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Modulation of Osteogenesis in Human Mesenchymal Stem Cells by Specific Pulsed Electromagnetic Field Stimulation

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Abstract

Human mesenchymal stem cells (hMSCs) are a promising candidate cell type for regenerative medicine and tissue engineering applications by virtue of their capacity for self-renewal and multipotent differentiation. Our intent was to characterize the effect of pulsed electromagnetic fields (PEMFs) on the proliferation and osteogenic differentiation of hMSCs in vitro. hMSCs isolated from the bone marrow of adult patients were cultured with osteogenic medium for up to 28 days and exposed to daily PEMF stimulation with single, narrow 300 μs quasi-rectangular pulses with a repetition rate of 7.5 Hz. Relatively greater cell numbers were observed at late stages of osteogenic culture with PEMF exposure. The production of alkaline phosphatase (ALP), an early marker of osteogenesis, was significantly enhanced at day 7 with PEMF treatment in both basal and osteogenic cultures as compared to untreated controls. Furthermore, the expressions of other early osteogenic genes, including Runx2/Cbfa1 and ALP, were also partially modulated by PEMF exposure, indicating that osteogenesis in hMSCs was associated with the specific PEMF stimulation. Based on ALP and alizarin red S staining, the accumulation of ALP protein produced by the hMSCs as well as calcium deposits reached their highest levels at day 28. Our results indicate that extremely low frequency PEMF stimulation may play a modulating role in hMSC osteogenesis. Taken together, these findings provide insights on the development of PEMF as an effective technology for regenerative medicine.

Keywords

pulsed electromagnetic field; *osteogenesis*; *human mesenchymal stem cells*; *proliferation*; *mineralization*

INTRODUCTION

Human mesenchymal stem cells (hMSCs) are a promising cell type for regenerative medicine and tissue engineering applications by virtue of their great capacity for self-renewal and potential for differentiation into cells of various types of tissues, such as bone, cartilage, and adipose.1 Consequently, great interest has recently been generated regarding

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characterization and control of hMSC differentiation. hMSC osteogenic differentiation is enhanced by appropriate growth factors2¯5 or chemical supplements.6 However, physical stimuli, such as pulsed electromagnetic fields (PEMFs) that have been widely used in orthopedics for at least three decades, have not been evaluated for their potential effects on hMSCs.

PEMF therapy is approved for bone disorders in animals and in patients, including ununited bone fracture healing,7 pseudoarthrosis,7›8 and osteoporosis.9¯11 PEMF treatment can also aid the healing of osteotomies.12¯14 PEMF affects osteoblast cellular proliferation and differentiation of bone cells in vitro by enhancing DNA synthesis,15¯17 increasing the expression of bone marker genes during differentiation and mineralization,18 and enhancing calcified matrix production.19 Moreover, PEMF inhibits bone resorption by osteoclasts20 by regulating osteoprotegerin and the receptor activator of NFκB-ligand.21

Osteoblasts are derived in vivo from osteoprogenitor cells, such as bone marrow MSCs. In light of the stimulatory effect of PEMF on osteoblasts, we sought to characterize its effect on the proliferation and osteogenic differentiation of hMSCs.

MATERIALS AND METHODS

Isolation, expansion, and culture of hMSCs

hMSCs were isolated from the bone marrow aspirate obtained from three donors (57-73 years old) undergoing total hip replacement (IRB approval, University of Washington, Seattle, WA) as previously described.22 Briefly, adherent cells derived from marrow stroma were maintained as monolayer cultures and expanded in basal medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA), 10% selected lots of fetal bovine serum (FBS; Hyclone, Logan UT, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells at passage 3-4 were cultured in 4-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) at initial seeding cell densities of 1,500 and 3,000 cells/cm^2. To induce osteogenic differentiation of hMSCs, cells were cultured in an osteogenic medium supplemented with 100 nM dexamethasone, 50 μg/ml L-ascorbic acid, and 10 mM β-glycerophosphate.23 Half of the conditioned medium was replaced with fresh medium every 2-3 days. After 1 day for cell attachment, cells were stimulated with PEMF for 2 hr/day for 14 days. All groups reached around 90% confluence at days 6-7 after seeding.

