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Histomorphometric and mechanical analysis of the hydroxyapatite-bone interface after electromagnetic stimulation

AN EXPERIMENTAL STUDY IN RABBITS

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We investigated the effect of stimulation with a pulsed electromagnetic field on the osseointegration of hydroxyapatite in cortical bone in rabbits. Implants were inserted into femoral cortical bone and were stimulated for six hours per day for three weeks.

Electromagnetic stimulation improved osseointegration of hydroxyapatite compared with animals which did not receive this treatment in terms of direct contact with the bone, the maturity of the bone and mechanical fixation. The highest values of maximum push-out force (F_{max}) and ultimate shear strength (σ_u) were observed in the treated group and differed significantly from those of the control group at three weeks (F_{max}; $p < 0.0001$; σ_u, $p < 0.0005$).

Various attempts have been made to modify the surfaces of joint prostheses in order to provide early fixation and to reduce loosening.[1] However, aseptic loosening is often related to subjective factors such as endogenous bone healing, rather than to the surface characteristics of biomaterials.[1]

Stimulation by pulsed electromagnetic field has been reported to improve cell-biomaterial interactions,[2] antibiotic efficacy in implant infections[3] and symptoms in patients with a loose hip prosthesis.[4] Recently, it has been shown that it also has significant biological effects in other tissues such as the heart and the skin[5] and alters the A2A adenosine receptor density and function in human neutrophils, thereby reducing inflammation.[6] Implants with increased surface porosity and roughness show more bone contact than those with a smooth surface. They are increasingly used in dental and orthopaedic surgery but they may promote inflammation.[7] There have, however, been only a few reports in the last 20 years on the effect of pulsed electromagnetic stimulation on the biomaterial-bone interface (Table I).[1,8-13]

In a previous study *in vivo* we showed the positive effect of pulsed electromagnetic stimulation on the osseointegration of hydroxyapatite (HA) cylinders implanted in trabecular bone in rabbits. Histomorphometric and microstructural data showed both an acceleration of osseointegration and an improvement in the quality of the bone at the interface.[9] A limitation of that study was the lack of mechanical testing. We have now investigated the histomorphometry, microhardness and mechanics of HA cylinders implanted in trabecular bone in rabbits.

Materials and Methods

The study was performed in compliance with European and Italian law on animal experimentation. It was approved by the Ethical Committee of the University of Bologna and the Ministry of Health. The animals were operated on under general anaesthesia. Antibiotics and analgesics were given in the immediate post-operative period as previously described.[9]

We used 12 adult male New Zealand rabbits of mean weight 3.250 kg (SD 0.35). The mid-diaphysis of the femur was exposed and two holes 2.9 mm in diameter were drilled bilaterally across the lateral cortices. An HA cylinder, 3 mm in diameter and 5 mm in length, was pressed into each hole. The skin was sutured in two layers. As a kinetic marker of bone mineralisation, oxytetracycline was injected intramuscularly at a dose of 30 to 35 mg/kg at days 10, 9, 2 and 1 before the animals were killed.

On the first post-operative day the animals were randomly divided into two groups: six were exposed to a pulsed electromagnetic field for six hours a day for three consecutive weeks and six were not and were the control group. Animals were killed three and six weeks after implantation. A total of 12 implants was studied at each experimental time. Six specimens were used for mechanical testing and six for histology, histomorphometry and studies on

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Table I. Main *in vivo* studies on biomaterial osseointegration after exposure to pulsed electromagnetic field

