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Investigation of *In Vitro* Bone Cell Adhesion and Proliferation on Ti Using Direct Current Stimulation

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Abstract

Our objective was to establish an *in vitro* cell culture protocol to improve bone cell attachment and proliferation on Ti substrate using direct current stimulation. For this purpose, a custom made electrical stimulator was developed and a varying range of direct currents, from 5 to 25 μ A, were used to study the current stimulation effect on bone cells cultured on conducting Ti samples *in vitro*. Cell–materials interaction was studied for a maximum of 5 days by culturing with human fetal osteoblast cells (hFOB). The direct current was applied in every 8 h time interval and the duration of electrical stimulation was kept constant at 15 min for all cases. *In vitro* results showed that direct current stimulation significantly favored bone cell attachment and proliferation in comparison to nonstimulated Ti surface. Immunohistochemistry and confocal microscopy results confirmed that the cell adhesion was most pronounced on 25 μ A direct current stimulated Ti surfaces as hFOB cells expressed higher vinculin protein with increasing amount of direct current. Furthermore, MTT assay results established that cells grew 30% higher in number under 25 μ A electrical stimulation as compared to nonstimulated Ti surface after 5 days of culture period. In this work we have successfully established a simple and cost effective *in vitro* protocol offering easy and rapid analysis of bone cell–materials interaction which can be used in promotion of bone cell attachment and growth on Ti substrate using direct current electrical stimulation in an *in vitro* model.

Keywords

Direct current stimulation; Human osteoblast cells; Bone cell adhesion and proliferation; Immunohistochemistry and confocal microscopy; MTT Assay

Introduction

Among many metallic biomaterials, titanium (Ti) and its alloys have been the most commonly used for load-bearing orthopedic implants due to excellent corrosion resistance and good mechanical properties along with high strength-to-weight ratio [1, 2]. However,

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being a metal, biocompatibility is a major concern for Ti based implants, which reduces Ti-based implant's osseointegration ability [3, 4]. After surgery, fibrous tissue encapsulation on bioinert Ti implants results in a weak interfacial bond, which reduces the *in vivo* lifetime of the implant. To address this limitation, several attempts have been made in recent years such as development of porous implants, alteration of biomaterials surface properties such as topography/roughness, chemistry, wettability, surface energy, surface charge, coatings as well as change in bulk material composition [4–10]. The main objective of those approaches is to engineer the biofunctionality at the implant-tissue interface with a specific aim to reduce the healing time as well as increase implant's lifespan.

Among many other approaches, electrical stimulation to assist bone remodeling and accelerate healing of bone nonunions and/or delayed union fracture has attracted growing attention in recent years in both experimentally as well as clinically [11–15]. It has been known for many years that all animals including human possess 'animal electricity' and can generate endogenous electrical signals of varying gradients which can potentially influence a number of biological processes including cell division, tissue regeneration, and wound healing [16, 17]. However, those earlier therapeutic approaches to stimulate bone regeneration with application of electrical energy were not fully understood and illustrated until the discovery of piezoelectricity phenomena of bone by Yasuda et al. [18]. In this pioneering work, direct current electrodes were implanted in a rabbit femur and demonstrated new bone formation in the vicinity of the cathode over a period of three weeks. Further in another study, Fukada et al. showed the significance of electric charge and polarity of bone electrical potentials on old bone resorption and new bone growth [19]. It has been shown that bone can grow more efficiently under compression when bone produce negative charge while under tension bone produces a positive charge and leads to bone resorption. Since that time, numerous modifications of electrical stimulation have been used to assist in bone healing. Primarily, three different forms of electrical stimulation techniques have been utilized for bone regeneration applications (a) direct current stimulation; (b) capacitively coupled (CC) electric field stimulation; and (c) inductively coupled electromagnetic field (IEMF) stimulation [20–24]. Among them direct current stimulation becomes very popular and commonly used approach due to its ease of operation and potential to enhance osteoblastic function by controlling of osteogenic gene expressions [22–26]. Bassett et al. showed that a direct current stimulation of 3 μ A through platinum electrodes placed into the medullary canal of the femur preferentially accelerated the new bone growth at the cathode location over a period a 21 days [25, 26]. Later in another study Aaron et al. reported similar observation in which osteogenesis was significantly stimulated by applying a direct current of 5 to 100 μ A in an *in vivo* experiment [23]. Recently, in an *in vitro* study, Supronowicz et al. showed that a current conducting carbon nanotube (CNT)/poly-lactic acid (PLA) composites exhibited 46% increments in osteoblast cell proliferation when exposed to alternating current stimulation at a dose of 10 μ A current for 6 h/day after 2 days of incubation period [27].

