

## Review Article

# The Use of Pulsed Electromagnetic Fields to Promote Bone Responses to Biomaterials *In Vitro* and *In Vivo*

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Implantable biomaterials are extensively used to promote bone regeneration or support endosseous prosthesis in orthopedics and dentistry. Their use, however, would benefit from additional strategies to improve bone responses. Pulsed Electromagnetic Fields (PEMFs) have long been known to act on osteoblasts and bone, affecting their metabolism, in spite of our poor understanding of the underlying mechanisms. Hence, we have the hypothesis that PEMFs may also ameliorate cell responses to biomaterials, improving their growth, differentiation, and the expression of a mature phenotype and therefore increasing the tissue integration of the implanted devices and their clinical success. A broad range of settings used for PEMFs stimulation still represents a hurdle to better define treatment protocols and extensive research is needed to overcome this issue. The present review includes studies that investigated the effects of PEMFs on the response of bone cells to different classes of biomaterials and the reports that focused on *in vivo* investigations of biomaterials implanted in bone.

## 1. Biomaterials and Bone Regeneration

Biomaterials play an important role in bone regenerative strategies [1] in both orthopedics and dentistry as scaffolds [2] or as a support for prosthesis, e.g., hip or dental implants [3]. In all these clinical situations the challenge biomaterials must face is to integrate in the host and promote bone healing along its surfaces [4], albeit with noticeable differences. Most scaffolds are made of resorbable materials, because common opinion dictates that scaffolds should progressively be replaced by native tissue [5], whereas prostheses are mostly permanent implants and their purpose is to last and function as long as possible in patients, usually while withstanding relevant mechanical forces in the process [6]. Thus, most scaffolds currently used in bone are made of bioceramics, predominantly calcium phosphates, because of their chemical similarity to the inorganic matrix of bone [7], which makes them osteoconductive [8, 9]. Furthermore, bioceramics are rigid and their mechanical properties have been shown to positively affect cell differentiation along the osteoblastic lineage [10, 11]. Last but not least, this class of biomaterials

is usually very biocompatible and resorbable within a time span that appears to quite closely meet the requirements for implantation into natural bone [8]. Although bioceramics can be loaded with biologically active ions [12] or biomolecules [13] to improve bone formation, they are not as versatile and customizable as polymers, whose structure can be modified almost *ad libitum*, enabling researchers to add functional groups and control their polymerization, their chemical behavior, their mechanical properties, and resorbability [14–16]. Polymers have opened up hitherto unexplored possibilities, such as injection of photopolymerizable compounds [17] or easy 3D printing [18].

In contrast, implantable prostheses are still mostly made of titanium and its alloys, although novel and highly resistant ceramics, i.e., zirconia, could represent a viable alternative [19, 20]. Titanium is a very biocompatible metal, which has been shown to represent an efficient material for orthopedic and dental implants [21]. A lot of effort has gone into investigating optimal surface treatments to optimize bone response and speed up tissue healing after surgery [4, 22]. What bioceramics, most polymers, and metals still

lack is, however, specific biochemical cues that can control cell behavior toward desired clinical goals, beside generic stimuli, such as calcium release from resorbable bioceramics or stiffness-related mechanical stimulation of cell differentiation, unless of course these materials are loaded with bioactive compounds [13, 23]. Most of these materials still offer the organism just a viable framework within which to heal or regenerate, supporting the process but fundamentally relying on the drive to healing that is intrinsic to many tissues, especially epithelia and bone. This means that those numerous clinical situations where the tissue regenerative potential has been compromised due to age or pathology are still a serious challenge and adjunctive or ancillary therapies are still an issue of interest and hot debate. This is where additional, physical therapies such as electromagnetic fields could play an important, if not vital, role.

## 2. Electromagnetic Fields and Bone

Electromagnetic fields (EMF) are created by the interaction of electrically charged objects and permeate our whole reality [24]. Our world is flooded with artificial EMFs created by electrical and electronic devices [25] and although these have become a source of potential health concerns [26–31], research has long sought a way to harness their therapeutic potential [32]. To this purpose, different sources of low frequency EMFs have been actively investigated. These can be further divided into Pulsed EMFs (PEMFs), where the EMF signal is delivered in pulses of different shape interspersed with gaps and sinusoidal EMFs (SEMFs), where the superposition of the EMF signal continuously and gradually varies along a sine waveform [33].

It is known that the effects of electromagnetic fields on living beings are complex. Organisms are composed of cells, which possess an electrically charged membrane and tightly regulate the concentration of ions, electrically charged particles, e.g.,  $\text{Ca}^{2+}$  or  $\text{Na}^+$ , which they use as potent signal mediators [34]. It is therefore likely that most of the effects of EMFs in cells occur or are triggered at the membrane level. There is abundant evidence suggesting that EMFs can act on  $\text{Ca}^{2+}$  concentration [35–37] and Ca-dependent pathways [38], and more recently Vincenzi et al. have convincingly shown a regulation of Adenosine receptors by PEMFs [39]. Actually the recent evidence by Yan et al. [40] and Xie et al. [41] of a role of primary cilia in transducing EMF effects in cells could be a part of a broader activity on membrane trafficking, including receptor trafficking. Further mechanisms are likely to be involved as PEMFs have been shown to modulate defenses against Reactive Oxygen Species [42] and the production of bioactive factors [40, 43–45] and to activate intracellular pathways such as the sAC–cAMP–PKA–CREB signaling pathway [46].

