Effects of Charcoal Stripped Fetal Bovine Serum and Titanium Surface Microtopography on Growth and Osteogenic Differentiation of Human Bone Marrow Stromal Cells

Chonticha Chookiartsiri

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Author	Ms.Chonticha Chookiartsiri
Major Program	Oral and Maxillofacial Surgery

Major Advisor :

……..……

(Assoc.Prof.Dr.Premjit Arpornmaeklong)

Examining Committee :

……..……..…Chairperson

(Prof.Dr.Prasit Pavasant)

……..……

(Assoc.Prof.Dr.Premjit Arpornmaeklong)

……..……

(Assoc.Prof.Boonsin Tangtrakuwanich)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Oral and Maxillofacial Surgery

……

(Assoc.Prof.Dr.Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……Signature

(Assoc.Prof.Dr.Premjit Arpornmaeklong)

Major Advisor

……Signature

(Ms.Chonticha Chookiartsiri)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……Signature

(Ms.Chonticha Chookiartsiri)

Candidate

ชื่อวิทยานิพนธ์ ผลของซีรั่มที่ผ่านการดูดซับด้วยถ่านและลักษณะของพื้นผิวไททาเนียมต่อการเจริญเติบโตและพัฒนาไปเป็นเซลล์สร้างกระดูกของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์

ผู้เขียน นางสาวชลธิชา ชูเกียรติศิริ

สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล

ปีการศึกษา 2556

บทคัดย่อ

วัตถุประสงค์: ศึกษาผลของลักษณะพื้นผิวไททาเนียมชนิดเรียบ (smooth) และไทเทเนียมที่ทำการปรับสภาพผิวด้วยการเป่าทรายและกรดกัด (sandblasted and acid-etched, SLA) ต่อการเจริญเติบโต (growth) และการพัฒนา (differentiation) ของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ (human bone marrow stromal cells) ในสภาวะคลาดแคลนเอสโทรเจน

วัสดุและวิธีการ: ทำการเลี้ยงเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ในน้ำเลี้ยงเซลล์ที่มีความจำเพาะต่อการเจริญเติบโตของเซลล์สร้างกระดูกบนแผ่นโลหะไทเทเนียมชนิดเรียบและชนิด SLA โดยเลี้ยงในน้ำเลี้ยงเพื่อการเจริญเติบโต (growth medium) และการเหนี่ยวนำการพัฒนาไปเป็นเซลล์สร้างกระดูก (osteogenic medium) 2 ชนิด คือ ชนิดที่มีการเติมซีรัมจากวัว (fetal bovine serum, FBS) และ ชนิดที่ขาดแคลนเอสโทรเจน (estrogen-deprived, ED) เป็นเวลา 21 วัน ทำการติดตามผลของน้ำเลี้ยงเซลล์ 2 ชนิด บนแผ่นโลหะไทเทเนียมทั้งสองแบบ ทำการย้อมสีเพื่อแสดงเซลล์ที่มีชีวิตและเซลล์ที่ตายแล้ว (live-dead staining) เพื่อศึกษาความมีชีวิต, การยึดเกาะและลักษณะของเซลล์บนพื้นผิวไทเทเนียมแล้วทำการตรวจสอบด้วยกล้องจุลทรรศน์แบบคอนโฟคอลชนิดที่ใช้เลเซอร์ในการสแกน (confocal laser scanning electron microscope, CLSM) และ กล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด (scanning electron microscope, SEM) ทำการตรวจวัดการเจริญเติบโตของเซลล์โดยการทำการทดสอบความมีชีวิตของเซลล์ (cell viability assay) และติดตามการพัฒนาการไปเป็นเซลล์สร้างกระดูกโดยทำการวัดระดับการทำงานของอัลคาไลน์ฟอสฟาเตส (alkaline phosphatase activity, ALP) และระดับของแคลเซียมสะสม (calcium contents) โดยทำการทดสอบในวันที่ 1 และ ทุก 7 วัน (จำนวนตัวอย่าง = 4, รายงานผลเป็น ค่าเฉลี่ย ± ส่วนเบี่ยงเบนมาตรฐาน)

