Investigating the effects of pulsed electromagnetic fields (PEMFs) on bovine nucleus pulposus cells

by

Oddný Björgvinsdóttir

Thesis of 60 ECTS credits

Master of Science in Biomedical Engineering

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Thesis of 60 ECTS credits submitted to the School of Science and Engineering
at Reykjavík University in partial fulfillment
of the requirements for the degree of
Master of Science in Biomedical Engineering

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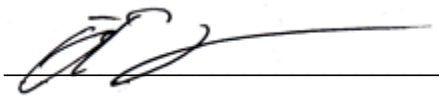

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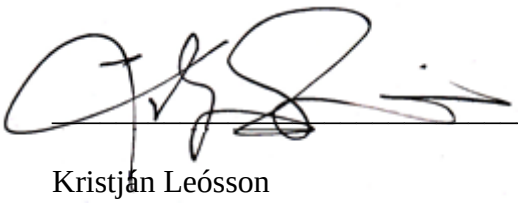

Kristján Leósson

Abstract

Low back pain is in many cases linked to the degeneration of the intervertebral disk (IVD). This medical condition has been identified as a major socio-economic problem. Despite the high prevalence of IVD degeneration, no therapeutic approach exists to date that can halt or reverse the degenerative processes in a non-invasive manner. Over the past decades, the therapeutic efficacy of pulsed electromagnetic fields (PEMFs) has been shown e.g. in the context of fracture healing of long bones, muscle regeneration and on cartilage healing but little is known about its effects on the IVD. In a recent study, low-intensity PEMFs (1.5 mT, 75 Hz) were shown to promote cell proliferation and modestly up-regulate the expression of anabolic genes. These effects of PEMF on the IVD is an indicator of possible usages of PEMF as a treatment of the degenerated disc and thus low back pain. In this project, low energy and pulsed electromagnetic field was applied on bovine nucleus pulposus IVD cells in the range of 0-2 mT and frequency of 15 Hz for either 5 or 10 minutes. The biological effects were analyzed by changes in gene expression of IVD-typical extracellular matrix proteins and mechano-sensitive ion channels. Cell viability and proliferation were also tested in response to PEMF.

No changes in the gene expression of the extracellular matrix proteins or the voltage gated ion channel were found. Viability of cells and the proliferation rate was not altered after treatment with PEMF. It is therefore clear that PEMFs with the specific parameters tested cannot be used as a method to assist in the regeneration of the IVD. However a longer exposure time, yet realistic in a clinical context, might stimulate the production of extracellular matrix. It is also possible that progenitor-like cells may be more responsive to PEMF but further research is needed to determine if that is the case.

Keywords: Pulsed electromagnetic fields, intervertebral disc, nucleus pulposus, voltage ion channels, disc degeneration, back pain.

Útdráttur

Verkir í baki eru stórt vandamál þar sem það dregur úr lífsgæðum einstaklinga og er kostnaðarsamt fyrir samfélagið. Sterk tengsl eru á milli verkja í mjóbaki og hrörnunar á hryggþófa. Þó að hrörnun á hryggþófa sé algengur sjúkdómur er enn sem komið er ekki til nein meðferð sem stöðvað getur hrörnunina eða snúið henni við án skurðaðgerðar. Síðustu áratugi hefur púlsað rafsegulsvið verið notað til lækninga á erfiðum beinbrotum og rannsóknir hafa sýnt að það hjálpar til við endurnýjun (e. regeneration) á vöðvum og brjóski. Lítið er hinsvegar vitað um áhrif púlsaðs rafsegulsviðs á hryggþófann. Í rannsóknum á þófakjarnafrumum úr nautgripum hefur komið í ljós að með púlsuðu rafsegulsviði (1.5 mT, 75 Hz) er hægt er að örva slíkar frumur til að skipta sér hraðar og örva tjáningu gena sem stjórna framleiðslu utanfrumuefnis próteina. Þessi áhrif af PEMF á þófakjarna frumur bendir til að hugsanlega væri hægt að nota PEMF sem meðferð við hrörnun á hryggþófa og bakverkjum. Í þessu verkefni var lágt púlsað rafsegulsvið á bilinu 0-2 mT við 15 Hz notað til að örva hryggþófafrumur úr nautgripum í annaðhvort 5 eða 10 mínútur. Líffræðileg áhrif segulsviðsins voru metin með því að skoða breytingu í tjáningu dæmigerðra utanfrumuefnis próteina og álagsnæmra jónagangna á frumuhimnu. Lífvænleiki og fjölgun frumna var einnig skoðuð í tengslum við segulsvið af mismunandi styrkleika. Engar marktækar breytingar sáust á tjáningu gena, hvorki á utanfrumuefnis próteinum né á spennustýrðum jóna göngum. Ekki sáust heldur marktækar breytingar á lífvænleika né á fjölgun frumna. PEMF með þeim stillingum sem valdar voru að rannsaka eru því ekki vænleg sem aðferð til lækningar á hrörnun hryggþófa. Þó er hugsanlegt að lengri meðferð í segulsviði geti skilað árangri eða að stofnfrumur í hryggþófa svari meðferð með segulsviði betur. Frekari rannsókna er þó þörf til að ákvarða ef það er raunin.

Lykilorð: Púlsað rafsegulsvið, hryggþófi, þófakjarni, spennustýrð jónagöng, hrörnun þófakjarna, bakverkir.

Ritgerðin heitir á íslensku: Áhrif púlsaðs rafsegulsviðs á þófkjarnafrumur úr nautgripum.

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Alginic acid sodium salt, from brow algae (Sigma-Aldrich, USA)

Anti-Anti (Sigma-Aldrich, USA)

Amphotericin β (Sigma-Aldrich, USA)

Betadine (Mundipharma, Switzerland)

CaCl2 x 2H2O (Sigma-Aldrich, USA)

Calcein-AM (Sigma-Aldrich, USA)

Collagenase NB4 (Serva, Germany)

Curcumin (Sigma-Aldrich, USA)

Dimethyl Sulfoxide (Sigma-Aldrich, USA)

Dispase II (Roche, Switzerland)

Dulbecco's modified Eagle's medium, F12 (Sigma-Aldrich, USA)

EDTA, 10mM (Sigma-Aldrich, USA)

Ethidium homodimer-1 (Sigma-Aldrich, USA)

Formaldehyde (Sigma-Aldrich, USA)

Glutaraldehyde (Sigma-Aldrich, USA)

L-ascorbate (Sigma-Aldrich, USA)

NaCl (Sigma-Aldrich, USA)

NaCl, 150 mM (Merek, USA)

TaqMan ® Fast Universal PCR Master Mix (Life Technologies, USA)

Tryzol (Life Technologies, USA)

Primers (Taqman, Live tehcnologies, USA)

Resveratrol (Sigma-Aldrich, USA)

Sodium citrate tribasic dihydrate (Sigma-Aldrich, USA)

TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA)

Trypan blue solution (Sigma-Aldrich)

List of equipment

Ca2 wafers (Edmund Optics, USA)

Hemocytometer (Digital Bio, Korea)

IQ5 Optical System (BioRad, USA)

IX51 inverted microscope (Olympus, USA)

PEMF machine (Institute of Electromagnetic Fields, ETH, Switzerland)

PL2241 Nd:YAG laser (EKSPLA, Lithuania)

T100™Thermal Cycler (Bio-Rad, USA)

Introduction

Clinical problem

Low back pain is a common health problem throughout the world that the majority of people experience at some point of time during their life. The back pain can influence and restrict work, mobility and other important activities of daily life but in most cases people recover within 3 months [4, 5]. For others the healing time is longer or it can be the beginning of a more serious conditions. About 10% of patients suffering from low back pain become chronically disabled [6], i.e. chronically meaning when the symptoms persists beyond 3 months of time [7]. Back and spine impairments are the most common impairments among young and middle-aged people [8], it is painful, costly and limits the quality of life. It causes disability and is to date the leading cause of global years lived with disability (YLDs) in all developed countries [1, 9] with a mean point prevalence of 11.9% [10]. The high prevalence of low back pain, the risk of disability and high direct and indirect cost make it a major economic and social burden around the world [11-13]. As more people are expected to reach higher age in the coming years the global burden of low back pain will continue to increase in the coming decades [10].

The onset and course of low back pain is influenced by biological, environmental and social factors. The biggest risk factors are considered to be age (with the highest incidence among individuals aged 20-29 and increasing prevalence until 60-65), low educational status, stress, anxiety, depression, job dissatisfaction, little social support in the workplace and whole-body vibration [14].

A significant cause of low back pain is degeneration of the intervertebral disc (IVD) [6, 15, 16]. The degeneration of the IVD can then lead to instability of the spinal column and can thus be a trigger for most spinal diseases [17]. Low back pain is primarily treated conservatively, most often with physical therapy, medication or injection. Surgery is considered if the conservative therapy did not prove to alleviate the pain. The gold standard is spinal arthrodesis, a fusion of two adjacent vertebrae and total disc replacement [18].

Goal of thesis

The treatment options available today are in most cases capable of relieving pain but do not halt or reverse the disc degeneration that is the most common underlying cause of back pain. In this project the effects of pulsed electromagnetic fields (PEMFs) are investigated in order to elucidate if PEMF can serve as a potential non-invasive treatment for patients with disc degeneration.

Background

Anatomy of the vertebral column

The human spine consist of 33 vertebrae. The 24 presacral vertebrae are divided into three anatomically distinct regions that from cranial to caudal consists of the cervical, thoracic and lumbar region [19]. They are made of 7, 12 and 5 vertebrae respectively. These anatomical parts are then followed by the sacrum, made of 5 fused vertebrae and the coccyx that most often consists of 4 fused coccygeal vertebrae [19] (figure 1) [2]. The intervertebral discs connecting the vertebrae in the presacral region are the main joints of the spine allowing movement like bending, flexion and torsion. The discs additionally function as a shock absorber [20]. Stability is provided by the surrounding muscles and ligament as well as the structure of the bone in the vertebral column. Four curvatures are in the vertebral column in the anterior-posterior plane, considered either kyphosis or lordosis [19]. The two curves that are convex anterior in the thoracic and sacral region are called kyphosis but the two concave in the lumbar and cervical

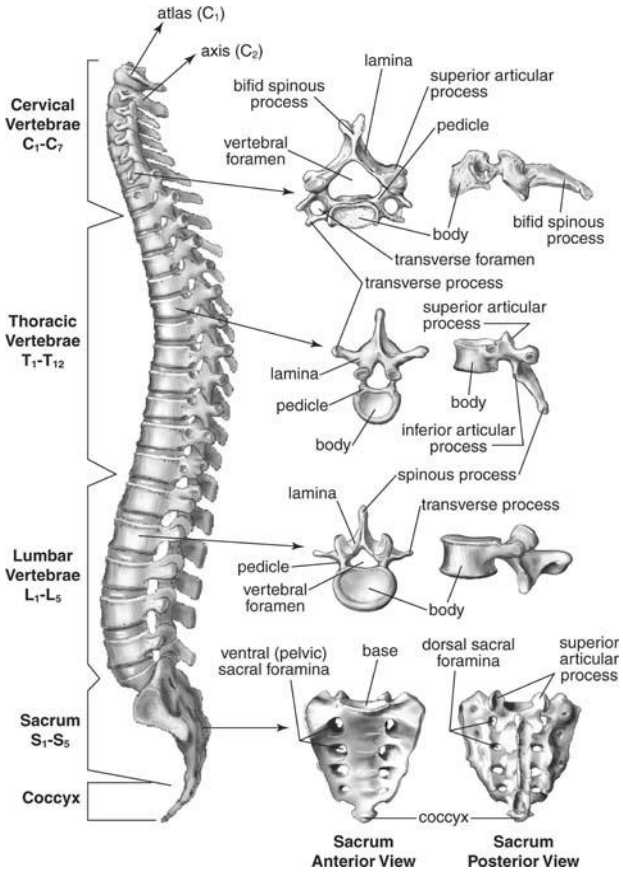

Fig. 1. The anatomy of the vertebral column [2].

regions are known as lordosis. The curvature aligns the head with a vertical line through the pelvis [21].