However, report on direct measurement of bone cell stimulation on Ti substrate using direct current in *in vitro* cell culture models system is limited in spite of several *in vivo* reports. Therefore, development of a simple and cost effective *in vitro* direct current electrical stimulation protocols for Ti substrate offering easy and rapid measurement of bone cell-materials interaction analysis are of great interest due to the escalating *in vivo* experimental costs. For this reason, the objective of our research was to establish an *in vitro* cell culture protocol to improve bone cell attachment and proliferation on Ti substrate using direct current stimulation. Further, such type of low cost and easy to operate *in vitro* direct current electrical stimulation protocols would also assist other researchers to initiate new investigations to combine the advantages of different established Ti surface modification

treatment accompanied with the original benefits of electrical stimulation to improve the osteoconduction property of Ti.

In our research, we have selected Ti as a conducting substrate which is most commonly used in metallic implant material and a varying range of direct current between 5 and 25 μ A for bone cell (hFOB) stimulation. The selection of direct current magnitude was based on previous investigation reports [23, 25–27]. In present report, we intended to provide data on cell adhesion by vinculin proteins expression as well as cell morphology and quantitatively determined viable cell proliferation results to unveil the bone cell–material interactions.

2. Materials and methods

2.1. Specimen preparation

99.7% commercially pure Ti plate was procured (Grade 2, President Titanium, MA, USA) and cut into disc shaped specimen with 3 mm thickness and 2 cm diameter. Each Ti disc specimen was first abraded with silicon carbide paper in successive grades from 600 to 1200 grit (Leco Corporation, MI, USA), then cloth polished with a 1 μ m alumina suspension. Following this the specimen was ultrasonically cleaned in acetone and 70% ethanol respectively, to remove any organic materials and finally sonicated in deionized water for 30 min in each steps. Afterwards, these surface cleaned Ti samples were used in *in vitro* cell culture experiments.

2.2. Electrical stimulator set-up

In our study a custom made electrical stimulator was used to stimulate the osteoblast cells on conducting Ti samples. Figure 1 shows the schematic of the uniquely designed electrical stimulator set up as prepared for a 12-well cell culture plate. The stimulator had two main components, a voltage generators and a cell culture plate electrode assembly. The plate cover is constructed from Ultem 1000 polythermide sheet with 0.6 cm thickness. The electrodes placed on the plate cover were connected to an external voltage source as shown in Figure 1b. During the experiment, the electrodes made a contact with the Ti samples as shown in Figure 1a. Each of the electrodes were constructed from 316 stainless steel hypo tube (internal diameter ~ 0.043 cm), with a discrete spring loaded contact swaged at the end. The electrode contacts were made of gold over nickel coated copper alloy sleeve and plunger. The spring was made of beryllium copper alloy. These electrodes were pressed into the polythermide sheet cover. The distance between two electrodes for each well was 1.5 cm. The current and voltage for each well in the 12-well cell culture plate was controlled by an individual multi-turn potentiometer. A range of current (from 0 to 30 μ A) can be applied for stimulation depending on the voltage adjustment (from 0 to 6 V, d.c. voltage) in the potentiometers. A 9 volt battery was used as a voltage source. Prior to start the *in vitro* experiment, the experimental voltage and current were adjusted by attaching a digital volt meter across each set of electrode contacts. During the adjustment procedure, all electrodes made a contact with the Ti samples and each well were filled with 3 ml of cell free Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM) culture media supplemented with 10% fetal bovine serum as used in cell culture experiment.

