Enhancing osteoblast function through pulsed electrical stimulation: implications for peri-implant healing and osseointegration

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Research Article

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Abstract

Electrical stimulation has been suggested as a mean for promoting the bonding of bone tissue to an implant, known as osseointegration. Previous work has investigated the impact of electrical stimulation in different models, both *in vitro* and *in vivo*, using various electrode configurations for delivering the electric field and with a wide range of stimulation parameters. However, there is no consensus on optimal electrode configuration nor stimulation parameters. Here, we investigated a novel approach of delivering electrical stimulation to a titanium implant using parameters clinically tested in a different application, namely peripheral nerve stimulation. We propose an *in vitro* model comprising of Ti6Al4V implants precultured with MC3T3-E1 preosteoblasts, stimulated for 72 h at two different pulse amplitudes (10 μ A and 20 μ A) and at two different frequencies (50 Hz and 100 Hz). We found that pulsed electrical stimulation enhances cell viability (and/or proliferation) and collagen production in an approximately dose-dependent manner. Our findings suggest that pulsed electrical stimulation with characteristics similar to peripheral nervous stimulation has the potential to accelerate osteoblast function and may provide a promising approach to improving peri-implant bone healing, particularly to neuromusculoskeletal interfaces in which implanted electrodes are readily available.

1. Introduction

The direct structural and functional bonding between an implant surface and bone, a phenomenon referred to as osseointegration [1], has revolutionised dental and orthopaedic rehabilitation. In comparison to suspending a limb prosthesis using a socket over soft-tissue, osseointegration allows for skeletal fixation resulting in a more comfortable and effective mechanical coupling to transfer load between an external prosthesis and the human body [2]. The external prosthesis is anchored to the bone via implant systems with entirely implanted and percutaneous components, also known as “fixture” and “abutment”, respectively. The fixture is the component that becomes osseointegrated within the bone tissue. The abutment extends from inside the fixture through the skin to provide the mechanical connection for the prosthesis, but it is not normally osseointegrated and therefore we reserve the term implant for the fixture hereafter. More recently, one of such systems has also included neuromuscular electrodes to record bioelectric signals for control of the prosthesis, and to deliver electrical stimulation to severed nerves for eliciting sensory feedback [3].

Commercially-pure titanium and Ti6Al4V are most frequently used in load-bearing orthopaedic implants due to their biocompatibility, mechanical strength, and high corrosion resistance [4], [5]. Despite being able to integrate with surrounding host bone, there is typically a healing period prior functional loading, approximately 3–12 months [2], [6], [7], in which one must allow for bone adaptation to the implant surface until the point that there is no progressive relative movement at the bone-implant interface [1]. Various factors affect peri-implant healing, including implant design and quality of the host bone [8]. In conditions where early implant loading is desired, or when the implant is placed in compromised healing conditions, there is a need to stimulate the osseointegration progression to a rapid and potentially better

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js n of function, and increased effective lifespan

of the prosthesis are the main driving forces behind enhancing biofunctionality at the bone-implant interface. To this end, various engineering approaches have been undertaken such as development of different metal alloys and macro-porous geometries, as well as alteration of implant surface properties, such as topographically and chemically [11].

Electrical stimulation has been instrumental in clinical treatments of a wide spectrum of disorders and disabilities [12]. Implants that deliver electrical stimulation have shown successful outcomes in applications such as cochlear implants to restore hearing function [13], wound-healing therapies aimed at achieving closure of chronic wounds [14], and in limb prostheses to restore sensory perception [3], [15]. The idea of promoting osteogenesis in bone fracture healing using electrical stimulation is known since the 1950's [16] and has since been explored in the clinical setting for bone injury treatments including bone healing of union and non-union fractures [17]. Furthermore, electrical stimulation has been investigated as a potential treatment for bone ingrowth into implants, both *in vitro* and *in vivo*, where different approaches have been developed by varying the electrode configuration, current type and source, and electrical stimulation parameters (e.g., amplitude and frequency) [18], [19]. Three modalities of electrical stimulation have commonly been used in this purpose: (i) direct stimulation, (ii) indirect stimulation (capacitive or inductive couplings), and (iii) combined stimulation [20]. Studies reveal significant increases in bone-implant contact [4], [9], [21]–[23], differentiation of preosteoblasts [12], [24], and increased cell proliferation [25] after applying direct current (DC) stimulation. However, the advantages of DC stimulation are not without major pitfalls and challenges, some of which include pH shifts, accumulation of charged proteins at the implant surface of opposite charge, and production of reactive oxygen species in the adjacent environment [25]. Pulsed electrical stimulation overcomes some of the challenges posed by DC stimulation [9] [25], particularly when pulses of opposite magnitudes are used to balance the displacement of charges [26]. Pulsed stimulation has shown beneficial effects on cell proliferation compared to unstimulated surfaces [25], as well as higher bone-implant contact compared to unstimulated/control specimens [9]. However, further investigation on the optimal electrical stimulation parameters is needed as no such values have been found yet [18], [19].