ผลการศึกษาและอภิปราย: ลักษณะพื้นผิวของไทเทเนียมและน้ำเลี้ยงเซลล์ที่ขาดแคลนเอสโทรเจนมีอิทธิพลต่อรูปร่าง ขนาดและการพัฒนาการไปเป็นเซลล์สร้างกระดูกของเซลล์ โดยเซลล์จากไข

กระดูกในชั้นสตอร์มาลของมนุษย์จะแสดงลักษณะ เป็นรูปกระสวยเมื่อเลี้ยงบน cell culture plate และ พื้นผิวไทเทเนียมชนิดเรียบ ในขณะที่เมื่อเลี้ยงเซลล์บนแผ่นไทเทเนียม SLA เซลล์จะมีรูปร่างเหมือนดาว (stellate-like cells) และการเพาะเลี้ยงเซลล์ในสภาวะที่ขาดแคลนเอสโทรเจนส่งผลให้มีการลดลงของการยึดเกาะของเซลล์, ขนาด, การเจริญเติบโตและการพัฒนาการไปเป็นเซลล์สร้างกระดูกของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ และอิทธิพลในทางลบของสภาวะการขาดแคลนเอสโทรเจนมีเพิ่มขึ้นเมื่อเลี้ยงเซลล์บนแผ่นไทเทเนียมชนิด SLA ในส่วนของระดับของ ALP และ แคลเซียมสะสมนั้นพบว่ามีค่าลดลงอย่างเห็นได้ชัดเมื่อเลี้ยงเซลล์ในน้ำเลี้ยงที่ขาดแคลนเอสโทรเจนบนทุกพื้นผิว พื้นผิวไทเทเนียมชนิด SLA ช่วยสนับสนุนการเจริญเติบโตและการพัฒนาการไปเป็นเซลล์สร้างกระดูกในน้ำเลี้ยงเซลล์ปกติที่มี FBS เท่านั้น ไม่พบผลการส่งเสริมดังกล่าวเมื่อเลี้ยงเซลล์บนแผ่นไทเทเนียมชนิด SLA ในสภาวะขาดแคลนเอสโทรเจน

สรุป: ลักษณะของพื้นผิวไททาเนียมและสภาวะขาดแคลนเอสโทรเจนมีอิทธิพลต่อการเกาะติด รูปร่างของเซลล์ การเจริญเติบโต และการพัฒนาไปเป็นเซลล์สร้างกระดูกของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ การเพาะเลี้ยงเซลล์ในสภาวะที่ขาดแคลนเอสโทรเจนลดการยึดเกาะ การเจริญเติบโตและการพัฒนาไปเป็นเซลล์สร้างกระดูกบนพื้นผิวไทเทียม พื้นผิวไทเทเนียมชนิด SLA ไม่สามารถช่วยกระตุ้นการพัฒนาไปเป็นเซลล์สร้างกระดูกของเซลล์จากไขกระดูกในชั้นสตอร์มาลของมนุษย์ในสภาวะขาดแคลนเอสโทรเจน ดังนั้นการปรับสภาพผิวของไทเทเนียมเพียงอย่างเดียวอาจจะไม่เพียงพอที่จะกระตุ้นการเกิดการเจริญของกระดูกเข้าสู่ผิวรากเทียม (osteointegration) ในผู้ป่วยที่มีภาวะกระดูกพรุน

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Author Miss Chonticha Chookiartsiri

Major Program Oral and Maxillofacial Surgery

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Abstract

Introduction: The study aimed to investigate effects of titanium surface topographies, smooth and sandblasted and acid-etched (SLA) titanium surfaces on growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs).

Materials and Methods: Human bone marrow stromal cells (hBMSCs) were seeded on cell culture plates and titanium disks, smooth and sandblasted and acid-etched (SLA) titanium surfaces and cultured in growth and osteogenic media for 21 days. The media were conventional media supplemented with fetal bovine serum (FBS) and estrogen-deprived (ED) media. Live-dead cell staining was performed to determine cell viability, attachment, growth and morphologies on titanium surfaces and the staining was observed under confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM). Cell viability assay was performed to determine cell growth. Alkaline phosphatase activity and calcium content levels were measured to determine osteogenic differentiation of hBMSCs. The investigations were performed on day 1 and every 7 day thereafter (n=4, Mean±SD).