Most life science and biomedical research has been focused on the biological effects of PEMFs of different waveform, frequency, and intensity on different tissues and in different clinical situations. Bone has long been recognized as a suitable target for EMF treatment [47].

Indeed EMFs have been investigated as a tool to promote bone healing in several preclinical studies of bone defect

healing in rodents, encompassing diverse defect models, e.g., limb or facial defects [48–56], bone loss due to (a) hyperparathyroidism [57], (b) glucocorticoids or ovariectomy [58–66], (c) disuse [67–69], or (d) diabetes [70], or even osteoporotic fractures [71] or osteoarthritis [72]. Different animal models, e.g., horses, were used as well for PEMF testing [73, 74], with positive results.

EMFs have also a long clinical story as an aid to reduce bone loss in osteoporosis [75–77], to improve osteotomies or nonunions [78–93], and different research groups have investigated frequencies, intensities, durations of exposure, pulses [94–97], or waveforms [98].

Actually EMFs can be administered in a vast range of modalities. Stimuli can be delivered as single pulses, or discrete pulses, or even complex arrays of pulse bursts, also known as Pulsed Radio Frequencies (PRF), similarly to FM radio receivers. In this case the single pulses that constitute the carrier frequency reach the kHz range, but these are modulated into sets or trains of pulses that cycle at slower frequency, often 15 Hz. Using high carrier frequency increases the penetration of EMFs throughout the body, which then is able to demodulate the signal and perceive the modulating frequency, which exerts the biological effect [99]. Intensities range across a wide spectrum as well, from  $\mu\text{T}$  to a few mTesla. However, a fundamental lack of understanding of the mechanisms of actions of EMFs on cells and tissues has been presented to reach a consensus on a set of clinical parameters to maximize the effects of EMFs [47].

To further compound this problem, it must be remembered that different biomaterials may require different stimulations to optimize the outcome and this has also hindered proving their clinical effectiveness, in spite of promising results [100–103].

Therefore, the present study will review the available literature on the effects of EMF treatment on osteoblasts and bone *in vitro* and in preclinical animal models *in vivo*.

## 3. The Effects of PEMFs on Osteoblasts

Several parameters have been shown to affect cell responses, e.g., PEMF waveform, its frequency, its intensity, or the duration of exposure. A study by J. Zhou et al. investigated the effects of EMF waveform on primary rat calvaria cells [98]. When comparing 50 Hz, 1.8 mT sinusoidal, triangular, square, or serrated EMFs on primary osteoblasts, the authors observed that only square waves significantly increased cell proliferation and that sinusoidal waves decreased it. Interestingly, only triangular and sinusoidal waves, however, significantly increased cell differentiation, as assessed by Alkaline Phosphatase activity or mineralization assays. Although the group by Zhang et al. reported similar findings [33], other studies report conflicting evidence.

Martino et al. [104] exposed human osteosarcoma SaOS-2 cells to 0.9 mT, 15 Hz PRF PEMF quasi square bursts of 4 kHz square pulses for 4 hours/day, and they observed an increase in ALP activity and the deposition of mineralized nodules although no effect on cell proliferation was reported. Their results were confirmed by Hannay et al., who applied a similar stimulation (15 Hz PRF bursts of trapezoidal pulses)

TABLE 1: The table summarizes the in vitro and in vivo studies on the effects of PEMF stimulation on osteoblastic primary cells and cell lines on calcium phosphate biomaterials. Studies are listed in chronological order.