The bovine vertebral column is often used as a model for humans even though there are some apparent anatomical differences like the curvature and length. The bovine vertebral column is composed of 7 cervical vertebrae, 13 thoracic, 6 lumbar, 5 sacral and 18-20 coccygeal vertebrae [22]. The total length of the bovine column is 209 cm longer that in humans and has only one lordotic and one kyphotic curve. The spinous processes in bovines are also longer in the thoracic region [23]. The geometry of the human intervertebral disc in the lumbar region is comparable in geometry as bovine discs in the coccyx region. This includes the shape and position of the nucleus pulposus, axial cross section and relative disc height [24].

Structure and function of the intervertebral disc

The IVD consists of distinct structures characterized by different mechanical properties, cell types and extracellular matrix (ECM). The central part, the nucleus pulposus is a gelatinous tissue that in the transverse plane is surrounded by the annulus fibrosus, a more fibrous cartilaginous tissue. The nucleus pulposus and the annulus fibrosus are located between two thin hyaline cartilage end plates. This interfaces the IVD and the vertebral body (figure 2) [3]. The intervertebral disc is an avascular tissue but the surrounding blood vessels provides necessary nutrients that is regulated by the endplates [25]. No nerves are in the tissue except in the outer part of the annulus fibrosus.

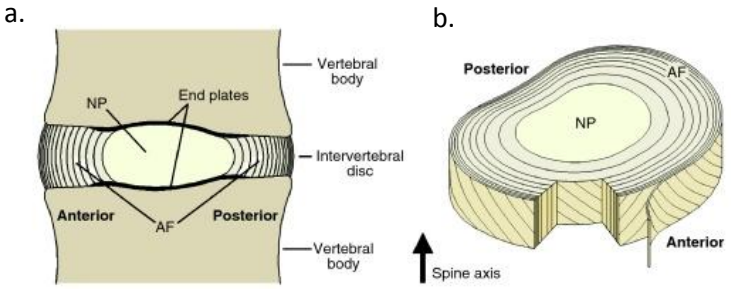

Fig. 2. Schematic drawing of the IVD. a) Sagittal cross section of the IVD and surrounding vertebrae. b) Transverse view of the structure of NP and AF [3].

The main function of the discs is mechanical as they transmit loads arising from body weight and activity through the spinal column. They are also the main joints of the spinal column

and they occupy about one third of the spinal height [6]. In the lumbar region they are approximately 7-10 mm thick and 4 cm in diameter [26].

IVD cells and extracellular matrix

The different structures of the intervertebral disc are characterized by different cell types and extracellular matrix (ECM). Three main cell types can be found in the tissue, nucleus pulposus cells (NP) cells, annulus fibrosus (AF) cells and notochordal cells (NC). Also a small population of progenitor cells are present in the disc. Cells occupy a relatively small part of the tissue compared to other tissue types, or less than 0.5% of the NP tissue volume [27]. These cells, by synthesis and degradation maintain the homeostasis of the EMC.

Nucleus Pulposus

The central zone of the disc, the nucleus pulposus contains a population of chondrocyte-like cells called nucleus pulposus cells. These cells are few and dispersed within the nucleus, with only approximately 5000 cells per mm^3 [28, 29]. The NP cells synthesize randomly organized collagen fibers [30] and radially organized elastin fibers [31] embedded in a highly hydrated gel rich of aggrecan [6]. Morphologically the NP cells are spherical and synthesize type II collagen and aggrecan as their main structural protein.

Annulus Fibrosus

The annulus fibrosus (AF) surrounds the NP in the transverse plane. The boundaries are clear in young individuals but become less distinct with age and degeneration of the disc. In the AF, cells are fibrocytic and elongated and aligned with the principal collagen fiber direction. These cells tend to become more like NP cells closer to the nucleus [6, 29]. The tissue is made up of 15-25 rings, or lamellae composed of bundles of collagen I fibers, which is the main structural protein in the AF. The fibers of the lamellae lies at 60° degrees to the vertical axis, alternating to left and right of it in adjacent lamellae [32]. Between lamellae is an intervening domain which is rich of elastin fibers. The function of these fibers is likely a

recovery mechanism that helps the disc conform its initial structure after bending. It also binds the lamellae together [6].

Notochordal cells

Notochordal cells are remnants from the embryonic notochord. They are larger than NP and NF cells and contain vacuole-like cytoplasmic structure [33]. These cells are rarely present in humans after adolescence and the decline of these cells are thought to be linked to disc degeneration [34, 35]. Hunter et al. have speculated that the NP population may be involved in the maintenance or repair of the disc and a loss of them would therefore initiate the degeneration of the disc [36].

Progenitor cells

A population of progenitor cells in the nucleus pulposus (Tie2 and disiaganglioside 2 positive) were recently discovered [17]. These cell types were found in both mouse and human NP tissue but decreased with age and degeneration of the disc. The cells proved to be clonally multipotent and differentiated into mesenchymal lineages [17]. Additionally, the cells were capable of inducing the reorganization of the tissue when implanted into mice [17]. Several studies can be found that use PEMF to induce the differentiation of mesenchymal stem cells (MSCs) [34, 35]. These studies however mainly focus on the differentiation of cells into osteocytes, probably since the main emphasis of PEMF research has been on bone [37, 38]. This indicates that stem cells and/or progenitor cells could be sensitive to PEMF, perhaps even more than other cell types.

End plates

The end plates, located superiorily and inferiorily to the disc consist of cartilage and vertebral end plates [39]. The cartilage end plates located next to the disc are approximately 600 µm thick and function as a mechanical barrier between the pressurized NP and the vertebral bone. They also regulate nutrient diffusion between the disc and the vertebral bodies supplied by the adjacent blood vessels [40, 41]. The vertebral end plates, located next to the vertebral bone are composed of cortical bone [25, 42].

Alternative cell source

Human IVD tissue is difficult to obtain because of ethical reasons, regulatory restriction and limited supply of surgical biopsies [43]. In biopsies obtained from patients undergoing spinal surgeries, NP from AF can be hard to distinguish reliably, especially for discs at later stages of degeneration. Furthermore, cell yields from human discs biopsies are relatively low, requiring protracted expansion times to obtain sufficient cells for biological analysis like gene expression as well as restricting the number of conditions that can be effectively tested. As a model to approximate the human IVDs, several animal tissues have been used. One of the animal models used as an approximation for human IVD is bovine disc tissue because the tissue is comparable to human IVD in both morphology, cell type and cytoskeletal properties [44]. Bovine NP tissue like human lose their NC cells in the NP early on and are rarely present in adulthood [45]. The similarity of the bovine and human tissue makes research on bovine IVD of greater clinical relevance than tissue from other less relevant species [43]. Disc tissues isolated from cow tails are also simple to obtain from recently slaughtered bovines and animals are not grown primarily for experimental but bought from the food industry.

Disc degeneration

The degeneration of the disc is correlated with age, genetic and environmental factors. Although the degeneration can be asymptomatic it has been shown to be strongly linked with back pain [16, 46]. The discs degenerates much earlier than other musculoskeletal tissues with the first evidence seen as early as in 11-16 years old individuals [47]. In fact 20% of people in their teens have disc with mild signs of degeneration, but then the degeneration increases with age and for people at the age of 70, 60% of the discs are severely degenerated [48]. One of the primary causes is thought to be failure of nutrient supply to disc cells. The disc is avascular and cells depend on nutrients by diffusion from capillaries that ends just above the cartilaginous end plate [49]. For nutrients to reach the cells, the diffusion must go undisturbed through the cartilaginous end plate and the dense extracellular matrix of the disc. Calcification of the cartilaginous end plate or diseases that affect the blood supply to the vertebral body affects this pathway and leads to accelerated disc degeneration [50-53]. Abnormal mechanical load and injury are also thought to be a trigger for disc degeneration and thus back pain [54].

With increasing age the degeneration of the disc progresses, becoming more and more disorganized (figure 3) [6]. Degeneration is characterized by apoptotic and necrotic cell death, enhanced cell proliferation, mucous degeneration, granular change and concentric tears of the lamellae [6, 55]. Additionally, nerves and blood vessels are increasingly found with degeneration [56].

Fig. 3. Healthy and degenerated human IVD from the lumbar region [1].

The main change seen in biochemistry is the loss of proteoglycan [57]. The aggrecan degrades and small fragments from the degraded tissue leach from the disc. This results in loss of glycosaminoglycans which causes the osmotic pressure of the disc to fall and thus a loss in hydration [57]. Also changes in the type and distribution of collagen with degeneration can be seen, mainly as an increase in the amount of denatured collagen type II [58, 59]. Other changes seen with degeneration is an increase in fibronectin formation, which has been shown in vitro to down-regulate aggrecan synthesis and up-regulate production of certain matrix metalloproteinases (MMPs), but the level of MMPs are higher in more degenerated discs [6].

With degeneration, the disc is less able to maintain hydration under load so it loses height and fluid more rapidly when loaded and the degenerated disc no longer behaves hydrostatically [60, 61]. Sciatica and disc herniation that is known to be associated with disc degeneration alters the disc height. This affects the mechanics of the disc as well as surrounding muscles and ligaments of the spinal column [62, 63].

Treatment of low back pain

Current treatment options

Low back pain is primarily treated conservatively, most often with physical therapy, medication or injection. A wide variety of medication is available, most aimed at reducing inflammation but others inhibiting the transmission of pain signals from reaching the brain. Epidural steroid injection is also given to reduce the inflammation but have the advantage over the medication that they reach the painful area directly. In fewer cases the back pain is treated invasively. This is only considered if the pain cannot be relieved with non-surgical methods. For sciatica (a set of symptoms caused by compression or irritation of the spinal nerve roots) laminectomy and microdisectomy have been used to relieve pain caused by the nerve roots [64]. Small portion of the bone and/or the disc material pressing the nerve is removed to relieve the compressed nerve from the surrounding compressing tissue [64]. Total disc replacement and spinal fusion are also common to treat degenerated discs. Spinal fusion is however the gold standard in surgical intervention [18]. In spinal fusion two or more vertebrae are fused together in order to stop the motion between two adjacent vertebrae. This can alleviate pain but does not restore the mechanism or the structure of the disc. Recurrent episodes of pain are common after spinal fusion and adjacent parts of the spine can degenerate faster because of it, increasing the need for additional surgical intervention [65, 66].

Future treatment

Biological solutions towards regeneration of the disc are still quite far from being clinically applicable. Many potential future therapies aim at stimulating the disc to produce more extracellular matrix. These methods all assume that the stimulated population of remaining cells are large enough to repair the matrix. Some research has been done with direct injection of growth factors or cytokines inhibitors but with very limited effect since these degrade fast in the tissue [67, 68]. Attempts to prolong the delivery are being studied, for example by embedding the substances in hydrogels prior to the injection into the disc or outside the lamellae for substances to diffuse into the disc. Some research has been aimed at gene therapy but the current status is far from clinical use. The regeneration or repair of IVD by injection of stem cells in hydrogel is also far from clinical application [69].