In this work, we investigated the response of MC3T3-E1 preosteoblasts (precursor cells to osteoblasts that allow for new bone formation) to pulsed electrical stimulation with similar parameters as those used for peripheral nerve stimulation to provide sensory feedback in artificial limbs [3], [15], [26]. We utilised parameters that have been used safely with implanted electrodes for several years [3] and are compatible with electronic embedded system for artificial limbs [27]. A versatile *in vitro* setup comprised of a bespoke, 3D-printed poly(lactic acid) (PLA) chamber was developed to minimise risk of inadvertent micromotion and enabled reproducible positioning of Ti6Al4V plates that simulated the implant to be osseointegrated, and circular plates that simulated implanted electrodes that serve as electrical reference. MC3T3-E1 preosteoblasts cultured on the titanium plates were exposed to different combinations of pulse amplitude and frequency over a continuous 72-hour period, followed by evaluations of pH, cell survival/proliferation, and collagen production compared to unstimulated controls. Our results showed, for the first time, that pulsed electrical stimulation significantly accelerates collagen production, which is

contingent on osteogenic differentiation. In addition, electrical stimulation significantly improved cell survival/proliferation compared to non-stimulated specimens without significantly imply any pH shifts.

2. Results

In vitro pulsed electrical stimulation experiments were conducted using a PLA chamber with integrated features for positioning the implants with precultured cells and reference electrodes (see materials and methods). No significant change in pH compared to unstimulated controls was found after 72 hours of pulsed electrical stimulation at different combinations of pulse amplitudes (10 μ A or 20 μ A) and frequencies (50 Hz or 100 Hz), namely, A10F50 (for amplitude of 10 μ A and frequency of 50 Hz, $p = 0.613$), A20F50 ($p = 0.126$), and A20F100 ($p = 0.55$).

No morphological changes after 72 hours of pulsed electrical stimulation

In the preculture group – the group cultured for 16 h prior to stimulation –, the osteoblasts appear in an elongated shape (Fig. 1a, e). Note that all specimens were included in the preculture group before they were assigned to either one of the stimulation groups or the control. After 72 h of pulsed electrical stimulation (16 h of preculture + 72 h of stimulation), the osteoblasts display a typical flattened morphology (Fig. 1f-h). The stimulated groups (A10F50, A20F50, and A20F100) displayed matrix structures (represented in Fig. 1b-d), of which the highest density was observed during scanning electron microscopy (SEM) imaging in the A20F50 group.

Pulsed electrical stimulation improves cell survival and collagen production

We found significantly improved cell survival compared to the control sample (Fig. 2) in the stimulated groups A10F50 ($p = 0.022$), A20F50 ($p < 0.001$), and A20F100 ($p < 0.001$). No significant difference in cell viability was observed between the cells pulsed with 50 Hz frequency (A10F50 vs. A20F50), however, a significant increase in cell viability was observed in group A20F100 compared to A20F50 ($p = 0.019$) (100 vs 50 Hz). Notably, the cell population recorded for A20F100 exceeded the number of cells at 0 h (preculture).

Compared to the control specimens, collagen production was significantly higher for A20F50 ($p = 0.033$) and A20F100 ($p < 0.001$), but not for A10F50 ($p > 0.05$), Fig. 3. There was a significant difference between A20F50 and A10F50 ($p = 0.039$), and A20F100 ($p = 0.0094$).

Interestingly, a nonlinear relationship was noted between cell survival and collagen production (Fig. 4), which warrants further investigation.