Authors	Implant site	Materials*	PEMF†	Main results*
Spadaro et al[1]	Moveable and stationary implants in the tibial and femoral medullary canal of rabbits	316 L stainless steel	Frequency: 15 Hz Burst width: 5000 μs 4 hrs/day for 2 wks	Improvement of bone formation in moveable femoral implants in PEMF-treated animals at 3 wks. Enlargement of femoral medullary canal containing moveable implants in PEMF-treated animals.
Buzzà et al[8]	Tibial metaphysis of rabbits	Titanium	Frequency: 20 Mc Pulse width: 85 μs 30 min/day for 3 and 6 wks	No significant differences in extraction torque values at 3 and 6 wks between PEMF-treated and control animals Similar histological features in both groups
Fini et al[9]	Distal end of the femur in rabbits	HA	Frequency: 75 Hz Intensity: 1.6 mT Pulse width: 1300 μs 6 hrs/day for 3 wks	Improvement of bone-HA contact ratio in PEMF-treated animals at 3 and 6 wks Increase in bone microhardness at the HA-bone interface in PEMF-treated animals at 3 wks
Ijiri et al[10]	Humeral medullary cavity of rabbits	Ti6A14V	Frequency: 10 Hz Intensity: 0.2 mT Pulse width: 25 μs 5 and 10 hrs/day for 2 wks	Improvement of new bone area in PEMF-treated animals at both 5- and 10-hr stimulation New bone area improvement in PEMF-treated animals at stimulation at 10 hrs *vs* that at 5 hrs
Matsumoto et al[11]	Distal end of the femur in rabbits	Ti6A14V	Frequency: 100 Hz Intensity: 0.2, 0.3, 0.8 mT Pulse width 25 μs 8 hrs/day/2 weeks	Improvement of bone contact ratio in PEMF-treated animal at 0.2, 0.3, 0.8 mT for 2 wks Improvement of bone area ratio in PEMF-treated animals at 0.2 and 0.3 mT for 2 wks
			Intensity: 0.2 mT 4 and 8 hrs/day for 2 weeks	Improvement of bone contact ratio and bone area ratio in PEMF-treated animals for 4 and 8 hrs/day for 2 wks No differences between 4- and 8-hour treatment
			Intensity: 0.2 mT 4 hrs/day for 1, 2, 4 wks	Improvement of bone contact ratio and bone area ratio in PEMF-treated animals for 1, 2 and 4 wks Improvement of bone contact ratio and bone area ratio in PEMF-treated animals for 2 wks *vs* those treated for 1 wk
Ottani et al[12]	Medial tibial cortex in the proximal tibia of rabbits	Natural and synthetic HA	Frequency: 50 Hz Intensity: 8 mT Pause: 2000 μs 30 min/day for 2, 4 wks	PEMF-treated animals showed more advanced bone formation in both forms of apatite at 4 wks *vs* controls
Shimizu et al[13]	Proximal tibial diaphysis of rabbits	HA and tricalcium phosphate	Frequency: 1.5 Hz Intensity: 0.18 mT Burst width: 26 000 μs 8 hrs/day for 1, 2, 3, 4, 6 wks	HA implants More bone formation in PEMF-treated animals at 3 and 4 wks Thicker bone trabeculae in PEMF-treated animals at 2, 3 and 4 wks HA surface area covered with bone in PEMF-treated animals at 2, 3 and 4 wks More bone formation in the medullary cavity area in PEMF-treated animals at 3 and 4 wks TCP implants PEMF did not show a similar beneficial effect

* HA, hydroxyapatite

† PEMF, pulsed electromagnetic field

microhardness. All the measurements for all the techniques were carried out by blinded operators.

Pulse electromagnetic field generator. Electromagnetic stimulators were provided by IGEA (Biostim; IGEA Srl, Carpi, Italy). They created an electromagnetic field with a frequency of 75 Hz, an intensity of 1.6 mT and a duty cycle of 1.35 ms.

Two coils were placed outside the cage so that they were positioned directly over the area to be stimulated, and were connected to a generator. The stimulators were turned on for six hours a day for three consecutive weeks. Each rabbit was held in a fixed position during stimulation inside the cage but was allowed free activity in the cage for 18 hours a day when not being treated. The same conditions were applied to the six control animals except that the coils were not energised.

Hydroxyapatite. HA powder was manufactured according to the mechanochemical method previously described.[9] A series of cylinders was produced with these powders by slip casting in a mould. The shaped samples obtained were treated for an hour at 1250°C and sterilised at 120°C for 20 minutes before implantation.

