Effects of Estrogen Deprivation and Titanium Surfaces on Osteogenic Differentiation of Human Bone Marrow Stromal Cells, an *in Vitro* Study

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Abstract

Objectives: The study aimed to investigate effects of estrogen deficiency and titanium surfaces on growth and osteogenic differentiation of hBMSCs.

Methods: Under a written informed consent, human bone marrow stromal cells (hBMSCs) were harvested, cultured and seeded on cell culture plates and titanium disks (Straumann, Switzerland), smooth and sandblasted acid-etched (SLA) titanium surfaces. Then cells were cultured in estrogen-deprived (ED) growth medium for 24 h. and subsequently in conventional (FBS-OS) and ED-osteogenic (ED-OS) media for 21 day. Examination under scanning electron microscope (SEM) was performed to assess cell viability, attachment, morphologies and growth. Cell viability assay was conducted to determine cell growth. Alkaline phosphatase activity and calcium contents levels were measured to evaluate osteogenic differentiation potential (n=4, Mean±SD).

Results: Titanium surface microtopographies and ED cell culture influenced cell morphology, attachment and growth. Human BMSCs were spindle-shaped cells on cell culture plate and smooth titanium surfaces, while on SLA titanium surface cells were stellate-like cells. Estrogen-deprived cell culture decreased cell attachment, growth and osteogenic differentiation potential of hBMSCs. Alkaline phosphatase activity and calcium content levels on all surfaces in ED-OS were markedly and significantly lower than FBS-OS media ($p<0.05$). Promoting effects of SLA surface on osteogenic differentiation, ALP activity and calcium contents, were found only in FBS-OS not ED-OS media.

Conclusions: Estrogen-deprived cell culture decreased cell growth and osteogenic differentiation of hBMSCs. A SLA surface could not promote osteogenic differentiation of hBMSCs in ED-condition. Thus, modification of titanium surface microtopographyalone might be insufficient to enhance osteointegration of dental implant in osteoporotic bone.

Key words: Osteoporosis, Sandblasted and acid etched titanium surface, Osteoblastic differentiation

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Introduction

Estrogen supports cell growth, decreases apoptosis and promotes osteogenic differentiation of human bone marrow stromal cells (hBMSCs) and osteoblasts [1]. Estrogen-deprivation (ED) in postmenopausal women causes imbalance bone remodeling [2] resulting in osteoporotic bone with thin trabecular bone and poor bone microarchitecture [3]. As a long life expectancy increases numbers of aging population [4], a higher number of elderly increases the demand for dental implant placements in osteoporotic bone.

Osteoporosis could be considered as a risk factor for survival of dental implant. Osteoporotic bone demonstrates negative effects on optimal osseointegration and implant stability in ovariectomizedrats and rabbits [5-7]. Based on the reports that titanium surface microtopography regulates cell growth and differentiation [8] and sand-blasted and acid-etched (SLA) titanium surface has been reported to enhance osteoblastic differentiation [9] and reduces healing time of the implant [10]. Sandblasted and acid-etched titanium surface might be able to enhance osteointegration of titanium implant in osteoporotic bone.

To mimic estrogen deprived condition in post-menopause cases, estrogen deprived cell culture can be established by utilizing charcoal stripped bovine serum and phenol red free culture medium to remove estrogenic activity of fetal bovine serum and cell culture medium. Phenol red in cell culture medium is able to stimulate cells growth and differentiation replicating estrogenic-like activity in cell culture [11]. Charcoal stripped bovine serum is estrogen and lipid-like components free serum that is useful for studying hormone and, growth factors responses in cell culture [12-14].

The current study aimed to investigate effects of SLA titanium surfaces on growth and osteogenic differentiation of hBMSCs in ED-cell culture.

Materials and Methods

A cell culture model:

Bone marrow cell culture: Under permission from an Ethical Committee of Songklanagarind Hospital and patient written informed consent, bone marrow was harvested from 3 healthy male patients undergoing orthopaedic surgery, age 16 – 45 years. Bone marrow was cultured as described [15]. At passage 1(Fig. 1A), human bone marrow stromal cells (hBMSCs) from 3 patients were pool and expanded for 3 passages for the experiment (Fig. 1B).