Biological effects of PEMF

Mechanism of action

Over the past decades, the therapeutic efficacy of pulsed electromagnetic fields (PEMFs) has been shown e.g. in the context of fracture healing [70]. In addition, positive effects of PEMF on cartilage healing have been described but still little is known about its effects on the IVD [71, 72]. The mechanism of action is not known but several hypothesis have been presented. What most of them have in common is to assume that the fields target the cell membrane [73, 74]. The magnetic field may affect ligand binding with membrane receptor and can also affect protein distribution in the membrane [75].

From studies on human osteoblast-like cells it was found that stimulation by magnetic fields raises the net Ca^{2+} flux in cells [76]. The increase was found to be frequency dependent, highest in the range of 15.3-16.3 Hz [77]. This increase in the cytosolic Ca^{2+} is then a starting point for signaling pathways targeting specific bone matrix genes like type I collagen and osteopontin [78].

Mechanosensitivity and TRP channels

Mechanical stimulation plays an important role during development, homeostasis and pathology of various tissues. Muscoskeletal tissues are known to response to mechanical force that is important in maintaining the metabolic balance of the tissue and mechanical factors are known to play a role in fracture healing [79]. For the IVD, moderate levels of mechanical stress produce anabolic responses [80-84] while unloading or hyper physiological loading has a negative effect on the disc [85, 86]. The exact mechano-pathology remains largely unknown but in tissues similar to the IVD, such as cartilage, the transient receptor potential (TRP) channels are thought to be fundamental in mechano-sensing [87-89]. They are ubiquitous and relevant in the sensing of osmotic, mechanical and inflammatory stress and play an important role in multiple diseases (e.g. osteoarthritis). TRPs represent a family of Ca-permeable cation channels, which can be classified into 6 subfamilies according to their structure: the TRPC, TRPV, TRPM, TRPA, TRPP and TRPML channel families. TRP channels act either as separate functional units or as multimeric complexes across and within the distinct subfamilies (e.g. TRPC1/4/5) [90-92]. There is substantial evidence that specifically TRPA1, TRPV1, TRPV2, TRPV4, TRPC1, TRPC5, TRPC6, TRPM2 and

TRPM7 are ubiquitously relevant in sensing of osmotic, mechanical and inflammatory stresses, with their importance having been demonstrated in a multitude of tissues, such as liver, endothelium, muscle, mucosa, cartilage, dental pulp, or neurons [87-89, 93-99]. Not only are they relevant in the sensing of these stresses but also in sensing PEMF. For muscle cells, low intensity PEMFs (<0.5 mT) in a frequency range of 10-100 Hz were shown to promote TRPC1 opening, thus permitting the entry of calcium in the absence of mechanical input. It has also been shown that the paracrine expression of IGF-1 is stimulated by PEMFs, leading to muscle regeneration [100]. Studies on human muscle cells demonstrate that not only is TRPC1 expressed but also TRPC6, TRPV2 and TRPV6 [101]. Not much is known about expression of the TPR channels in the disc but what is known for muscle cell could apply to NP cells in a similar way. If that is the case a change in the behavior of the mechanosensitive TRP channels (TRPC1, TRPC6, TRPV2 and TRPV6) are likely to be evident after PEMF treatment.

Regardless the mechanism of PEMF cells can possibly be stimulated at the gene level. Held et.al. found that 1.5 mT field strength and 75 Hz on bovine NP cells promoted cell proliferation and modestly up-regulated the expression of anabolic genes if exposed 18 hours per day [102]. In this study cells were embedded in agarose and cultured up to 28 days. Cells however only showed up-regulation of genes (collagen I, collagen II and aggrecan) at the end of the culture time. In order to consider PEMF as a treatment option that stimulates the expression of the anabolic genes and thus promoting disc regeneration, a reasonable treatment option in terms of time, setup and cost has to be developed. If NP cells responded to PEMF by up-regulation of the same genes but after shorter time and proved to have sufficient regenerative potential, PEMF therapy for degenerative disc disease could be evolved and implemented into future clinical practices.

Hypothesis and aims

Hypothesis

Based on findings in the literature and preliminary studies on muscle cells and bovine NP cells it is hypothesized that PEMF exposure will cause an anabolic change in cell behavior in the disc cells by influencing mechanically regulated calcium channels.

Aims

The aim of this project is to investigate the effect of PEMFs on bovine nucleus pulposus cells driven by the need of a new and effective treatment of the degenerated IVD. The aims are as follows:

1. To analyze changes in cell viability and proliferation in response to PEMF.
2. To analyze changes in mRNA expression of collagen I, collagen II and aggrecan in response to PEMF.
3. To determine the role of calcium channels in the response to PEMF.

Methods

Isolation and digestion of bovine NP cells

Recently slaughtered bovine tails were obtained from a local slaughterhouse (Angst, Zurich). The muscles and tendons were removed from the vertebral column and the outer surface then sterilized with 70% EtOH (in experiments that started with expansion of cells in monolayer in growth medium containing antibiotics) or with betadine (for experiments where no antibiotics were used). The nucleus pulposus was removed from the vertebral column by a surgical knife and cut into 2x2 mm pieces. The dissected tissue was enzymatically digested over night at 37°C and 5% CO2 in a solution containing 0.3% collagenase NB4 and 02% dispase II in phosphate buffered saline (PBS). In the following methods 3% anti-anti was added to the digestion solution when cells were expanded in 2D prior to 3D culture, but amphotericin β was used for cells in 2D experiments. After digestion overnight the solution was poured through a 70 µm filter, centrifuged at 1000 rpm for 5 minutes, then washed once in medium and centrifuged again at 1000 rpm for 5 minutes. The cells were finally seeded and cultured according to given protocol for various experiments.

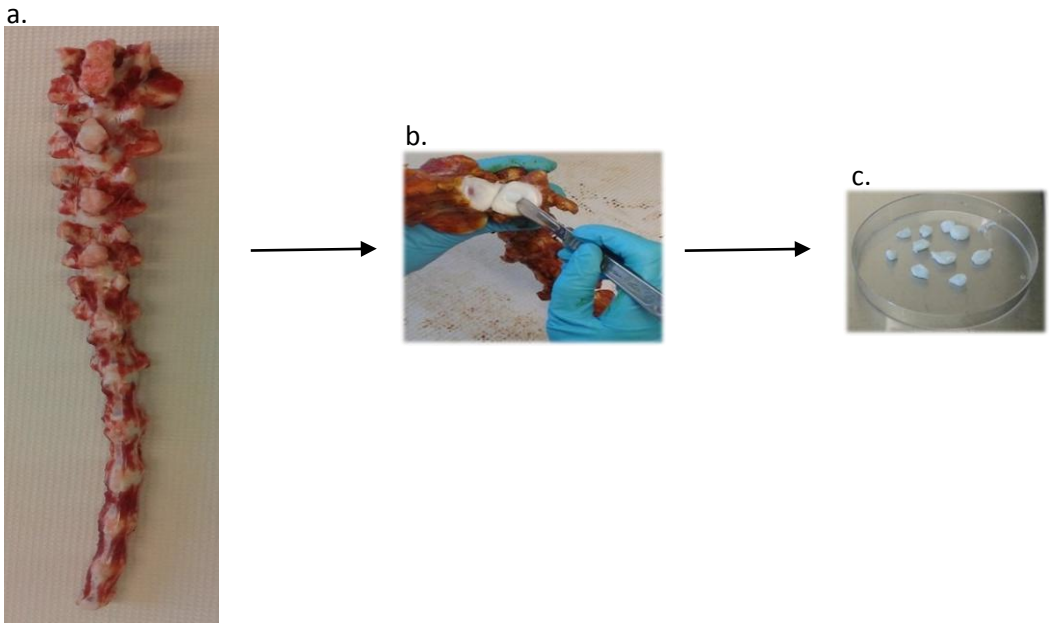

Fig. 4. a) Bovine tail after the tissue around the discs has been removed for a more clean isolation of NP tissue b) Isolation of nucleus pulposus from the disc c) Nucleus pulposus on a petri dish after isolation.

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Alginate beads

After serial expansion in monolayer, the cells were imbedded in alginate. Cells were suspended in sterile 0.9 % NaCl containing low-viscosity alginate gel (1.2%) and then encapsulated in alginate beads at a density of $4x10^6$ cells/ml. The cell suspension was slowly pressed out of a syringe through a 21 gauge needle in a drop wise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize for 5 minutes in $CaCl_2$ solution. The beads were thereafter filtered from the solution and washed with 0.9 % NaCl solution and thereafter PBS. After the beads had been washed, they were transferred into a 6-well plate. Each bead contained approximately $145 \cdot 10^3$ cells. 5 beads per well were cultured in 5 ml of Dulbecco's modified Eagle's medium: nutrient mixture F-12, 10% fetal calf serum (FCS) in a presence of 50 µM vitamin C for either 6 or 10 days depending on experiment. The cultures were incubated in humidified atmosphere of 5% CO_2 in air at 37°C with medium change once during the culture time. To isolate the cells from the alginate beads at the end of the culture time, 12 beads were dissolved in 1 ml of sodium citrate solution.

PEMF treatment of bovine NP cells in 3D culture

Bovine NP cells were isolated as described before and expanded in monolayers. The cells were then embedded in alginate and cultured for 7 days in dulbecco's modified eagle's medium: nutrient mixture F-12, 10% FCS in a presence of 50 µM vitamin C. This was done in order to reverse the dedifferentiation of the NP cells that is known to occur in 2D culture [103]. The beads were cultured in a 6-well plate with 5 beads per well. After 6 days, the cell-seeded beads were exposed to PEMF at 15 Hz at intensities of 0, 0.3, 0.5, 1 and 2 mT for 10 minutes. Cells were harvested 18 hours after exposure and cell viability tested with ethidium homodimer and calcein staining. Gene expression of collagen I, collagen II and aggrecan were analyzed by qPCR.

PEMF treatment with chemicals of bovine NP cells in 3D culture

Bovine NP cells were isolated and expanded in 2D as previously described. The cells were embedded in alginate and cultured for 10 days. Then two different substances, curcumin and

resveratrol, were added to each culture medium for 24 hours at the concentration seen in table 1. The solvent, dimethyl sulfoxide (DMSO) was added to the control group to avoid bias of the results. The approach of adding curcumin and resveratrol was undertaken in order to reduce the stress level of the cells and thus increase its potential to respond to the applied stimulus [104, 105]. After 24 hours in culture with the substances, cells were treated with PEMF for 10 min at 0, 0.3, 0.5, 1 and 2 mT. Viability staining (by ethidium homodimer and calcein) and gene expression analysis (by qPCR) was done 18 hours after PEMF. In this 3D experiment, the gene expression of TRPC1, TRPC6, TRPV2 and TRPV6 were also tested in addition to collagen I, collagen II and aggrecan.

Name of chemical	***Concentration of solvent (DMSO)***	***Concentration of substance***
Control (DMSO+)	41.1 µM	0 µM
Curcumin	41.1 µM	20 µM
Resveratrol	11.3 µM	100 µM

Table 1. Concentraion of the substances used prior to PEMF exposure.

PEMF treatment of bovine NP cells after 3 days in 2D culture

Bovine NP tissue was enzymatically digested as previously described but additionally with 5 µg/ml of Amphotericin β in the digestion solution. 300.000 cells were seeded in two groups containing different concentration of FCS, 2.5% or 10% FCS in order to test whether the FCS content influences cellular responses to PEMF. The medium used (DMEM/F12) was supplemented with Amphotericin β (5 µg/ml). Streptomycin is thought to possibly alter TRP functionality, hence Amphotericin β was used to decrease the risk of contamination. After 3 days in culture, the cells were treated with PEMF for 10 minutes at 0.3, 0.5 and 1 mT and RNA was isolated after 18 hours for gene expression analysis. Due to the limited number of cells after 3 days expansion, the 2 mT group was excluded in this set-up.