PEMF Apparatus

The apparatus designed for this study included PEMF stimulators and solenoid coils (Fig. 1). Repetitive single quasi-rectangular pulses with pulse duration of 300 μs, and a repetitive rate of 7.5 Hz were generated by the pulse generator (Model PIC/16C54; Microchip Tech., Chandler, AZ). The 7.5 Hz repetitive pulsed waveform was based on our previous investigations.20›21›17 Output signals from the generator were amplified by the power amplifier (Model PA-950; Union Electronics, Tainan, Taiwan) to drive the solenoid coils.21 The solenoids were hollow cylinders (8 cm diam × 19 cm long), fitting up to two 4-well chamber slides inside for spatially uniform magnetic fields exposure for 2 h per day (Fig. 1). The average flux density of the magnetic field produced within the solenoids was set at 0.13 mT rms, corresponding to an induced electric field of 2 mV/cm (as determined and adjusted by a search coil and an oscilloscope, Fig. 1). Solenoids were shielded to avoid magnetic field interference generated by other coils. The untreated controls were maintained in another incubator. The incubators were maintained under identical conditions, and the temperature within the solenoids and incubators was monitored and controlled at 37°C ± 0.1°C (Fig. 1).

Cell Proliferation Assay

Proliferation of hMSCs was determined by a quantitative DNA assay. Cells were lysed in 2% Triton X-100 solution (Sigma, St. Louis, MO), and the Quanti-iT™ PicoGreen dsDNA Kit (Invitrogen, Carlsbad, CA) was used to determine the DNA amount in each culture based on a DNA standard curve.

Alkaline Phosphatase (ALP) Assay

hMSCs cultured in osteogenic medium were collected from each well at different time points and assayed for ALP activity with a reagent kit (Sigma) by measuring the formation of p-nitrophenol (p-NP) from p-nitrophenyl phosphate following the manufacturer's instructions. Cell cultures were lysed in a buffer containing 1.5 M Tris-HCl, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, pH 9.0, containing 2% Triton X-100, and reacted with phosphatase substrate reagent (2 mg/ml) in a microplate. p-NP was quantified based on the spectrophotometric absorbance at 405 nm, and enzymatic activity was expressed as mmoles of p-NP/min/μg DNA.

Histochemistry Assay

Osteogenic differentiation was evaluated on the basis of ALP production histochemically detected in cultures fixed with 2% paraformaldehyde in methanol using the Leukocyte Alkaline Phosphatase Kit (Kit No. 86C, Sigma) according to the manufacturer's protocol. Matrix mineralization was visualized by staining cultures fixed in 60% isopropanol with 2% alizarin red (Sigma) solution (pH 4.1-4.3) for 5 min at room temperature.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

mRNA expressions of Runx2/Cbfa1, ALP, collagen type I α2 (Col I), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) during osteogenic culture with or without PEMF exposure were determined by RT-PCR assays. Cells were cultured in 6-cm Petri dishes, and total RNA was extracted by using the RNeasy Micro Kit (Qiagen, Valencia, CA) at days 3, 7, and 10. First-strand cDNA was synthesized by using the SuperScript First-Strand Synthesis System (Invitrogen), and cDNA product was amplified by using Platinum Taq DNA Polymerase (Invitrogen). The sense and antisense sequences of primers used for semi-quantitative RT-PCR reactions were as follows: human Runx2/Cbfa1 (forward, 5′-CCG CAC GAC AAC CGC ACC AT-3′; reverse, 5′-CGC TCC GGC CCA CAA ATC TC-3′), human ALP (forward, 5′-TGG AGC TTC AGA AGC TCA ACA CCA-3′; reverse, 5′-ATC TCG TTG TCT GAG TAC CAG TC-3′), human Col I (forward, 5′-GGA CAC AAT GGA TTG CAA GG-3′; reverse, 5′-TAA CCA CTG CTC CAC TCT GG-3′), and human GAPDH (forward, 5′-GGG CTG CTT TTA ACT CTG GT-3′; reverse, 5′-GCA GGT TTT TCT AGA CGG-3′). The reaction conditions were as follows: incubation at 94°C for 2 min, denaturation at 94°C for 45 s, annealing at 51°C (Runx2/Cbfa1) or 58°C (ALP and Col I) for 45 s, and polymerization at 72°C for 60 s for 32 cycles, followed by a final extension at 72°C for 5 min. The amplified products were analyzed twice by means of 2% agarose gel electrophoresis and ethidium bromide staining. All electrophoresis images were quantitatively analyzed by using NIH Image J software and normalized to their respective GAPDH values.

Statistical Analysis

All data are presented as mean ± std. dev. from two independent experiments conducted with eight samples per assay, unless stated otherwise. Statistical analysis was performed by one-way ANOVA.