Histomorphometry. The retrieved femora were stripped of soft tissues, fixed in 4% buffered paraformaldehyde and dehydrated in graded series of alcohols for undecalcified bone and embedded in polymethylmethacrylate. The blocks

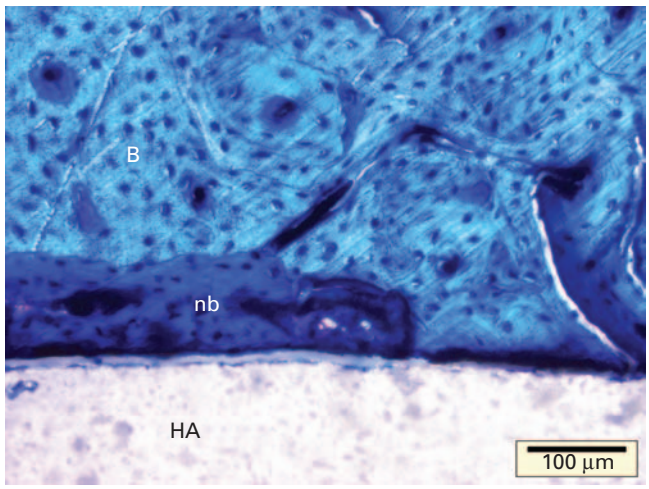

Fig. 1a

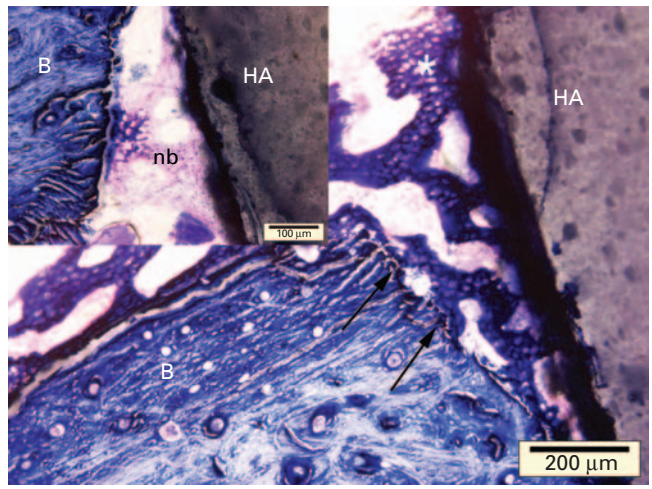

Fig. 1b

Photomicrographs of hydroxyapatite (HA) implants in the diaphysis of the rabbit femur at three weeks. There is a regular architectural pattern of the cortical bone surrounding the implants without fibrous interposition. Figure 1a – The pulsed electromagnetic field treated group. New lamellar bone tissue (nb) is seen at the HA-bone interface. Figure 1b – The control group. New woven bone (*) is growing and filling the gap between the implant and the original cortical bone (B) which shows signs of post-operative necrosis along the margin of the hole (arrows). The new bone is easily distinguished from the old by its trabecular aspect and colouration. A detail of new bone (nb) apposition around the HA implant is shown in the inset (basic fuchsia, Methylene Blue and fast green x16; inset x8).