Culture mediums: Growth medium comprised of DMEM-F12, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.5% fungizone. Osteogenic (OS) medium was growth medium supplemented with 50 mM ascorbic acid, 10 mMβ-glycerophosphate and 100 nM dexamethasone. For estrogen-deprived (ED) culture media, phenol red free DMEM-F12 and charcoal-stripped FBS were used (all from Sigma, USA) [16].

Cell seeding: Titanium disks, smooth and SLA titanium surfaces, size 15x1 mm, were kindly provided by Straumann (Straumann, Switzerland) (Fig. 1C & D). Expanded hBMSCs in FBS-growth medium were seeded on cell culture plates (Corning, USA) and smooth and SLA titanium disks. Cell seeding density for cell growth study was $1x10^4$ cells/cm^2 and osteogenic differentiation study was $2x10^4$ cells/cm^2. At 3 hr. after cell seeding, cells were cultured

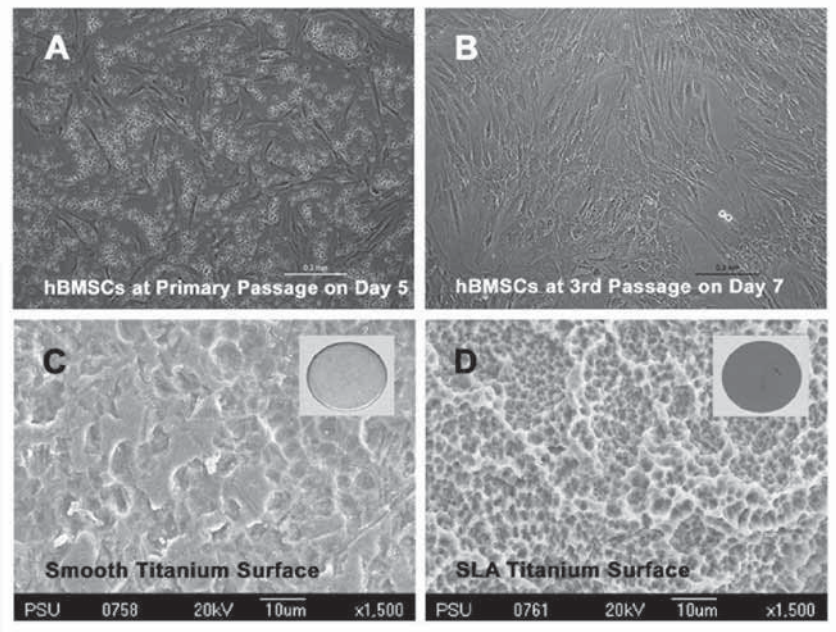

Figure 1. Demonstrating morphologies of hBMSCs (A) at primary, (B) 3rd passage, and scanning electron microscope images of (C) smooth and (D) sandblasted and acid-etched (SLA) titanium surfaces. Insets represent (C) smooth and (D) SLA titanium disks.

in ED-growth medium for 24 hr. After that, culture medium was changed to regular (FBS) and ED-growth media for cell growth and regular (FBS-OS) and ED-osteogenic media (ED-OS) for osteogenic differentiation studies, constituting 6 groups of study [17].

Investigations

Cell attachment and morphology observations

Scanning electron microscope: Cell morphology and attachment on titanium disks were assessed optically on days 7 and 21 using scanning electron microscope (SEM) (5800LV, JEOL, Japan). Cells were fixed in 4% glutaraldehyde and 10% formaldehyde, dehydrated in ethanol series of 30-100%, air dried, gold sputter-coated and imaged (n=2 for each investigation time) [17].

Cell growth

Cell viability assay: Cell viability was measured to represent cell growth. On day 21, cells were incubated in 20% CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA) in phenol red free culture medium with 0.5% FBS for 2 hr. in a humidified incubator at 37°C with 5% CO_2, then the formazan dye was quantified at 440 nm absorbance on duplicate samples (Multiskan GO, Thermo Scientific, Finland) (n=4, mean±SD) [17].