2.3. *In vitro* bone cell-materials interaction under electrical stimulation

The influence of direct current stimulation on *in vitro* bone cell-materials interactions was evaluated for a maximum of 5 days using human fetal osteoblast cells (hFOB 1.19). In our study, three different direct currents i.e., 5, 15 and 25 μ A were used to study the stimulation effects. The hFOB cells used were derived from an immortalized, osteoblastic cell line, established from human fetal bone tissue. Prior to cell culture experiments Ti samples were sterilized by autoclaving at 121°C for 20 mins. The polythermide cell culture plate assembly

with attached electrodes was also sterilized by autoclaving under similar condition. Following this, sterilized Ti samples were placed into the well of a 12- well cell culture plate and cells were seeded onto the Ti samples with a cell density of 2.0×10^4 cells/well. Following this, 3 ml Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM) culture media supplemented with 10% fetal bovine serum was added to each well. Cultures were maintained at 37.4°C under an atmosphere of 5% CO₂ and 95% air in an incubator. The first electrical stimulation was conducted 2 h after the cells were seeded onto the Ti samples. The direct current was applied in every 8 h time interval and the duration of electrical stimulation was kept constant at 15 min for all cases. Triplicate samples per group were evaluated for all experiments. Figure 1c shows a schematic of the experimental set up of 12-well cell culture plate with different applied direct current condition. Three rows (2nd, 3rd and 4th) in a 12-well plate was used for three different amount of applied direct current i.e., 5, 15 and 25 μ A while in each well of any particular row similar magnitude of direct current was applied. The 1st row of the 12-well plate was used as control well where no direct current was applied as shown in Figure 1c. The culture media (DMEM) were changed every alternate day during the duration of the experiment.

2.3.1. Cell morphology—Cell morphology was assessed under SEM after 5 days of incubation period to qualitatively analyze the hFOB cell attachment and spreading on different electrically stimulated and nonstimulated Ti samples. Cultured samples for SEM observation were collected and rinsed with 0.1 M phosphate-buffered saline (PBS) and subsequently fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. Following this, post fixation for each sample was made with 2% Osmium tetroxide (OsO₄) for 2h at room temperature. Fixed samples were then dehydrated in an ethanol series 30%, 50%, 70%, 95% and 100% three times, followed by a hexamethyldisilane (HMDS) drying procedure. Dried samples were then mounted on aluminum stubs, gold coated and observed under a field emission SEM (FEI Inc., OR, USA).

2.3.2. Immunochemical analysis and confocal microscopy—Vinculin, the specific protein expressions relevant to focal adhesion formation was assayed for both stimulated and nonstimulated Ti samples by fluorescent staining and confocal laser scanning microscopy (CLSM) observation. Different test samples after 5 days of incubation, were collected and rinsed with 0.1M PBS, fixed in 4% paraformaldehyde in 0.1M phosphate buffer and kept for 24h at 4°C for overnight. Following these, samples were incubated in 0.5% Triton X-100 in 0.1M PBS for 10min and blocked with TBST/BSA (Tris-buffered saline with 1% bovine serum albumin, 250mMNaCl, pH 8.3) for 1h at room temperature. Primary antibody against vinculin (Sigma, MO, USA) was added at a 1:100 dilution and incubated at room temperature for 2h. The samples were then rinsed three times with TBST/BSA for 10min. Following this, the secondary antibody, goat antimouse (GAM) oregon green (Molecular Probes, OR, USA), was added at a 1:100 dilution and incubated for 1h. Samples were then mounted on coverslips with Vectashield mounting medium (Vector Labs, Burlingame, CA) with propidium iodide (PI) and kept at 4°C for future CLSM imaging. The microscopical examinations were performed on a Zeiss 510 laser scanning microscope (LSM 510 META, Carl Zeiss MicroImaging, Inc., NY, USA). The images (1024×1024 pixels) were taken using a 10× objective with 0.25 numerical aperture and stored in a workstation computer. In the present study, the specific absorption of vinculin identified by the expression of green fluorescence and nuclei counterstained with the chemical dye propidium iodide (PI) present in the mounting medium were expressed as red fluorescence. For the green fluorescence, an excitation Ar ion (488nm, 30mW) laser was used at 50% of maximum output at full power, and for the red fluorescence excitation, a He-Ne (543nm, 1.2mW) laser was used at full output and full power. A combination of a band-