RESULTS

The effect of PEMF on cell proliferation was carried out on hMSCs cultured on 4-well chamber slides with different initial cell seeding densities (1,500 and 3,000 cells/cm^2) in basal medium. Both control and PEMF-treated cultures showed a steady proliferation rate consistent with their initial plating densities during the culture period (Fig. 2). The higher density group had greater cell numbers than the lower density group, especially at days 3 and 5, although cell number appeared to decrease at day 7. In general, PEMF stimulation at 2 mV/cm did not increase the proliferation of hMSCs cultured in basal medium.

In osteogenic induction experiments, cultures of hMSCs with initial seeding densities of 1,500 and 3,000 cells/cm^2 were treated with osteogenic medium and collected at various time points for cell number and ALP activity assays. The lower density, untreated group showed a significant increase in cell number (75%; $p < 0.05$) compared to the PEMF-treated group at day 7 (Fig. 3A). Interestingly, at day 10, PEMF-treated cultures showed significantly higher cell number (62%; $p < 0.05$) as compared to the untreated control. In this higher density group, the untreated group also showed a higher cell number, especially from days 7 to 10 (Fig. 3B). Specifically, although the cell number of the untreated group was significantly higher (84%, $p < 0.05$) as compared to the PEMF-treated cells at day 7, cell proliferation in the PEMF-treated cultures rapidly increased between days 7 and 10. Thus, PEMF stimulation of cell proliferation occurred from days 7 to 10 and was specific for hMSCs undergoing osteogenic differentiation.

PEMF treatment appeared to stimulate earlier onset of osteogenic differentiation of hMSCs on the basis of ALP activity. Specifically, PEMF-treated cells seeded at low density exhibited significantly higher ALP activity (82%, $p < 0.01$) compared to the untreated control at day 7 (Fig. 3C). Conversely, the untreated group showed increased ALP (123%, $p < 0.05$) as compared to PEMF-treated cells at day 10. The earlier increase in ALP activity was also seen at day 7 in high density cultures exposed to PEMF (121%, $p < 0.01$) as compared to the untreated cells (Fig. 3D). PEMF exposure apparently accelerated osteogenic differentiation of hMSCs by stimulating ALP protein production early in the osteogenic induction program.

Runx2/Cbfa1 is a key transcription factor for osteogenesis. Runx2/Cbfa1, ALP, and Col I were evaluated as early hMSC osteogenic markers (Fig. 4A). In the PEMF-treated group with low initial seeding density, mRNA levels of Runx2/Cbfa1 were significantly higher at day 7 and lower at day 10, compared to the untreated group (Fig. 4B). The mRNA expression of ALP in PEMF-treated groups at both low and high initial cell seeding densities were consistent with the ALP activity data shown in Figs. 3C and 3D. No significant difference existed in osteogenic gene expression in PEMF-treated and untreated cultures with high initial cell density during the culture period, except a significant increase in ALP at day 7 (Fig. 4B). PEMF did not strongly enhance Col I gene expression during hMSC osteogenesis.

hMSCs were histochemically stained for ALP activity and with alizarin red S to assess osteogenic differentiation and mineralization. ALP staining was first detectable at day 7 in the control and PEMF-treated groups (data not shown), and increased during the entire culture period, especially at day 28 (Fig. 5: e and f; E and F). Alizarin red S staining indicated that calcium deposits accumulated over time in both groups (Fig. 5: g and h; G and H). No positive alizarin red S staining was seen in hMSC cultures maintained in basal medium without any osteogenic supplement (data not shown).

DISCUSSION

We demonstrated that PEMF-treated hMSCs not only maintained regularly increased DNA content in basal medium, but also a relatively higher proliferation rate and significant ALP activity compared to untreated cells at the mid-late stages of an osteogenic culture period. Early gene expression of osteoblast markers, such as Runx2/Cbfa1 and ALP, were also up-modulated by PEMF exposure at the mid stages of culture during hMSC osteogenesis. Finally, ALP activity and calcium accumulation reached their highest levels at the end of the culture period in both PEMF-treated and untreated groups.

