

## Original Article

# A qualitative and quantitative analysis of various parameters of electric current and its effect on biomarkers (receptor activator of nuclear factor kappa-B ligand, osteoprotegerin): A *in vitro* study

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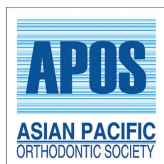
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## ABSTRACT

**Objectives:** The objectives of the study are to examine the qualitative and quantitative effects of exogenous electric current (square waveform with varying pulsed frequencies and current intensities) on sarcoma osteogenic-2 (Saos-2) cells, focusing on the expression of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL).

**Material and Methods:** The experimental group was divided into two groups: Group A with a 1s pulsed frequency and Group B with a 10s pulsed frequency. Each experimental group (i.e., Group A and Group B) was further subdivided into five subgroups (A1–A5, B1–B5) based on the current levels applied (10–30  $\mu$ A). Saos2 cells seeded in 6-well plates were subjected to initial compression forces using glass coverslips for 1 h. Reverse piezoelectricity was applied continuously for 24 h a day over 3 days. A total of 2  $\mu$ L of media was collected at specific time points of 1, 3, 5, 7, 12, 24, 48, and 72 h, and analyzed using an enzyme-linked immunosorbent assay for OPG and RANKL concentrations. The cells underwent histological examination for qualitative analysis after 72 h. All experiments were performed in triplicate. The data obtained were analyzed statistically.

**Results:** Maximum OPG (0.090 ng/mL,  $P < 0.001$ ) and RANKL (0.091 pg/mL,  $P < 0.001$ ) expression occurred at 30  $\mu$ A and 24 h in Group B; in Group A, the peak was delayed to 48 h with decreased expression levels (OPG: 0.078 ng/mL,  $P < 0.001$ ; RANKL: 0.077pg/mL,  $P < 0.001$ ). OPG levels showed a progressive increase at earlier time points. OPG levels rose early and were influenced by the timing of media collection, intensity, and frequency. RANKL was influenced only by the time points ( $P < 0.001$ ). Phase contrast microscopy revealed an enlarged size of Saos-2 cells at 30  $\mu$ A with a 10s frequency.

**Conclusion:** The findings of this study show that bioelectric stimulation can directly modulate both OPG and RANKL. There are unique frequencies that may be applied to bone cells to regulate, alter, and optimize the expression of OPG and RANKL. These findings suggest that bone cells, and possibly other cells, possess biochemical pathways that respond reliably to specific frequencies of electrical stimulation.

**Keywords:** Bio-markers, Bone remodeling, Osteoprotegerin, Piezoelectricity, Receptor activator of nuclear factor kappa-B ligand

## INTRODUCTION

For decades, the orthodontic community has been concerned about the duration of orthodontic treatment. Long-term treatment not only increases the risk of side effects such as root

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resorption<sup>[1]</sup> and dental caries<sup>[2]</sup> but also reduces patient compliance.<sup>[3]</sup> Researchers have documented various procedures to enhance the rate of tooth movement. Surgical methods<sup>[4]</sup> include corticotomies, modified corticotomies (piezocision), osteotomies, micro-perforation, distraction osteogenesis, and others. Non-surgical methods include a variety of transmucosal interventions, low-level laser therapy, pharmacological measures, pulsed electromagnetic field (PEMF), extracorporeal shock wave therapy, and exogenous electric current application.<sup>[5]</sup>

Periodontal defects,<sup>[6]</sup> bony fractures,<sup>[7]</sup> an increased rate of tooth movement,<sup>[8]</sup> and a reduction in alveolar ridge resorption following extraction<sup>[9]</sup> all benefit from electric stimulation (EStim). Two hypotheses have been proposed for alveolar bone remodeling, i.e., piezoelectricity (inorganic crystals, including those in bone and organic crystals, like collagen crystals in the periodontal ligament [PDL]) generated in alveolar bone bending and pressure tension in the PDL.

An electric field can cause a crystal to deform and produce a force, creating a reverse piezoelectric effect. Reverse piezoelectricity has no place in natural control systems. EStim's pro-healing effect is attributed to its influence on the behavior and function of bone-forming stem cells, including migration,<sup>[10]</sup> proliferation,<sup>[11]</sup> differentiation,<sup>[12]</sup> mineralization,<sup>[13]</sup> extracellular matrix deposition, and attachment to scaffold materials.<sup>[14]</sup>

When cells are exposed to an exogenous electric stimulus, a response known as electrocoupling is generated, which is caused by the high resistance of the plasma membrane. This high resistance prevents the penetration of electric stimuli, independent of the cytoplasmic conductive capacity.<sup>[15]</sup> Asymmetric redistribution or diffusion of electrically charged cell membrane receptors in response to electric fields activates numerous downstream signaling cascades.<sup>[16]</sup> The cell membrane depolarization is due to the direct activation of voltage-gated cytosolic calcium ( $Ca_2+$ ) channels, leading to an increase in intracellular calcium ion concentration.<sup>[17]</sup> Other mechanisms that play a role include mechanotransduction<sup>[18]</sup> surface receptor redistribution, adenosine triphosphate synthesis stimulation<sup>[19]</sup> activation of heat shock protein<sup>[20]</sup> generation of reactive oxygen species<sup>[21]</sup> and redistribution of lipid rafts.<sup>[22]</sup>

