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Manifestation Pattern of the Extracorporeal Shock Wave Therapy Using Mechanotransduction

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Manifestation Pattern of the Extracorporeal Shock Wave Therapy Using Mechanotransduction

Taken from the ZES Kronberg, and the pharmaceutical institute of the University of Hamburg (Manager: Prof. H.J. Duchstein)

Introduction

Extracorporeal shock waves can be seen as a mechanical stressor that is able to induce biochemical changes in living tissue and which can ultimately have an influence on the gene expression of cells at molecular level; consequently, when used selectively, they can produce a specific tissue reaction. This process is referred to as mechanotransduction.

Mechanical stimuli affect almost all cellular functions of living tissue such as growth, cell differentiation, cell migration, protein synthesis, physiological apoptosis and tissue necrosis. Mechanical forces have a direct influence on the form and thereby on the function of tissue. Cellular structures are subjected to a constant mechanical stress level of 0.01 to 0.1 ATM in vivo. This corresponds to 1 to 0.1 nN per cellular contact. Even the smallest of changes in the strength and distribution of these forces can lead to a compensatory restructuring of the cell matrix and cell-cell contacts, thereby bringing about a whole range of changes in cellular behaviour. In everyday life, their influences are most strongly seen in hearing, taste and touch.

As we assume that this transformation of mechanical stimuli into biochemical reactions does not take place through cellular activity that can be evoked by ligands via receptors on the surface of the cells, but rather through general changes to the form of the cellular membrane, mechanotransduction is distinguished in the literature from other biological signalling pathways. Membrane and secreting proteins and peptides, small lipophilic molecules, such as steroid hormones or thyroxin, small hydrophilic molecules derived from amino acids, such as catecholamine, and gases take part in these signalling pathways. The extraordinarily large variety of receptors on the surface of cells can be placed into one of three main groups: G protein coupled receptors, ionic channels with cytosolic tyrosine-kinase coupled receptors with their own catalytic activity.

All signalling pathways are a focussed flow of information whose signals can be seen as biological information units. They produce specific biochemical changes in the cell itself for which the signal is destined. This also applies to mechanotransduction and its resulting reactions, which however have their own independent physiological process.

If we assume that mechanochemical conversion on the whole takes place on the surface of the cell, there must be corresponding structures that convey that process. Very recently, stress-sensitive mechanoreceptors have been found all over the membranes on the surface of cells. When they are stimulated using mechanic stimulation, the ion flow can then be regulated up or down, resulting in stimulation of the cell nucleus.

The structures that make this transfer possible are called the cytoskeleton and the extracellular matrix. Both of these are connected to each other through cell-cell and cell-matrix bonds whose main structural task is to

hold cellular tissue together. These connections fulfil this function by connecting the inner cytoskeleton directly with the cell exterior – either with another cell, or with the extracellular matrix. There are essentially two groups of so-called cell adhesion molecules (CAM), cadherin and integrin, that take part in this process.

The extracellular matrix is a usually insoluble network of polysaccharides such as glucosamineglycans, fibrous proteins such as collagen, laminin, fibronectin and other adhesive proteins that can be excreted from animal cells. It stabilises the tissue and affects the biochemical performance of cells. Laminin is particularly important for mechanotransduction. Laminin is a multi-adhesive protein located in the basal lamina which bonds with heparan sulphate, type IV collagen and specific cell surface receptors. Fibronectins which are also multi-adhesive proteins connect collagen and other matrix proteins with integrin, thereby forming a bond between the cell and the matrix.

The cytoskeleton is a three-dimensional network of fibrous proteins in the cytoplasma of eukaryotic cells which serves as carrier material for their structure. It also stabilises the cells and the position of the intracellular organelles and is at the same time responsible for the movement of the organelles and chromosomes during mitosis as well as of the cell itself. It is comprised of three different components: microtubules (diameter 25 nm), actin-microfilaments (6-9 nm) and intermediate filaments (10 nm).

Summary

Key words: extracorporal shock waves – mechanotransduction – extracellular matrix – integrin – cadherin – cytoskeleton – second messenger – mesenchymal stem cell – heat shock proteins

Extracorporal Shock Waves Manifest Themselves as Biological Mechanotransduction

The impact of extracorporal shockwaves (ESWs) on living tissue results in the conversion of mechanical stimuli into biochemical and/or molecular-biological signals. These signals in turn induce a certain flow of information. Subsequent signals are viewed as a biological information unit that brings about certain biological changes in the cell itself for which the signals are meant. This sequence is referred to as mechanotransduction.

The tissue structures mainly involved in mechanotransduction are part of the extracellular matrix that transfers information via so-called adhesion molecules, as connecting links to the cytoskeleton. The signals are transmitted to the cell nucleus via the constituent components of the cytoskeleton, thereby inducing gene transcription and expression.