pass filter transmitting 505–570nm and a long pass filter at 560nm was applied to obtain fluorescence images. Since the test samples were opaque, the confocal pinholes were opened up to 560 μ m to pass more fluorescence light to improve the quality of the fluorescence image.

2.3.3. Cell proliferation using MTT assay—The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma Inc., St Louis, MO) was performed after 1, 3 and 5 days of incubation to quantitatively determine hFOB cell proliferation on electrically stimulated and nonstimulated Ti samples. Triplicate samples per group were evaluated and three data points were measured from each sample. The MTT (Sigma, St. Louis, MO) solution of 5 mg/ml was prepared by dissolving MTT in PBS, and was filter-sterilized using a filter paper of 0.2 micron pore. The MTT was diluted (0.3 ml into 2.7 ml) in DMEM/F12 medium. 3 mL diluted MTT solution was then added to each sample kept in 12-well plates. After 2h of incubation, samples were transferred carefully to another 12-well plate and 3 ml of solubilization solution made up of 10% Triton X-100, 0.1N HCl, and isopropanol was added to dissolve the formazan crystals. 100 μ L of the resulting supernatant was transferred into a 96-well plate, and read by an optical plate reader at 570 nm.

2.4. Statistical analysis

The MTT assay results are presented as mean $\pm$ standard deviation. Statistical analysis was performed on the MTT assay results using Student's *t*-test, and a *p* value of <0.05 was considered significant.

3. Results and discussion

3.1. Morphology of hFOB cells

In Figure 2, sample surfaces imaged after 5 days of culture to investigate the influence of different magnitude of direct current stimulation on bone cell attachment, and spreading on Ti surfaces. Figure 2a revealed that relatively fewer hFOB cells were observed on nonstimulated Ti surfaces. From Figure 2a it can be seen that cells have assumed a more round shape morphology with diffused or under developed cellular microextension on nonstimulated bioinert Ti surfaces, which indicates poor cell-materials interactions. However, upon application of direct current stimulation, a significant difference in cell attachment and growth behavior among Ti samples can be noticed. Interestingly, cells were found to assume more flat spread shape morphology and grew in higher numbers with increasing direct current from 5 μ A to 25 μ A (Figure 2b-d) in comparison to nonstimulated Ti (Figure 2a). Evidently, significant improvement in cell-material interactions can be observed for 25 μ A direct current stimulated Ti samples (Figure 2d) where cells appeared to be more elongated and confluent on Ti surface. In particular, among all samples, the highest level of cytocompatibility was clearly observed on 25 μ A treated Ti surfaces as cells were seen to adhere to each other with cellular micro extensions and connected to substrate in addition to the neighboring cells. This observation is in good agreement with previous reports which reportedly showed that electrical stimulation has strong positive influence on osteoblast attachment and growth [24–26]. It is generally believed that the application of electrical energy promotes different osteogenic gene expressions and enhances intracellular free calcium ion concentration [22]. Wang et al. showed that direct current stimulation of 100 μ A/cm² significantly accelerated proliferation of rat calvariae-like cells after 24 h. Additionally, the intracellular calcium level increased to 2.3 times, which helped in cell signaling and subsequent osteogenesis process [28]. However, direct comparison among these reported results is very difficult due to the difference in design parameters of biological experiments, cell lines used as well as different forms of electrical stimulation techniques and delivery modes.