Osteogenic differentiation

For alkaline phosphatase (ALP) activity measurement: On day 21, cells were lyzed in 1% Triton X 100 (Sigma, USA) and amount of total protein content was measured using DC Protein

Assay Kit (Bio-Rad, USA) following manufacturer's instruction. Cell lysate was incubated with 0.2% para-nitrophenol phosphates (Sigma) yielding a yellow substrate of p-nitrophenol. Color intensity was read at 405 nm absorbance in duplicate (Multiskan GO) [17].

For calcium content assay: Cell pellets from cell lysates were demineralized overnight in 0.5M HCL at RT, then levels of calcium contents were measured using Calcium Colorimetric Assay kit (Biovision, USA) following a manufacturer's instruction. Concentrations of p-nitrophenol and calcium contents were extrapolated with standard curves of serial dilutions of p-nitrophenol and calcium standard, respectively, then normalized by the amount of protein contents of the same samples and reported as nM p-nitrophenol/ mg protein and ng calcium/mg protein, (n=4, Mean±SD) [18].

Data analysis

When the data were normally distributed, one-way analysis of variance (ANOVA) was used with either the Tukey HSD or Dunnette T3 methods as appropriate. Otherwise, the Kruskal-Wallis analysis with Mann-Whitney test was performed at $p<0.05$.

Results

Cell shapes, attachment and growth were influenced by titanium surface microtopography and estrogen-deprived condition.

Scanning electron microscope images demonstrated different cell shapes on different titanium surfaces and a lower cell density in ED- than FBS-OS culture media, particularly on SLA titanium surface (Fig. 2A-D). On smooth surface, hBMSCs spread out and forming cell sheet covering the substrate surface (Fig. 2A& B), while on SLA, cells stretched cell body across rough surfaces by extending small cytoplasmic processes to attach on porous surface creating intercellular network (Fig. 2C & D). Lower cell density on smooth and SLA surfaces in ED-OS(Fig. 2B& D) than FBS-OS media (Fig. 2A & C) suggested inhibitory effects of ED-cell culture on cell growth. Cell sheets and density were thinner on smooth and lower on SLA titanium surfaces, respectively, in ED-OS (Fig. 2 B & D) than FBS-OS (Fig. 2 A& C). The inhibitory effects of ED-cell culture on cell growth was worsen on the SLA surface in ED-OS (Fig. 2D).

Inhibitory effects of ED-cell culture on cell growth was further highlighted by cell viability assay. It was clearly shown that growth of cells on all surfaces in ED-growth was significantly lower than FBS-growth media ($p<0.05$). The highest level of cell growth was found on cell culture plate in FBS-and ED-growth media ($p<0.05$). Effects of cell culture surface substrates were greater distinct in ED-than FBS-growth media. In FBS-growth medium, growth of cells on smooth and SLA titanium surfaces was not significantly different ($p>0.05$), but in ED-growth medium, growth of cells on SLA surface was significantly lower than smooth surface ($p<0.05$) (Fig. 3).

Estrogen-deprived cell culture decreased osteogenic differentiation of hBMSCs and enhancing effect of SLA on the differentiation in ED-cell culture was not found.