In case of destruction of the cytoskeleton mechanotransduction is rendered impossible.

Specific so-called mechanogated membrane ion channels which belong to the DEG/ENAC super-family are responsible for initial and fast prompting of mechanotransduction.

Relevant for the mechanotransduction are the frequency, amplitude, intensity and duration of the extracorporal stimuli which determine – as if by code – the concentration of certain second messengers and thus turn on the gene expression.

Cadherins form a family of Ca2+ dependent CAMs that take on a crucial role during cell-cell adhesion and are extremely important for the differentiation and structure of various tissues.

Integrins are the most important adhesive proteins between cell and matrix. They are made of heterodimers formed of alpha and beta sub-units that bond with the cell-fixation areas of fibronectin, laminin, collagen type II and IV and other matrix molecules. Via the integrins, among other things, autocrine stimulation of interleukin 4 receptors takes place, which in turn affects gene expression via the janus kinase or phospholipase C path; phospholipase C is responsible for the intracellular release of CA2+ ions, which in turn help to control gene expression through intermittently producing changes in cytosolic concentration levels.

Mechanoreceptors are cation channels (Na and Na/Ca channels) that control genes that are activated when the cell membrane is stretched or touched. It is suspected that the stretch receptors belong mainly to the initial protein receptors which came into being during the evolution of the organism. Very recent molecular biology and electrophysiological tests have shown that the rapid,

initial impetus for mechanotransduction takes place via specific, so-called "mechano-gated membrane ion channels" which belong to the DEG/ENaC super family (degenerin/epithelial sodium channel). Degenerins were originally identified during a genetic screening of touch-sensitive worms (caenorhabditis elegans) which are known as the so-called mec-mutants (mechanosensory defect). They form a bond between the cytoskeleton and the extracellular matrix (ECM).
Mec-18 is very similar to the tubulins, which are monomer components of microtubules that extend through the cytoplasma as long, stiff polymers and control the position of the organelles surrounded by a membrane and other cellular components. Mec-2 is 75 % similar to mammalian stomatin, a commonly found integral membrane protein. The carboxy terminus of stomatin has been proven to bond with cytoskeletal proteins. Mec-9 is excreted from sensitive nerve endings and is combined with mec-5, which resembles collagen.
The form, duration and amplitude of the stimulation are of great importance during mechanicochemical conversion taking place in living tissue. Hydrostatic, shear force and tension account for the greater part of this mechanical effect. It is known that hydrostatic compression of the cell over a long period of time down-regulates its biosynthesis ability. If dynamic, intermittent compression is applied, however, biosynthesis is regulated upwards. Too high a frequency appears also to have a negative impact on the biosynthesis of cells.
It is also known that the individual tissue structures react differently to mechanical forces. Bone and cartilage tissue are affected by hydrostatic forces, while shear force has an impact on tendon and muscle tissue.

Mechanotransduction: extracorporeal shock waves

The effect of extracorporeal shock waves (ESW) on living tissue constitutes a complex, signal-giving stress situation that manifests itself at various interacting levels.
We can firstly assume that the mechanical stimuli that have an effect on the cellular structures of the corresponding tissue by way of ESW are converted into chemical signals. For the most part, these signalling pathways correspond to the general processes that we were already aware of, and which are described in the introduction. In order to operate as a single integrated whole, multi-cellular organisms had to develop complex mechanisms in order to transmit these signals throughout the whole body. This overall coordination relies on one main mechanism, namely the transfer of mechanical forces through viscoelastic tissue, various cellular structures and fluids.
The mechanisms of mechanotransduction are essentially the same for the various types of tissue, but there are various signalling paths for each individual type of tissue which bring about the desired cellular reaction.
Of particular importance, in the signal cascade is the activation of so-called second messengers.
Second messengers are low molecular intracellular signalling molecules whose concentration increases or decreases in reaction to the activation of certain cell surface receptors. Examples for this are cAMP, cGMP, Ca2+, diacylglycerol (DAG), inositol 1,4,5, triphosphate (IP3) and nitrogen monoxide. In the case of mechanochemical conversion of extracorporeal shock waves, our own experiments appear to show that Ca2+ and nitrogen monoxide are attributed an outstanding role.
As a slightly chelate-forming ion, Ca2+ helps proteins and lipids in cell membranes to reach structural stability in organelles and chromosomes. Ca2+ bonds with great affinity to tubulin and is necessary to enable the cell to enter the S-phase of the cell cycle. Contractility, chemotaxis, and aggregation are regulated by Ca2+ and the arachidonic acid metabolism. Ca2+ plays an important role in transmitting nerve impulses and in muscle contraction. After a mechanical stimulus of the cell membrane, the mechanogated membrane ion channels are activated, allowing Ca2+ ions into the cell; at the same time, 4,5 diphosphinositol is hydrolysed to inositol 1,5,5 triphosphate and diacylglycerol (DAG). DAG itself is also a second messenger that contributes indirectly to - among other things - the release of intracellular Ca2+ through the phosphorylation of various proteins. This causes the intracellular Ca level to rise briefly to around 1 µmol. Thanks to this concentration, Ca2+ activates some proteins directly and others indirectly by binding