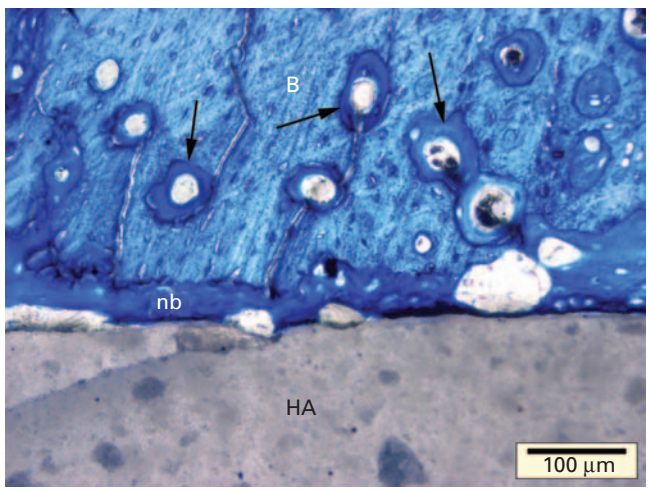

Fig. 2a

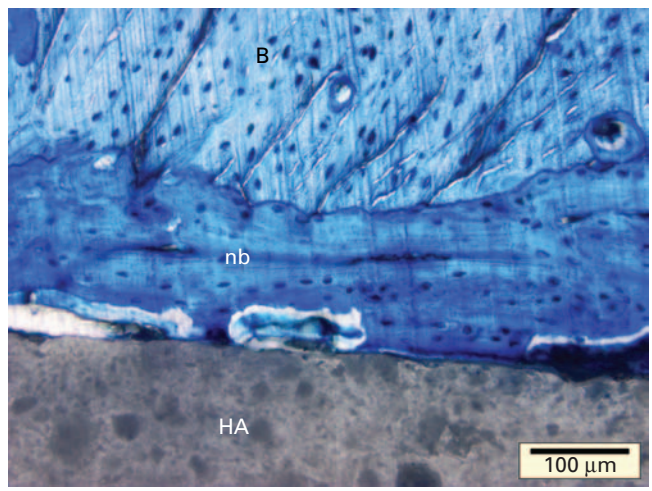

Fig. 2b

Photomicrographs of hydroxyapatite (HA) implants in the diaphysis of the rabbit femur at six weeks, showing bone apposition around the implants. Figure 2a – The pulsed electromagnetic field treated group. Secondary osteons (arrows) start to remodel the original cortical bone nearest the HA implant and tend to be orientated perpendicular to the plane of the section in the *in vivo* specimen. There is thinning of new bone at the interface with HA; Figure 2b – Control group. Dense bone is seen around the implant and the new bone (nb) can clearly be identified interposed between the original cortical bone (B) and the HA implant (basic fuchsia, Methylene Blue and fast green x16).

were sectioned along a plane parallel to the major axis of the cylinder using a Leica 1600 diamond saw microtome (Leica SpA, Milan, Italy). To allow comparison between the areas under investigation, a series of 40 µm thick sections and spaced 200 µm apart were obtained. Unstained sections were viewed under fluorescent light to evaluate the deposition of new bone around the implants. The sections were stained with 2% basic fuchsin, Methylene Blue and fast green. Routine histology and histomorphometric analyses were made using transmission and polarised light microscopy (Axioskop Carl Zeiss GmbH, Jena, Germany) and image-analysis software (KS 300; Kontron Electronic GmbH, Eiching bei Munchen, Germany).

We calculated the affinity index, which is the length of the region where bone is in direct contact with the implant divided by the total length of the interface multiplied by 100. Dynamic histomorphometry by tetracycline labelling of the newly-formed bone at the HA-bone interface gave measurement of the rate of mineral apposition (µm/day) and the rate of bone formation ($\mu m^2/\mu m$/day).

Table II. Mean (SEM) static and dynamic histomorphometric results of the control and pulsed electromagnetic field (PEMF)-treated group at three and six weeks

	Control		PEMF-treated	
Results*	**3 wks**	**6 wks**	**3 wks**	**6 wks**
AI (%)	54.9 (*8.4*)	53.5 (*5.0*)	61.0 (*5.1*)†	63.6 (*6.1*)†
MAR (μm/day)	3.39 (*0.41*)	4.24 (*0.29*)	5.64 (*0.24*)†§	4.36 (*0.49*)
BFR/BS ($\mu m^2/\mu m$/day)	1.48 (*0.06*)	1.15 (*0.05*)	1.54 (*0.11*)‡¶	1.46 (*0.08*)

* AI, affinity index; MAR, mineral apposition rate; BFR/BS, bone formation rate

† Wilcoxon *t* tests between PEMF-treated and control groups within each experimental time $p < 0.05$

‡ Wilcoxon *t* tests between PEMF-treated and control groups within each experimental time $p < 0.001$

§ Mann-Whitney U test at three and six weeks within each treatment $p < 0.01$

¶ Mann-Whitney U test at three and six weeks within each treatment $p < 0.05$

These are reported as indices of osteoblast teams and their activity following the nomenclature and methodology approved by the American Society of Bone and Mineral Research.[14]

Microhardness. The same blocks containing the residual part of the implanted HA were used to measure the hardness of bone by means of an indentation test (Microhardness VHMT 30; Leica, Wien, Austria). The detailed method has been described previously.[9] Briefly, measurements of microhardness were made tangential to the interface with a Vickers indenter applied to the bone at a load of 0.05 Kgf and at a dwell time of 5 s. The Vickers hardness degree (HV) was calculated by dividing the indentation force by the surface of the imprint (four pyramid surfaces) observed under the microscope. The mean value for each sample was calculated as a mean of ten for each area at two sites: a) in the regrown bone, within 200 μm from the interface (HV_{200}) and b) in the pre-existing host bone, at 1000 μm from the HA-bone interface (HV_{1000}). Finally, the bone maturation index (BMI) was calculated as a percentage of the ratio HV_{200}/HV_{1000}.

