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Original article

Effect of pulsed electromagnetic field stimulation on knee cartilage, subchondral and epyphiseal trabecular bone of aged Dunkin Hartley guinea pigs

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Abstract

It has been demonstrated that pulsed electromagnetic field (PEMF) stimulation has a chondroprotective effect on osteoarthritis (OA) progression in the knee joints of the 12-month-old guinea pigs. The aim of the present study was to discover whether the therapeutic efficacy of PEMFs was maintained in older animals also in more severe OA lesions.

PEMFs were administered daily (6 h/day for 6 months) to 15-month-old guinea pigs. The knee joints (medial and lateral tibial plateaus, medial and lateral femoral condyles) were evaluated by means of a histological/histochemical Mankin modified by Carlsson grading score and histomorphometric measurements of cartilage thickness (CT), fibrillation index (FI), subchondral bone thickness (SBT) and epiphyseal bone microarchitecture (bone volume: BV/TV; trabecular thickness: Tb.Th; trabecular number: Tb.N; trabecular separation: Tb.SP). Periarticular knee bone was also evaluated with dual X-ray absorptiometry (DXA).

PEMF stimulation significantly changed the progression of OA lesions in all examined knee areas. In the most affected area of the knee joint (medial tibial plateau), significant lower histochemical score ($p < 0.0005$), FI ($p < 0.005$), SBT ($p < 0.05$), BV/TV ($p < 0.0005$), Tb.Th ($p < 0.05$) and Tb.N ($p < 0.05$) were observed while CT ($p < 0.05$) and Tb.Sp ($p < 0.0005$) were significantly higher than in SHAM-treated animals. DXA confirmed the significantly higher bone density in SHAM-treated animals. Even in the presence of severe OA lesions PEMFs maintained a significant efficacy in reducing lesion progression.

Keywords: Osteoarthritis; Pulsed electromagnetic fields; Guinea pig; Chondroprotection

1. Introduction

Osteoarthritis (OA) is a multifactorial disease affecting millions of people. Aging decreases the ability of chondrocytes to maintain articular cartilage mechanical competence and restore its integrity after minor damage, resulting in tissue degeneration and loss of function [1]. Currently, treatment is aimed at relieving symptoms and in most instances it is unable to prevent disease progression. Therefore, the increasing

Abbreviations: OA, osteoarthritis; PEMF, pulsed electromagnetic field; CT, cartilage thickness; FI, fibrillation index; SBT, subchondral bone thickness; BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; DXA, dual X-ray absorptiometry; BMD, bone mineral density; BMC, bone mineral content; DFBMD, distal femur bone mineral density; DFBMC, distal femur bone mineral content; PTBMD, proximal tibia bone mineral density; PTBMC, proximal tibia bone mineral content.

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severity of OA often leads to hip or knee replacement surgery, as is well known in clinical practice. The ideal therapy of OA should be aimed at preventing or stabilising the progression of OA by acting on the underlying pathophysiological processes [2,3].

Recent findings have opened new perspectives for the understanding of the fundamental mechanism of OA and its treatment. Two recent studies by Stanton et al. [4] and Glasson et al. [5] demonstrated that the deletion of active ADAMTS5 (aggrecanase-2) prevents cartilage degradation in a murine model of OA. Cohen et al. [6] demonstrated that septic joint destruction could be prevented by treating rabbits with an adenosine receptor agonist. Finally, electromagnetic stimulation of Dunkin Hartley guinea pigs was effective in preventing OA progression in the knee joint [7]. In all the above studies it was hypothesized that a key role was played by the control of inflammatory cytokines.

Although not considered a traditional inflammatory disease, inflammation processes are closely involved in OA pathogenesis. Symptoms of inflammation and synovitis are present in many patients affected by OA [8,9]. The presence of increased levels of pro-inflammatory cytokines and chemokines (IL-1, TNF-alpha, IL-6, IL-18, IL-17) has been demonstrated in the synovial fluid and it has been shown that pro-inflammatory cytokines stimulate the expression of inflammatory matrix degrading enzymes in an OA joint [2,9–12].