to calmodulin. Many enzymes, pumps, membrane transport proteins and other target proteins are regulated by Ca2+ / CaM, but most effects are not triggered directly by calmodulin, rather by protein kinases that are dependent on Ca2+ / CaM. Protein kinase C is subject to coordinated regulation by Ca2+ and DAG. While Ca2+ is fixing the enzyme in the membrane, it is activated by DAG.
We might consider the influence of the migratory activity of adult mesenchymal stem cells - demonstrated by us for the first time - as an excellent example of mechanotransduction brought about by ESW. We were able to show that through focussed extracorporeal shock waves, the migration of mesenchymal stem cells (MSC) can be significantly increased.
Another factor that can be cited as an effect of extracorporeal shock waves as a mechanical stressor is the generation of free radicals, to which we had basically attributed the release of so-called oxidative stress in the past. We have since learned however that they play a significant role as signalling and modulator molecules. Free radicals are taken to be atoms or molecules that have one or several unpaired electrons, and as a result are highly chemically reactive. The most important examples of this are superoxide, hydroxyl, and nitric oxide radicals. We turned our attention particularly to the generation of NO after ESW induction. We have been able to prove this in vitro using electron paramagnetic resonance spectroscopy (EPR) and in vivo with a modified NO analyser. The three most important qualities of NO as a signalling molecule are:

1. the activation of guanyl cyclase through the reaction of NO with the heme iron of this enzyme, with cGMP formation and subsequent vasodilatation
2. the neuroprotection of NO observed under oxidative conditions, whereby the neuroprotection is attributed to the interaction of NO with thiolate ions in the ion channel of an NMDA receptor. The inactivation of the excitatory amino acids results from this.
3. Protection against foreign bodies through the activation of macrophages with the simultaneous presence of reactive oxygen species (respiratory burst).

The activation of a very old - in evolutionary terms - protection and defence system of living organisms is also part of the complex mechanism of action of extracorporeal shock waves. The cellular response of an organism to a stress exerted from the outside, be it of a physical or chemical nature, is to form heat shock proteins (HSP), quickly and in large quantities. They occur in almost all pro and eukaryotic cells, and are accordingly divided into groups according to their molecular mass, where the number given in the abbreviation indicates the approximate molecular mass in kilodaltons (kDa). We have managed through mechanically stimulating muscle tissue by way of extracorporeal shock waves to prove the expression of HSP.

Summary and outlook

The biological and molecular biological mode of action of extracorporeal show waves (ESW) is based on the conversion of mechanical stimuli into biochemical signals. Generally speaking, an energy transfer takes place, from mechanical into chemical energy.
In order for ESWT to work, the stimulus applied must produce its effect on the target site – the target cell or the cell nucleus - in such a form that the corresponding targeted flow of information leads demonstrably to the desired biochemical changes in the cell itself.
As ESWT is used as a therapeutic measure, two effects should be produced: specific substances within the body such as cytokines, growth factors, neurotransmitters, heat shock proteins and RONS (reactive oxygen nitrogen species) should be activated to incite the organise to generate new tissue; and the gene expression of the cell should be affected at molecular level.
As in music, where it is possible to create pieces of music that sound different using the same instruments, by changing the oscillation, tempo and rhythm in order to produce another tone, individual tissues respond differently to the frequency and amplitude of mechanical stimulation, as well as to the type of forces released. The cellular morphology, that is to say the sound body of the cell, appears to play a role in mechanotransduction.
In order to find the right tone for the various areas of application for ESWT, various parameters are available to

set the frequency and the magnitude of the shear force to stimulate bone cells for example. These parameters could be determined by the amount of intracellular calcium, the release of prostaglandin, and in molecular biology by the bone-specific protein, osteopontin.

When mechanically stimulating the cartilage, in addition to the frequency and the amplitude of the compression, the duration of the stimulation also plays a decisive role. In this case too, the intermittent release of Ca2+ appears to play a decisive role, since, by changing the frequency of the concentration of Ca2+ in the cytoplasmic matrix, the gene expression can be controlled, as if applying a code.