PEMF treatment of bovine NP cells after 18 hours in 2D culture

Identical to the 3 days set-up above, bovine NP tissue was enzymatically digested with 5 µg/ml amphotericin β in the digestion solution. The cells were cultured for 18 hours in DMEM/F12 medium, supplemented with 10% FCS and amphotericin β (5 µg/ml). The cells were treated with PEMF for 10 minutes at 0.3 mT. RNA was isolated after 18 hours for gene expression analysis. Due to the limited number of cells after 18 hours expansion, only one group could be tested (0.3 mT). The FCS content was kept constant at 10%.

The PEMF exposure system

For the treatment of cells with PEMF an exposure system prototype was used made by Cristian Beyer and Jürg Fröhlich at the Institute of Electromagnetic Fields (IEF), ETH, Zurich (fig. 5). The PEMF exposure system consists of a field generator, two amplifiers and an exposure chamber. The exposure system is controlled by a computer that allows real time monitoring of the magnetic field strength and the temperature within the exposure chamber (figure 6).

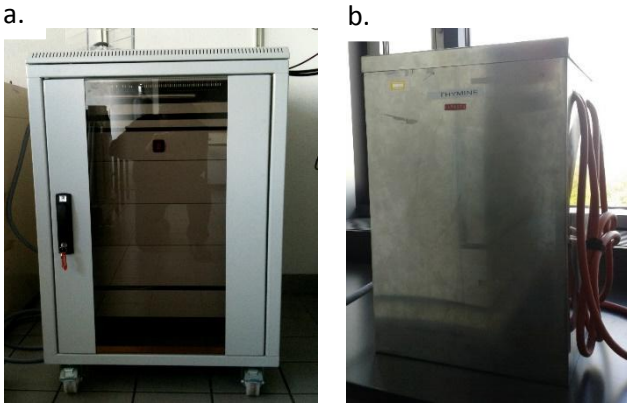

Fig. 5. The PEMF exposure system. a) The signal generator, amplifiers and control of the device b) The exposure chamber.

In a treatment, samples are placed inside the exposure chamber made of a 1.3 mm thick µ-metal that shields the samples form the external magnetic fields (shielding factor of 1:100.000, inside versus out). The system allows exposure with frequencies ranging from 0-50 Hz and magnetic field strength up to 5 mT. NP cells were exposed with asymmetric pulsed magnetic fields for 6 ms at a repetition rate of 15 Hz (asymmetric meaning that the

signal is not symmetric about the x-axis (figure 8)). The applied flux densities were 0, 0.3, 0.5, 1 or 2 mT and the exposure time was either 5 or 10 minutes depending on experiment. The control (non-exposed cells) were placed in the chamber for identical periods but without any exposure. Due to the short exposure time, the chamber was maintained at room temperature.

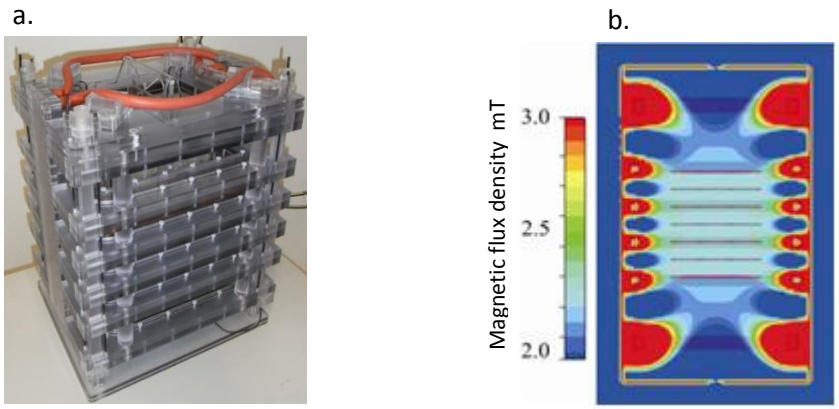

Fig. 6. a) Inside the µ-metal box. Exposure chamber made of coils connected to a polycarbonate frame. A sample holder (also made of polycarbonate for easy sterilization by autoclave) can be seen in the middle inside the exposure volume. b) Simulation of the magnetic flux density.

The stimulating waveform consists of a pulse train of 20 quasi-rectangular pulses per 150 µs with a repetition frequency of 15 Hz (figures 7 and 8).

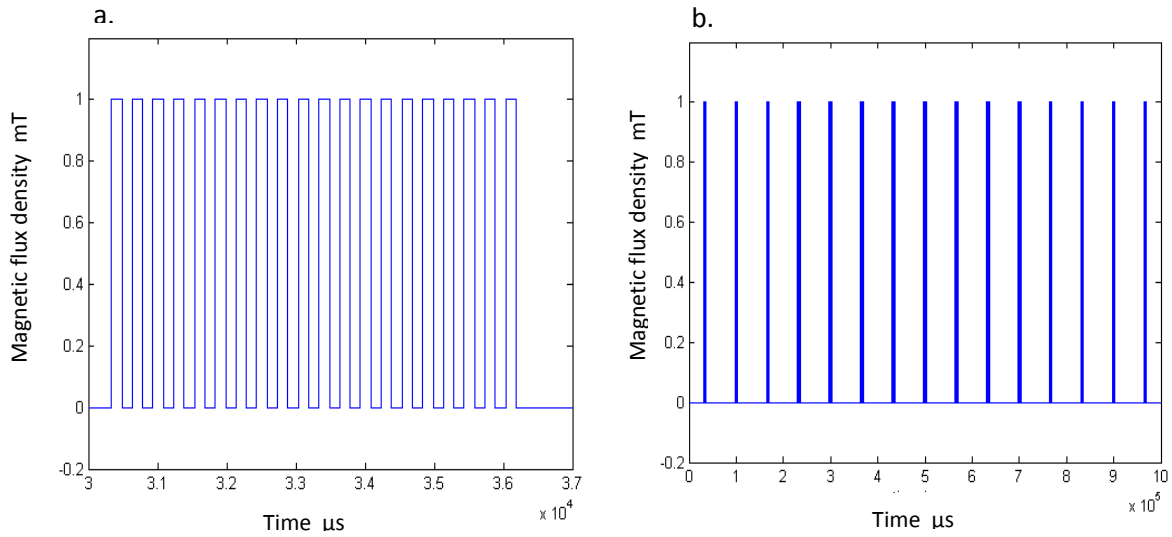

Fig. 7. Representation of the pulsed signal used for the stimulation of cells. a) Each 6 ms long train of pulses in figure a) is repeated at 15 Hz frequency (figure b).

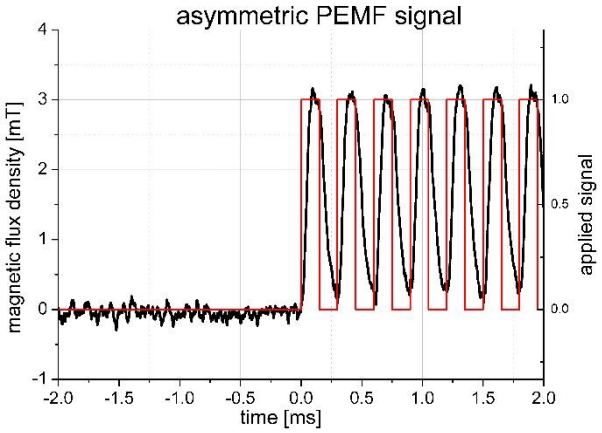

Fig. 8. Signal characteristics. The quasi-rectangular pulses that represent the measured signal is shown in black, the theoretical signal is shown in red.

The dimensions inside the chamber were cells are treated are: 16 cm (length), 12.5 cm (width) and 29.5 cm (height), or a volume of 5900 cm^3. This chamber size allows up to seven 75 cm^2 cell culture flasks to be stacked and exposed at the same time. Free flow of air through holes on the metal chamber cover ventilates the box so that temperature, air composition and pressure is the same as in the room where the device is located. Reduction of the mechanical vibration was done by connecting both the holder and the coils to a structural frame with elastic strands made of silicon rubber (MVQ). Six coils are within the chamber, with 68, 38, 34, 34, 38 and 68 windings from top to bottom of size 191x242, 208x271, 208 x 271 mm^2 (lower to higher number of windings). A central processing unit uses 5 different magnetic Hall sensors (Allegro A 1321) in the exposure chamber to monitor and control the magnetic flux via feedback loop to the signal generator and the amplifiers. Field non-uniformity can hence be kept below 4% within the exposed culture volume and the background magnetic field strength is less than the measurement accuracy of 1 µT [106].

Cell viability of bovine NP by Calcein/Ethidium Homodimer staining

After culture in 3D, cells were simultaneously stained with green fluorescent calcein-AM and red fluorescent ethidium homodimer-1 to determine the cell viability. The staining solution contained 1.25 µl of 2mM ethidium homodimer, 0.5 µl of 4mM calcein in 1250 µl PBS. The calcein staining indicates intracellular esterase activity (hence staining live cells) and the ethidium homodimer indicates the loss of plasma membrane integrity (hence staining dead cells). After incubation in the staining solution for 30 min, 2 beads per condition were

flattened on microscope slides and cells were visualized by fluorescence microscopy and photographed (each bead was photographed at three different location under the slides). By using the image processing program Fiji, life and dead cells were counted and the live/dead ratio of the cell population was determined.

Cell proliferation in 2D cultures

In order to determine cell proliferation in 2D cultures, 5.000 primary NP cells were seeded per well in a 6-well plate and cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 containing 10% FCS and 5 µg/ml of Amphotericin β. 18 hours after seeding, the cultures were treated with PEMF for 10 minutes at 0.3, 0.5, 1 or 2 mT field strength. The control cells were not exposed to magnetic field but placed in the chamber for same time as the treated samples. Cells were counted at three different time points or 2, 4 and 6 days after treatment with PEMF, using hemocytometer slides.

Cell proliferation in 3D cultures

To determine the cell proliferation in 3D cultures, primary NP cells were first expanded in 2D culture and then embedded in alginate beads. 5 beads per well in a 6-well plate were cultured in 5 ml of Dulbecco's modified Eagle's medium: nutrient mixture F-12, containing 10% FCS and 50 µM vitamin C. For each condition, a total of 10 beads was cultured. After 6 days in culture, beads were exposed to PEMF at 0, 0.3, 0.5, 1 or 2 mT field strength for 10 minutes. Cells were dissolved in 1 ml of sodium citrate (for 20 min at room temperature) and centrifuged for 5 min at 1000 rcf. Non-viable cells were stained with 0.4% trypan blue (1:1 of trypan blue vs. cell suspension). Viable cells (unstained by the trypan blue viability assay) from 10 beads were counted right before PEMF treatment and then 2, 4 and 6 days after exposure.