The positive effect of pulsed electromagnetic field (PEMF) stimulation on OA prevention has been attributed to an anti-inflammatory effect mediated by the adenosine receptor agonist effect observed *in vitro* [13]. Moreover, PEMF stimulation was shown to increase chondrocyte proliferation *in vitro*, augment proteoglycan synthesis in full-thickness cartilage explants, prevent the catabolic effect of IL-1 on extracellular matrix, and up-regulate gene expression of the TGF-beta superfamily members *in vivo* [14–20]. These effects of PEMFs are largely dependent on exposure length and the stimulation parameter used.

PEMF stimulation has been used in clinical studies in patients suffering from OA and a significant improvement in pain and disability in PEMF-treated patients in comparison with SHAM-treated ones has been described according to different physical parameters and exposure times [21–26].

In previous studies the chondroprotective effect of PEMFs was demonstrated in 12-month-old Dunkin Hartley (DH) guinea pigs [7,27]. It is well known that OA lesion progression and pathogenesis in the DH guinea pig are very similar to those of the human form of this disease. The earliest histological signs of OA in DH guinea pigs appear at 3–6 months of age. They are more relevant in the medial tibial compartment and progress to moderate and severe degenerative changes with aging [28–33]. Particularly in the medial tibial plateau of 15-month-old animals, the present authors observed a quantifiable progressive worsening of OA lesions in comparison with 12-months aged animals with significant increase of cartilage surface irregularities, decrease of cartilage thickness and increase in epiphyseal trabecular bone eburnization (unpublished data). Therefore, in order to obtain additional information in the efficacy of PEMF stimulation in the treatment of OA lesions, the aim of the present study was to find out whether the positive effect of PEMFs on articular cartilage could be observed in more advanced stages of cartilage degeneration than previously studied [7,27]. The severity of OA lesions, in fact, is of extreme relevance in evaluating the therapeutic efficacy of any proposed treatment for OA [34].

Dunkin Hartley guinea pigs aged 15 months at the beginning of the study were used and were treated for 6 months (until the age of 21 months) with PEMFs (6 h/day).

The effects of PEMF stimulation were analysed in all areas of the knee joints: the medial and lateral tibial plateaus, and the medial and lateral femoral condyles. The cartilage was evaluated by means of a histomorphometric/histochemical score and measuring articular surface regularity, and cartilage thickness. We focussed also on subchondral and epiphyseal trabecular bone as parameters of cartilage deterioration. Cartilage degeneration causes the direct transfer of mechanical stress to the articular bone that increases its thickness and its density. Subchondral bone thickness and microarchitecture of epiphyseal femoral and tibial trabecular bone were evaluated with histomorphometry and dual X-ray absorptiometry (DXA).

2. Methods and materials

2.1. Animals and PEMF stimulator

A pre-hoc power analysis at 95% power, $p = 0.01$, was performed and it was determined that 5 guinea pigs in each group were necessary to detect a decrease in cartilage histochemical score of about 65% when comparing PEMF-treated to SHAM-treated animals [7].

The animal study was approved by the Italian Ministry of Health and was performed following Italian Law on animal experimentation. Ten DH guinea pigs (Charles River, Calco, Lecco, Italy) aged 15 months at the beginning of the study were used. The animals were housed individually in Plexiglas cages (40 × 25 × 18 cm) and nourished with standard food (Piccioni, Settimo Milanese, Italy) and given tap water *ad libitum*. Experimental conditions were set up at a temperature 20 ± 1 °C with a relative humidity of 55% and 12 h of illumination alternated with 12 h of darkness. Ten animals aged 15 months were randomly divided into two groups of five: the PEMF-treated group underwent PEMF stimulation for 6 h/day for 6 months while the SHAM-treated group underwent treatment simulation. At the end of the study, the animals (21 months old) were euthanized via intravenous injection of Tanax (Hoechst Roussel Vet, Milan, Italy) under general anaesthesia (ketamine 87 mg/kg and xylazine 13 mg/kg). Right and left knee joints were cut 1 cm above and below the joint line, stripped of muscle, and post-fixed. A total of 10 PEMF-treated and 10 SHAM-treated knees were examined.