In epithelial tissue, the modulation of the force applied is of importance. Shear forces, that among other things initiate NO generation and the ensuing vasodilatation, are of prime importance here.

The fact that it is possible to activate mesenchymal stems cells in a targeted and controlled manner opens up the possibility of observing and monitoring other signalling and differentiation paths of MSC. This is not only of huge importance in the orthopaedic and surgical field with regard to the therapeutic use of ESW, but is hugely beneficial for cardiovascular regeneration.

The modes of action sketched out here of extracorporeal shock waves are certainly still incomplete. The list of biochemical and molecular biological effects is long. The main issues are on the one hand finding the optimal frequency, amplitude and duration of ESWT stimulation and the amount of force that has to be delivered to induce the regeneration of the various tissue structures, and on the other hand the objective documentation and visualisation of the molecular biological changes. This latter appears to have moved nearer to being within our grasp thanks to the possibility of molecular magnetic resonance tomography (mMRT).

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Induction of Adult (Tissue-specific) Mesenchymal Stem Cells through Extracorporeal Shock Waves to Regenerate Muscoskeletal Tissue

Taken from the ZES Kronberg, and the institute for cell and molecular biology, Sporthochschule Köln (Manager: Prof. W. Block)

Introduction

The so-called stem cells are the original cells of highly differentiated cells. After division, the daughter cells can either turn back into stem cells (self-renewal) or differentiate into tissue-specific cells such into cardiac or skeletomusclular cells, cartilage-bone cells, nerve, skin, conjunctive tissue or fat cells; whereas, precursor cells can only differentiate into specialised cells, e.g. neutrophils or blood cells (fig. 1).

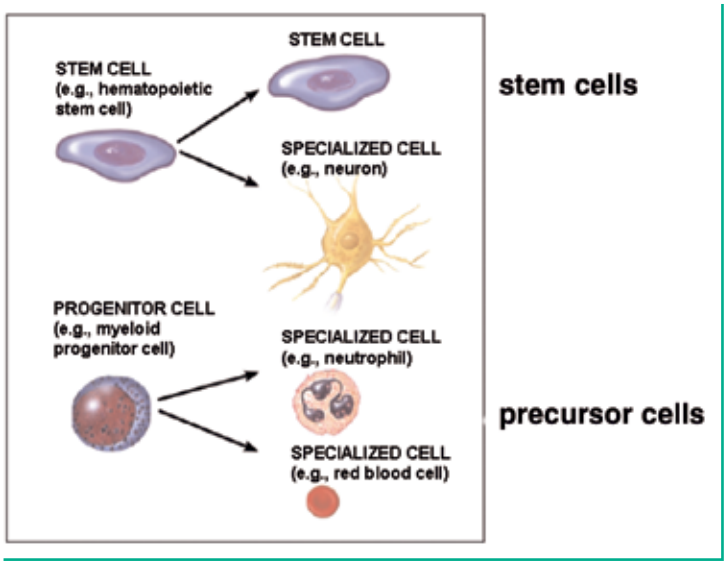

Fig 1: Stem cells and precursor cells.

Stem cells occur for the first time during early embryonic development, where the fertilised egg (zygote) constitutes a totipotent stem cell that goes through the early embryonic stages and from which later on all tissue of the human body is formed (fig. 2).

The more the daughter cells of stem cells become specialised, the more severely limited the subsequent potential for differentiation into different type of tissue will be. Even in adults, there are stem cells in many types of tissue throughout the person's life, albeit in decreasing number as the person grows older. They have an important task in tissue regeneration and repair, and keep tissue and organs in working order through providing differentiated cells, replacing damaged and dead cells.

Usually, we make a distinction between adult and embryonic stem cells, although it would be more accurate to talk of tissue-specific instead of adult stem cells.

Adult stem cells can differentiate into different types of tissue; they are multipotent cells and occur mainly in bone marrow and in the CNS (fig. 3).
Bone marrow contains two types of stem cells: hematopoietic stem cells (HSC) and stromal stem cells (mesenchymal stem cells, MSC).
Mesenchymal stem cells have the ability to develop both in vivo and ex vivo into many types of connective tissue. The differentiation of mesenchymal stem cells into various types of connective tissue has similarities with haematopoiesis and is therefore often referred to as mesengenesis.
The treatment carried out by us over the past two years on post-traumatic and degenerative cartilage damage, especially in the knee and hip joints, using extracorporeal shock waves led to a significant, subjective improvement in pain levels in the patient. This treatment has also been proven to work through observations of changes to the cartilage using MRT in specific cartilage sequences. This inevitably led to the question as to whether mechanical stress exerted from the outside, in our case in the form of extracorporeal shock waves, can boost the mobilisation of stem and precursor cells, or whether as a result of this, differentiation processes are induced, producing stem and precursor cells (fig 4).