Gene expression analysis

The effect of PEMF on the expression of extra cellular matrix synthesis and ion channel activity were tested in 2D and 3D culture systems. RNA was extracted with the

trizol/chloroform method 18 hours after being treated with PEMF i.e. 18 hours after treatment 1 ml of tryzol was added to cells and keep at -80°C for further analysis. At the day of RNA isolation, the samples were thawed and 200 µl of chloroform was added to samples. The content was mixed by shaking for 15 min but then the tube was left at room temperature for 5 minutes in order to separate the different phases i.e. RNA, DNA and protein. Then the solution containing the different phases was centrifuged at 12000 rcf for 15 min at 4°C and 400 µl of the upper phase (RNA) was transferred to a new tube. 500 µl of isopropanol was added to the RNA and mixed by inverting the tube 10 times. After 5 min the content was centrifuged at 12000 rcf for 10 min at 4°C. Supernatant were removed from the pellet and 1 ml of 75% EtOH added to each sample. After a brief vortex, the pellet in EtoH was centrifuged at 7500 rcf for 5 min at 4°C. The supernatant were removed and pellet dried at room temperature. The pellet was resuspended in RNase-free water and RNA concentration measured with Nanodrop. The ratio 260/280 above 1.8 was considered of sufficient quality.

Up to 3 µg of RNA (amount depending on number of target genes) was reverse transcribed in 30 µl with the TaqMan MicroRNA Reverse Transcription Kit to complementary DNA. The transcription was run in T100™Thermal Cycler with the following settings: 25°C (10 min), 37°C (2 hours), 10°C (∞).

Gene expression was measured using real-time PCR. For each reaction 5 µl of TaqMan ® Fast Universal PCR Master Mix was mixed with 30 ng of cDNA in 4.5 µl of water (10 ng cDNA for the first experiment in 3D when the TRP channel were not analyzed) and 0.5 µl primer. The samples was tested in duplicates. For the qPCR, the following settings were used: denaturation: 95° C (20 s, 1 cycle), 39 amplification cycles: 95° C (1 s) and finally 60° C (20 s).

Data was analyzed by the comparative Cq method (2ΔΔCq method, housekeeping gene: β-actin). Results are presented as a gene expression relative to untreated group. The primer sequence number of the Taqman primers used are listed in table 2. Data was normalized to β-actin and expressed as fold change compared to control group.

Gene	*Primer sequence number*
βACT	Bt03279174_g1
COL1	Bt03214883_m1
COL2	Bt03251861_m1
AGG	Bt03212186_m1
TRPC1	Bt03214652_m1
TRPC6	Bt04301403_m1
TRPV2	Bt03210780_m1
TRPV6	Bt04290621_g1

Table 2. Primer sequences number for real-time PCR. βACT, β-actin;COL1, collagen-I; COL2, collagen-II; AGG, aggrecan; TRPC1, Transient receptor potential channel, subfamily C, member 1; TRPC2, Transient receptor potential channel, subfamily C, member 6; TRPV2, transient receptor potential cation channel, subfamily V, member 2; TRPV6, transient receptor potential cation channel, subfamily V, member 6.

PEMF treated human NP cells on CaF_2 Wavers

In order to determine the effect of PEMF on the protein expression of IVD cells (specifically on collagen expression), collaborative experiments were performed with Prof. Dr. Patrick Koelsch from the University of Washington. Human IVD cells (rather than bovine IVD cells) were used in order to adhere to the collaborative research plan, but all other parameters were identical to the thus far described experiments.

In detail, 5000 human IVD were seeded on 25.3 mm diameter x 1 mm thick calcium fluoride wafer. Prior to seeding the wafers were dipped in anti-anti and thereafter in PBS and left under a UV light for 15 minutes. Cell suspension (5000 cells in 100 µl antibiotic free medium) was seeded onto each wafer (one wafer per well in a 6-well plate) and allowed to attach for 30 minutes in an incubator. Then 5 ml of medium was added to wells with wafers. After 24 hours in culture, cells were treated with PEMF for either 5 or 10 minutes. Field strengths of 0, 0.3, 0.5, 1 and 2 mT were tested. 18 hour after the PEMF exposure, the cells-seeded wafers were fixed with 1% formaldehyde and 2% glutaraldehyde overnight. The fixed samples were washed with water, dried and sent to Prof. Dr. Patrick Koelsch, for analysis by vibrational sum-frequency-generation (SFG) spectroscopy. SFG spectroscopy, a method first

presented in 1987 probes the vibrational spectra of molecular adsorbates on an interface [107]. In the recent years the method has been used to detect substrates on an interface through a layer of cells in order to investigate various biomolecules including peptides, proteins and DNA [108-111].

For the analysis of samples, SFG spectra acquired by using a broadband SFG spectrometer (described elsewhere) [112]. Visible light with 532 nm wavelength and tunable IR pulses (100 fs pulses, repetition rate of 1 kHz) were overlapped at the samples substrate/liquid interface. The laser beams were applied to the substrate under the cell monolayer on the CaF_2 wafer from above to probe the interface. The visible light and the tunable IR pulses were overlapped at the sample with incidence angles of 67° and 55° relative to the surface normal [113]. The energy of the beams in the CH spectral region were around 200 μJ per pulse but 70 μJ per pulse in the amide region. For the ppp polarization combination between 2800 and 3000 cm^{-1}, the spectral resolution was 2 cm^{-1} with 200 shots accumulated at each wavenumber. For the ssp polarization combination in the range of 2800 - 3850 cm^{-1} and 1000 - 1100 cm^{-1}, the spectral resolution was 4 cm^{-1} with 100 shots accumulated at each wavenumber [113]. The intensity of the generated SF signal is given through the following expression:

$$I_{SF} \propto \left|x^{(2)}\right|^2 I_{IR} I_{VIS} \tag{1}$$

Where I_{IR} and I_{VIS} are the intensities of the infrared and visible beams, respectively and:

$$X^{(2)} = X_{NR}^{(2)} + X_R^{(2)} = \left|X_{NR}^{(2)}\right| e^{i\phi_{NR}} + \sum_k \left|\frac{A_k}{(\omega_{IR}-\omega_k)+i\Gamma_k}\right| e^{i\phi_{R,k}} \tag{2}$$

Where $X_{NR}^{(2)}$ and $X_R^{(2)}$ are the non-resonant and resonant contributions to the second-order surface nonlinear susceptibility but ϕ_{NR} and $\phi_{R,k}$ are the phase difference between the non-resonant and resonant contributions, respectively. A_k and Γ_k represents the amplitude and linewidth of the k^{th} surface vibrational mode with frequency ω_k [113]. All SFG spectra were analyzed using equation 2 and normalized to a non-resonant background.

Statistical analysis

For the gene expression analysis, t-test was used to compare treated samples to control. For cell proliferation a single factor analysis of variance (ANOVA) was used to determine if one group was statistically different from other. Differences were considered significant at a value of $P \leq 0.05$. Data from gene expression analysis are represented as mean ± SEM.

Results

PEMF treatment of bovine NP cells in 3D culture

PEMF treatment of 3D-cultured NP cells (10 min after 6 days in culture) did not result in any significant changes in the gene expression of collagen I, collagen II or aggrecan compared to untreated controls (Figure 9). Some trends could be observed for the 1 mT group for collagen I and collagen II, but these were minor (max 1.57) and statistically not significant, so that their biological relevance is questionable.

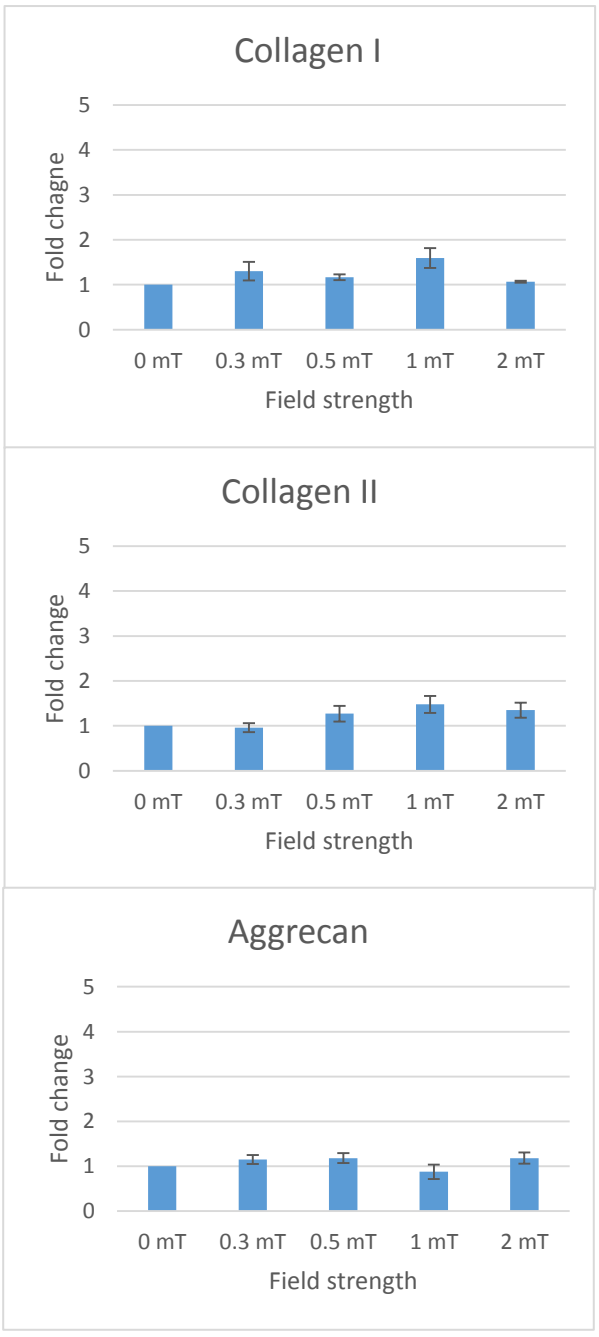

Fig. 9. Changes in gene expression in 3D cultured NP cells after exposure to PEMF (n=3). Mean ± SEM. $p<0.05$.

PEMF treated bovine NP cells after 3 days in 2D culture

PEMF treatment of 2D-cultured NP cells (10 min after 3 days in culture) did not result in any significant changes in the gene expression of collagen I, collagen II, aggrecan or TRPC1 at any field strength compared to untreated controls (Figure 10). Lower FCS concentration (2.5% vs. 10%) did not result in any significant changes in gene expression either, indicating that FCS does not influence cellular responses to PEMF. The expression of TRPC6, TRPV2 and TRPV6 was below the detection limit when using 30 ng cDNA per reaction. The fold change of PEMF exposed samples were in all cases minor (max 1.47) and statistically not significant.