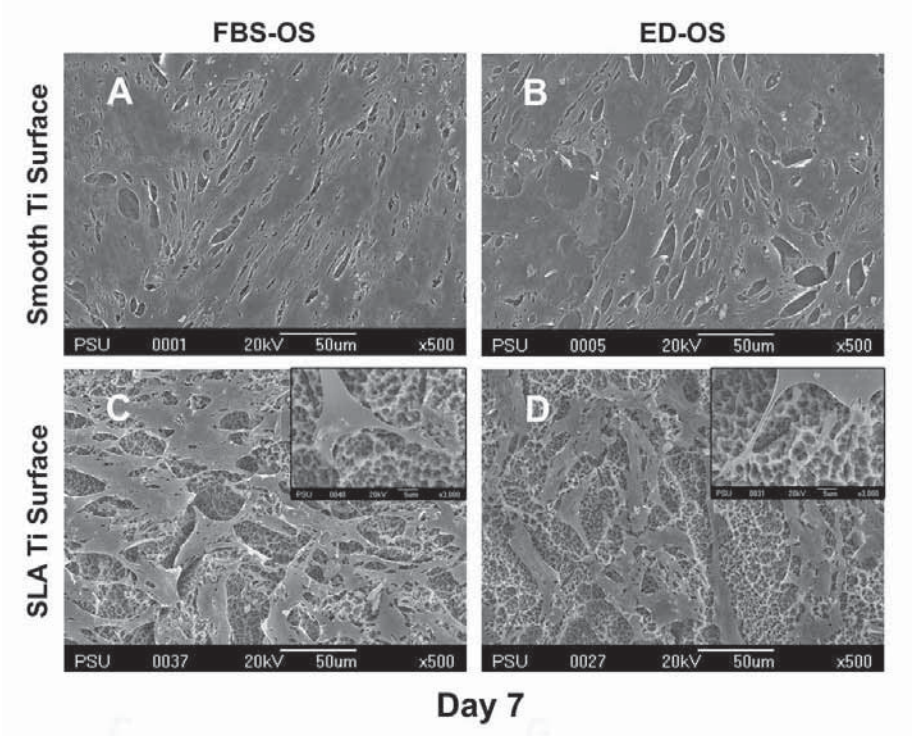

Figure 2. Scanning electron microscope images of hBMSCs on smooth and SLA titanium surfaces on day 7 (A&B) on smooth and (C&D) sandblasted and acid-etched (SLA) titanium surfaces, (A&C) in conventional (FBS-OS) and (B&D) estrogen-deprived osteogenic media (ED-OS). Inset images on C and D exhibit cell attachment on the SLA titanium surface.

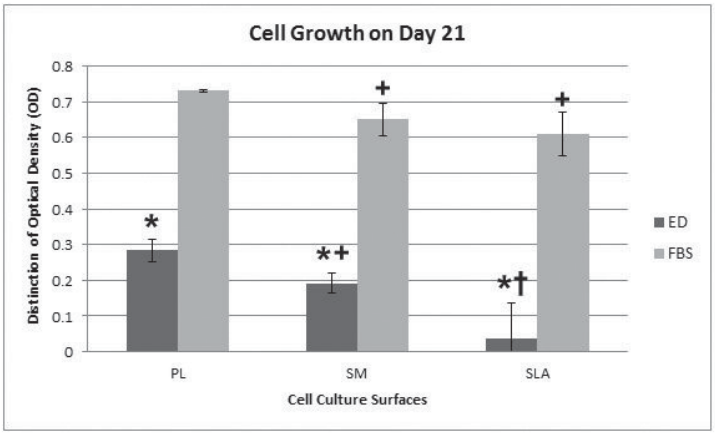

Figure 3. Demonstrating cell growth derived from cell viability assay of hBMSCson day 21, on cell culture plate (PI) and smooth (SM) and sandblasted and acid-etched (SLA) titanium surfaces in conventional (FBS) and estrogen-deprived (ED) growth media. Symbols + represents significantly lower than cell culture plate, *, significantly lower than FBS-OS on the same cell culture substrates, and † significantly lower than other surfaces in ED-OS and FBS-OS media, at $p<0.05$ (n=4, Mean±SD).

Alkaline phosphatase (ALP) activity and calcium content levels of hBMSCs on all surfaces in ED-OS were significantly lower than FBS-OS media ($p<0.05$) (Fig. 4 A&B). Alkaline phosphatase activity on SLA in FBS-OS tended to be higher than the activities cell culture plates and smooth titanium surface ($p>0.05$). The lowest ALP activity was found on SLA in ED-OS medium ($p<0.05$) (Fig. 4A).

The highest level of calcium content was found on SLA titanium surface in FBS-OS and the lowest level was on cell culture plate in ED-OS media ($p<0.05$). Levels of calcium contents on cell culture plate and smooth titanium surface in FBS-OS were not different ($p>0.05$), but significantly lower than the level on SLA titanium surface ($p<0.05$). In ED-OS medium calcium levels on smooth and SLA surfaces were not significantly different ($p>0.05$) but were significantly higher than the activity on cell culture plate ($p<0.05$) (Fig. 4B).