For most applications, purpose-built chambers can be used to apply *in vitro* electrical stimulation (ES) to cells grown in 2-D or 3-D cultures. These chambers encompass a metallic electrode EStim chamber, a salt bridge EStim chamber, and microfluidic chip EStim chambers.<sup>[23]</sup>

Direct, capacitive, or inductive coupling can apply EStim *in vivo*. Several *in vitro* studies<sup>[12,24-26]</sup> have demonstrated that direct current (DC) and PEMFs enhance proliferation,

differentiation factors such as osteocalcin, osteopontin, and Runt-related transcription factor 2, alkaline phosphatase activity, and calcium deposition in osteoblasts and mesenchymal stem cells. In their *in vitro* study on osteosarcoma cell lines, Fidan *et al.* found an increase in cell number following exposure to 0.5 kV/cm and 2.3 kV/cm DC electric fields; however, the authors did not consider the effect of current frequency and waveform.<sup>[27]</sup>

Andrew and Bassett found that an electric current of 5–20  $\mu$ A has been proven to aid osteogenesis in studies. The studies also reveal, however, that electric currents <5  $\mu$ A do not promote bone formation, and that electric currents >20  $\mu$ A cause cellular necrosis and bone death.<sup>[28]</sup> France *et al.* have found it to be efficient in repair and bone strength.<sup>[29]</sup> Davidovitch *et al.*, in their animal study, applied 15  $\mu$ A of DC. to the gingival tissues (near one maxillary canine) in a cat model.<sup>[30]</sup> There was an intense staining of cyclic adenosine monophosphate and cyclic guanosine monophosphate evident, which suggested that ES enhances cellular enzymatic phosphorylation activities. However, none of the above studies have considered the waveform or frequency of the applied current.

Active bone cells produce bone markers such as tartrate-resistant acid phosphatase, osteoprotegerin (OPG), and receptor activator of nuclear factor kappa beta ligand (RANKL), which provide an estimate of the level of bone remodeling in serum or plasma. Therefore, we designed a study to understand the impact of applying exogenous electric current with a square waveform, varying in parameters such as pulsed frequency (seconds) and current- $\mu$ A, on Saos2 cells, by estimating OPG and RANKL both qualitatively (histologically) and quantitatively enzyme-linked immunosorbent assay (ELISA). The clinical relevance of the study could pave the way for the development of adjunctive bioelectric stimulation therapies in orthodontics, accelerating tooth movement and reducing treatment duration.<sup>[31]</sup> By identifying effective stimulation parameters, personalized protocols could be designed for different age groups or clinical conditions.<sup>[32]</sup> The results of this study would provide a foundation for future *in vivo* and clinical trials, potentially extending to applications in bone regeneration, implantology, and periodontal therapy.<sup>[33]</sup> Moreover, the use of bone markers like OPG and RANKL provides a basis for biomarker-based monitoring of bone remodeling, enabling more precise and adaptive treatment strategies in clinical practice.<sup>[34]</sup>

## MATERIAL AND METHODS

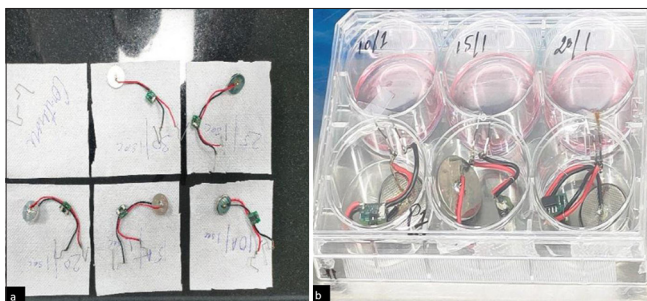
### Group

An experiment was designed [Figure 1] to compare EStim Saos2 cells (Experimental) versus non-stimulated (Control)

cell groups. The experimental group was divided into Group A with a 1s pulsed frequency and Group B with a 10s pulsed frequency. Each experimental group was further subdivided into five subgroups (A1–A5, B1–B5) based on the current levels subjected, i.e., 10, 15, 20, 25, and 30  $\mu\text{A}$ . The control group did not receive any ES; however, the electrodes ( $0.17 \times 0.25$  inches) were placed similarly to those in the experimental groups. The cells in the control were subjected to the same environmental conditions and procedures as those for the experimental groups, except for the application of exogenous EStim.