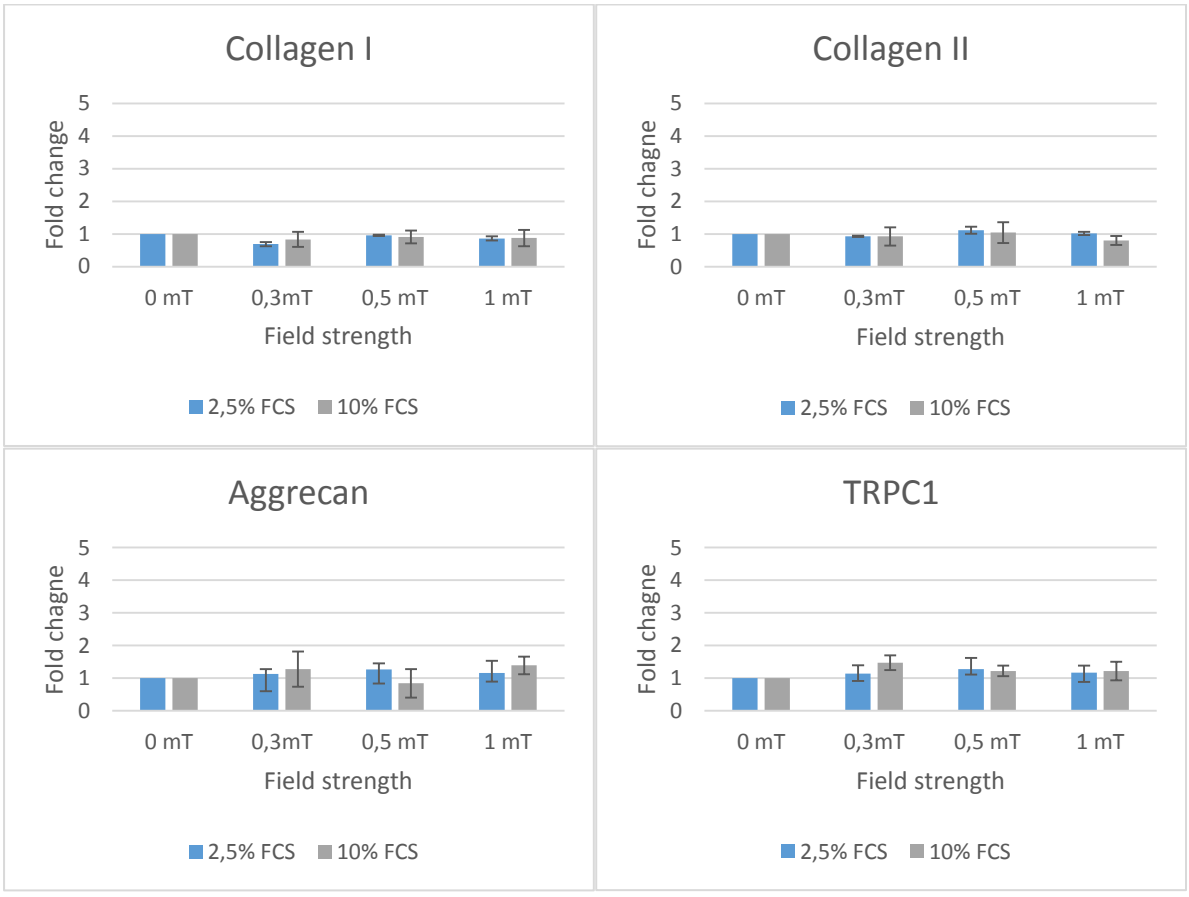

Fig. 10. Changes in gene expression in 2D cultured NP cells after exposure to PEMF at the indicated field strengths. Mean ± SEM, (n=3). $p<0.05$.

Changes in gene expression for higher sample number (n=5) for one specific field strength only (0.3 mT) can be seen in Figure 11. Only one condition was tested because of limited cell number gained from the tissue, also the short culture time allowed only limited expansion of cells.

No significant changes in the expression of collagen I, collagen II, aggrecan or TRPC1 compared to untreated controls can be seen. Neither had a lower FCS concentration (2.5% vs. 10% FCS) a significant influence. As before, the expression of TRPC6, TRPV2 and TRPV6 was below the detection limit when using 30 ng cDNA per reaction.

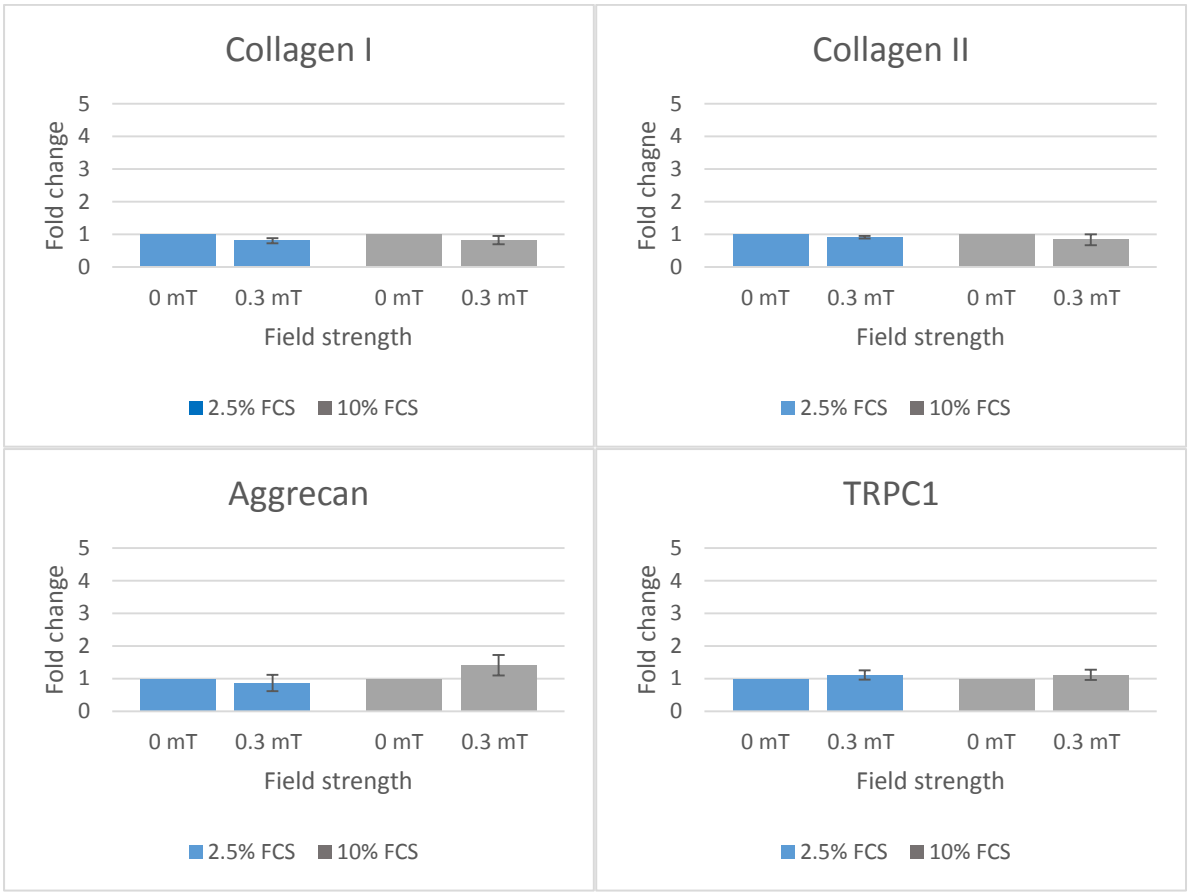

Fig. 11. Gene expression of PEMF treated NP cells after 3 days in culture at 0.3 mT field strength. Mean ± SEM, (n=5). $p<0.05$.

PEMF treated bovine NP cells after 18 hours in 2D culture

PEMF treatment of 2D-cultured NP cells (10 min after 18 vs. 72 hours in culture) did not result in any significant changes in the gene expression of collagen I, collagen II, aggrecan or

TRPC1 compared to untreated controls (Figure 12). The culture time was reduced as much as possible to fully prevent that dedifferentiation of cells did not change their responsiveness to PEMF. As in previous experiments the TRPC6, TRPV2 and TRPV6 were below the detection limit when 30 ng cDNA per reaction was used.

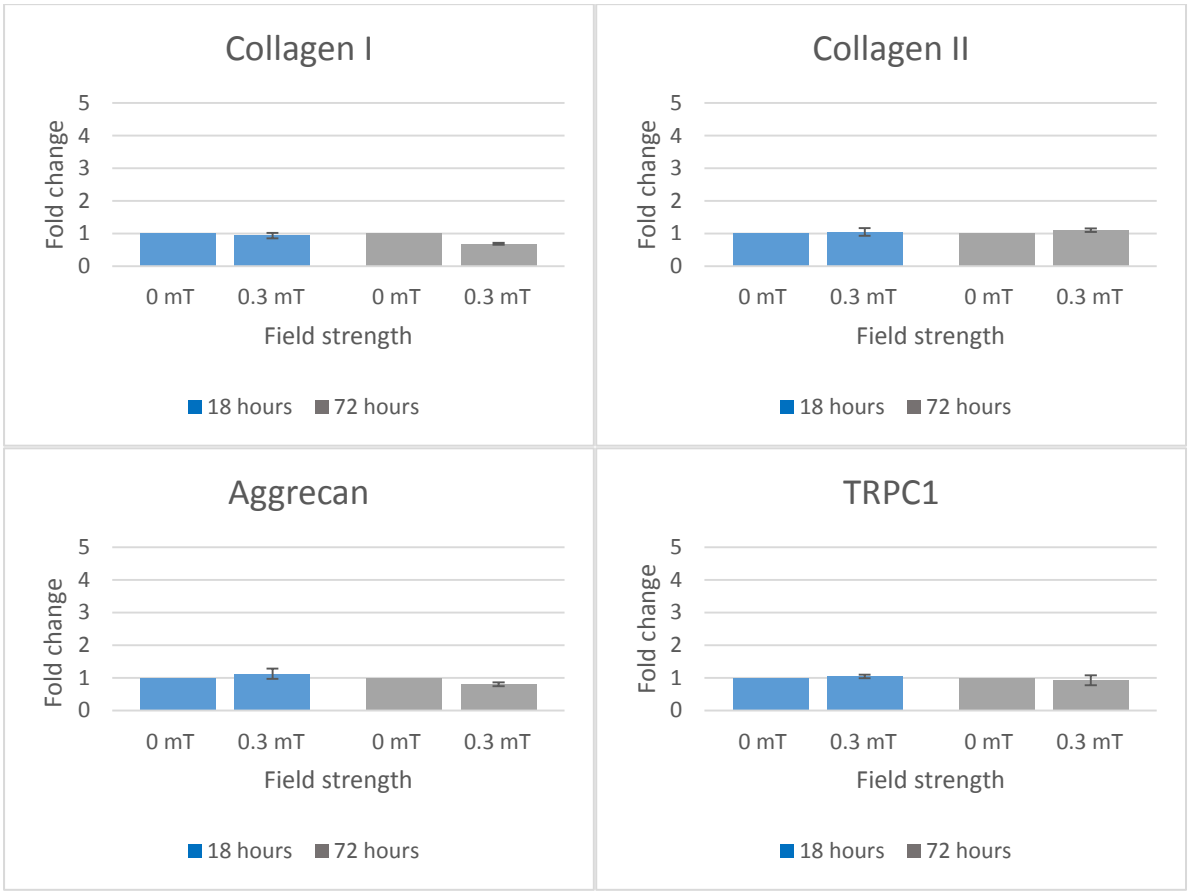

Fig. 12. Gene expression of PEMF treated NP cells after 18 hours in culture (n=3). Mean ± SEM. $p<0.05$.

Cell proliferation in 2D cultures

In figure 13 the proliferation of cells in 2D culture can be seen (n=1). Cells were treated with PEMF 18 hours after seeding (5000 cells per well, 6 wells per condition) and counted at 3 different time points after the PEMFs were applied. Statistical analysis by single factor ANOVA showed that the proliferation of cells were not significantly different (p-value = 0.99).