Experimental model	Biomaterial	PEMF	Field intensity (mT unless otherwise specified)	PEMF waveform	Exposure	PEMF Generator	Reference
Defects in proximal tibia of rabbits	Porous hydroxyapatite (HA) or tricalcium phosphate (TCP) nails	1.5 Hz, 26 ms-long PEMF bursts of 3.8 kHz pulses	0.18	Quasi square	8 hours/day for up to 6 weeks	American Medical Electronics (Dallas, TX, U.S.A.)	(Shimizu et al., 1988)
Defects in rabbit tibia	Natural or synthetic hydroxyapatite granules	50 Hz	8	Triangular	30 min/12 hours for up to 4 weeks	In-house built generator	(Ottani et al., 2002)
Defects in rabbit femur (condyles)	Synthetic HA rods obtained by granule sintering	1.3 ms-long, 75 Hz	1.6	Trapezoidal	6 hours/day for 3 weeks	BIOSTIM, Igea, Carpi, Italy	(Milena Fini et al., 2002)
Defects in rabbit femurs (cortical bone, mid-diaphysis)	Synthetic HA rods obtained by granule sintering	1.3 ms-long, 75 Hz	1.6	Trapezoidal	6 hours/day for 3 weeks	BIOSTIM, Igea, Carpi, Italy	(M. Fini, Giavaresi, Giardino, Cavani, & Cadossi, 2006)
Commercially available human mesenchymal stem cells	Commercially available calcium phosphate discs	4.5 ms-long, 15 Hz bursts of 4.4 kHz, 225 $\mu$ s-long pulses	1.6	Quasi-square (with trapezoidal pulses)	8 hours/day	Electro-Biology Inc., Parsippany, NJ	(Z. Schwartz et al., 2008)
Commercially available mesenchymal stem cells, normal human osteoblasts, MG-63 or Saos-2	Commercially available calcium phosphate discs	4.5 ms-long, 15 Hz bursts of 4.4 kHz, 225 $\mu$ s-long pulses	1.6	Quasi-square (with trapezoidal pulses)	8 hours/day	Electro-Biology Inc., Parsippany, NJ	(Zvi Schwartz, Fisher, Lohmann, Simon, & Boyan, 2009)
Human osteosarcoma Saos-2 cells	Commercially available discs of porous bovine natural apatite	1.3 ms pulses at 75 Hz	2	Trapezoidal	24 hours/day for 22 days	BIOSTIM, Igea, Carpi, Italy	(Lorenzo Fassina et al., 2010)

with a 1.6 mT intensity to Saos-2 and observed significant increase in ALP activity [105]. Other cell models, such as human osteosarcoma MG-63 [43, 106–108], mouse calvaria osteoblastic cell line MC3T3-E1 [36, 95, 109–114], rat primary calvaria cells [37, 40, 41, 45, 115, 116], primary human osteoblasts [42, 117–119], adipocyte-derived mesenchymal stem cells [118, 120–122], or bone marrow stromal cells [120, 123–133] were tested as well. As anticipated, most studies on osteoblast-related cell models rely on the 50–75 Hz range of stimulation [40, 41, 107, 108, 134–137] or, alternatively, on the use of 15 Hz PRF burst system [43–45, 105, 111, 112, 132, 138, 139]. The spectrum of intensities used is quite broad but, taken together, most works focus on the 0.6–2 mT [40, 41, 110, 137].

When osteoblastic cells grow on biomaterials however, a further layer of complexity is added. For the sake of simplicity, these studies were divided according to the nature of the biomaterial used.

#### 4. PEMFs and Calcium Phosphate Scaffolds

All the studies on EMFs and calcium phosphate scaffolds included in the present review are listed in Table 1. One of the first studies to investigate the effects of PEMFs on bone response to bioceramics was performed by Shimizu et al. who implanted porous hydroxyapatite (HA) or tricalcium phosphate (TCP) cylinders in the proximal tibia of rabbits,

which were then exposed to 1.5 Hz, 26 ms-long PFR PEMF bursts at 0.18 mT intensity for 8 hours/day. They were able to demonstrate a beneficial effect of PEMF stimulation on bone ingrowth into HA samples, with a higher amount of newly formed bone in and around HA, in both the cortical and medullary area, up to 4 weeks after surgery, but not around TCP implants [140]. A morphological evaluation of bone ingrowth into natural or synthetic hydroxyapatite granules implanted into rabbit tibia defects was conducted by Ottani et al. using 50 Hz triangular-shaped PEMF pulses at an intensity of 8 mT for 30'-long sessions twice a day. The sacrifice and subsequent TEM and SEM observation with electron backscattering at 2 and 4 weeks after surgery showed that PEMF treatment promoted a more advanced bone formation around the granules, which appeared cemented into the healing defect [141]. In the same year a study by Fini et al. was published, which investigated the effects of PEMFs on the integration of synthetic HA rods obtained by granule sintering in bone defects created in rabbit femoral condyles. The group used 1.35 ms-long trapezoidal PEMF pulses, repeated at a 75 Hz frequency, with an intensity of 1.6 mT for 6 hours/day for 3 weeks. Although histomorphometry did not reveal any increase in bone architectural parameters after PEMF stimulation at either 3 or 6 weeks after surgery, the bone-to-implant contact (BIC) was increased in the PEMF-treated group at both time points. The same happened with the mechanical properties of the treated bones, as assessed by hardness to microindentation [142]. The same research group adopted this stimulation model again to evaluate the integration of synthetic HA rods in the cortical bone of rabbit femurs and observed that PEMFs were able to significantly increase bone-to-implant contact, Mineral Apposition Rate (MAR), and Bone Formation Rate (BFR) at both time points. They also confirmed that the mechanical properties of treated bones were increased by PEMFs, using both indentation and push-out tests [143]. The cellular effects of PEMFs on the response of human Saos-2 osteosarcoma cells to discs of porous bovine natural apatite were investigated by Fassina et al., who exposed cells to 1.3 ms trapezoidal pulses at 75 Hz, 2 mT in bioreactors for 24 hours/day for 22 days [144]. In response to PEMFs the authors observed an increase in cell proliferation and the deposition of components of the extracellular matrix.