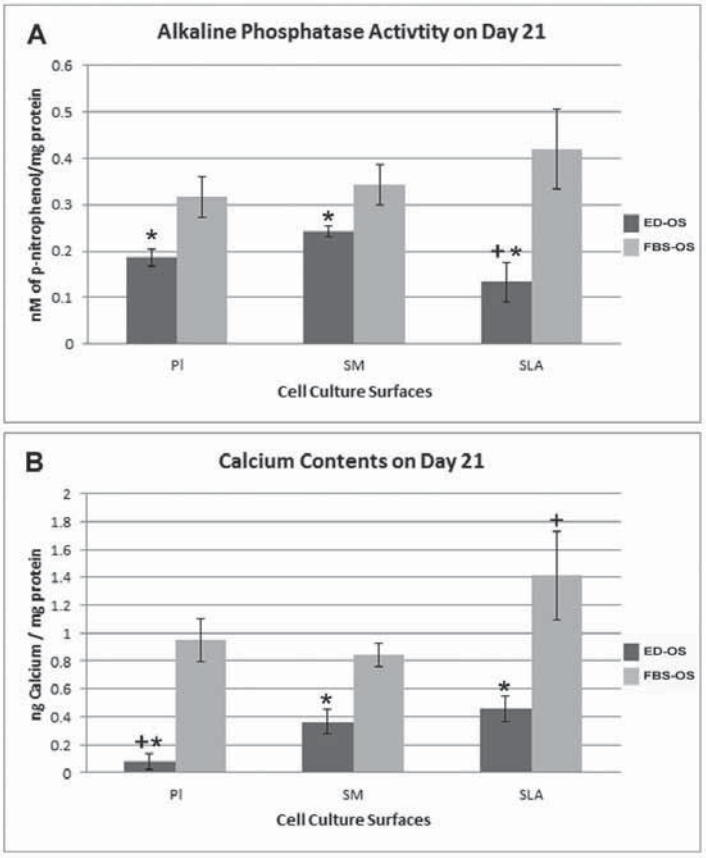

Figure 4. Demonstrating osteogenic differentiation markers of hBMSCs, (A) alkaline phosphatase activity and (B) calcium contents on cell culture plate (Pl) and smooth (SM) and sandblasted and acid-etched (SLA) titanium surfaces in conventional (FBS-OS) and estrogen-deprived osteogenic media (ED-OS) on day 21. Symbols *represents significantly lower than FBS and +, significantly different from other groups at $p<0.05$ (n=5, Mean±SD).

Discussion

To elucidate effects of estrogen deficiency on osteointegration of dental implant, the current study investigated growth and osteogenic differentiation of hBMSCs on titanium surfaces in a long term ED-cell culture. Adverse effects of ED on growth and osteogenic differentiation of hBMSCs found in the current study emphasized important roles of estrogen on growth and osteogenic differentiation of hBMSCsand osteoblasts [1] and highlighted deteriorating effects of post-menopausal estrogen deficiency on osteogenesis of osteoporotic bone [19].

Differences of cell morphology and growth on smooth and SLA titanium surfaces (Fig. 2&3) and the higher levels of osteoblastic differentiation in FBS-OS than ED-OS media of hBMSCs (Fig. 4) support previous reports [20, 21] that titanium surface microtopography influence cell biological responses [22] and estrogen is essential to cell growth and oseogenic differentiation [23].

As cell attachment to the ECM providing external signals regulates growth and survival of hBMSCs [24], a decreasing of ECM synthesis, particularly collagen type I in ED-condition [25] might be an underline cause that altered cell-substrate interaction and thus cell growth and differentiation. An assumption was supported by the current findings that SLA titanium surface in ED-OS medium could not enhance osteogenic differentiation of hBMSCs. In contrast, the supporting of osteogenic differentiation was found on SLA surface in FBS-OS (Fig. 4) and promoting effects of SLA titanium surface on osteogenic differentiation of osteoblasts and hBMSCs in regular osteogenic medium has been reported [20, 21].

Taken together, the findings supported hypotheses that ED condition decreased growth and osteogenic differentiation of hBMSCs but in contrast SLA titanium surface could not promote osteogenic differentiation of hBMSCs in estrogen deprived condition. Thus titanium surface modification alone might be insufficient to enhance osteointegration of dental implant in osteoporotic bone.

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