3. Discussion

Electrical stimulation has been regarded as a potential approach for promoting peri-implant osteogenesis [19]. Here, we investigated the impact of pulsed electrical stimulation of similar characteristics as used in

peripheral nerve stimulation to restore sensory feedback in osseointegrated artificial limbs [3], [15], [28]. We employ an *in vitro* setup comprising Ti6Al4V implants that were precultured with MC3T3-E1 preosteoblasts, stimulated for 72 h at two different pulse amplitudes (A; 10 μ A and 20 μ A) at two different frequencies (F; 50 Hz and 100 Hz). We demonstrated that pulsed electrical stimulation enhances cell viability (and/or proliferation) and collagen production compared to non-stimulated surfaces in an approximately dose-dependent manner.

SEM images displayed healthy-looking osteoblasts where the cells have a flattened appearance and are stretched across the implant surface. There were no significant dissimilarities in cell morphology between the various stimulated groups, indicating that the different electrical stimulation combinations did not have a visual impact on the cell morphology compared to each other. Some extracellular matrix-like features were noted on the implant surface in the stimulated specimens, but further investigation is required to better understand the structural and functional characteristics of extra cellular matrix produced under pulsed electrical stimulation.

Clinically-relevant dimensions (4 cm in length $\times$ 4 mm in diameter) of implants used for direct skeletal attachment of upper limb prosthesis was used for the study-specific implant [29]. We used a flat/plate design of the implant for technical reasons, that is, to facilitate imaging. However, a cylindrical implant can be used with minor design modifications. The model was limited to a 2D culture surrounded by a homogenous environment and it was not currently known up to which distance from the implant surface cells will retain their responsiveness to pulsed electrical stimulation.

Cell survival decreased after 72 h in groups A10F50, A20F50, and control in relation to time point 0 h (start of stimulation). This may be explained by the use of HEPES buffer, for periods outside CO₂ incubators, which potentially could have led to non-physiological changes in pH [30]. In addition, other contributing factors to reduced cell survival could have been poor cell attachment to the implant after 16 h of preculture and physical cell removal during transfer from preculturing tube to chamber prior to stimulation start. However, the decrease in cell number was significantly less in the stimulated groups compared to control, meaning significantly higher cell survival in stimulated groups. The mean value in A20F50 was larger compared to A10F50, although there was no significant difference between the amplitude values. Comparing the two frequencies, 50 and 100 Hz, there was a significant increase in cell survival in A20F100 compared to A20F50, and notably A20F100 was the only group where the population had increased in number compared to time point 0 h (start of stimulation). Applying higher frequency of the pulse train means that the time period between each pulse event decreases, and the more the stimulation resembles DC.

The stimulated groups A20F50 and A20F100 showed significantly accelerated collagen production compared to A10F50 and control. Soluble collagen production is produced by osteoblasts [31] and the cell line used in this study, MC3T3-E1, was preosteoblastic. Therefore, the result also implied that stimulation triggered differentiation of preosteoblasts to osteoblasts. Interesting future directions from this work could be to investigate whether the electrical stimulation itself stimulates differentiation without

osteoblastic differentiation medium, and also if electrical stimulation drives differentiation of mesenchymal stem cells to osteoblasts. Additional future directions include model development with implant fixtures from clinical settings, as well as to investigate parameters such as pulse width, duty cycle, and stimulation duration longer than 72 h.

4. Conclusions

In summary, pulsed electrical stimulation exhibited a strong positive influence on osteoblast survival/proliferation, collagen production, attachment and spreading on Ti6Al4V surfaces, which are important processes in osseointegration. Our results showed enhanced cell survival with stimulation of 10 and 20 μA and bone cells grew in higher numbers on stimulated Ti6Al4V as compared to non-stimulated Ti6Al4V surfaces. Among all test conditions, 20 μA indicated as the most beneficial amplitude, although not significantly higher compared to 10 μA . Regarding frequency, 100 Hz was found to favour cell proliferation compared to 50 Hz and no stimulation (control). The highest osteoblast density was measured at 20 μA and 100 Hz after 72 h where cells produced 5 times more collagen and proliferated 120% as compared to non-stimulated surfaces.