Electromagnetic stimulators generated a pulsed electromagnetic field with the following characteristics: frequency = 75 Hz, intensity of electromagnetic field = 1.6 mT and duty cycle = 1.3 ms. The coil was placed outside the cage

and then connected to the pulsed generator. The stimulators were turned on for 6 h a day for 6 months. The same conditions were applied to the five animals housed in separate non-energized cages. This constituted the control of the experiment (SHAM-treated, Control group). When the pulsed generator was switched off, the non-static electromagnetic field background, measured by Emdex II (EnertechQ2), was of 5 ± 0.2 μT in both experimental and control cages. The instruments used to evaluate the magnetic field and the induced voltage were a Gaussmeter DG50 (Teslameter, Electrophysical Laboratory, Nerviano, Milano, Italy) and a Tektronix 720A oscilloscope (Tektronix, Inc., Beaverton). Temperature was measured in both active and SHAM conditions with a probe having a sensitivity of 0.1 °C (Hygrometer HD 8501H, Deltaohm, Padova, Italy) and no differences were observed between the 2 groups. In the exposure conditions, coils and cages were not in direct contact but separated by a 1 mm distance. The PEMF generator system was kindly provided by IGEA (ONE, IGEA Srl, Carpi, Modena, Italy).

2.2. Histology and histomorphometry

Specimens were fixed in 4% buffered paraformaldehyde and dehydrated in a graded series of alcohols for undecalcified bone processing in polymethylmethacrylate. Blocks were sectioned along a sagittal plane with a Leica 1600 diamond saw microtome (Leica SpA, Milan, Italy). A series of 10 sections, 300 μm in thickness and spaced 300 μm apart, were obtained and sliced to about 5 μm. After staining with toluidine blue and fast green, the joints were processed for histological and histomorphometric analysis with a transmission and polarized light Axioskop microscope (Carl Zeiss GmbH, Jena, Germany) and image analysis Kontron KS 300 software (Kontron Electronic GmbH, Eiching bei Munchen, Germany). The middle third of 6 central sections, defined as the central portion of the articular bone surfaces corresponding to an area of 3–4 mm^2, was analyzed for each knee and all measurements were made in this central portion [32].

The semi-quantitative histological grading criteria of Mankin modified by Carlsson [35] was used for chondropathy evaluation at a magnification of 100×. The histological score represented the sum of articular cartilage structure (from 0 in normal cartilage to 8 in the presence of clefts, extending to the zone of calcified cartilage), proteoglycan loss (from 0 in uniform staining throughout articular cartilage to 6 in case of loss of staining in all 3 zones for half the length or greater of the condyle or plateau), cellularity (from 0 in normal to 3 in hypocellularity) and tidemark integrity (0 in intact/single tidemark and 1 when tidemark was crossed by vessels and reduplicated). Cartilage thickness (CT) was measured in μm at a magnification of 20× and the cartilage surface fibrillation index (FI) was calculated according to the method developed by Pastoreau et al. [36] by dividing the length of the cartilage surface border by the length of a standardized measured area ×100 (expressed in %) at a magnification of 80×. The subchondral bone plate thickness (SBT) was measured in μm from the cartilage-bone interface to the top of the epiphyseal marrow space. The mean of 10 measurements for each section perpendicular to the articular surface was calculated.

Histomorphometric evaluation of epiphyseal trabecular bone underlying subchondral bone plate was measured on the same samples where the cartilage was evaluated. Measurements were performed on the superior half of the epiphysis (from the end of the subchondral bone plate to a distance of 450 ± 50 μm from it) as suggested by Pastoreau et al. [36]. The following parameters were calculated: trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm), and trabecular separation (Tb.Sp, μm) at a magnification of 50×.

All sections were read blindly by an experienced investigator.