Migration of mesenchymal stem cells (MSCs)

Cell cultivation and manipulation

The mesenchymal stem cells required for this experiment

Summary

Key words: stem cells – embryonic and adult – migration activity – stem cell cultivation and manipulation – Boyden-chamber

The Effect of Focused Extracorporal Shock Waves on Migration Activity of Mesenchymal Stem Cells (MSCs) Ex vivo

In contrast to the omnipotent embryonic stem cells which can differentiate into all types of tissue, the so-called adult MSCs, which are primarily found in bone marrow, exhibit a limited potential of differentiation. They can mainly differentiate into muscle, cartilage and bone tissue as well as into connective and fatty tissue. Thus, to regenerate these tissues, MSCs are required. The question arises as to which mechanism provides the route to the locus where they are needed.
Up to now, a direct effect on the migration activity of MSCs through high physical strain in sports activities was established only by one research team at the Sports University of Cologne. Based on these findings we set out to produce a similar effect through impact of external mechanical stress. And indeed, through the use of focused extracorporal shock waves (ESWs) we were the first to increase significantly the migration of MSCs. The proof was obtained by means of the Boyden-Chamber assay in the pig skin model.
The fact that targeted and well-defined activation of MSCs is possible opens up the possibility to observe and monitor further signal and differentiation paths of MSCs. This potentially provides tremendous scope not only for therapeutic benefits of ESWs in the orthopedic surgical domain, but also for cardiovascular regeneration.

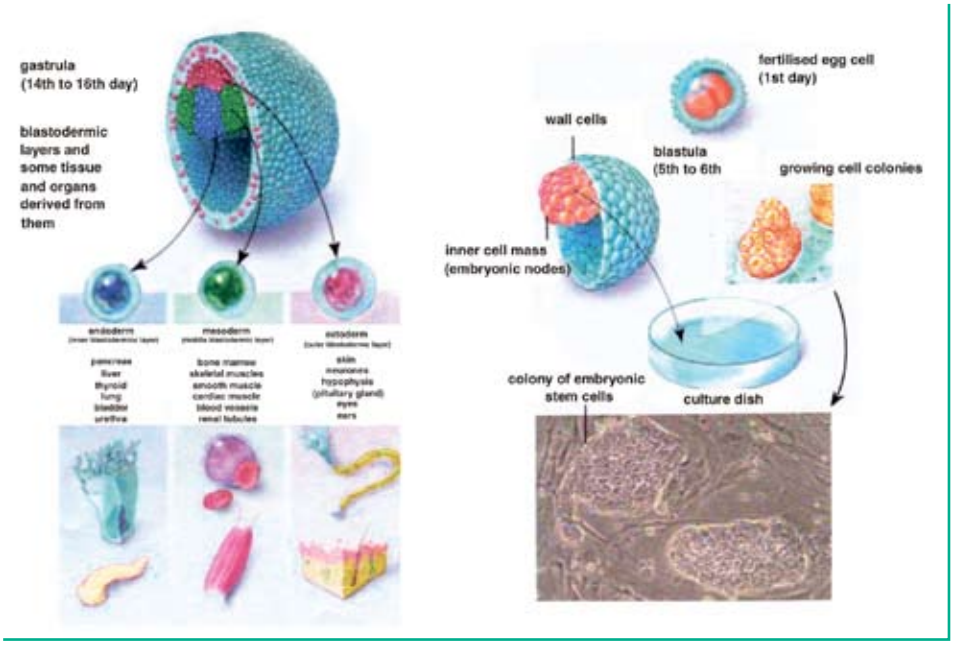

Fig. 2: Development of stem cells.

were obtained through aspiration both from the marrow of the femur and from the head of the femur. Bone marrow was required from ten people who at the time of extraction were aged between 49 and 84 years old, coming to an average of 66.2 years. The bone marrow was then filtered (mesh of 70 µm) and then centrifuged (Ficoll Paque PLUS density gradient centrifugation, Amersham Pharmacia Biotech, Uppsala, Sweden). The first change of medium (Alpha MEM, 20 % [v/v] FCS, 200 µ M L – glutamine, 100 U/ml penicillin, U/ml streptomycin) was carried out two days after cultivation (95 % humidity, 5 % CO2). For each test, 2,000 cells per cm2 were plated. The medium was changed twice a week (fig 5).

The study was authorised by the local ethics committee and is compliant with the declaration of Helsinki.