### Cell preparation and culture

Cells were procured from the National Center for Cell Science, Pune. The cells were cultured in Dulbecco's Modified Eagles Medium (MP Biomedicals California Cat # 091233354) which contains 4 times greater concentrations of amino acids, vitamins, and supplementary components with L-glutamine and 4.5 G/L of glucose and 10% v/v fetal bovine serum (MP Biomedicals California Cat # 092910154) and 1% penicillin/streptomycin (10.00 U/mL) (MP Biomedicals California Cat # 1670049). Proliferative cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator, and subculture was carried out by washing the cell monolayers twice with calcium and magnesium-free phosphate-buffered saline (G-Bioscience Lot # 190305). Cells were collected by the addition of 1X trypsin/ethylenediaminetetraacetic acid solution (Sigma Aldrich) and incubated at 37°C until the cells detached. Trypsin was inactivated by the addition of a growth medium before seeding in fresh flasks at densities of  $1.5\text{--}2 \times 10^4$  cells/cm<sup>2</sup>. After 70–80% confluency, cells were seeded into six-well plates at densities of  $1.5 \times 10^4$  cells/cm<sup>2</sup> and incubated at 37°C in humidified 5% CO<sub>2</sub> for 24 h. The cells were harvested simultaneously for both experimental and control groups. Once 60–70% confluency was achieved, cells were stimulated using sterilized electrodes to which an electric current was applied. For sterilization, electrodes were submerged in 70% ethanol for 10 min and washed with sterile calcium–magnesium-free phosphate buffer saline and were finally exposed under ultraviolet light overnight. The cells that received ES were exposed to 3V DC EStim for 24 h for 3 days [Video 1]. 2  $\mu\text{L}$  of medium was collected

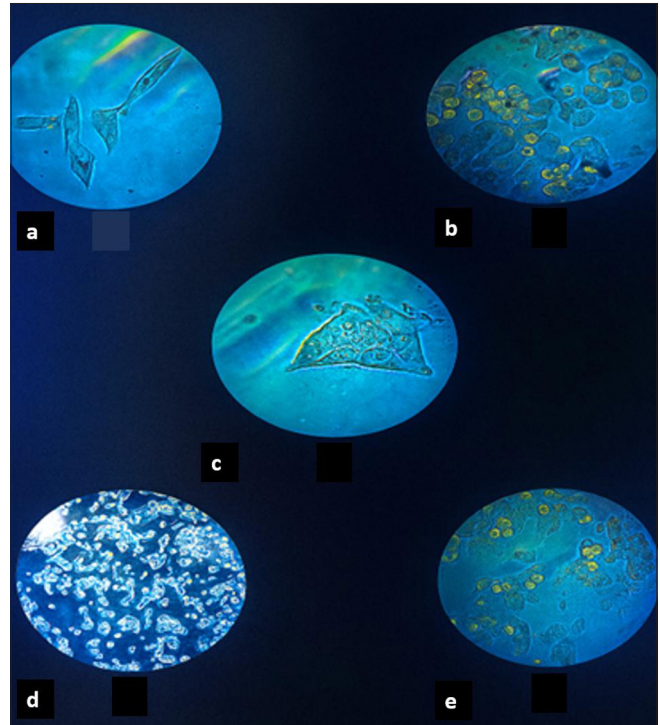


**Figure 1:** (a and b) Experimental setup.

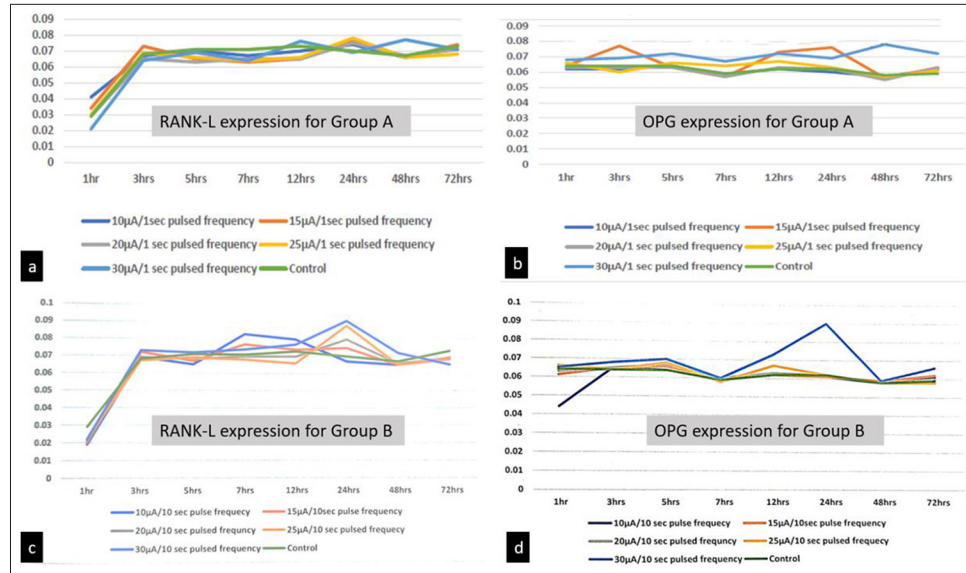
from each of the 10 experimental and one control Well of 4 six-well plates at 1, 3, 5, 7, 12, 24, 48, and 72 h. The liquid thus collected was subjected to an ELISA test for OPG and RANKL concentration. All the experiments were run in triplicate.

### Statistical analysis

Data were collected and compiled using Microsoft Excel 16 software. Analysis was carried out using the Statistical Package for the Social Sciences software version 23.0. The level of significance was kept at 5%. The normality of the data was assessed using the Shapiro–Wilk test. Results of the normality assessment show that the data followed a normal distribution. One-way and three-way analysis of variance were applied to analyze the effect of various parameters on the expression of RANK and OPG levels.