Therefore, it can be concluded that pulsed electrical stimulation with similar properties to sensory feedback stimulation in artificial limbs, has a beneficial impact on osteoblast function (e.g., cell survival and collagen production). These preliminary findings offer insight into a promising novel approach towards improving peri-implant bone healing, i.e., osseointegration. An important application would be stimulation to regain osseointegration of failing bone-anchored implants.

5. Materials And Methods

Experimental setup

The *in vitro* experimental setup included a 3D-printed chamber made of PLA, a plate and two discs made of Ti6Al4V, a bipolar constant current stimulator (DS5, Digitimer) and an Arbitrary Function Generator (AFG-2112–12MHz, Gwinstek).

The PLA chamber was a rectangular box with integrated design features (Fig. 5a-b). The chamber contained two different positioners, one for the implant and two for the electrodes. The implant positioner had two separated components that allowed the implant to stand up by sliding into two slots. The slot nearest the chamber wall was designed with an output that allowed the wire to exit the chamber. The electrode positioner contained a cylindrical extrusion with an opening closest to the wall in order to let the wire exit. The chamber had three rectangular slots in the upper part of the wall, two located at the long side and one positioned at the short side. Those slots were designed to prevent rotation and restrict movements of the implant and the electrodes during the experiment.

The Ti6Al4V plate was chosen to imitate the implant fixture with a size of 40x4 mm and thickness of 1 mm. The wires had a diameter of 4 mm and height of 3 mm. The wires

that connected the plate and the discs to the current generator were made of titanium grade 1 (Sargenta AB) with a length of 10 mm where the part of the wire that was in the cell culture medium was isolated within a silicone tube. To prevent corrosion between the wire and the implant, and leakage due to the capillary effect in the silicone tube, a small droplet of silicone glue (Med-1037, Nusil) was used to cover the welding and prevent the media from being sucked out through the tube. The function generator was used to control the bipolar constant current generator that sent out the pulse with desired settings. The implant was connected to the negative output, thus functioning as a cathode, and the electrodes were connected to the positive output, thereby serving as anodes (Fig. 5c). In order to produce replicates with the same amount of applied current, the chambers were coupled in series where the implant in one chamber was connected to the electrodes in another chamber.

Expansion of MC3T3-E1 cells and preculture on Ti6Al4V

The same vial of passage 10 osteoblastic cell line MC3T3-E1, established from C57BL/6 mouse calvaria, was used for every experimental cycle. Cells were precultured on the implant surface in a 2 mL Eppendorf tube at 37°C in 95% humidity and 5% CO₂ for 16 h with Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, USA) containing 4.5 g/L D-glucose, L-glutamine, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, and 0.25 mg/mL amphotericin-B (sDMEM). The implant surface facing the electrodes in the experimental setup was placed upwards and a seeding density of 10⁵ cells per implant was applied. Six randomly-selected implants were counted at 16 h to determine number of cells attached to the surface prior to start of stimulation and two randomly-selected implants were qualitatively investigated by SEM imaging after 16 h of preculturing before stimulation.

The implant with precultured cells was removed from the Eppendorf tube and carefully placed into position in the PLA chamber. The electrodes were placed in their positioners and connected to the generators. 12 mL osteogenic differentiation media (sDMEM supplemented with 1% L-ascorbic acid 4.5 mM, 1% dexamethasone 1 mM and 2% β-glycerophosphate 1 M) were added to the chamber before placement in a non-CO₂ incubator (Heratherm IMC 18, Thermo Scientific). The experiment started when electrical stimulation was applied.

Pulsed electrical stimulation

The electrical stimulation consisted of charge-balanced, cathodic, rectangular, biphasic asymmetric (10:1), current-controlled pulses (Fig. 5d). The cathodic phase (negative pulse) was followed by an inter-pulse break (zero amplitude) and a recovery phase (positive pulse) that was 10x smaller in amplitude and 10x longer in duration than the cathodic phase. Each stimulation pulse was followed by a charge recovery phase where any residual charge was recovered back to zero to ensure that charge accumulation cannot occur.