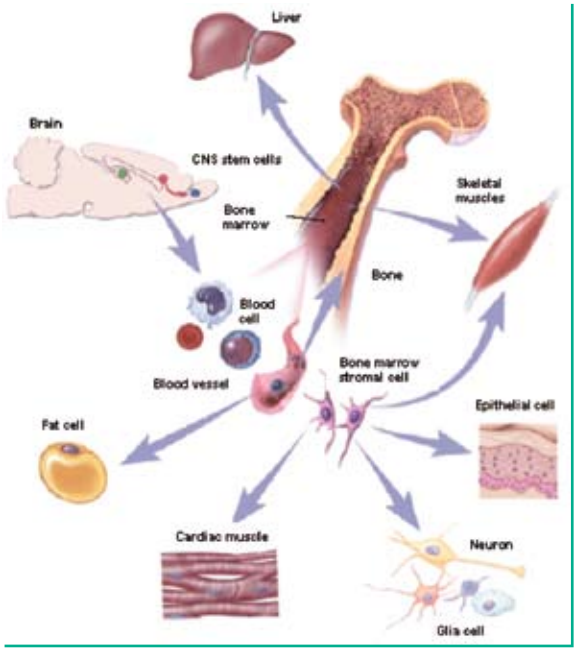

Fig. 3: Adult stem cells.

Quality control of the MSCs cell culture

The quality of the cultivated MSCs was checked through the microscopic evaluation of the cells' morphology, flow cytometry, CFU-F assays, and differentiation assays. The cells were stained for the flow cytometry analysis in accordance with predetermined volumes of CD 106 – FITC, CD 105 – PE (Ancell, Bayport, USA), CD 45 – ECD, CD 14 – PC 5 and CD 34 – PC 5 (Beckman Coulter, Krefeld, Germany) for twenty minutes at room temperature, then washed twice and obtained using a Beckman Coulter Epics XL with Expo 32 software (Beckman Coulter see above). To determine the proliferation potential of the cultivated MSCs, the CFU-F assay was used (colony forming unit fibroblast assay). Tests were carried out to check the potential for chondrogenic, osteogenic and adipogenic differentiation of the mesenchymal stem cells under appropriate conditions and could be monitored histologically, immunohistochemically and using real-time PCR at gene expression level (fig 6).

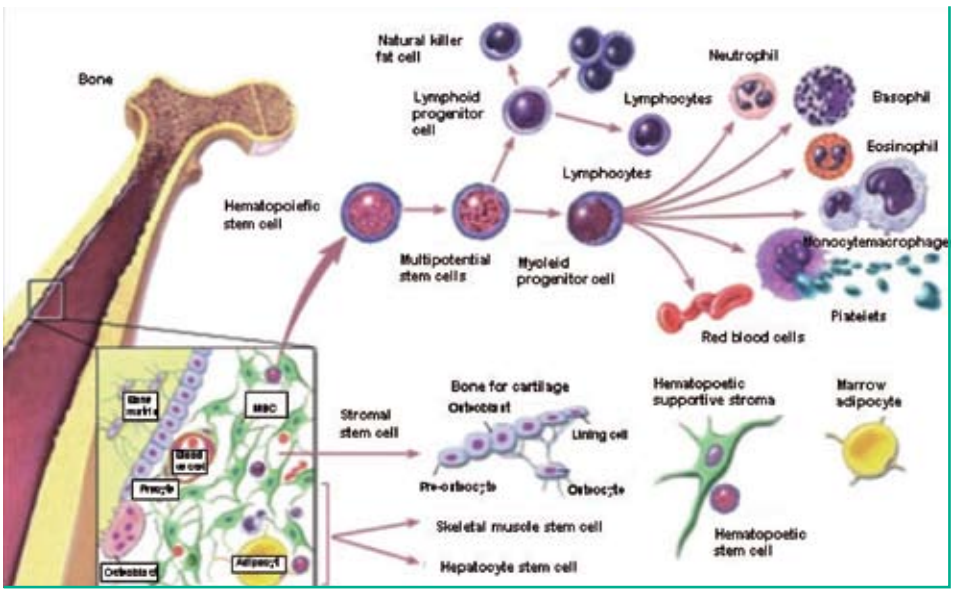

Fig. 4: Stem cells in bone marrow.

Migration/Boyden Chamber

Before the start of migration, the MSCs were subjected to focussed extracorporeal shock waves (PiezoSon, Wolf, Knittlingen, Germany) (fig 7).

To apply the ESW to the cultivated MSCs, a semi-permeable membrane (fresh pig skin) was used, and ultrasound transmission gel was used for coupling (Aquasonic 100, PARKER).

The penetration depth of the ESW came to exactly 5 cm. The cells from sample I were treated with 2,000 impulses, and sample II was treated with 1,000 impulses, and sample III with 500 impulses. Intensity level 6 was selected for the PiezoSon 100 for all conditions.