Mechanical push-out test. Periosteal overgrowth was removed from the specimens by grinding to obtain proper alignment. They were kept wet with normal saline at 4°C until the mechanical tests were carried out at no more than 36 hours after retrieval. The specimens were then warmed in 0.9% NaCl at room temperature for at least three hours and finally conditioned in 0.9% NaCl at 37°C during the subsequent stages of the mechanical testing which was performed within an hour.

The push-out test was carried out by placing the femoral segments on a support jig using an MTS apparatus (Sintech-1/M; MTS Adamel Lhomargy, Ivry sur Seine, France). The force was applied to the implant from the medullary side at a constant cross-head speed of 1 mm per minute. We measured the maximum push-out force (F_{max}) and the ultimate shear strength at the interface (σ_u) defined as $\sigma_u = F_{max}/(\pi\ OD\ \bar{t})$, where OD is the outer diameter of the cylinder (mm) and $\bar{t}$ is the thickness of the cortical bone (mm).

After the push-out tests, random samples collected from each group were fixed in 2% glutaraldehyde/0.1 M phosphate buffer, dehydrated in ethanol, dried at CO_2 (top critical point 30) mounted on aluminium stubs using a carbon tape and coated with a 20 nm Au/Pd layer (Coating Unit Polaron; Polaron Equipment Ltd, Watford, UK). The specimens were then examined by SEM (J840A; Jeol Tokyo, Japan) in secondary-electrons mode to determine the failure mode.[15]

Statistical analysis. This was performed using SPSS 10.1 software (SPSS/PC Inc., Chicago, Illinois). Data were reported as the median (SEM) at a level of significance of $p < 0.05$. The non-parametric Mann-Whitney U and Wilcoxon *t* tests, followed by the Monte Carlo methods to compute probability, were used to highlight any significant difference for affinity index, microhardness and mechanical findings between the pulsed electromagnetic field and control groups within each experimental time and between the three- and six-week groups.

Results

Histological findings. There was ingrowth of bone tissue in both the pulsed electromagnetic field and control groups without fibrous interposition. Bone apposition was higher in the pulsed electromagnetic field group at three weeks when small areas of bone not in contact with the HA surface were still visible. At three weeks trabecular endosteal and periosteal hypertrophy was seen in the pulsed electromagnetic field group compared with the control animals. New lamellar bone tissue formed at the HA-bone interface of the pulsed electromagnetic field animals and was intimately bound to HA (Fig. 1a). New woven bone was still growing and filling the gap between the host bone and HA in the control group at three weeks (Fig. 1b).

At six weeks, newly-formed lamellar bone containing mature Haversian systems was seen in the pulsed electromagnetic field group. In the control group, the interface was filled with young bone tissue (Fig. 2a). Bone remodelling was more advanced in the pulsed electromagnetic field than in the control animals (Fig. 2b).

Osseointegration of HA samples was significantly increased in the pulsed electromagnetic field group compared with the control group in terms of the affinity index, mineral apposition rate and bone formation rate ($p < 0.05$, Table II).

Microhardness. The microhardness of the regrown bone (HV_{200}) was increased by electromagnetic treatment at

Table III. Mean (SEM) microhardness and mechanical results of the control and pulsed electomagnetic field (PEMF)-treated groups at three and six weeks

	Control		PEMF-treated	
Results*	**3 wks**	**6 wks**	**3 wks**	**6 wks**
HV_{200}	49.7 (*5.5*)	55.6 (*0.2*)	67.6 (*2.6*)†	54.2 (*0.9*)
BMI (%)	54 (*5*)	58 (*3*)	77 (*2*)†	70 (*1*)
F_{max} (N)	87 (*3*)	132 (*12*)	234 (*21*)‡§	167 (*17*)
σ_u (MPa)	6.3 (*0.1*)	7.9 (*0.5*)	14.6 (*0.8*)‡¶	8.9 (*0.5*)

* HV_{200}, Vickers hardness degree within 200 μm from the interface; BMI, body mass index; F_{max}, maximum push-out force; σ_u, ultimate sheer strength at the interface

† Wilcoxon *t* tests between PEMF-treated and control groups within each experimental time $p < 0.01$

‡ Wilcoxon *t* tests between PEMF-treated and control groups within each experimental time $p < 0.001$

§ Mann-Whitney U test at three and six weeks within each treatment $p < 0.05$

¶ Mann-Whitney U test at three and six weeks within each treatment $p < 0.0005$

three weeks (Table III) but as expected, treatment did not have any effect on that of pre-existing host bone.