The group by Schwartz et al. investigated the effects of electromagnetic fields on human mesenchymal stem cells, using an established stimulation model of 4.5 ms PEMF bursts at 15 Hz frequency, with each burst composed of 225  $\mu$ s-long pulses. Cells were grown on commercially available calcium phosphate discs and were exposed to PEMFs for 8 hours/day. Although, in their model, they did not observe significant effects of PEMFs on cell number or differentiation markers, the group found that electromagnetic fields synergistically stimulated cell responses to BMP-2 and promoted Alkaline Phosphatase (ALP) activity, Osteocalcin expression, and the release of TGF $\beta$ 1 [145].

Interestingly, BMPs have been shown to be involved in the responses of rat calvaria osteoblasts to PEMFs in a study by Bodamyali et al. [45] and by Yan et al. [40]. Selvamurugan et al. demonstrated that PEMFs and BMP-2 may act

synergistically in rat osteoblasts and this could be indicative of similar or overlapping signaling pathways in bone cells [115]. The group by Schwartz et al. also investigated the response of mesenchymal stem cells, commercially available normal human osteoblasts, or osteoblastic cells from two well established cell lines (MG-63 and Saos-2 cells) to 8-hour long exposures to 4.5 ms-long pulse bursts repeated at 15 Hz [146]. Their study showed that PEMFs were able to increase OPG expression in cell lines when cultured on calcium phosphate discs and synergistically increase OPG when administered together with BMP-2 in mesenchymal stem cells, while not affecting RANKL. Given the relevance of the OPG-RANKL system in bone, the effects of PEMFs on these molecular effects have been extensively studied in several osteoblastic models, also in the absence of biomaterials, and most studies agree with the results from Schwartz's groups in observing an increase in OPG following PEMF exposure. This is of obvious interest to bone researcher, because of the role of OPG and RANKL for tissue metabolism [147–150]. Schwartz's results were confirmed in cell cultures on plastic by Borsje et al. and similarly by Jansen et al. using BMMSCs [129] and even in human marrow macrophages cultures [132]. The group by Chang et al. showed that 7.5 Hz 0.3 ms long PEMF pulses increased OPG secretion [151] in mouse bone marrow cells [151]. They also observed that PEMFs enhanced OPG and hampered RANKL expression in mouse primary calvaria cells [152].

## 5. PEMFs and Titanium Surfaces or Implantable Devices

The effects of PEMFs on metal devices have been investigated in several studies. Though stainless steel implants in rabbit tibia and femurs were investigated by Spadaro et al., who observed an increase in the amount of formed bone in the medullary canal of femurs around moveable steel wires after 15 Hz PRF PEMF stimulation [153], most of the subsequent research focused on titanium and titanium alloy-based biomaterials. Saos-2 cells were used as a model of osteoblastic cells on titanium fiber-mesh scaffolds and continuously stimulated with 1.3 ms trapezoidal pulses at 75 Hz, 2 mT in bioreactors for 22 days. It was shown that PEMFs increased the expression of TGF- $\beta$  and upregulated the deposition of matrix on the scaffolds, by increasing the expression of Decorin, Osteopontin, and Type I collagen [154]. The same group investigated the effects of PEMFs using the same cell and stimulation model on sintered titanium grids [155], observing similar findings. Wang et al. stimulated primary rat calvaria cells with 15 Hz, 5 ms long bursts of 4.5 kHz pulses, 0.9 mT, on polished, sand-blasted/acid-etched or anodized nanotubular titanium surfaces [156]. Interestingly, PEMF stimulation increased protein adsorption and cell adhesion on all titanium surfaces, cell proliferation up to 7 days, and cell mineralization on all surfaces. PEMF also affected cell morphology and induced more pseudopodia and cytoskeletal reorganization that aligned cells along their main axis. Interestingly, PEMFs also increased BMP-2 expression, beside differentiation markers. Bloise et al. [157] recently stimulated human BMMSCs nanostructured TiO<sub>2</sub> surfaces

obtained through cluster-assembly by a pulsed microplasma cluster source [158, 159] with 1.3 ms long, 75 Hz PEMFs at 2 mT intensity for 10 min/day. The authors observed an increase in osteogenic differentiation in PEMF-stimulated cells, an increase in the intracellular levels of  $\text{Ca}^{2+}$ , and an increase in the extracellular  $\text{Ca}^{2+}$  deposition.

Using TiZr or titanium discs with different topography, Atalay et al. showed that the proliferative response of primary calvaria cells to 100 Hz PEMFs was clearly dependent on the microgeometry and physicochemical properties of the substrate [160].