The control cell group of MSCs not treated with ESW was also was also taken out of the incubator with pig skin after the same period of time as the three samples that were treated.
A modified Boyden chamber with a 24 well HTS Fluoro Blok insert system with 8 µm pores (Falcon Becton Dickson GmbH, Heidelberg, Germany) was used for the migration analysis of the MSCs. After 8 hours of incubation, the cells were fixed with 4 % (w/v) paraformaldehyde (fig 8).
Finally, the membranes were spread over a cover slide and stained. The number of migrated cells was counted (fig 9).
Statistical analysis: all data is shown as an average +/- SD. The data analysis was carried out using a student t test to check for inconsistent data. The data is considered significant if is p value is under 0.05.

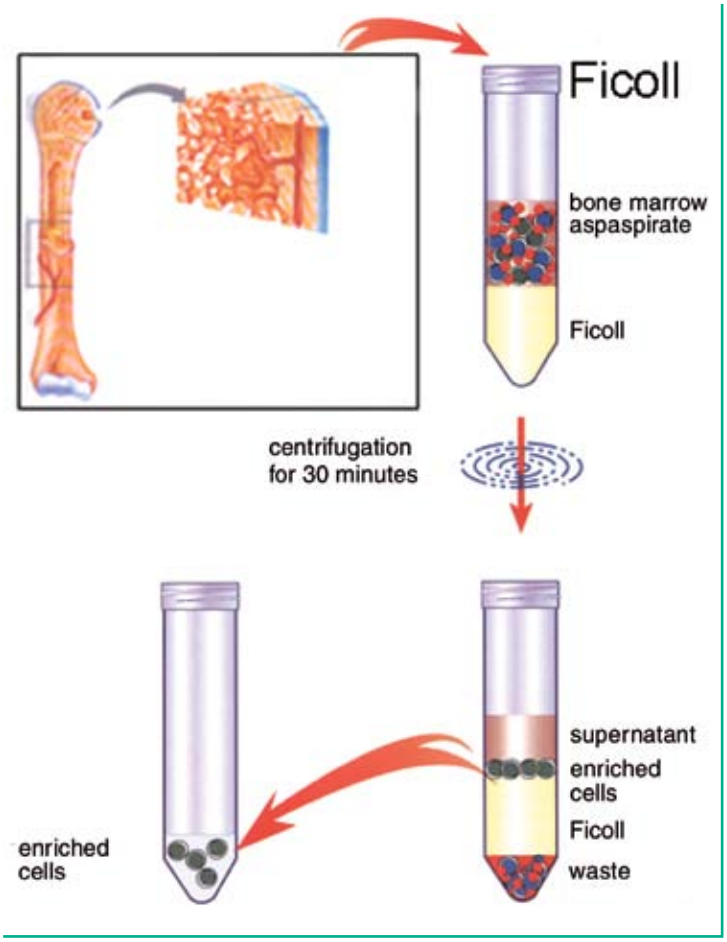

Fig. 5: Ficoll.

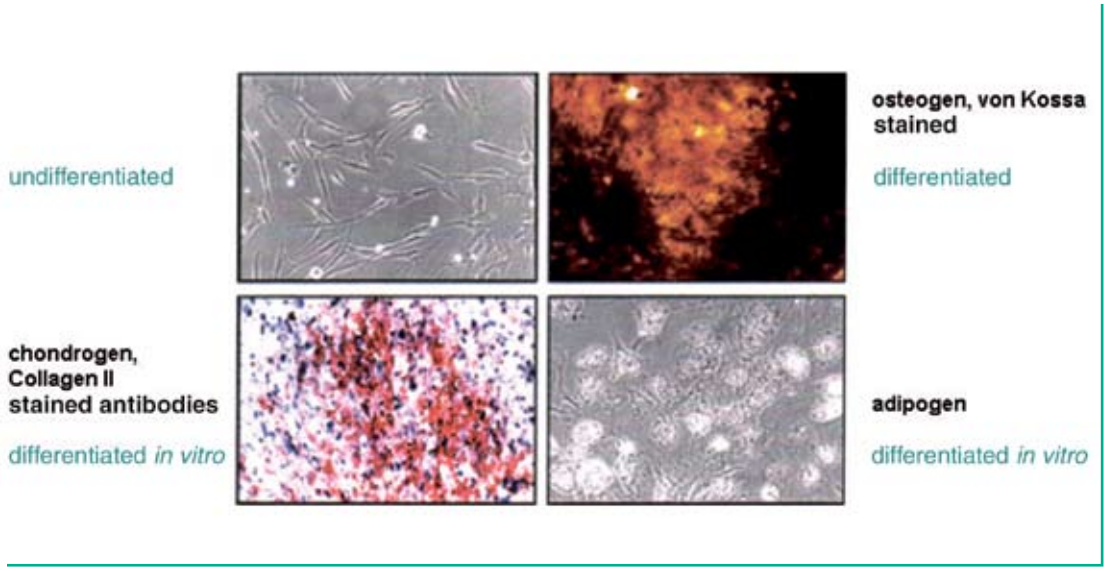

Fig. 6: Histology.