Since PEMF is efficacious in preventing osteoporotic bone loss as well as facilitating ununited fracture repair in animals and humans, the biological effect of PEMF on bone cells in vitro has received considerable attention. Extremely low frequency PEMF has been reported to not only significantly enhance osteoblast proliferation in vitro,15،16،24 but also suppress both the formation and bone resorption of osteoclasts in bone marrow culture.20،21 Furthermore, PEMF stimulation can efficiently accelerate osteoclastic apoptosis during bone marrow culture at different intensities.25 However, controversy exists on possible mechanisms of PEMF on bone formation in vitro. De Mattei et al. reported that two human osteosarcoma cell lines, TE-85 and MG-63, showed increased DNA synthesis when exposed to PEMF stimulation for at least 30 min (75 Hz single pulse, 2.3 mT peak).15 Upon at least 9-hour PEMF exposure, normal human osteoblastic cells showed increased DNA synthesis, but only when cultured in the presence of 10% serum. A later study by Diniz et al. reported that although PEMF treatment (15 Hz pulse burst, 0.7 mT peak, 24h/day treatment) accelerated the proliferation and differentiation of murine MC3T3-E1 cells during the active proliferation stage, the stimulatory effect of PEMF on enhancing bone formation was most likely associated with the enhancement of cellular differentiation, but not with an increase in the number of cells.16 However, Chang et al. showed that PEMF stimulation (15 Hz pulse burst, 0.1 mT peak, 8 hr/day treatment) enhanced proliferation of murine primary cultured osteoblast proliferation but not their differentiation in the presence of 1% serum culture 24 The controversy arising from these studies may be due to differences in cell types, serum volume, and PEMF parameters. Thus, PEMF may influence proliferation and osteogenic differentiation in different cell types via dissimilar pathways. The differences in experimental conditions notwithstanding, these results strongly suggest that exposure to PEMF with 7.5 Hz repetitive pulsed waveform and specific selected parameters can contribute to bone formation by modulating cell activities.

Although the stimulatory effect of PEMF on bone cells has been demonstrated, the effect of PEMF on MSCs, the precursors of osteoblasts, has not been characterized in detail. Our findings demonstrated the effect of PEMF on hMSC proliferation and osteogenic differentiation in vitro, suggesting that this specific stimulation may play a modulating role in hMSC osteogenesis.

It is interesting that PEMF-treated hMSCs exhibited opposite effects on cell number and ALP activity during the mid-late stages of the osteogenic culture period (Fig. 3). Thus, PEMF exposure may exert a delayed response in increasing cell number during hMSC osteogenesis. Upon PEMF treatment, although gene expression of Runx2/Cbfa1 in both low- and high-density culture groups was inconsistent during the osteogenic culture, levels of ALP mRNA and ALP activity in all groups showed parallel increases during the entire culture period. Taken together, these results suggest that PEMF exposure may induce an earlier osteogenic induction in hMSC by modulating ALP activity and the early osteoblastic gene expression of Runx2/Cbfa1 and ALP, accompanied by a delayed increase in cell proliferation.

The time-dependent effects of PEMF on cell proliferation and osteogenic differentiation of hMSCs is likely a function of the differentiation and maturation state of the cells undergoing osteogenesis (i.e., proliferation, differentiation, or mineralization).16 The parameters of PEMF stimulation, such as frequency and electric or magnetic field, may be required to maintain and prolong the earlier response during hMSC osteogenesis in vitro.

In conclusion, our data suggest that hMSCs respond to a 7.5 Hz repetitive pulse PEMF exposure with a delayed increase of cell proliferation as well as an earlier increase of ALP activity during osteogenic culture. In the lower-density group, early gene expression of Runx2/Cbfa1 and ALP is enhanced by PEMF at the mid stages of osteogenic culture period, followed by a subsequent decrease. In addition, PEMF-treated hMSCs progressively increase ALP and calcium accumulation in both low- and high-density cultures. The observed effects of PEMF on hMSC osteogenesis at extremely low frequency and low amplitude via modulation of cell proliferation and osteogenic differentiation provides additional information on the clinical application of PEMF in regenerative medicine and bone tissue engineering.