Stimulation treatment

Pulsed electrical stimulation was applied for a continuous duration of 72 h at 3 combinations of negative pulse amplitude (denoted “A”, 10 and 20 μ A) and frequency (denoted “F”, 50 and 100 Hz), e.g., A10F50, A20F50, A20F100. Fixed pulse parameters included negative pulse width (500 μ s), inter-pulse break (50 μ s) and sample frequency (100 kSPS). To adjust for evaporation, 2 mL fresh medium was added per chamber every 24 h. The three first replicates in each stimulated group were evaluated for cell count and the two last replicates were prepared for SEM imaging. Every replicate was evaluated for collagen production.

EVALUATION ASSAYS

Cell distribution, morphology, and attachment

Distribution, morphology, and attachment of cells on the titanium implant were qualitatively evaluated using SEM imaging ($n = 2$ per group). Samples were fixed in 4% paraformaldehyde for 2 h at room temperature and stained with 1% OsO₄ for 2 h. After rinsing with buffer, the samples were briefly dehydrated in a graded ethanol series for 5 min per cycle (50, 70, 80, 90, 95 and 100% ethanol) and allowed to air dry. The samples were sputter-coated with gold before examination in an Ultra 55 FEG SEM (Leo Electron Microscopy Ltd, UK) with settings of 5 kV accelerating voltage, 5 mm working distance and 30 μ m aperture size.

Cell proliferation

Number of cells attached to the implant were counted using a NucleoCounter at 72 h of stimulation. Briefly, each implant was removed from the PLA chamber and placed into a 2 mL Eppendorf tube. Lysis buffer (200 μ L; Reagent A100, Chemometec) was added and the tube was vortexed for 30 s to detach cells. Next, stabilisation buffer (200 μ L; Reagent B, Chemometec) was added and the tube was vortexed again for 30 s. The solution (detached cells and both buffer solutions) was taken up in a NucleoCounter cassette (NucleoCassette™, 941-0002) for counting.

Collagen production

The amount of soluble collagen present in the cell culture medium at 72 h was measured using a collagen detection kit (Sircol Soluble Collagen Assay, Biocolor). The medium for every replicate in each experimental group was collected and diluted to 11.5 mL in consideration of uneven evaporation. Samples were prepared according to the manufacturer's protocol and absorbance measurements were performed at 555 nm by a microplate reader (FLUOstar Omega, BMG LABTECH). OD_{555nm} values were transformed to μ g collagen by the standard curve function, $y = 5.1528 \cdot x - 0.7766$, $R^2 = 0.9665$. Three technical replicates per sample were measured and each sample is presented as the mean value of the technical replicates.

pH measurement

Cell culture media pH was measured using a pH meter (Beckman, USA). Two technical replicates per sample were measured, and each replicate is presented as the mean of the technical replicates.

Statistical analyses

For all statistical analyses, the unpaired, two-tailed, homoscedastic Student's *t*-test was used and *p* values < 0.05 were considered statistically significant.

Declarations

Author contribution

All authors contributed to the study design; EP conducted the study experiments. EP and FAS collected and analysed data. EP and FAS drafted the manuscript. EP, FAS and MOC edited and revised the manuscript. All authors approved the final version of the manuscript.

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Competing interests

EP and FAS declare no competing interests. MOC has served as a consultant for a company commercialising orthopaedic implants.

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Figures

Figure 1

MC3T3-E1 preosteoblast morphology before (preculture) and after 72 h pulsed electrical stimulation (scanning electron microscopy).

Figure 2

MC3T3-E1 cell survival at 72 h. The average number of implant-adherent cells at 16 h preculture (~24600 cells per implant) is taken as 100% survival at 0 h. Ctrl, n = 5; A10F50, A20F50, and A20F100, n = 3.

Figure 3

Figure 4

Linear regression plot and polynomial plot. Mean collagen production (μg) in each group is plotted against mean cell survival (%) in each group. Linear regression: $y = 0.00018 + 0.25x$, $R^2 = 0.7873$; polynomial plot: $Y = 8 \times 10^{-9}x^2 - 6 \times 10^{-5}x + 1.4232$, $R^2 = 0.8985$.

Figure 5

Experimental setup. A) CAD sketch of the 3D-printed chamber where specific design features are indicated by arrows. B) Image of the experimental setup during the experiments where the implant and electrodes are placed in their positioners and the chamber is filled with cell culture medium. C) The experimental electrical circuit where the implant is the cathode and the electrodes function as anodes connected to the constant current generator, which in turn is connected to the function generator. D) Pulse characteristics.