3.2. Immunocytochemistry and confocal microscopy

It can be recalled that osteoblasts are attachment-dependent cells, i.e., they must attach first on the surface then spread and reach confluence. Therefore, it is important to understand the influence of current stimulation on bone cell adhesion to unveil the *in vitro* bone cell-materials interactions. For this purpose, expression of vinculin protein which aids in the assembly of focal contacts by cross-linking and recruiting other proteins to form adhesive plaques has been analyzed for different direct current stimulated Ti samples and compared with control nonstimulated Ti. From Figure 3a nonspecific absorption of vinculin protein can be noticed on nonstimulated Ti surfaces as identified by poor expression of green fluorescence indicating weak cellular attachment and limited spreading on bioinert Ti surface. In comparison to nonstimulated Ti samples, significantly better osteoblast adhesion was observed for direct current stimulated Ti samples, which evidently showed a positive immunostaining for vinculin, a protein expressed by osteoblast cells which forms at focal adhesion sites (Figure 3b-c). However, the intensity and distribution of vinculin expression were found different for different samples depending on stimulating current amount. Interestingly, increasing direct current amount has been found to promote formation of more focal contacts and the cell adhesion. It can be noticed that the intensity of green fluorescence as well as specific expression of vinculin increases with increasing the current from 5 μA (Figure 3b) to 10 μA (Figure 3c). Evidently, the highest level of green fluorescence was observed for 25 μA current stimulation (Figure 3d). This clearly suggests that 25 μA direct current stimulation is the most effective and favors the formation of the highest number of focal adhesion sites on 25 μA stimulated Ti surface as well as allows cytoskeleton to establish spread shape morphology (Figure 3d). This observation is well supported by the SEM cell morphology analysis results and evidently confirms the benefits of current stimulation. Similar observation was also reported by Hartig et al. and showed that electrical stimulation helped osteoblast-like cells to reach confluent state and favored extra cellular matrix (ECM) related protein synthesis [22]. In an *in vitro* experiment, it was demonstrated that application of capacitively coupled electrical field (6kV/m) enhanced osteoblast-like cells proliferation, alkaline phosphatase and other osteogenic gene expression, which further led to ECM maturation within a period of 18 days.

3.3. Cell proliferation

MTT assay was used to quantitatively determine the proliferation of viable hFOB cells on direct current stimulated and nonstimulated Ti samples. Figure 4 shows a comparison of viable cell densities for different samples after 1, 3 and 5 days of culture. It can be recalled that a higher number of living cells densities relates to superior biocompatibility and/or cytocompatibility of a material. Evidently for all culture durations, cells were proliferated in higher numbers under direct current stimulation compared to that measured on nonstimulated Ti samples and with increasing culture days higher numbers of osteoblast densities were measured with increasing stimulating current level. Further, statistical analysis also confirmed that the differences in cell densities are significant (P value < 0.05) among electrically stimulated Ti and nonstimulated Ti control for all culture periods. However, statistical analysis revealed that after 1 day of incubation period, the difference in cell densities was not significant (P value > 0.05) between 5 μA and 15 μA direct current stimulated samples as well as in between 15 μA and 25 μA direct current stimulated samples although significant difference (P value < 0.05) in cell densities was measured between 5 μA and 25 μA direct current stimulated samples. This indicates that cell proliferation increases with increasing stimulated current amount. After 3 and 5 days of culture, significantly higher cell densities was counted on Ti surface stimulated at 25 μA direct current in comparison to all other samples (P value < 0.05); although the similar comparison was not statistically significant between 5 μA and 15 μA direct current stimulated Ti samples until 5 days of culture. The highest number of viable osteoblast density was measured on 25 μA electrically