**Figure 2:** Qualitative analysis: Inverted cone microscopic view. (a) Control - normally dividing cells, no exogenous current. (b) 30  $\mu\text{A}/10$  s pulse frequency shows the cells are larger and are increased in number compared (e) to the 30  $\mu\text{A}/1$  s pulse frequency. (c) Rapidly dividing cells under the influence of exogenous electric stimulation. (d) 15  $\mu\text{A}/10$  s pulse frequency showing the cells are alive and spindle-shaped, but in comparison to (b) and (e), the exogenous current has not brought any significant changes. (e) 30 -  $\mu\text{A}/1$  s pulse frequently shows a reduced number of cells as compared to (b) the 30  $\mu\text{A}/10$  s pulse frequency. The overall size of the cells is smaller compared to (b). No staining was used. The 6-well plate was directly taken under an inverted cone microscope to view the cells under 300X magnification.



**Figure 3:** Pictorial representation of Graph (group a and b). (a) The graph represents the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) under various direct current (DC) applications at 1 s pulsed frequency (SPF). The expression reaches a peak at 48 h, 0.077 pg/mL under a 30 μA current level, and the control lags. (b) The graph represents the expression of osteoprotegerin (OPG) under various DC applications at 1 SPF; the peak is reached again at 48 h, 0.078 ng/mL, with control lagging. (c and d) Both graphs showed that the expression of RANKL and OPG was maximum at 30 μA, as seen in (a) and (b); however, the expression increased at 10 s pulsed frequency, which was earlier at 24 h (RANKL – 0.091 pg/mL, OPG – 0.090 ng/mL). The X-axis in all line diagrams represents the time points of media extraction, Y axis in: RANKL line diagram represents – measurements in pg/ml OPG line diagram represents – measurements in ng/ml

## RESULTS

Different pulse frequencies (1 s pulsed frequency and 10 s pulsed frequency), current levels (10–30 μA), and time duration (media collected at various intervals) individually had a significant influence on the expression of OPG [Table 1]. Interaction between pulse frequency and time, pulse frequency and current levels, and current level and time duration also showed a significant influence on the expression of OPG. The influence of interaction between pulse frequency, current level, and time on OPG expression was also found to be significant [Figure 2].

Similarly, different time intervals also had a significant influence on the expression of RANKL [Table 2]; however, different pulse frequencies and current levels did not have a significant influence on the expression of RANKL. The effect of interaction between pulse frequency and time, pulse frequency and current levels, and current level and time duration did not show a significant influence on the expression of RANKL. Finally, the effect of interaction between pulse frequency, current level, and time on RANKL expression was also non-significant.

OPG secretion peaked at 48 h (0.078 ng/mL,  $P < 0.001$ ) [Table 3a] under a 1s pulsed frequency using a 30 μA current. A similar increase in OPG levels was observed

at the 3 h time point with the application of 15 μA at the same frequency. Compared to the control group, OPG secretion consistently increased from 1 h to 72 h across the experimental timeline. Similarly, OPG expression reached its maximum at 24 h (0.090 ng/mL,  $P < 0.001$ ) [Table 3b] with a 30 μA current under a 10 s pulsed frequency. Quantitative analysis revealed that overall OPG expression was higher at the 10 s pulsed frequency than at the 1 s frequency, with peak expression occurring 24 h earlier in the 10 s group. RANKL [Figure 3] expression at a 1s pulsed frequency [Table 3c] showed no consistent pattern of secretion across different current levels. In contrast, RANKL secretion was highest at 24 h (0.091 pg/mL,  $P < 0.001$ ) [Table 3d] post-stimulation with a 30 μA current under the 10 s pulsed frequency, earlier than the peak observed under the same current at 1 s pulsed frequency.

## DISCUSSION

The bioelectrical cues found in natural living bone, such as piezoelectricity, pyroelectricity, and ferroelectricity, have been identified as an important factor in regulating metabolic activities, including growth, structural remodeling, and fracture healing.<sup>[35,36]</sup> Researchers have suggested that the non-centrosymmetric collagen molecule is the primary reason behind bioelectricity in living bone.<sup>[37-39]</sup> The collagen

**Table 1:** Three-way ANOVA test to assess the effect of the interaction of current, pulse frequency, current levels, and time on OPG expression.

Source	Type III sum of squares	df	Mean square	F	Sig.	Partial eta squared
Corrected model	0.009 <sup>a</sup>	95	9.921E-005	42.081	<0.001*	0.954
Intercept	1.154	1	1.154	489659.434	<0.001*	1.000
Pulse frequency	0.000	1	0.000	48.784	<0.001*	0.203
Time points	0.002	7	0.000	117.530	<0.001*	0.811
Current level	0.003	5	0.001	252.371	<0.001*	0.868
Pulse frequency * time	0.000	7	2.366E-005	10.037	<0.001*	0.268
Pulse frequency * current level	0.000	5	4.302E-005	18.248	<0.001*	0.322
Time * current level	0.002	35	4.809E-005	20.398	<0.001*	0.788
Pulse frequency * time * current level	0.002	35	6.662E-005	28.255	<0.001*	0.837
Error	0.000	192	2.358E-006			
Total	1.164	288				
Corrected total	0.010	287				

<sup>a</sup>R Squared=0.954 (Adjusted R Squared=0.931). ANOVA: Analysis of variance, OPG: Osteoprotegerin, \*denotes highly significant, P-value ≤ 0.05. df: Degrees of freedom, Sig: Significance (P-value), F: Fisher-Snedecor F distribution

**Table 2:** Three-way ANOVA test to assess the effect of the interaction of current, pulse frequency, current levels, and time on Rank-L expression.