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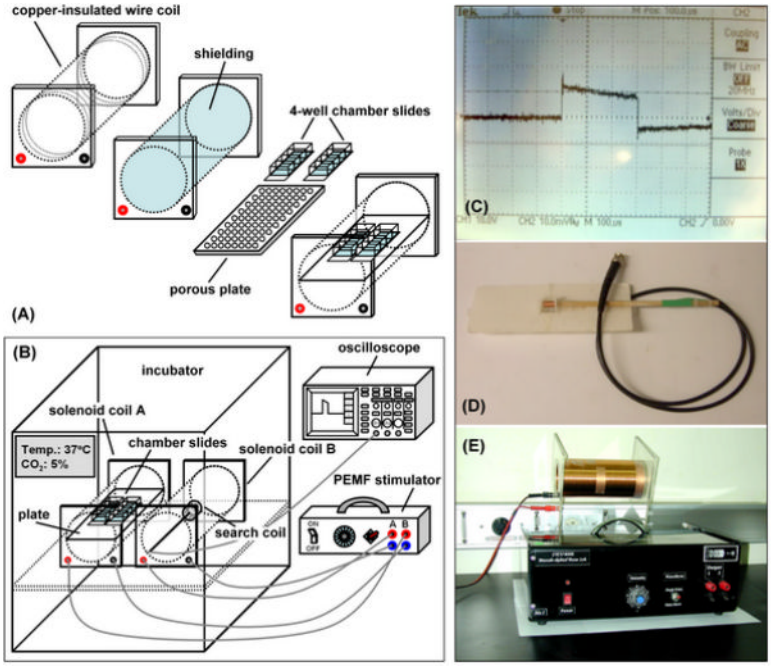

Figure 1.
The PEMF stimulation system: (A) solenoid coils with porous plastic board placed horizontally in the center; (B) control units for regulating and monitoring the signals derived from solenoids placed inside an incubator; (C) induced electric field with single quasi-rectangular pulse; (D) a search coil for detecting induced electric fields; and (E) PEMF stimulator and solenoid coil.

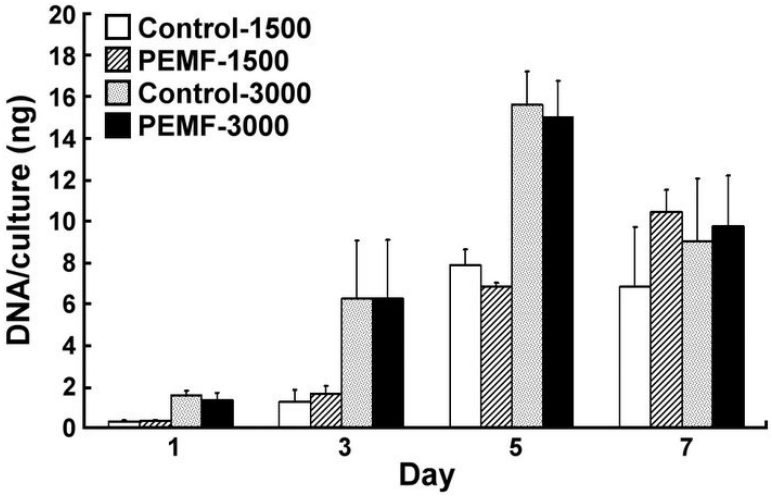

Figure 2.

DNA quantitation of hMSCs cultured in basal medium without the addition of osteogenic supplement for 7 days. hMSCs were cultured at seeding densities of 1,500 and 3,000 cells/cm^2. The PEMF-treated cells were exposed for 2 h per day. Data are from two independent experiments, with two 4-well chamber slides per group in each (N=16 total) *, $p<0.05$.

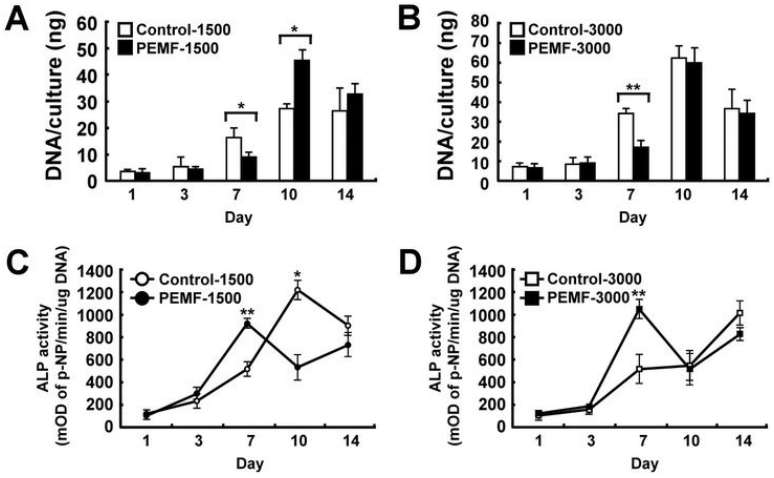

Figure 3.