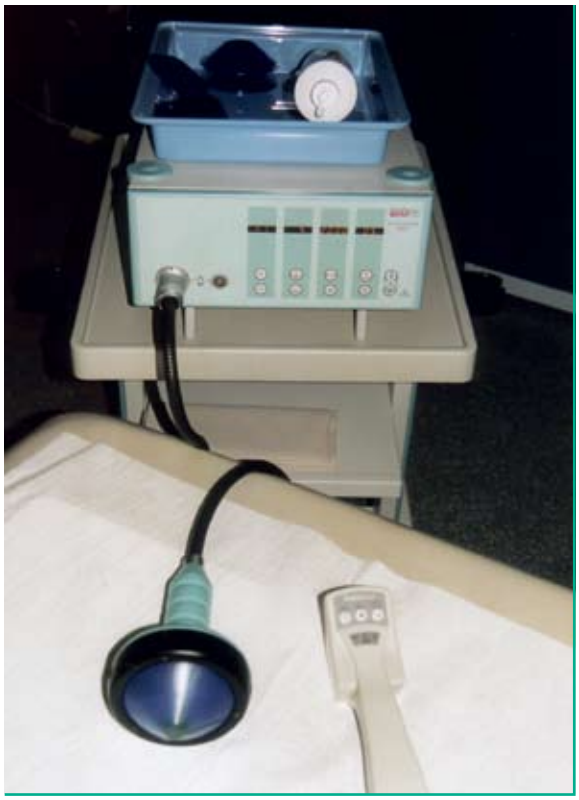

Fig. 7: Piezoson 100.

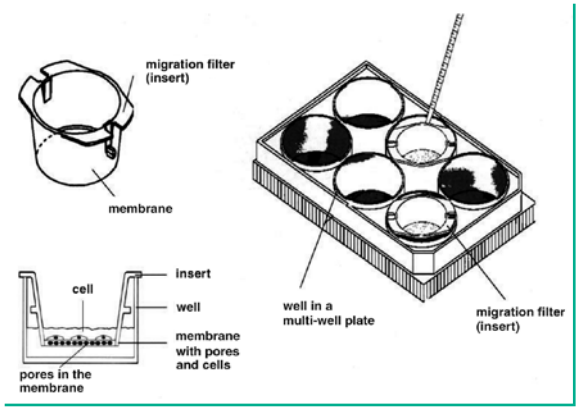

Fig. 8: Boyden Chamber.

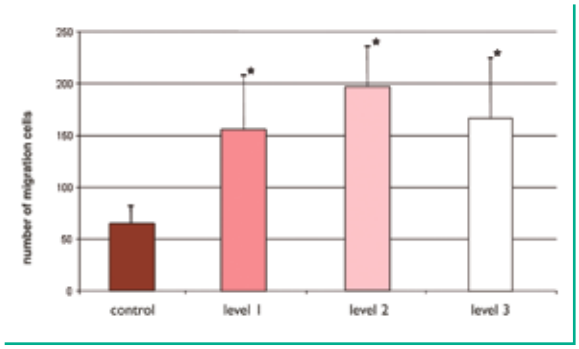

Fig. 9: Result.

Summary and evaluation

Human mesenchymal stem cells were taken from the bone marrow of the femur and head of the femur using aspiration from ten patients with an average age of 66.2 years. The bone marrow was first of all filtered and then centrifuged. The quality of the cultivated MSCs was checked by way of microscopic analysis of the cell morphology, as well as flow cytology, and CFU-F and differentiation assays. Before the start of migration, the MSCs were treated with focussed extracorporeal shock waves, and a modified Boyden chamber was used for migration analysis. The analysis showed a significant increase in migration activity in treated MSCs compared with the non-treated control group. The first experiment was confirmed by way of a control test carried out in exactly the same way.

Outlook

The targeted, controlled extracorporeal induction of migration activity of MSCs with simultaneous mechanical stimulation of locally damaged tissue to activate fixed growth factors, cytokines and other morphogenic factors opens up the possibility of tissue engineering that is bloodless and free of side-effects. We have been able to provide some degree proof of this through the above-mentioned treatment of degenerative and post-traumatic cartilage damage. Particular attention with regard to the effect of chemotactic factors to "attract" MSCs to the repair or regeneration site should be given to the heat shock protein system (Neuland et al, 2005).

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