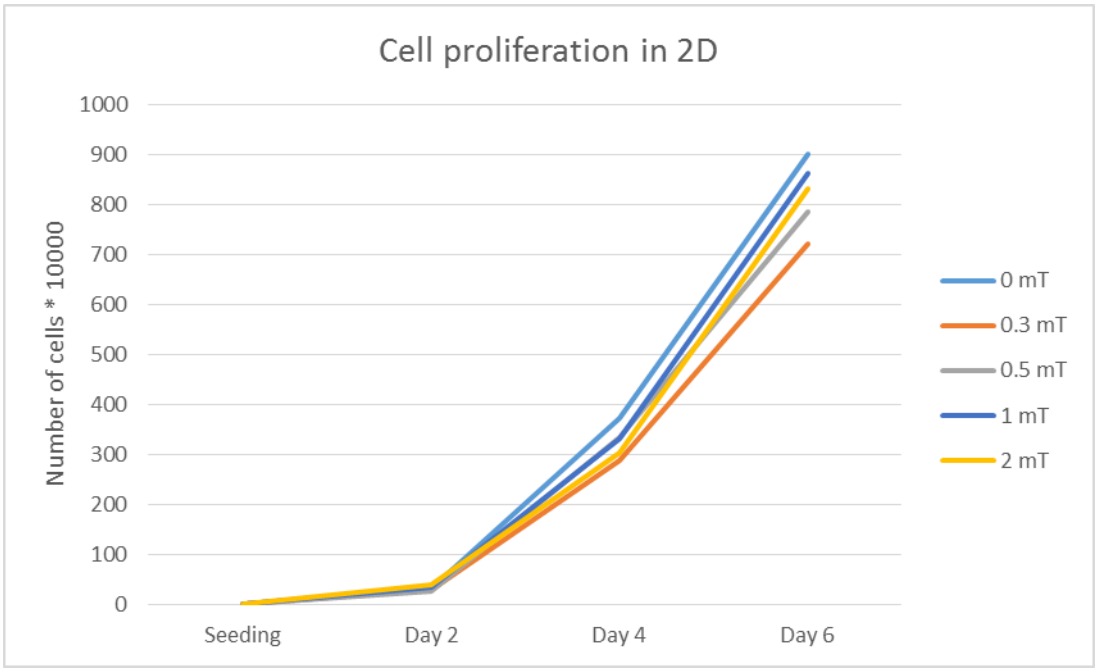

Fig. 13. Cell proliferation of 2D cultures treated with PEMF 18 hours after seeding (n=1).

Cell proliferation of PEMF treated NP cells in 3D culture

In figure 14 the proliferation of cells in 3D culture can be seen (n=1). Cells were treated with PEMF after 10 days in culture and counted at 4 different time points. One just before PEMF treatment and then 2, 4 and 6 days after treatment. Even though 10 beads were dissolved for counting per condition the variation in cell number is high. High variation is already seen when cells were counted before PEMF exposure, and continues to be high throughout all time points of counting. No trend towards a general increase in cell number can be seen. Statistical analysis by single factor ANOVA showed that the proliferation of cells were not significantly different (p-value = 0.054).

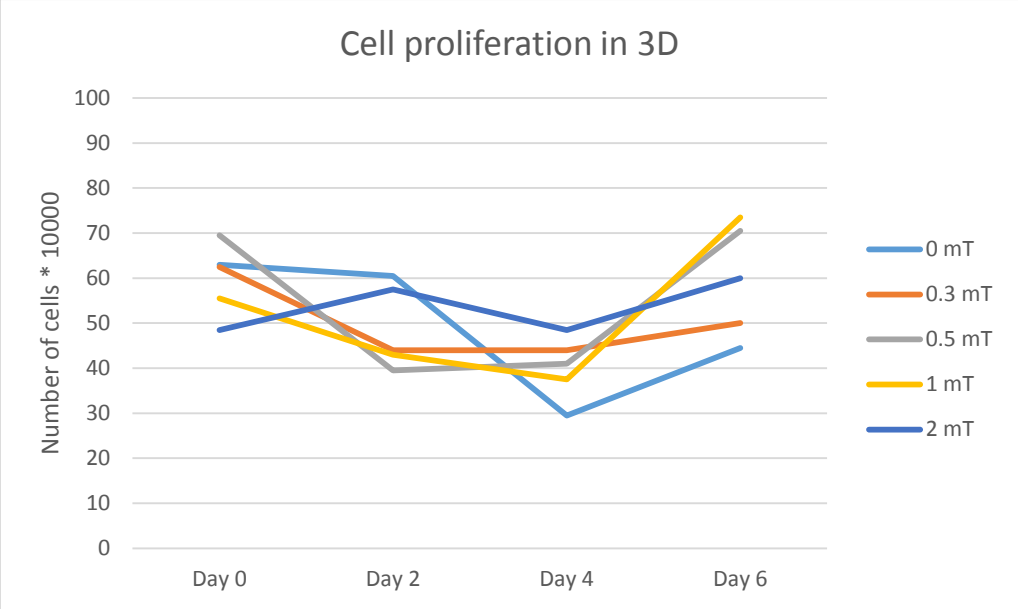

Fig. 14. Cell proliferation of 3D cultures treated with PEMF after 10 days in culture (n=1)

PEMF treatment with chemicals of bovine NP cells in 3D culture

PEMF treatment of 3D-cultured NP cells (10 min after 10 days in culture) did show changes above 2 fold for two test conditions (figure 15). The expression of collagen I was 2.67 fold at 1 mT for cells pretreated with curcumin and the expression of aggrecan was 2.18 fold at 1 mT (cells not pretreated with substances). However, the data represent only one donor so the biological relevance is questionable.

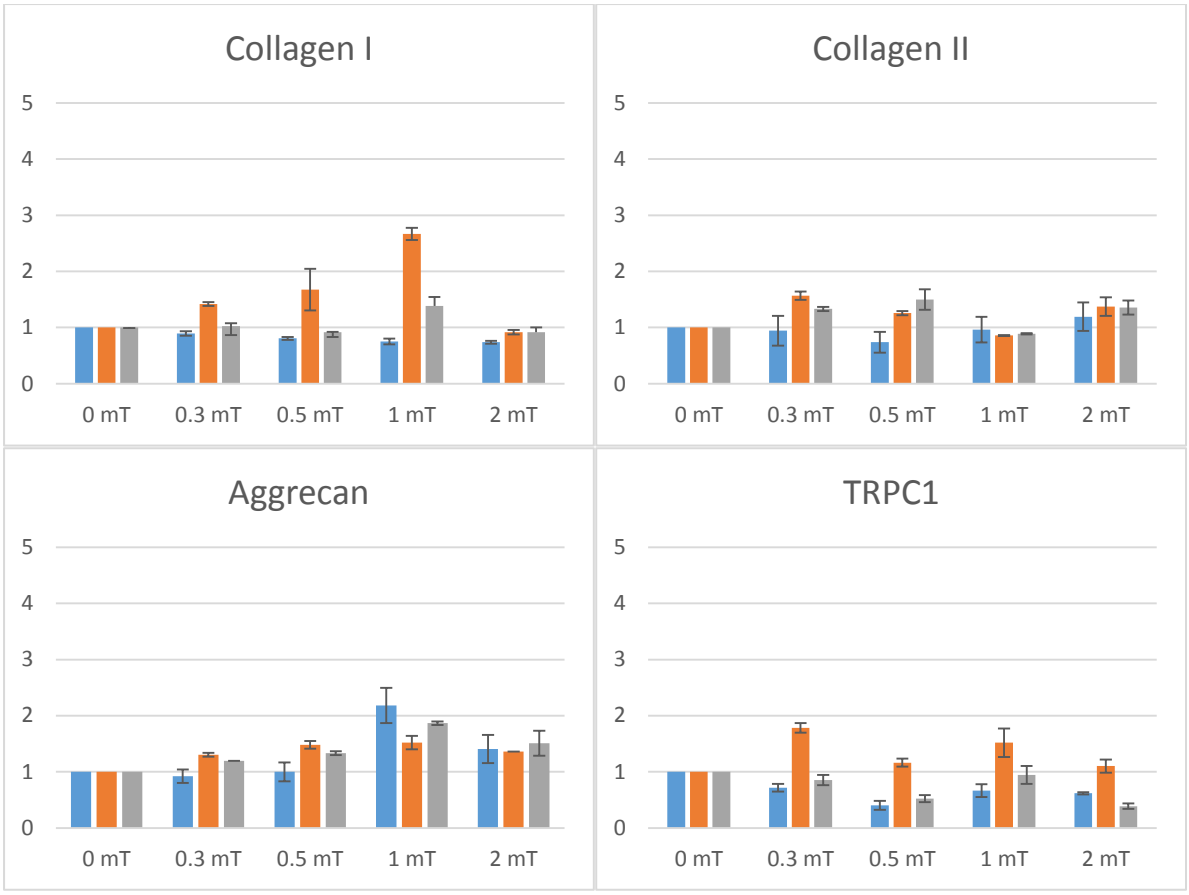

Fig. 15. Gene expression of PEMF treated NP cells after 10 days in 3D culture. Blue bars represent the control, red and grey bars show pretreated samples with chemicals. Red with curcumin and grey with resveratrol. Mean ± SEM. (n=1)

Cell viability of bovine NP by Calcein/Ethidium Homodimer staining

Table 3 and 4 show the viability of cells in 3D culture, tested with calcein and ethidium homodimer staining. No change in viability was seen for cells after 6 days in culture (table 3).

	0 mT	*0.3 mT*	*0.5 mT*	*1 mT*	*2 mT*
Viable cells (% ± SEM)	99.3 ± 0.4	98.0 ± 1.3	99.3 ± 0.7	97.6 ± 0.2	98.3 ± 0.7

Table 3. Viable cells after 6 days in 3D culture (n=1).

Change in cell viability was not altered either after 10 days in culture, neither did the pretreatment with 20 µM curcumin or 100 µM resveratrol affect the viability of the cells (table 4).

Viable cells (% ± SEM)	*0 mT*	*0.3 mT*	*0.5 mT*	*1 mT*	*2 mT*
Control	99.2 ± 0.2	99.0 ± 0.3	98.6 ± 0.4	99.0 ± 0.2	98.9 ± 0.2
Curcumin	98.3 ± 0.4	97.2 ± 1.0	98.2 ± 0.6	98.4 ± 0.3	98.6 ± 0.7
Resveratrol	97.8 ± 0.4	96.7 ± 1.3	98.6 ± 0.4	98.4 ±0.5	98.2 ± 0.4

Table 4. Viable cells after 10 days in 3D culture (n=1).

PEMF treated human NP cells on CaF_2 Wavers

Preliminary data from the SFG analysis show stronger normalized SFG intensity at 1 mT after 5 min PEMF exposure (see graph representing signal from achiral molecules) and 10 min exposure (from chiral molecules). The sample containing cells treated with PEMF at 0.3 mT for 5 minutes got damaged and could therefore not be analyzed. The control on the 5 min, chiral graph is missing. As data only show first preliminary results for only one donor, it should be interpreted with caution.

