up to 6; if more than 6, list the first 3 followed by et al.

– Journal: Kibler WB. The role of the scapula in athletic shoulder function. Am J Sports Med. 1998;26(2):325-337.

– Book: Perry J. Biomechanics of the shoulder. In: Rowe CR, ed. The shoulder. London: Churchill Livingstone, 1988:1-15.

- Footnotes should be avoided.

REVIEW PROCESS

Contributions will be reviewed by the editorial board for scientific research, review papers, case reports, clinical lessons, and abstracts. Manuscripts should meet the following criteria: material is original; writing is clear; study methods are appropriate; the data are valid; conclusions are reasonable and supported by the data; information is important; and topic has general shockwave therapy interest.

Manuscripts with insufficient priority or quality for publication are rejected promptly. Other manuscripts are sent to expert consultants for peer review. Peer reviewer identities are kept confidential, but author identities are known by reviewers. The existence of a manuscript under review is not revealed to anyone other than peer reviewers and editorial staff.

INTELLECTUAL PROPERTY

- The article must be your own original work.
- If the article contains any photographs, figures, diagrams, summary tables, graphs or other non-textual elements that are not your own original work, you must ensure that you have obtained written permission from the copyright owner to include their work in your article for publication in Journal of Extracorporeal Shockwave Therapy. Permission letters must be submitted with your article.