Mechanical push-out tests. There was an improvement in the osseointegration of HA implants in the pulsed electromagnetic field treated group, in terms of the maximum push-out force (F_{max}) and ultimate shear strength (σ_u) (Table III). The highest values of F_{max} and σ_u were observed in the pulsed electromagnetic field treated group at three weeks and differed significantly from those of the control group at the same experimental time ($p < 0.0005$). SEM observations revealed that bone was detached from the implant surface by the push-out test at both experimental times and fractures were located at the implant-bone interface. Microfractures were also observed.

Finally, significant ($p < 0.0005$) correlations were found between microhardness and the mechanical parameters: BMI – F_{max}, Spearman, $p = 0.895$ and BMI – σ_u, Spearman, $p = 0.900$.

Discussion

Enhancement of bone ingrowth is needed to improve the rate of success in terms of the reliability and longevity of orthopaedic implants. Adjuvant therapies could improve osseointegration, particularly when endogenous osteogenetic potential is expected to be low.[16] Our *in vivo* study aimed at evaluating the effect of electromagnetic stimulation on the fixation of HA implants in the cortical bone of rabbits during the initial phases of bone healing.

We found a positive effect of electromagnetic stimulation on bone apposition, mineralisation and mechanical attachment to HA. The dynamic histomorphometric parameters showed a positive effect of treatment on the formation and mineralisation of bone at three weeks. The data strongly confirm those obtained by Canè, Botti and Soana[17] when studying the healing process of transcortical holes in diaphyseal compact bone. They observed a significant increase in mineral apposition rate in electromagnetically treated cases in comparison with the control group and concluded that treatment stimulated the activity of osteoblasts.[17] At six weeks HA osseointegration was not increased compared with that at three weeks and this steady state was accompanied by a decrease in mineral apposition rate and BFR.

At the HA-bone interface the greatest mineralisation and microhardness were observed after three weeks of electromagnetic stimulation. Finally, the mechanical results paralleled those obtained from measurement of microhardness. Electromagnetic stimulation produced a strong implant-bone fixation at three weeks, which was reduced at six weeks but gave mechanical values higher than those of the control group at the same time. Because of the lack of data on the osteogenic effect of electromagnetism after the stimulation is turned off, and on implants in cortical bone, we suggest that there is a different temporal pattern of bone ingrowth and remodelling between treated and untreated animals. Bone remodelling started earlier in the treated animals when bone apposition to HA was almost complete and could have been responsible for the observed results. Push-out data were, at least in part, influenced by the characteristics of bone around the implants. The remodelling phase of bone is already at an advanced stage in electromagnetically-treated animals, while in untreated animals at six weeks bone density is increased, as reported by other authors.[18] When studying biomaterials with different rates of osseointegration, they observed a lower percentage of bone apposition combined with a higher bone density. In untreated animals, the lower rate of osseointegration of HA observed at both three and six weeks may have resulted in an increased bone mass around the implants.[18]

Good correlation has been reported between the microhardness and the mechanical parameters and could explain the linear relationship between findings of the biomechanical push-out tests and the data on microhardness.[9,19] No correlation between mechanical (microhardness and push-out) and the affinity index was observed. It may be explained by the unequal distribution of stresses along the interface during the push-out tests as well as by the variations in the biomechanical characteristics of the bone tissue in contact with the implant.[15,20]

The physical parameters of the electromagnetic field were the same as those of our previous study because of the positive results obtained for HA osseointegration and in previous *in vitro* and *in vivo* studies on bone healing.[2,9,17,21-23] Other studies supported the choice of a treat-

ment period of three weeks because of evidence of the major differences between the treated and untreated animals at this time.[1,9,13]

In summary, electromagnetic stimulation appears to be a promising treatment for accelerating HA osseointegration in both trabecular and cortical bone. However, the weakness of our transcortical non-weight-bearing model is that it does not reflect the microenvironment around hip prostheses and it uses small animals.[24] Issues such as biomechanical loading, physical forces, micromovement at the interface, fluid pressure waves, the related host response or changes which occur at the implant-bone interface may be further investigated in joint replacement models in larger animals.

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