2.3. Dual X-ray absorptiometry

Immediately after animal euthanasia, the bone mineral density (BMD) and content (BMC) of the proximal tibiae and distal femurs were measured using dual X-ray absorptiometry (Norland XR 26 Mark, Norland Scientific Instruments, Fort Atkinson, WI), with a scan speed of 1 mm/s and a resolution of 0.5×0.5 mm. Before taking the measurements, the instrument was calibrated by means of a Norland phantom. The BMD (mg/cm^2) and BMC (mg) were determined by the analysis of two regions of interest (ROI): the distal femur (DFBMD, DFBMC) and the proximal tibia (PTBMD, PTBMC) in an area of about 0.50 cm^2.

2.4. Statistical analysis

Statistical analysis was performed using SPSS v.12 software (SPSS Inc., Chicago, IL). Data are reported as mean ± SD at a significance level of $p < 0.05$. After testing data for normal distribution, Student's unpaired *t*-test was used to assess the significant difference between PEMF-treated and SHAM-treated animals.

3. Results

The morphological findings in the cartilage were significantly different between the PEMF- and SHAM-treated animals. Fig. 1 shows histological images of the medial tibial plateau of SHAM (a) and PEMF (b)-treated animals. Surface irregularities and loss of proteoglycan in superficial and medial cartilage zones are well visible in SHAM-treated compared to PEMF-treated animals. It is also possible to observe the increased subchondral bone thickness and epiphyseal trabecular bone eburnization in SHAM-treated animals versus PEMF-treated ones.

Fig. 2 shows images of the knee medial compartment (tibial medial plateau and medial femoral condyle) in SHAM (a) and PEMF (b)-treated animals. In SHAM-treated joints (a) the complete absence of proteoglycan in superficial, medial and deep cartilage zones of both tibial and femoral surfaces are well evident together with a high degree of hypocellularity.

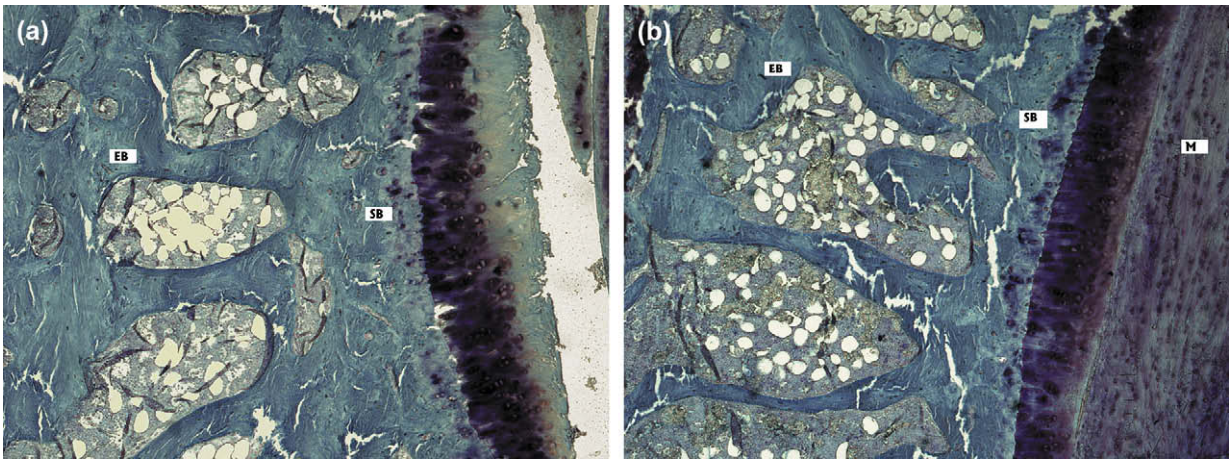

Fig. 1. a. Medial tibial plateau of a SHAM-treated animal. Surface irregularities and loss of staining in the superficial and medial cartilage surfaces are visible (Toluidine Blue staining, 50×). (SB: subchondral bone; EB: epiphyseal bone). b. Medial tibial plateau of a PEMF-treated animal. Articular cartilage maintains a normal structure and staining (Toluidine Blue staining, 50×) (SB: subchondral bone; EB: epyphiseal bone; M: meniscus). (For interpretation of the references to colour in figure legends, the reader is refered to web version of this article).