Results & Discussion: Titanium surface microtopographies and ED cell culture influenced cell shapes, size and osteogenic differentiation. Human BMSCs exhibited spindle-shaped cells on cell culture plates and smooth titanium surfaces, while on the SLA titanium surface cells were stellate-like cells. Estrogen-deprived cell culture decreased cell attachment, size, growth and osteogenic differentiation potential of hBMSCs. Adverse effects of ED condition on cell growth were aggravated on SLA titanium surface. Alkaline phosphatase activity and calcium content levels were markedly decreased in ED-osteogenic medium on all surfaces. Promoting effects of SLA surface was found only in conventional not in ED osteogenic media.

Conclusion: Titanium surface microtopography and estrogen-deprived cell culture influenced attachment, morphology, growth and osteogenic differentiation of hBMSCs. Estrogen-deprived condition decreased cell attachment, growth and osteogenic differentiation on the titanium surfaces. Sandblasted and acid-etched titanium surface could not promote osteogenic differentiation potential of ED-hBMSCs. Therefore, titanium surface modification only might be inadequate to enhance osteointegration of dental implant in osteoporotic bone.

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List of Abbreviations and Symbols

α-DMEM	= Alpha- Dulbecco's Modified Eagle medium
µg	= Microgram
µl	= Microliter
µM	= Micromolar
°c	= Degree celcius
ALP	= Alkaline phosphatase
ANOVA	= One-way analysis of variance
BMP	= Bone morphogenetic protein
BMSCs	= Bone marrow stromal cells
Cat No.	= Catalog number
CLSM	= Confocal laser scanning microscope
cm	= centimeter
d.	= day
DMSO	= Dimethyl sulfoxide
ECM	= Extracellular matrix
ELISA	= enzyme-linked immunosorbent assay
FBS	= Fetal bovine serum
FDA	= Fluorescein diacetate
Fig.	= Figure
g	= gravitation force
g.	= gram
hr.	= hour, hours
L	= Liter
M	= Molar
mg.	= Milligram
min	= minutes
ml	= Milliter
mM	= Millimolar

List of Abbreviatons and symbols (continued)

mm	= millimeter
mol	= mole
MSCs	= Mesenchymal stem cells
MW	= Molecular weight
N	= number
NaoH	= Sodium hydroxide
nm	= Nanometer
OD	= Optical density
PBS	= Phosphate buffer saline
rpm	= round per minute
RT	= room temperature
s.	= seconds
SD	= Standard deviation
SE	= Standard error of mean
SEM	= Scanning electron microscope
Vol	= volume
w/v	= weight by volume

Chapter 1

Introduction

Osteoporosis, one of the public health problems associated with aging, might be considered as a risk in implant therapy. In senile osteoporosis, estrogen deficiency decreases bone formation but enhances adipogenic differentiation of mesenchymal stem cells (MSCs)[1]. Unbalanced bone remodeling and deteriorating microarchitecture of bone tissue demonstrate negative effects on optimal ossteointegration and implant stability in ovarectomized rats and rabbits[2-4]. A high demand for implant therapy in aging population necessitates the development of strategies to maximize osteointegration in osteoporotic condition. Surface microtopography of titanium surfaces regulates cell growth and differentiation by influencing up-regulation of the signaling associating with osteogenesis[5]. Sandblasted and acid-etched titanium surface (SLA) is an accepted surface that offers reduced healing time of the implant. Surface roughness of SLA is shown to have an effect on the proliferation, differentiation, and protein synthesis of human osteoblast-like cells[6]. Surface roughness of SLA titanium surface promotes early differentiation, production of prostaglandin E2 of MG63 osteoblast cell line, bone formation and implant integration[7]. A higher removal torque of SLA titanium implant in miniature pigs is reported[8]. The current study aimed to investigate effects of titanium surface microtopographies, smooth and SLA titanium surfaces on growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs). It was hypothesized that microtopographies of treated titanium surface such as SLA titanium surfaces would be able to reverse adverse effects of estrogen deficiency on osteogenic differentiation of MSCs.