The group of Jing et al. used 15 Hz, 5 ms long PEMF bursts with 2 mT intensity to stimulate MC3T3-E1 cells on porous titanium scaffolds (70% porosity, 750  $\mu\text{m}$  pore size) for 2 hours/day for 3 days [161]. Besides observing an increase in cell proliferation and expression of differentiation markers Runx2 and Osterix, two important transcription factors activated in osteoblasts, the group reported that PEMF treatment increased  $\beta$ -catenin, Lrp6, and Wnt1 expression, important components of the canonical Wnt pathway, at the mRNA and protein levels. Remarkably, these findings were confirmed *in vivo* after implanting porous titanium scaffolds in cylindrical defects in the femur of rabbits, which were then treated for up to 12 weeks with PEMFs. MicroCT analysis of the defects showed that PEMF treatment significantly improved bone architectural parameters, e.g., BV/TV, Trabecular Number (Tb.N), and spacing (Tb.Sp), and dynamic histomorphometry demonstrated that MAR, Mineralizing Surface, and BFR were significantly higher in rabbits treated with PEMFs than control animals. Moreover, real time PCR indicated an increase in the expression of BMP-2, consistently with Lohmann [145, 146], but also Wnt1, Lrp6 and  $\beta$ -catenin as observed *in vitro*.

These results are in agreement with Single Pulsed EMF (sPEMF) exposure of MC3T3 cells on plastic culture substrates [114]. The authors exposed this cell line to 0.2 Hz, 5 ms long, 1 T PEMF pulses for up to 20 days and observed an increase in the expression of Wnt1, Wnt3a, Wnt10b, and Wnt receptor frizzled 9 and an increase, albeit not significant, of the Wnt coreceptor Lrp6. Similarly, Zhai et al. [110] observed that 2 mT, 15 Hz bursts of 4.5 kHz PEMF pulses for 2h/day for 3 days increased the expression of Wnt1, Lrp6, and  $\beta$ -catenin in MC3T3-E1 cells.

Buzzà et al. used 85  $\mu\text{s}$  long pulses at 20 MHz for 30'/day for up to 42 days to stimulate titanium implants in rabbit tibias but failed to observe any significant increase in removal torque [162]. A slightly lower PEMF frequency (1 MHz, 25  $\mu\text{s}$  long pulses, 0.8 mT) was used by do Nascimento et al. for 20'/day for 2 weeks to stimulate postextractive dental implants in dog mandibles. The authors observed a slight increase in bone tissue formed around the implants, although no quantification was provided [163]. Matsumoto et al. investigated the effects of 100 Hz, 25  $\mu\text{s}$  PEMFs at 0.2, 0.3, or 0.8 mT for 4 or 8 hours/day on the integration of Ti-6Al-4V dental implants with anodized surface into rabbit femurs and reported that BIC was higher after exposure to 0.2 or 0.3 mT PEMFs for 4 or 8 hours [164]. This stimulation model was also used with Ti-6Al-4V dental implants inserted in

rabbit mandibles. The animals were stimulated with PEMFs for 2 weeks and sacrificed right after 2 weeks or 6 more weeks (without PEMF application). Remarkably, although no differences were observed at 2 weeks and 6 weeks after PEMF stimulation a dramatic increase in labial and lingual bone was observed in treated animals, together with higher osteoblast counts, indicating that PEMF could promote a long-acting bone formation [165]. A similar PEMF stimulation model was used by Akca et al. to investigate the effects of PEMFs on the integration of cylindrical titanium implants in tibias of ovariectomized rats. The animals were stimulated for 4 hours/day for 14 days and PEMF stimulation increased Bone Volume and trabecular number in the peri-implant bone, as determined by microCT [166]. A study by Grana et al. investigated the effects of 60 ms, 1.9 Hz PEMF bursts of 50 Hz sinusoidal trains at an intensity of 72 mT administered for 30'/twice a day on bone healing around titanium mini implants in rat tibias and found a significant increase in the amount of newly formed bone around implants at 10 and 20 days after surgeries [167]. Ten Hz, 0.4 mT PEMFs were investigated as a tool to improve the bone integration of commercially available titanium dental implants inserted in rabbit tibias in a more recent study [168]. Most noticeably, PEMFs were generated by a portable device which was installed on the implant, via a screw-retained connection, not unlike common prosthetic components. The device generated a magnetic field that was concentrated around the coronal area of the implant and steeply decreased in the surrounding areas. When considering the coronal area alone, where the signal was stronger, Bone Volume/Total Volume around test implants was 56% and 68% significantly higher than control implants at 2 and 4 weeks of healing, respectively, with corresponding increased Tb.N and smaller Tb.Sp. Moreover, by 2 weeks BIC was 15% higher around stimulated implants [168]. The idea of installing intraoral devices to stimulate implants with PEMFs was explored in several papers, as devices generating 10 Hz PRF PEMF bursts at 2 mT were proposed [169] (or even neodymium-iron-bore magnets placed in the implants and generating static magnetic fields [170]). Twenty-five  $\mu\text{s}$  PEMFs at 10 Hz and 0.2 mT were also investigated as a tool to promote the integration of porous titanium implants in the diaphysis of rabbit humerus bones for 5 or 10 hours/day and shown to increase bone ingrowth by a 14-day stimulation [171]. Cai et al. showed that 15 Hz, 5 ms PEMF bursts of 4.5 kHz pulses 2 hours/day for 8 weeks improved bone turnover serum markers and bone architecture parameters in rabbits with alloxan-induced type 1 diabetes mellitus (T1DM). More importantly for our current review, when cylindrical sintered Ti2448 implants were inserted into the lateral condyle of these rabbits, the 8-week treatment improved bone ingrowth into the scaffold and MAR around and inside the implants, which caused an increase in the mechanical properties of the trabecular bone around the implants [172]. For a list of the studies on EMFs and titanium biomaterials included in the present review, please see Table 2.