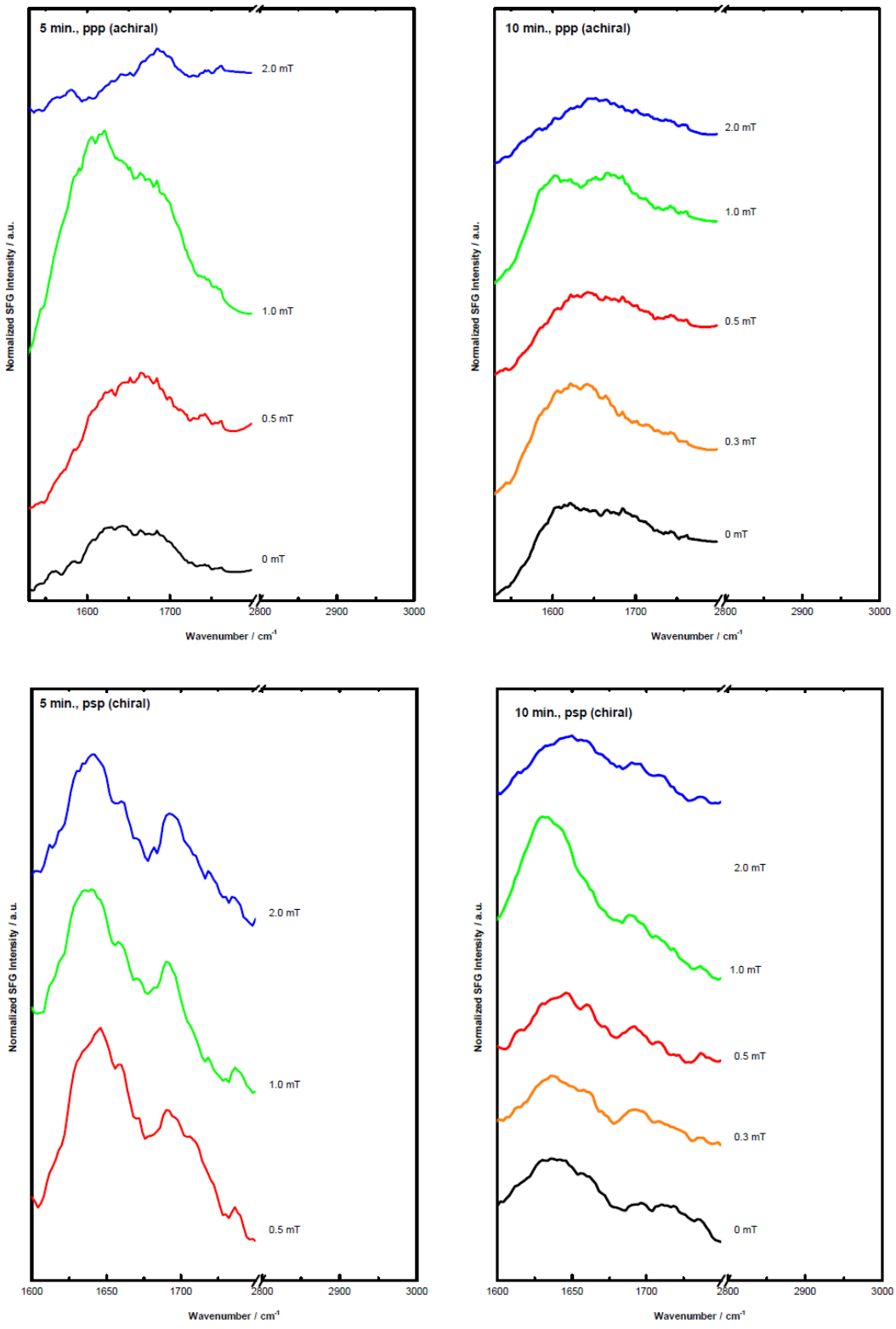

Fig. 16. Preliminary result of normalized SFG intensities (5 and 10 min PEMF exposure) after 24 hours in culture (n=1)

Discussion

In summary, results of this study demonstrate that PEMF treatment at the chosen conditions does not have a negative impact on the viability of nucleus pulposus cells. However, no beneficial effect of PEMF treatment could be observed on cell proliferation and the mRNA expression of IVD-relevant matrix proteins or the TRP channels. While cell proliferation was not increased upon PEMF treatment in our experiments, PEMF has been shown to promote the proliferation of various cell types in other studies, such as osteoblasts and chondrocytes [114, 115]. However, compared to these cell types, per se proliferation of IVD cells is relatively low [72], with bovine disc cells (which are used in this study) proliferating faster than human disc cells [116, 117]. Importantly, Held et al. described that PEMF induced proliferation of bovine NP cells [102] but after much longer exposure times (1.5 mT, 75 Hz, 18 hours per day for 2-3 weeks) which is clinically not applicable. For our study, short exposures of 10 minutes proved to be sufficient to induce cellular changes in myoblasts (unpublished data). The short exposure time was chosen due to its suitability for a clinical setting, saving both time and costs. In the future, it will be essential to determine the lowest exposure time that will result in beneficial effect while ensuring compliance from patients and health care providers. Although our data demonstrated overall no effect of PEMF on proliferation, results of cells in 3D are less clear than of cells in 2D due to high variation in 3D. The reason for the high variation could originate from insufficient mixing of cells with the alginate solution which results in inhomogeneous beads containing different number of cells. Cells could also be lost in the process of dissolving the beads. The data for both 2D and 3D should be interpreted with caution as only one donor was tested.

After the first experiment in 3D (figure 9), the changes in gene expression were small and not significant. Hence, it was felt that optimization was needed. Cellular responses can be influenced by many aspects, such as high stress level of cells, culture time or dedifferentiation status of the cells. The following experiments tested if any of these factors could influence the cellular responsiveness. It is known that chondrocytes and chondrocyte-like cells like NP cells that are expanded in monolayers gradually dedifferentiate. They lose their phenotype with time and become more fibrocytic. The synthesis of cartilage specific collagen (II, IX and XI) and aggrecan also changes towards synthesis typical for fibroblast (like collagen I, III and V) [103, 118, 119]. To be able to treat the NP cells in a state as close

to they are naturally (not in the dedifferentiated state), the treatment has either to be applied within short time after seeding or after culture in a 3D system. Culturing cells in a 3D culture systems like alginate enables redifferentiation toward their natural phenotype, thus reversing the processes occurring during culture and expansion of cells in monolayers [119]. However, no effects of PEMF treatment were observed in NP cells cultured in alginate beads, but the culture time of 6 days before PEMF may not have been long enough for proper redifferentiation of the cells. Short culture time in 2D was therefore tested (3 days in culture before treatment with PEMF) and additionally with two different types of FCS concentration. This culture time was considered short enough for minimal dedifferention of cells but still long enough to allow cells to attach and proliferate, resulting in sufficient cell numbers for several experimental conditions. Two different FCS concentrations were used as experiments on PEMF exposed myoblaststs (15 Hz, 1 mT) showed that cells were more responsive to PEMF in lower FCS concentration (unpublished data). However in the case of NP cells, no changes in gene expression could be seen. Importantly, the FCS concentration did not have any impact on the cellular responsiveness. Even when further reducing the culture time (18 hours) in order to fully prevent dedifferentiation of cells (while giving sufficient time for attachment), no alterations in the gene expression levels of matrix proteins could be seen. However, it has to be noted that large parts of the cell population had not attached at that point, which may have affected the gene expression results. Another point was that the cell didn‘t respond to the magnetic field because of too high stress level. To reduce the stress levels of the cells, they were pretreated with substances (curcumin or resveratrol) 24 hours before PEMF treatment. Curcumin and resveratrol both have been shown to have positive effects (anti-imflammatory and anti-catabolic) on NP cells [104, 105]. Extended redifferentiation phases in 3D (10 days instead of 6) and pretreatment with curcumin or resveratrol seems to have stimulating effect as higher peaks was seen as before (figure 15). As the data in figure 15 represent the gene expression of only one donor, repetitions of the experiment are needed for more reliable results.

Despite several adjustments in the protocol that aimed to control the dedifferentiation status and stress status of the cells, no significant change in gene expression were observed. While some minor trends could be observed, statistical significance was never reached due to donor-donor variation and small effect size (around 1.5 fold maximum). However, the magnitude observed is in a similar range as seen in other IVD studies on ECM genes, although they used longer exposure time [102, 120].

TRP channels were analyzed in addition to matrix proteins as there is first indication that these channels may be responsible for the observed PEMF effects in other cell types. As PEMF did not induce any biological response in NP cells in our setting, a more detailed analysis of the role of TRP channels was not suitable. However, if a suitable experimental protocol will be found in the future (e.g. with longer or repetitive PEMF exposure or different cell confluency or culture time), the role of TRP channels will be investigated again. So far, we only detected expression of TRP channels and found TRPC1 to be expressed, yet not altered by PEMF treatment. In future studies, the expression of TRP channels itself will not be sufficient to draw conclusions about their involvement. Functional studies (e.g. the use of TRP inhibitors or siRNA) will be required to determine the functional role of TRP channels with PEMF treatment [121, 122]. Even though four TRP channels were tested, only TRPC1 could be detected. The expression level of TRPC6, TRPV2 and TRPV6 may be low, so more cDNA may be needed to reach the detection limit in qPCR. Interestingly, human IVD cells express all of these channels (unpublished data), indicating that their expression level is species-specific or depends on the age or degree of differentiation. While human disc tissue was excised from patients at the age of 35 years or higher, with underlying degeneration, bovine discs were obtained from young cows with an approximate age of 1 year.

The preliminary results from the SFG analysis indicate that field strength of 1 mT promotes the production of extracellular matrix. The normalized SFG intensities were higher at 1 mT (ppp data) after 5 min exposure. This was also the case for cells treated at 1 mT (psp data) for 10 minutes indicating that PEMF can in fact influence mechanisms of the cells even though it cannot be detected on the gene level i.e. PEMF can influence cell mechanism that amplify on protein level. This data should however be interpreted with caution as these are only preliminary SFG results. In total, 3 donors were treated with PEMF but the analysis of the other two donors are still ongoing. At this time point the data can only indicate that some changes had taken place, but the analysis of all sets of samples has to be completed to get a stronger indication that this is the case.

It is clear that the evidence that we have so far is not strong enough to establish a new treatment for disc degeneration. However a longer exposure time, yet realistic in a clinical context, might be able to stimulate the production of extracellular matrix. Studies on disc cells have found small but statistically significant changes in the gene expression of extracellular matrix proteins after being exposed to PEMF [102, 120]. The numerous

parameters that needs to be controlled (cell culture conditions, field strength, frequency, exposure time etc.) makes it complex to find the exact parameters that optimizes the production of ECM for potential use in clinics.

Further experiments are needed to determine if longer treatment time than used in this project but still relevant for clinical application can promote the synthesis of extracellular matrix. Then the approach of applying PEMF as a treatment, either alone or in combination with other methods can be established.

It is possible that progenitor-like cells may be more responsive to PEMF than NP cells. As stem cells become less capable of responding to mechanical signals with age [100] it would be interesting to see how adult stem cells from NP tissue responded to PEMF by age. Recently two markers for NP progenitor cells were discovered in human and mouse [17]. This makes it possible to sort the progenitor cells from the NP cell population to test the responsiveness to PEMF. The cells need however to be expanded to some extent prior to treatment to gain enough RNA for analysis by qPCR. Also, the mesenchymal stem cell properties of these cells in the bovine system have to be tested and confirmed. Preliminary experiments with cell sorting were done in this project, but the method is still in the process of being established. The research started in the project on IVD progenitor cells will continue after this project in order to get more knowledge on their responsiveness to PEMF.

Conclusion

PEMF treatment on bovine NP cells with the parameters chosen in this study i.e. field strengths of 0, 0.3, 0.5, 1 and 2 mT applied at 15 Hz for 10 min does not negatively impact cell viability. However, the PEMF treatment has no effect on proliferation. Furthermore, for gene expression, only minor trends could be observed. These were not significant and the magnitude of the effect was small. The current knowledge about the effect of PEMF from the literature and from the results presented in this study are not sufficient to establish a practical and effective therapy for disc degeneration. Further research including experiments with longer exposure time or progenitor cells as well as analysis on protein are needed to determine whether and how PEMF can be used in future therapeutic applications on the degenerated disc.

Appendix I – Solutions

Alginate solution (1.2%)
120 mg Alginic acid sodium salt
10 ml Sodium chloride solution (0.9%)

Sodium chloride solution (0.9%)
9.0 g NaCl
1000 ml Aqua dest.

Calcium cloride solution (102 mM)
15.0 g $CaCl_2$ x $2H_2O$
1000 ml Aqua dest.

Sodium citrate solution (55 mM)
8.0 g Sodium citrate tribasic dihydrate
4.38 g NaCl (150 mM)
1.86 g EDTA (10mM)
500 ml Aqua dest.

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