Source	Type III sum of squares	df	Mean square	F	Sig.	Partial eta squared
Corrected model	0.197 <sup>a</sup>	95	0.002	1.604	0.003*	0.442
Intercept	1.287	1	1.287	992.938	<0.001*	0.838
Pulse frequency	0.002	1	0.002	1.491	0.224	0.008
Time points	0.076	7	0.011	8.403	<0.001*	0.235
Current level	0.005	5	0.001	0.769	0.573	0.020
Pulse frequency * Time points	0.011	7	0.002	1.262	0.271	0.044
Pulse frequency * Current level	0.007	5	0.001	1.095	0.365	0.028
Time points * Current level	0.048	35	0.001	1.049	0.404	0.160
Pulse frequency * Time points * Current level	0.048	35	0.001	1.063	0.383	0.162
Error	0.249	192	0.001			
Total	1.733	288				
Corrected Total	0.446	287				

<sup>a</sup>R Squared=0.954 (Adjusted R Squared=0.931). ANOVA: Analysis of variance, \*denotes highly significant, P-value ≤ 0.05. df: Degrees of freedom, Sig: Significance (P-value), F: Fisher-Snedecor F distribution

**Table 3a:** Comparison of OPG expression within group A among different periods of media extraction.

Time points of media extraction	10 μA/1 SPF		15 μA/1 SPF		20 μA/1 SPF		25 μA/1 SPF		30 μA/1 SPF		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1 h	0.062	0.003	0.064	0.001	0.063	0.002	0.066	0.001	0.068	0.002	0.010*
3 h	0.062	0.002	0.077	0.005	0.063	0.002	0.060	0.002	0.067	0.002	<0.001*
5 h	0.064	0.001	0.063	0.001	0.063	0.001	0.066	0.001	0.072	0.001	<0.001*
7 h	0.059	0.002	0.057	0.001	0.057	0.002	0.064	0.001	0.067	0.000	<0.001*
12 h	0.062	0.001	0.073	0.002	0.063	0.002	0.067	0.001	0.066	0.001	<0.001*
24 h	0.060	0.002	0.076	0.002	0.062	0.000	0.063	0.002	0.069	0.001	<0.001*
48 h	0.057	0.002	0.056	0.003	0.055	0.001	0.057	0.001	0.078	0.001	<0.001*
72 h	0.062	0.002	0.063	0.002	0.063	0.001	0.061	0.002	0.067	0.001	<0.001*

P-value was determined as a part of one-way ANOVA. \*Indicates Significance level  $P \leq 0.05$ . P-value was determined using the F-distribution with degrees of freedom (k - 1, N - k), the probability of obtaining the observed F or larger under the null hypothesis (no mean difference) is the p-value. If  $p < 0.05 \rightarrow$  significant difference among groups. If  $p \geq 0.05 \rightarrow$  no significant difference. SPF: Second pulsed frequency, Measurement in-ng/mL. ANOVA: Analysis of variance, SD: Standard deviation, OPG: Osteoprotegerin

**Table 3b:** Comparison of OPG expression within Group B among different periods of media extraction.

Time points of media extraction	10 $\mu$ A/10 SPF		15 $\mu$ A/10 SPF		20 $\mu$ A/10 SPF		25 $\mu$ A/10 SPF		30 $\mu$ A/10SPF		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1 h	0.044	0.001	0.061	0.001	0.063	0.001	0.066	0.005	0.065	0.001	<0.001*
3 h	0.065	0.002	0.065	0.001	0.065	0.001	0.064	0.002	0.068	0.002	0.113
5 h	0.064	0.001	0.066	0.001	0.067	0.001	0.068	0.001	0.070	0.002	<0.001*
7 h	0.059	0.001	0.060	0.001	0.060	0.001	0.058	0.001	0.060	0.002	0.261
12 h	0.062	0.002	0.062	0.001	0.063	0.001	0.067	0.001	0.073	0.002	<0.001*
24 h	0.061	0.000	0.061	0.001	0.062	0.001	0.062	0.001	0.090	0.002	<0.001*
48 h	0.058	0.002	0.059	0.001	0.058	0.000	0.058	0.002	0.059	0.002	0.422
72 h	0.061	0.002	0.062	0.001	0.060	0.001	0.058	0.002	0.066	0.001	<0.001*

P-value was determined as a part of one-way ANOVA. \*Indicates significance level  $P \leq 0.05$ . P-value was determined using the F-distribution with degrees of freedom ( $k - 1, N - k$ ), the probability of obtaining the observed F or larger under the null hypothesis (no mean difference) is the p-value. If  $p < 0.05 \rightarrow$  significant difference among groups. If  $p \geq 0.05 \rightarrow$  no significant difference. SPF: Second pulsed frequency. ANOVA: Analysis of variance, SD: Standard deviation. Measurement in ng/mL, OPG: Osteoprotegerin