stimulated Ti surfaces after 5 days of incubation for all current stimulation conditions. MTT assay results quantitatively determined that cells grew 30% higher in number upon 25 μ A electrical stimulation compared to nonstimulated Ti surface after 5 days of culture period. Therefore, our results provide evidence that direct current stimulation significantly promotes bone cell attachment, spreading and proliferation on Ti surface in comparison to nonstimulated control Ti. Further, it can be concluded that the benefit of current stimulation can be better utilized with higher amounts of current level (i.e., 25 μ A) while for lower amounts of current stimulation such as 5 μ A and 15 μ A the advantage is rather limited during the initial cell growth period. Based on the previous reports it is hypothesized that the direct current stimulation possibly increases the concentration of extracellular calcium and promotes cell signaling and the expression of different cell adhesive proteins as well as other ECM marker proteins (i.e., vinculin, alkaline phosphatase, osteocalcin etc.), which accelerates growth and progressive maturation of osteoblast cells [22–27].

4. Conclusions

In summary, direct current stimulation exhibited strong positive influence on osteoblast attachment, spreading and proliferation on Ti surfaces. Research results showed enhanced bone cell-materials interactions with increasing amount of direct current stimulation from 5 μ A to 25 μ A and bone cells grew in higher numbers on stimulated Ti as compared to nonstimulated Ti surfaces. Among all test condition, 25 μ A direct current stimulation was found the most beneficial favoring the formation of highest number of focal adhesion sites and thereby helped the cytoskeleton to establish spread shape morphology. The highest viable osteoblast cell density was measured on 25 μ A electrically stimulated Ti surfaces after 5 days of incubation where cells grew almost 30% higher in number as compared to nonstimulated Ti surface. Therefore, it can be concluded that a simple and cost effective *in vitro* direct current electrical stimulation protocols for Ti substrate offering easy and rapid measurement of bone cell -materials interaction analysis has been established and the efficacy of direct current stimulation for promotion of bone cell adhesion and growth in *in vitro* cell culture models system has been successfully demonstrated.

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Highlights

- The aim of our work was to establish a simple and cost-effective *in vitro* cell culture protocol offering easy and rapid analysis of bone cell-materials interaction using direct current electrical stimulation.
- Bone cells (hFOB) cultured on conducting Ti samples were stimulated by varying range of direct currents of 5 to 25 μ A.
- Bone cell attachment and proliferation enhances with increasing stimulated current amount.
- 25 μ A direct current stimulation was found the most beneficial for promotion of bone cell adhesion and growth.
- Our research findings demonstrated the efficacy of direct current stimulation for promotion of bone cell adhesion and growth in a simple and cost-effective *in vitro* cell culture models system.

Figure 1.

(a) Schematic of the electrical stimulation set up as prepared for a 12-well cell culture plate, (b) schematic of the 12-well cell culture plate electrode assembly showing a uniquely designed polythermide sheet culture plate cover. The electrodes placed on the plate cover were connected to an external direct current/voltage source, (c) schematic of the experimental set up of 12-well cell culture plate with different applied pulse direct current condition. Commercially pure Ti was used as test samples in all experiments.

Figure 2. SEM micrographs illustrating the cell adhesion and proliferation after 5 days of culture on control (a) nonstimulated Ti control, and different test Ti surfaces stimulated with (b) 5 μ A, (c) 15 μ A, and (d) 25 μ A direct current stimulation.

Figure 3.

Immunolocalization of adhesive molecule vinculin in hFOB cells after 5 days hFOB cell culture on (a) nonstimulated Ti control, and different test Ti surfaces stimulated with (b) 5 μA , (c) 15 μA , and (d) 25 μA pulse direct current stimulation. Green fluorescence indicating antibody bound to vinculin and cell nuclei were contrast-labeled in red by adding propidium iodide (PI).

Figure 4. Optical density measurement illustrating bone cell (hFOB) proliferation on Ti surfaces stimulated with different external direct current as well as nonstimulated Ti control.