Table 1 reports the results of histochemical score, FI, CT and SBT in all knee examined areas.

In Fig. 3, data on bone histomorphometric parameters of epiphyseal trabecular bone in DH guinea pigs PEMF-treated in comparison to SHAM-treated from the age of 15 to 21 months are reported.

Six months of PEMF stimulation produced a significant delay in OA lesion severity compared to that of the SHAM-treated animals. In all examined surfaces the quite totality of bone and cartilage values measured were significantly better in stimulated versus SHAM-treated animals, demonstrating that OA was more advanced in the untreated animals.

The medial tibial plateau, as expected, was the most affected area compared to the other surfaces of the knee joint, as demonstrated by the cartilage histochemical score (cartilage structure, proteoglycan loss, cellularity, tidemark integrity), surface irregularities and clefts (FI), and epiphyseal trabecular bone microarchitecture (BV/TV, Tb.Th, Tb.Sp).

As a result of increased subchondral bone thickness and epiphyseal bone eburnization, BMD and BMC measured in the knee periarticular bone of the proximal tibia (PTBMD, PT BMC) and of the distal femur (DFBMD, DFBMC) confirmed histomorphometric results. PTBMD and DFBMD of PEMF-treated animals (0.252 ± 0.019 and 0.266 ± 0.03 mg/cm^2, respectively) were significantly lower ($p < 0.05$) than PTBMD and DFBMD of SHAM-treated animals (0.292 ± 0.036 and 0.310 ± 0.039 mg/cm^2, respectively). Finally, also PTBMC of PEMF-treated animals (0.111 ± 0.030 mg) was significantly lower ($p < 0.005$) than PTBMC of SHAM-treated animals (0.159 ± 0.032 mg).

4. Discussion

Interest in the effects of PEMFs on articular hyaline cartilage to prevent degeneration or possibly favour its repair is increasing. Both *in vitro* and *in vivo* data are quite convincing

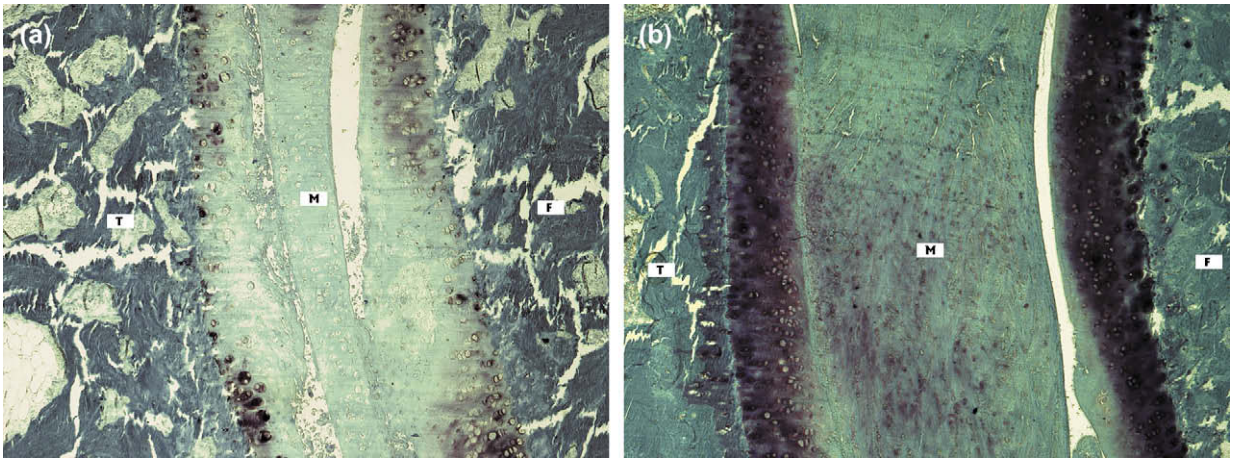

Fig. 2. a. Medial articular compartment of the knee in a SHAM-treated animal. The almost complete absence of staining and chondrocytes in articular cartilage are shown (Toluidine Blue staining, 50×) (T: medial tibial plateau; F: medial femoral condyle; M: meniscus). b. Medial articular compartment of the knee in a PEMF-treated animal. Presence of cells and proteoglycan in both femoral condyle and tibial plateau (Toluidine Blue staining, 50×) (T: medial tibial plateau; F: medial femoral condyle; M: meniscus). (For interpretation of the references to colour in figure legends, the reader is refered to web version of this article).