TABLE 2: The table summarizes the in vitro and in vivo studies on the effects of PEMF stimulation on osteoblastic primary cells and cell lines on titanium-based biomaterials. Studies are listed in chronological order.

Experimental model	Biomaterial	PEMF	Field intensity (mT)	PEMF waveform	Exposure	PEMF Generator	Reference
Placement in the medullary canal of femur and tibia in rabbits	Implants of 316 L stainless steel wire	5 ms, 15 Hz PEMF bursts of 4 kHz pulses	n/a	Quasi-square (trapezoidal pulses)	4 hours/day for 2 weeks	American Medical Electronics (Dallas, TX, U.S.A.)	(Spadaro et al., 1990)
Diaphysis of rabbit humerus	Bead-covered titanium implants	25 $\mu$ s PEMF pulses at 10 Hz	0.2	n/a	5-10 hours/day for 2 weeks	n/a	(Ijiri et al., 1996)
Placement in rabbit femurs	Commercially available Ti-6Al-4V dental implants with anodized surface	100 Hz, 25 $\mu$ s PEMFs	0.2, 0.3, 0.8	n/a	4 or 8 hours/day for up to 4 weeks	Riken Electromagnetic Field Pulse Generator, Institute of Physical and Chemical Research, Saitama, Japan	(Matsumoto et al., 2000)
Placement in rabbit tibias	Commercially available titanium dental implants	85 $\mu$ s-long pulses at 20 MHz	1 W	n/a	30 minutes/day for 21 or 42 days	Healtec-Celular, Healtec Eletromedicina Ltd., Brazil	(Buzzá et al., 2003)
Placement in rabbit mandibles	Custom Ti-6Al-4V dental implants	100 Hz, 25 $\mu$ s PEMFs	0.2	n/a	4 hours/day for 14 days	In-house built	(Özen et al., 2004)
Placement in tibias of ovariectomized rats	Cylindrical titanium implants	100 Hz, 25 $\mu$ s PEMFs	0.2	n/a	4 hours/day for 14 days	In-house built	(Akca et al., 2007)
Human osteosarcoma Saos-2 cells	Titanium fiber-mesh sheets	1.3 ms pulses at 75 Hz	2	Trapezoidal	24 hours/day for 22 days	BIOSTIM, Igea, Carpi, Italy	(Fassina et al., 2008b)
Human osteosarcoma Saos-2 cells	Sintered titanium grids	1.3 ms pulses at 75 Hz	2	Trapezoidal	24 hours/day for 22 days	BIOSTIM, Igea, Carpi, Italy	(Fassina et al., 2008a)
Placement in rat tibias	Custom cylindrical threaded titanium implants	60 ms, 1.9 Hz PEMF bursts of 50 Hz trains	72	Quasi-square (with sinusoidal pulses)	30 minutes/twice a day	Magnetherp (Meditea Electromédica, Buenos Aires, Argentina)	(Grana et al., 2008)
Dog mandibles, immediate post-extraction placement	Commercially available titanium dental implants	1 MHz, 25 $\mu$ s-long pulses	0.8	n/a	20 minutes/day for 2 weeks	n/a	(do Nascimento et al., 2012)
Primary rat calvaria cells	Commercially pure titanium or TiZr discs	100 Hz, 25 $\mu$ s PEMFs	0.2	n/a	2 hours/day for up to 72 hours	In-house built	(Atalay et al., 2013)

TABLE 2: Continued.

