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Synergy of substrate conductivity and intermittent electrical stimulation towards osteogenic differentiation of human mesenchymal stem cells

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ABSTRACT

Human Mesenchymal Stem cells (hMSCs) have the unique potential to differentiate into multiple cell types. Depending on the cellular microenvironment (physical and biochemical cues), hMSCs can be directed to differentiate into osteogenic, chondrogenic, myogenic and adipogenic lineages. Among the strategies available to direct stem cell fate processes, electrical stimulation based approach has been extensively investigated in recent studies. In the present study, the conducting Hydroxyapatite-$CaTiO_3$ (HA-CT) composites are used as electroconductive platforms to support the differentiation of hMSCs, *in vitro*. During culture without osteogenic supplements, intermittent electrical stimulation is provided every 24 h over a period of 4 weeks through parallel plate electrodes separated by a distance of 15 mm and maintained at a static potential of 15 V for 10 min. In addition to cell morphological changes, the differentiation behavior of hMSCs after electrical stimulation is evaluated by mRNA expression analysis through polymerase chain reaction (PCR). Importantly, specific bone markers, in particular ALP, Col IA and Osteocalcin are expressed more significantly due to electrical stimulation, which also enhances the extent of extracellular matrix mineralization. Taken together, this study establishes the effectiveness of electroconductive HA-CT composites together with intermittent electrical stimulation to direct osteogenesis of hMSCs.

1. Introduction

Human Mesenchymal Stem cells (hMSCs) are multipotent cells, which can differentiate into osteogenic, adipogenic, myogenic and chondrogenic lineages [1]. The differentiation potential and non-immunogenic nature of hMSCs makes them a highly preferred starting point for stem-cell based regenerative therapies and tissue engineering applications. Among the applications of hMSCs, the therapies to induce repair and regeneration of bone tissue to treat bone diseases and injuries are most commonly studied [2]. Due to the potential of stem cells for multi-lineage commitment, it is necessary to direct the stem cell differentiation towards a particular lineage as necessary for the required therapy, *e.g.* osteogenic lineage for bone cell therapies, myogenic and neurogenic lineages for muscle and nerve cell treatments respectively. The differentiation of hMSCs towards a particular lineage can be directed by various factors, including biochemical agents, physical and environmental cues, both *in vitro* and *in vivo*. Several growth factors such as bone morphogenetic protein (BMP-2), transforming growth factor-β (TGF-β) and chemical supplements (*e.g.* dexamethasone, hyaluronic acid, ascorbic acid and β-glycerol phosphate), when available either as a part of the culture medium or as a coating on the substrate is reported to induce stem cell differentiation into osteoblasts [3,4].

On the other hand, physical cues encompass a number of factors including substrate properties such as stiffness, conductivity and topography, which can be altered to elicit differential cellular responses. The influence of substrate stiffness on the proliferation and differentiation of hMSCs has been reported extensively in recent studies [5,6,7]. In particular, the control in stiffness of the substrates can direct hMSC differentiation into osteogenic, myogenic and neurogenic lineages [8,9]. In addition, surface topography is found to have a significant impact not only on the osteogenic differentiation of hMSCs, but also in directing the differentiation towards neuronal, myocardial lineage *in vitro* [10–14].

The aforementioned strategies to direct the stem cell fate processes might not be viable in all cases. Moreover, experimental evidence suggests that the usage of chemical supplements and growth factors may have systemic effects *in vivo* [15–16]. This has led to the investigation of the effect of altering environmental cues in directing the differentiation of hMSCs to a particular lineage. In the above backdrop, electromagnetic fields have been used successfully in various cases to alter the stem cell microenvironment and thereby the cell fate processes, such as proliferation and differentiation [17]. Many studies indicate that electromagnetic fields, when applied at low frequencies, can induce osteogenic

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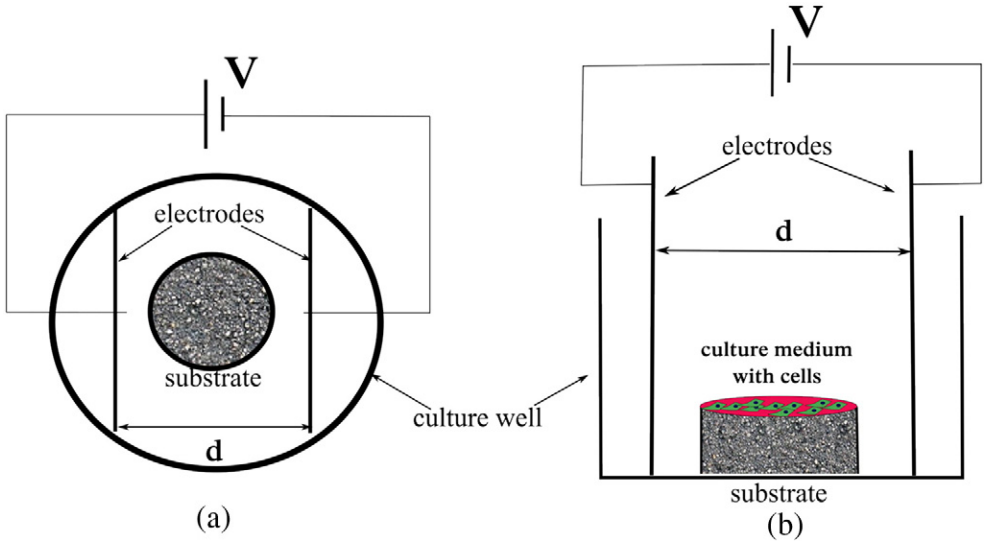
differentiation in hMSCs [17–18]. Also, pulsed electromagnetic fields are known to cause bone augmentation, when coupled inductively to the skin [19] and have been used to treat fractures, musculoskeletal disorders and osteoporosis [20–23]. In the study by Hronik-Tupaj et al. [24], low intensity (20 mV/cm), high frequency (60 kHz) electric field (EF) when exposed to hMSCs resulted in significant morphological changes and differentiation towards osteogenic lineage was confirmed with upregulation of type I collagen and heat shock protein 27 (hsp 27), a stress marker after day 15 and day 10 in culture respectively. However, differentiation factors were used in addition to electrical stimulation in the study. The current hypothesis is that electric and magnetic stimulation of hMSCs induces cytosolic Ca^{2+} oscillations, thereby enhancing the Ca^{2+} ion flux and acts as molecular cues for differentiation of hMSCs [25].