Table 1
Histochemical score (Mankin modified by Carlsson), fibrillation index (FI), cartilage thickness (CT) and subchondral bone thickness (SBT) in the medial tibial plateau, medial femoral condyle, lateral tibial plateau and lateral femoral condyle of Dunkin Hartley guinea pigs SHAM and PEMF-treated from the age of 15 to 21 months

Anatomic site	Histochemical score		FI		CT		SBT	
	SHAM	PEMFs	SHAM	PEMFs	SHAM	PEMFs	SHAM	PEMFs
Medial tibial plateau	13.8 ± 1.1	4.6 ± 1.5***	173 ± 5	111 ± 6**	218 ± 11	243 ± 26*	329 ± 82	263 ± 18*
Medial femoral condyle	6.3 ± 1.1	2.3 ± 1.2***	107 ± 7	102 ± 1**	150 ± 41	163 ± 14*	377 ± 44	320 ± 39*
Lateral tibial plateau	5.0 ± 1.3	2.3 ± 1.5***	101 ± 1	102 ± 2**	146 ± 8	180 ± 41*	361 ± 43	271 ± 10*
Lateral femoral condyle	4.6 ± 2.0	2.0 ± 1.4***	102 ± 1	102 ± 2	133 ± 14	144 ± 32*	355 ± 83	291 ± 31*

Differences according to Student's *t*-test between PEMF-treated and SHAM-treated animals: *, $p < 0.05$; **, $p < 0.005$; ***; $p < 0.0005$. Mean ± SD, $n = 5$.

and the being able to supply the treatment locally and non-invasively without side effects is certainly appealing also for human applications [37].

To the authors' knowledge, to date 2 experimental *in vivo* studies into the effect of PEMFs on OA have been performed and in both studies 12-month-old DH guinea pigs were used. The results of these studies demonstrated a chondroprotective effect of PEMFs on OA lesion progression in the animals' knees [7,27].

In this study, we assessed whether PEMFs could protect the joint against OA degeneration by diminishing cartilage damage progression and subchondral bone sclerosis even in more severe disease than that studied previously [7,27]. To do this, previously the spontaneous age-related lesion progression was studied in DH guinea pigs.

In fact, focussing the attention on the medial tibial plateau that is the most affected area by OA of the knee joint, the increase in OA severity in DH guinea pigs from the age of 12 months to the age of 15 months was previously demonstrated with a significant increase of cartilage surface irregularities (FI, +23%, $p < 0.005$), a significant decrease of CT (−13%, $p < 0.01$), and a significant higher density of epiphyseal trabecular bone as demonstrated by the increase of BV/TV (+42%, $p < 0.001$) and Tb.Th (+79%, $p < 0.0005$) accompanied by the decrease of Tb.N (−19%, $p < 0.005$) and Tb.Sp (−43%, $p < 0.05$) (unpublished data). Therefore, the adopted experimental model include the pathophysiological steps of human knee OA progression and data on knee joint conditions in 15 month-aged animals substained the rationale of the present study.

All cartilage parameters considered in both tibia and femoral joint surfaces demonstrated that the progression of OA was significantly delayed by the exposure to PEMFs over a 6-month period. The average modified Mankin score was 2 to 3 times higher in control animals compared to PEMF-treated ones. In fact, stimulation with PEMFs had striking effects on the cartilage degeneration that is normally characterized by a progressive loosening of structure with formation of clefts and fibrillations, loss of glycosaminoglycans and cells, and thinning.