**Table 3c:** Comparison of RANKL expression within Group A among different periods of media extraction.

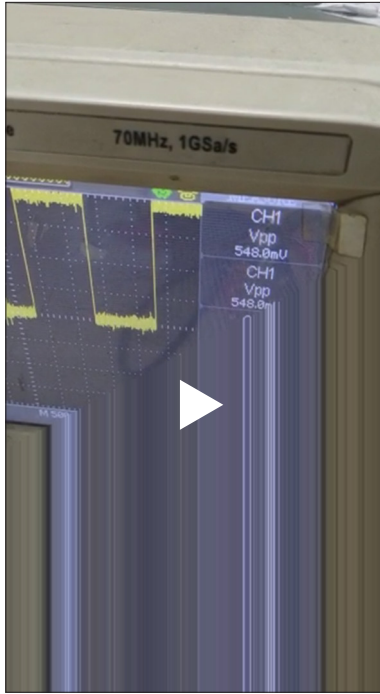
Time points of media extraction	10 $\mu$ A/1 SPF		15 $\mu$ A/1 SPF		20 $\mu$ A/1 SPF		25 $\mu$ A/1 SPF		30 $\mu$ A/1 SPF		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1 h	0.041	0.002	0.034	0.004	0.029	0.002	0.03	0.002	0.021	0.001	<0.001*
3 h	0.067	0.001	0.073	0.001	0.065	0.001	0.069	0.002	0.064	0.002	<0.001*
5 h	0.07	0.001	0.065	0.001	0.063	0.001	0.066	0.001	0.069	0.001	<0.001*
7 h	0.067	0.001	0.063	0.002	0.065	0.002	0.064	0.001	0.064	0.001	0.053
12 h	0.07	0.001	0.065	0.001	0.065	0.001	0.066	0.001	0.076	0.002	<0.001*
24 h	0.074	0.001	0.076	0.002	0.075	0.002	0.078	0.002	0.069	0.001	<0.001*
48 h	0.066	0.001	0.066	0.001	0.067	0.001	0.066	0.001	0.077	0.002	<0.001*
72 h	0.073	0.001	0.074	0.001	0.071	0.002	0.068	0.001	0.071	0.001	0.002*

P-value was determined as a part of one-way ANOVA. \*Indicates Significance level  $P \leq 0.05$ . P-value was determined using the F-distribution with degrees of freedom ( $k - 1, N - k$ ), the probability of obtaining the observed F or larger under the null hypothesis (no mean difference) is the p-value. If  $p < 0.05 \rightarrow$  significant difference among groups. If  $p \geq 0.05 \rightarrow$  no significant difference. SPF: Second pulsed frequency. RANKL: Receptor activator of nuclear factor kappa-B ligand, ANOVA: Analysis of variance, SD: Standard deviation. Measurement in pg/mL

**Table 3d:** Comparison of RANKL expression within Group B among different periods of media extraction.

Time points of media extraction	10 $\mu$ A/10 SPF		15 $\mu$ A/10 SPF		20 $\mu$ A/10 SPF		25 $\mu$ A/10 SPF		30 $\mu$ A/10 SPF		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1 h	0.019	0.001	0.02	0.002	0.021	0.001	0.022	0	0.022	0.003	0.038*
3 h	0.069	0.002	0.072	0.001	0.069	0.001	0.067	0.002	0.073	0.001	0.455
5 h	0.065	0.002	0.067	0.001	0.068	0.001	0.069	0.001	0.072	0.001	<0.001*
7 h	0.083	0.003	0.077	0.002	0.07	0.001	0.068	0.002	0.074	0.002	<0.001*
12 h	0.08	0.002	0.074	0.001	0.07	0.001	0.066	0.001	0.077	0.001	<0.001*
24 h	0.067	0.002	0.075	0.001	0.08	0.002	0.088	0.002	0.091	0.004	<0.001*
48 h	0.065	0.001	0.065	0.001	0.066	0.001	0.065	0.001	0.072	0.001	<0.001*
72 h	0.069	0.001	0.069	0.001	0.068	0.001	0.068	0.002	0.065	0.001	0.004*

P-value was determined as a part of one-way ANOVA. \*Indicates Significance level  $P \leq 0.05$ . P-value was determined using the F-distribution with degrees of freedom ( $k - 1, N - k$ ), the probability of obtaining the observed F or larger under the null hypothesis (no mean difference) is the p-value. If  $p < 0.05 \rightarrow$  significant difference among groups. If  $p \geq 0.05 \rightarrow$  no significant difference. SPF: Second pulsed frequency. RANKL: Receptor activator of nuclear factor kappa-B ligand, ANOVA: Analysis of variance, SD: Standard deviation. Measurement in pg/mL



**Video 1:** Working of an innovative micro printed circuit board device with square waveform and varying current levels, pulse frequency on an oscilloscope. Video available on: [https://doi.org/10.25259/APOS\\_215\\_2024](https://doi.org/10.25259/APOS_215_2024)

present in bone exhibits piezoelectric properties, generating a streaming potential. When subjected to stress, it leads to a decrease in hydraulic permeability and an increase in stiffness.<sup>[40]</sup>