However, electric and magnetic fields alone are not sufficient for applications involving bone repair or regeneration. The dependence of cell response on substrate conductivity also has been reported [26]. It is evident then, that a suitable substrate, which supports the functionality of bone like cells provides additional driving force for hMSC differentiation towards osteogenic lineage. Recently, this has been demonstrated in hMSCs on moderately conducting Polyaniline films coated with hyaluronic acid (conductivity 10^{-4}–10^{-3} $S \cdot cm^{-1}$) exposed to pulsed EF of intensity 3 mV/cm (with pulse width 7 ms and frequency 10 Hz) [27]. In the current study, Hydroxyapatite (HA)-based substrates have been used with DC electrical stimulation to achieve osteogenic differentiation in hMSCs. Among the materials for bone replacement/regeneration applications, HA is an excellent choice owing to the fact that its composition is chemically similar to the inorganic component of bone and well known to support bone mineralization and ingrowth into the host tissue [28–29]. Although HA is known to enhance osteoblast cell activity, there are relatively fewer reports on osteogenic differentiation of hMSCs on HA-based materials [30–32]. As far as the physical properties are concerned, natural bone has a moderate conductivity, piezoelectricity, mechanical strength and toughness properties [33–36], whereas monolithic HA is an insulator, piezo-inactive and has poor strength and toughness [37]. According to the well-established fact, human bone has good fracture toughness (2–12 $MPa \cdot m^{1/2}$) and a moderate conductivity of the order of 10^{-12}–10^{-10} $S \cdot cm^{-1}$. It is also known that these properties aid in maintaining the balance between bone formation and resorption *in vivo* [33]. In order to impart additional multi-functional properties, $CaTiO_3$ has been added to HA for improving the conductivity (10^{-11}–10^{-9} $S \cdot cm^{-1}$) and mechanical properties (fracture toughness-1.7 $MPa \cdot m^{1/2}$ and flexural strength-155 MPa) [38–39]. Recent studies with electroactive Hydroxyapatite-$CaTiO_3$ (HA-CT) composites show a conductivity dependent myoblast cell response *in vitro* [26]. Sintered HA and CT both show excellent resistance to dissolution (non-biodegradable) *in vivo* and the use of these composites as implants in femoral bone defects in a rabbit animal model has been shown to improve early stage osseointegration [40–41]. With a background of firmly established characterization of HA-CT composites, *in vitro* cytocompatibility and *in vivo* biocompatibility, the efficacy of intermittent electrical stimulation on the differentiation of hMSCs towards osteogenic lineage is explored *in vitro*. The assessment of the osteogenic differentiation is made by quantifying the expression of multiple bone markers such as RunX2, Alkaline phosphatase activity (ALP), Collagen (total collagen and Col IA) and Osteocalcin (OCN) with the help of biochemical assays, polymerase chain reaction (PCR) and immunofluorescence studies.

2. Materials & methods

2.1. Processing

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ powders were synthesized using wet precipitation route with precursors (CaO and orthophosphoric acid) to obtain fine particle sizes (D_{50} ~ 1.12 μm) [42]. Calcium titanate (CT) (~5 μm) was synthesized using the mechanochemical activation of CaO and TiO_2 (anatase) mixture, followed by calcination at 900 °C for 2 h [43]. HA-CT powder mix with varying amounts of CT (20, 40, 60 and 80 wt%) was obtained by ball milling (Fritsch, Pulverisette P6, Germany) for 16 h using agate balls and jars as grinding media. The powders of different compositions were consolidated using Multistage spark plasma sintering technique (MSSPS) (Dr. Sinter, Model 515S, SPS Syntax Inc., Japan). The optimization of sintering parameters and the heating cycle along with phase analysis is described in detail elsewhere [38]. To summarize, the graphite die-punch assembly was heated by pulsed direct current to a temperature of 850 °C and held for 5 min. In the same heating cycle, the powder compact was subsequently heated to a temperature of 950 °C with a dwell time of 5 min, followed by final stage of sintering at temperature of 1200 °C for holding time of 5 min. During the entire heating cycle, the powder compact was under uniaxial pressure of 50 MPa.



Schematic 1. Top view (a) and front view (b) of the electrical stimulation setup used in the experiment showing the position of the parallel electrodes in the culture well and the substrate with culture medium (colored on the substrate) and cells on the substrate (cell sizes are not to scale). The electrodes are maintained at a potential difference of 15 V and the separation distance 'd' fixed at 15 mm.