Proliferation and ALP activity of hMSCs at seeding densities of 1,500 and 3,000 cells/cm^2 during 14 days of osteogenic culture. (A) DNA amount and (C) ALP activity in low-density hMSCs cultures; and (B) DNA amount and (D) ALP activity in high-density hMSCs cultures. Data are from two independent experiments, with two 4-well chamber slides per group in each experiment (N=16 total). *, $p<0.05$; **, $p < 0.01$.

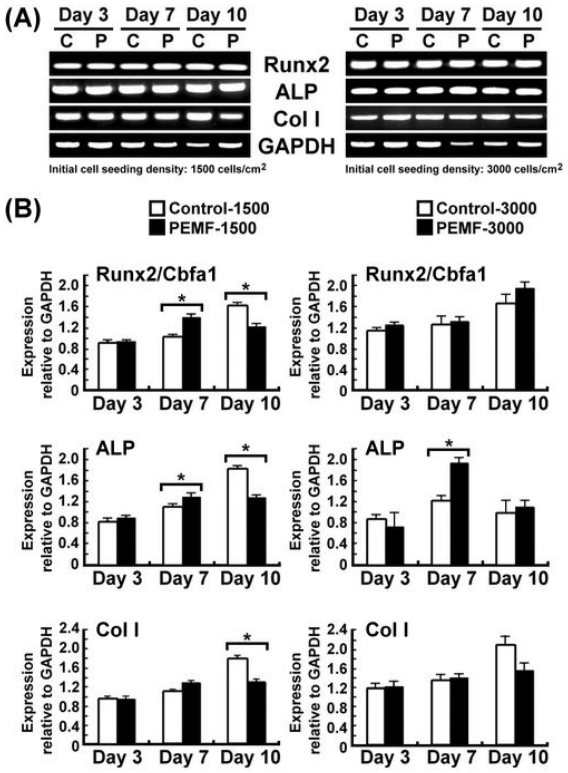

Figure 4.

Early osteoblastic gene expression of Runx2/Cbfa1, ALP, and collagen type I during hMSC osteogenesis. (A) Electrophoretic RT-PCR analysis of RNA samples extracted at day 3, 7, and 10 (C, control; P, PEMF-treated). (B) Densitometric quantification of RT-PCR results. Data presented are from two independent experiments, with triplicate samples (6-cm Petri dish) per experiment (N=6 total). Electrophoresis was carried out twice for each sample for gel image analysis. *, $p < 0.05$.

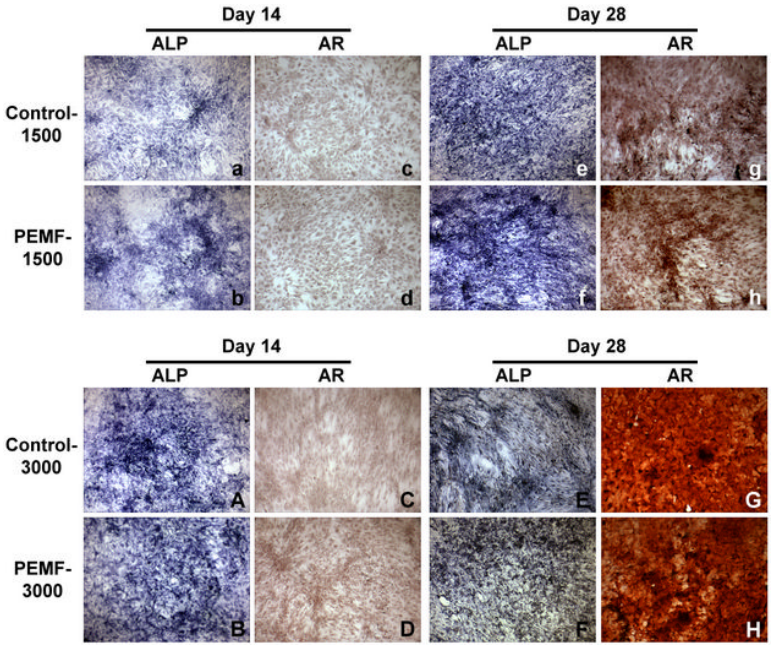

Figure 5.

Osteogenic differentiation and matrix mineralization of hMSCs under PEMF exposure during 28 days of osteogenic culture. Cells were stained for ALP and with alizarin red S at different time points for osteogenic differentiation and matrix mineralization, respectively. ALP activity increased in a time-dependent manner, reached the highest level at day 28 in both groups (e and f; E and F). Matrix mineralization accumulated over time, reaching its highest level at day 28 (g and h; G and H). Magnification, 50×.