Bioelectric stimulation can be applied via exogenous sources and various techniques, including DC stimulation, alternating current stimulation, PEMF therapy, and others. The present study analyzes the effect of various parameters of direct electrical (amount of current-- $\mu\text{A}$ , (sec) pulsed frequency) stimulation of Saos2 cells on the *in vitro* secretion of RANKL and OPG. Saos2 cells are a widely used human osteosarcoma cell line in biomedical research. We chose Saos2 cells as they are widely used in research related to bone biology, including studies on osteoblast differentiation, bone mineralization, bone-related diseases, drug screening, and evaluating the effects of various factors on bone cell behavior.<sup>[41,42]</sup> Despite being a valuable tool in bone-related research, it is important to note that Saos2 cells originate from a cancerous origin (osteosarcoma). Therefore, one should exercise caution when extrapolating findings from Saos2 cells to normal physiological conditions. In this study, we used six-well culture plates for the experimental setup, adhering Saos2 bone cells to the bottom of the polytetrafluoroethylene (PTFE) culture well and bathing them in bone cell-specific culture media. The DC source device required electrodes to transmit

electric current to the media and cells. The choice of electrode material is a crucial factor that can significantly impact the effectiveness of ES in biological tissues. The electrodes generate and inject charges (electrons) through the anode's oxidation process.<sup>[43]</sup> The charge-injection mechanism is dependent on the characteristics of the electrode. In both *in vitro* and *in vivo* stimulation experiments, the electrode material should exhibit satisfactory biocompatibility, ensuring it does not induce irreversible faradaic corrosion reactions in the culture medium during ES.<sup>[44]</sup> These materials include carbon (graphite rods), platinum (Pt), iridium (Ir), gold, titanium (Ti), tungsten (W), Pt-Ir alloys, Ti nitride, stainless steel (SS), indium tin oxide, and others.<sup>[45]</sup> We used SS electrodes in this study, as it is readily available, bio-inert, have good mechanical strength, and possess a passive iron oxide layer that protects the surface. Cells behave in different ways when subjected to various external or internal stimuli. For instance, studies have reported that extracellular physical stimulation of osteoblasts inhibits osteoblast differentiation and cell proliferation. However, it has also been observed that sustained stimulation activates p38, a molecule involved in the mitogen-activated protein kinase cascade, thereby promoting osteoblast differentiation.<sup>[46]</sup> ES can also start intracellular signaling through cell adhesion factors and increase the expression of the Src family kinases (Src) family<sup>[47]</sup> in fibroblasts. This study observed dynamic changes in the expression of RANKL and OPG in controlled, electrically stimulated Saos2 cells (experimental groups) compared to the control group, which did not receive any ES. We also observed a significant increase in RANKL and OPG expression compared to the control group.

ES increases  $\text{Ca}^{2+}$  levels and activates cytoskeletal calmodulin. ES and mechanical strain trigger cellular responses that involve the modulation of calcium signaling pathways.<sup>[48]</sup> Katoh has demonstrated that cells increase staining with phosphotyrosine antibodies, tyrosine-phosphorylated focal adhesion kinase, and tyrosine-phosphorylated c-Scr, indicating that ES affects signal transduction-related proteins.<sup>[49]</sup> Stephan *et al.* used continuous DC at 2.5–3.5 mV/m and 0.24–0.35 mV/m (60 kHz) for 3 days: 0.1 V improved metabolic rate, increased tissue inhibitor of metalloproteinases-1 (TIMP1) and OPG messenger ribonucleic acid.<sup>[50]</sup> Dauben *et al.* used *in vitro* DC ES with 0.2 V or 1.4 V, 20 Hz, 45 min  $\times$  3/day over 3 days, on human osteoblasts–0.2 V increased procollagen type I expression, and 1.4 V enhanced osteocalcin transcription.<sup>[51]</sup> Authors in the past have used different amounts of current/voltages in their *in vivo* experiments, but none of them have elucidated the reason for opting for such current levels.<sup>[5]</sup> We therefore set up an *in vitro* study to investigate the various aspects of electric current and its impact on bone cells, utilizing ELISA to measure RANKL and OPG levels.

RANKL is a transmembrane protein available in two forms:

Membrane-bound and secreted. The membrane-bound form can be converted into the secreted form through proteolytic cleavage or alternative splicing.<sup>[52]</sup> Various factors promote osteoclast formation and activity and stimulate the expression of RANKL in osteoblasts or stromal cells. The expression of RANK and RANKL increases bone resorption through osteoclastic differentiation, which starts bone softening and leads to tooth movement (Ikebuchi *et al.*, 2018).<sup>[53]</sup> In our study, comparing RANKL expression, the available 10s pulsed frequency group showed that a current level of 30- $\mu$ A increased RANKL expression, which peaked at 24 h and then decreased below the control level by 72 h. The 1-s frequency interval group experienced a 48 h delay in peak expression, whereas the 10s frequency interval group experienced it at 24 h. We observed significant alteration in expression within the 1s pulsed frequency group after 12 and 24 h. When we compared different current intensities, we found that 30  $\mu$ A significantly increased RANKL expression compared to all other current levels. Similarly, within the 10s frequency interval group, significant differences were observed between the 10  $\mu$ A and 30  $\mu$ A current levels. Therefore, we could comment that a current level of 30  $\mu$ A at 10s pulsed frequency showed the most pronounced effects in enhancing RANKL expression.