Experimental model	Biomaterial	PEMF	Field intensity (mT)	PEMF waveform	Exposure	PEMF Generator	Reference
Primary rat calvaria cells	Polished, sand-blasted/acid-etched or anodized nanotubular titanium surfaces	15 Hz, 5 ms-long bursts of 4.5 kHz pulses	0.96	Quasi-square (with square pulses)	Up to 7 days	GHY-III, FMMU, Xi'an, China	(Wang et al., 2014)
Placement in rabbit tibias	Commercially available titanium dental implants	10 Hz	0.4-0.2	n/a	24 hours/day for 2 or 4 weeks	n/a	(Barak et al., 2016)
Murine MC3T3-E1 osteoblastic cells	Porous titanium scaffolds by electron beam melting system	15 Hz, 5 ms-long bursts of 4.5 kHz pulses	2	Quasi-square (with square pulses)	2 hours/day for 3 days	GHY-III, FMMU, Xi'an, China	(Jing et al., 2016)
Defects in rabbit femurs (condyles)	Porous titanium scaffolds by electron beam melting system	15 Hz, 5 ms-long bursts of 4.5 kHz pulses	2	Quasi-square (with square pulses)	2 hours/day for 6 or 12 weeks	GHY-III, FMMU, Xi'an, China	(Jing et al., 2016)
Placement in rabbit femurs (condyles)	Cylindrical sintered Ti2448 implants	5 Hz, 5 ms PEMF bursts of 4.5 kHz pulses	2	Quasi-square (with square pulses)	2 hours/day for 8 weeks	GHY-III, FMMU, Xi'an, China	(Cai et al., 2018)
Human BMMSCs	Nano-TiO <sub>2</sub> surfaces	1.3 ms-long, 75 Hz	2	Trapezoidal	10 min/day	BIOSTIM, Igea, Carpi, Italy	(Bloise et al., 2018)

## 6. PEMFs and Polymers

Table 3 summarizes all the studies on polymer scaffolds and EMFs that were included in the present review. Polymer scaffolds were tested for cell responses to PEMFs as well. Electrospun poly(caprolactone) nanofibrous scaffolds were used as substrate to culture adipose tissue-derived stem cells, which were then stimulated with 50 Hz, 1 mT PEMFs for 6 hours/day in normal or osteogenic medium [173]. PEMFs increased cell proliferation, mineralization, and the expression of differentiation markers, such as Runx2, Osteocalcin, Osteonectin, and ALP activity. The group of Tsai et al. cultured rat calvaria osteoblasts on highly porous poly(DL-lactic-co-glycolic acid) (PLGA) scaffolds in bioreactors and stimulated them for 2 or 8 hours/day with 300  $\mu$ s long rectangular pulses at 75 Hz. The magnetic field they used had an intensity of 0.13, 0.24, or 0.32 mT. Interestingly, stimulation with 0.13 mT PEMFs was able to significantly increase cell number on the scaffolds up to day 12 of culture, while more intense 0.32 mT PEMFs significantly decreased cell number compared to the control group up to day 18 of culture. However, not surprisingly, the highest intensity

was also most effective in increasing ALP activity and thus cell differentiation [174]. Lin et al. used an *in vitro* inflammation model to study the effects of 75 Hz, 1.5 mT PEMFs, using previously well described instrumentation [108] in 7F2 murine osteoblasts cultured on 3D chitosan scaffolds exposed to 9 hours of treatment [135]. The osteoblastic cells were cocultured with LPS-activated RAW 264.7 macrophages. The investigators detected higher Nitric Oxide levels after PEMF treatment, consistently with the previous literature [112, 175, 176], but increased osteoblast viability and collagen expression, although reduced differentiation, as measured by ALP activity and Osteocalcin levels. In agreement with their observations, Ehnert et al. [42] exposed primary human osteoblasts to 16 Hz 0.28 mT PEMF bursts for 7 minutes/day and demonstrated an increase in defenses against reactive oxygen species after PEMF stimulation [119], which actually appears necessary for PEMF effect [42].

The response of human osteosarcoma MG-63 cells to trapezoidal 1.3 ms long, 75 Hz, 2.3. mT PEMF pulses [134] when cultured on poly-methylmethacrylate (PMMA) scaffolds or PMMA-alpha Tricalcium Phosphate ( $\alpha$ -TCP) composite scaffolds was investigated by Torricelli et al. [177].

TABLE 3: The table summarizes the in vitro and in vivo studies on the effects of PEMF stimulation on osteoblastic primary cells and cell lines on polymer-based biomaterials. Studies are listed in chronological order.