Because of the persistence of the articular cartilage degeneration, bone metabolism parameters are more and more markedly altered; this fact could be explained by the current knowledge on OA, which suggests that chondrocytes play an important role in OA process initiation, while subchondral bone response may play a role in disease progression and is secondary to cartilage degeneration [38,39]. The loss of function of the degenerated articular cartilage exposes over time

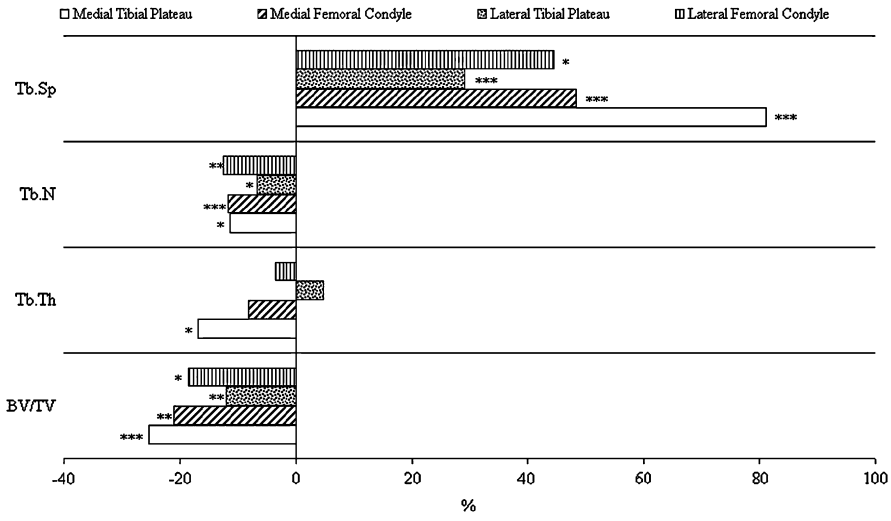

Fig. 3. Percentage variations of bone histomorphometric parameters of DH guinea pigs PEMF-Treated in comparison to SHAM-Treated from the age of 15 to 21 months in all examined knee areas (medial tibial plateau, medial femoral condyle, lateral tibial plateau and lateral femoral condyle). Student's *t*-test: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

the subchondral and cancellous bone to increased mechanical loads, thus resulting in altered metabolism. On the other side, a reduction of subchondral bone compliance may results in greater stresses being sustained by the articular cartilage leading to overloading and, consequently, breakdown [40].

The increased thickening of both subchondral bone and epiphyseal trabecular bone density is reflected in the BMD and BMC values, which are significantly higher in control SHAM-Treated animals. Increased BMD is often associated to juxta-articular bone sclerosis in the OA knee [41]. The role of DXA in the osteoarthritic distal femur of meniscectomized animals was emphasized by other authors, who found significantly increased BMD in operated animals in comparison to control ones [42].

Our results support the positive effect of PEMF against articular degeneration both in cartilage and bone in late-stage lesions. The effect of stimulation on subchondral and epiphyseal bone was not evident in previous studies involving 12 month old animals probably because the OA was not so dated and advanced [7].

In vitro studies indicate that PEMF can prevent cartilage degeneration through an adenosine receptor agonist effect that can control locally the inflammatory processes that are always associated with OA progression [13]. Drugs with adenosine receptor agonist have been shown to prevent the cartilage degeneration associated to OA [6]. *In vitro* in full-thickness cartilage explants PEMFs are able to favour proteoglycan synthesis both directly and in the presence of IGF-1 [18,19]. Furthermore, *in-vivo* PEMF stimulation favours the expression of TGF-β in DH guinea pig articular cartilage [27]. The capability to favour anabolic activity at the level of articular cartilage can be of great value in maintaining the extracellular matrix homeostasis and therefore its mechanical competence, which can ultimately protect the subchondral bone from direct mechanical stress.

These results are relevant for the human pathology and may open therapeutic perspectives for the local treatment of individual joints whenever the OA process may be accelerated after trauma, in local chronic inflammatory processes, torsion, shear stress to joint cartilage, and in professional athletes.

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