Osteoblasts express OPG in various tissues, including the heart, kidney, liver, spleen, and bone marrow.<sup>[54]</sup> Bone softening is prevented by inhibiting osteoclastogenesis and promoting bone hardening through enhanced OPG expression (Baud'huin *et al.*, 2013).<sup>[55]</sup> The same factors that induce RANKL expression in osteoblasts also regulate OPG expression, similar to RANKL. OPG expression was highest under the influence of a 30  $\mu$ A current within a 1-s time interval. Compared to the 10 s time interval, OPG showed an increasing trend from 3 h to 24 h and decreased at 72 h with 30- $\mu$ A current. This experiment has consistently demonstrated that OPG expression peaked with a 30- $\mu$ A current and a 10-s time interval. Pair-wise comparisons of OPG expression within the 1-s pulse frequency (5–72 h) showed significant statistical differences between the control group and the 30- $\mu$ A current group. These effects are primarily due to changes in cellular activity and the microenvironment. Changes in oxygen concentration and pH near the negative electrode directly affect cellular activity. An alkaline environment, induced by ES, promotes the release of calcium, which in turn promotes osteogenesis. These mechanisms contribute to the observed changes in cellular behavior and bone-related activity.<sup>[56]</sup> Kuehne *et al.*<sup>[57]</sup> advocated that ES at 100 mV, 60 kHz significantly increased OPG expression in MG-63 cells cultured on biphasic calcium phosphate scaffolds, enhancing osteogenic differentiation. In contrast, 1 V showed no added benefit and slightly reduced cell viability. The RANKL/OPG ratio was favorably altered, indicating a potential role in regulating bone remodeling.<sup>[57]</sup>

Under physiological conditions, the interaction between RANKL (produced by osteoblasts) and RANK (present on the surface of osteoclast precursors) plays a crucial role in osteoclastogenesis regulation. When RANKL binds to RANK, it recruits the adaptor protein TNF receptor-associated factor 6 (TRAF6), which initiates a signaling cascade leading to the activation of nuclear factor kappa-B (NF- $\kappa$ B) and its subsequent translocation to the nucleus. Turning on NF- $\kappa$ B increases the production of c-Fos, a transcription factor that collaborates with (nuclear factor of activated T cells [NFATc1], cytoplasmic 1). The interaction between c-Fos and NFATc1 is essential for the transcription of genes involved in osteoclast differentiation and function. Together, they trigger osteoclastogenic gene expression, promoting osteoclast formation and activity. However, OPG acts as a regulatory factor by binding to RANKL and inhibiting its interaction with RANK. By inhibiting this interaction, OPG interferes with the initiation of the osteoclastogenesis process. As a result, OPG serves as a counterbalance to RANKL, maintaining bone remodeling homeostasis by controlling osteoclast activation and differentiation.<sup>[58]</sup> An increase in the ratio of RANKL to OPG favors osteoclastogenesis, promoting osteoclast formation and activity. The RANKL/OPG ratio has been widely recognized as a significant determinant of bone mass.<sup>[59]</sup>

The findings of this study show that bioelectric stimulation can directly modulate both OPG and RANKL. OPG expression was on the rise at intervals of 1, 5, 12, and 24 h, and OPG expression increased with an increase in the current levels. As the current levels increased from 10  $\mu$ A to 30- $\mu$ A, we observed an increasing trend in the amount of RANKL content at 1, 5, and 24 h in groups with a 10 s time interval. These outcomes cannot be considered just a universal onset of cellular protein expression; instead, there are unique electrical frequencies that may be applied to bone cells to regulate, alter, and optimize the expression of OPG and RANKL.

### Limitations of the study

1. The exposure of bone cells to electric current was continuous throughout the experiment, which may have contributed to cell death. This approach was critical for identifying the specific current level that maximizes the expression of OPG and RANKL. Interestingly, existing literature appears to overlook this aspect, as most studies have empirically selected exposure durations and current parameters without providing a clear rationale.
2. We used Saos-2 osteosarcoma-derived cells, which may not fully reflect primary human osteoblast behavior, limiting generalizability.
3. Only short-term stimulation durations and current intensities were tested, leaving optimal clinical parameters unidentified.
4. As an *in vitro* study, it cannot replicate the bone

microenvironment's complexity, including vascular

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## CONCLUSION

Our results show that square-wave EStim modulates OPG and RANKL expression in Saos-2 cells, with peak expression dependent on pulse frequency (earlier at 24 h for the 10s interval, and delayed for the 1-s interval), time duration, and current levels. These findings suggest that specific EStim parameters can fine-tune osteogenic signaling, supporting their therapeutic potential. Future studies may include validating findings in primary human cells, extending stimulation protocols, and conducting *in vivo* studies to assess long-term biological effects.

**Author contributions:** AA: Made substantial contributions to the conception or design of the work, drafted the work, revised it critically for important intellectual content, and approved the version to be published; NM: Supervised the work being done; MZ: Helped in cellular experiments and laboratory work. All authors collectively agreed on the manuscript and its final draft.

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