Experimental model	Biomaterial	PEMF	Field Intensity (mT)	PEMF waveform	Exposure	PEMF Generator	Reference
Human osteosarcoma MG-63 cells	poly-methyl methacrylate (PMMA) scaffolds or PMMA- $\alpha$ Tricalcium Phosphate ( $\alpha$ -TCP) composite scaffolds	1.3 ms-long, 75 Hz	2.3	Trapezoidal	12 hours/day for 3 days	Igea, Carpi, Italy	(Torricelli et al., 2003)
Primary rat calvaria osteoblasts	Porous poly(DL-lactic-co-glycolic acid) (PLGA) scaffolds	300 $\mu$ s-long pulses at 7.5 Hz	0.13, 0.24 or 0.32	Rectangular	2 or 8 hours/day	PIC/16C54 series, Microchip Technology Inc., AZ	(Tsai et al., 2007)
7F2+ RAW 264.7	3D chitosan scaffolds	1.3 ms-long, 75 Hz	1.5	Trapezoidal	9 hours	BIOSTIM, Igea, Carpi, Italy	(Lin and Lin, 2011)
Human osteosarcoma Saos-2 cells	Methacrylamide-modified gelatin type B scaffolds	1.3 ms pulses at 75 Hz	2	Trapezoidal	24 hours/day for 22 days	BIOSTIM, Igea, Carpi, Italy	(Fassina et al., 2012)
Osteochondral defects in rabbit medial femoral condyles.	Commercially available equine collagen scaffolds with or w/o BMC	1.3 ms-long, 75 Hz	1.5	Trapezoidal	4 hours/day for 40 days	I-ONE, Igea, Carpi, Italy	(Veronesi et al., 2015)
Rat calvaria defects	Commercially available collagen sponges loaded with 2.5-10 $\mu$ g rhBMP-2	12 $\mu$ s pulses, 60 Hz	1	n/a	8 hours/day for 5 days	In-house built	(Yang et al., 2015)
Human adipose tissue-derived stem cells	Electrospun poly( $\epsilon$ -caprolactone) nanofibrous scaffolds	50 Hz	1	n/a	6 hours/day for up to 21 days	n/a	(Arjmand et al., 2018)

Cells were stimulated for 12 hours/day for 3 days, and PEMFs were able to increase the expression of Osteocalcin, C-terminal procollagen type 1, and TGF $\beta$ 1 in cells on composite scaffolds, while decreasing IL-6 expression by 6 days of culture. An involvement of TGF- $\beta$  in PEMF stimulation was highlighted by several researches in MG63 cells [43], in serum-starved MC3T3 cells [111], and in human BMMSCs, where PEMFs increased Smad-2 and miRNA21, a microRNA targeting Smad-7, a TGF- $\beta$  signaling inhibitor [131].

Veronesi et al. showed that 75 Hz, 1.5 mT PEMF stimulation for 4 hours/day improved 40-day healing in osteochondral defects in rabbit knees, when used together with collagen scaffolds [178]. Collagen sponges loaded with increasing doses of recombinant human BMP-2 were also implanted in calvaria defects in rats and treated with 1 mT, 60 Hz PEMF stimulation for 8 hours/day for 5 days [179]. Computer microtomography 4 weeks after surgery revealed that PEMF stimulation increased Bone Volume and Bone Mineral Density in the absence or in the presence of rhBMP-2 but not with the highest, 10  $\mu$ g, dose, where no additional effect was observed. In the samples implanted with 2.5 micrograms as well PEMF stimulation significantly increased also Tb.N. and decreased Tb.Sp. Similarly, histology showed that PEMFs were able to increase bone regeneration in the central area of the defect without the addition of rhBMP-2.

Hydrogels were also explored together with PEMF exposure. Fassina et al. [180] cultured Saos-2 cells in bioreactors on methacrylamide-modified gelatin type B using the same exposure model as previously described [144, 181] and observed an increase in the deposition of Extracellular Matrix. Some research groups are also creating EMF-responsive hydrogels, which can release their bioactive load under EMD stimulation, e.g., methacrylated chondroitin sulfate (MA-CS) hydrogels coated with iron-based magnetic nanoparticles for PDGF release [182] and Ca<sup>2+</sup>-crosslinked Alginate/Xanthan gum hydrogels with magnetite particles for dopamine delivery [183, 184], although these studies were not included in the present review as EMFs were used only as a release-triggering stimulus and not to elicit biological effects.

## 7. Conclusions

The world of biomaterials is as diverse as the clinical applications that rely on them; therefore it stands to reason that there is no easy solution to improve their performance and the responses of the organisms to implanted material and devices. We nevertheless attempted at simplifying the wealth of available materials by dividing them into three main categories, which are however broad as well. A few conclusions can be drawn.

PEMFs have been repeatedly shown to possess the potential to affect osteoblast behavior on different biomaterials and thus represent a potential tool to improve the clinical outcome of several regenerative and prosthetic therapies in orthopedics and dentistry and should be more thoroughly investigated by proper clinical trials.

The response of cells and tissues to PEMF in the presence of titanium devices, for orthopedic or dental use, has been investigated using a vast range of PEMF approaches and

settings but besides a few attempts in the early 2000s with 100 Hz PEMF pulses with very light intensities, around 0.2 mT (following the seminal work by Matsumoto et al. [164]), most recent studies are narrowing down their focus to 15 Hz PRF PEMF stimulation or 75 Hz trapezoidal stimuli, with higher intensity, around 1-2 mT. Similar conclusions can be achieved considering the biological responses to bioceramic and polymer scaffolds. However broader screening studies testing cell or tissue responses across a spectrum of frequencies are still missing, though they would be sorely needed to better understand and possibly overcome the differences that exist among schools, with the purpose of establishing better and more reliable clinical protocols for this powerful technology.

## Data Availability

The data that were mentioned in this review are from previously reported studies and datasets, which have been cited. Please see the reference list and Tables 1–3.

## Conflicts of Interest

The authors have no conflicts of interest to disclose.

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