Literature Review

Bone Marrow Stromal Cells

Bone Marrow Stromal stem cells (BMSCs) are multipotent stromal cells that can differentiate into a variety of cell types including osteoblasts[9], chondrocytes[10] and adipocytes[11]. Friedenstein and colleagues initially isolated and cultured BMSCs *in vitro* and later established their osteogenic potential[12, 13]. When these cells are plated, BMSCs rapidly adhere and can be easily separated from the nonadherent hemataopoietic cells by repeated washing. One of stemness characteristics of BMSCs is an ability of a single precursor cell to multiply to form a distinct colony known as colony forming unit-fibroblasts (CFU-F). Colony forming unit-fibroblasts (CFU-F) represents the cell proliferation and differentiation capacity of BMSCs[13] and are later identified as progenitors by their ability to differentiate into osteoblasts, chondroblasts, and myoblasts[14, 15]. However, growth and differentiation potential of each CFU-F are remarkably varied. Terms BMSCs and MSCs have been interchangeably used[16].

Osteoblastic differentiation of mesenchymal stem cells

Osteoblastic differentiation is a sequential process starting from osteoprogenitor cells to pre-osteoblasts and osteoblasts and then cell lining and osteocytes[17]. The differentiation stages are characterized by the temporal sequence of osteoblastic phenotype exhibition and osteoblast-related gene expression which can be categorized into three phases: (i) growth and extracellular matrix biosynthesis; (ii) extracellular matrix maturation and (iii) extracellular matrix mineralization[18]. Pre-osteoblasts are characterized by expression of alkaline phosphatase (ALP) followed by secreting type I collagen, while mature osteoblasts secret osteocalcin and mineralized extracellular matrix[19].

Expression of ALP, found in pre-osteoblasts and osteoblasts, indicates beginning of extracellular matrix maturation stage, when become osteocytes embedded in mineralized bone matrix, ALP enzyme is decreased and absence. Alkaline phosphatase activity is commonly used as a relative marker of osteoblastic differentiation *in vitro*[19, 20]. As osteocalcin and *in vitro* mineralization are

closely related to mineralization of the extracellular matrix and thus markers of osteoblastic differentiation or mature osteoblasts[18, 20].

Osteointegration of titanium implant

Osseointegration is defined by Brånemark[21] quoted "as a direct anchorage of an implant by the formation of a living bony tissue surrounding the implant without growth of fibrous tissue at the bone-implant interface". Osteointegration allows the implant to transmit occlusal forces directly to the bone to support the prostheses[21].

Osseointegration is influenced by primary and secondary stabilities regulated by mechanical and biologic factors, respectively. Primary stability is an absence of mobility in the bone bed upon insertion of the implant and is influenced by quantity and quality of bone, surgical technique and implant design. On the other hand, a secondary stability is controlled by a nature of bone formation and remodeling at the implant-bone interface which is regulated by the implant surface and the wound-healing process[22].

Osseous wound healing adjacent to dental implants are similar to regular events of bone healing. Blood clot is formed immediately after the insertion of a dental implant into an alveolar bone causing platelet activation. The degranulated platelets release large number of osteogenic growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) promoting angiogenesis, chemotaxis and proliferation of various cell types including osteoprogenitor cells and undifferentiated mesenchymal stem cells[23]. Following a hemostasis and clot formation, fibrinolysis occurs with formation of loose connective tissue stroma that supports angiogenesis, subsequently, a recruitment of osteoprogenitor cells and osteoblasts from marrow stem cells and endosteal or periosteal[24]. While osteoclasts and macrophages remove necrotic bone and hematoma to prepare the implant surface for deposition of osteoid, newly differentiated osteoblasts, derived from recruited osteoprogenitor cells and undifferentiated mesenchymal stem cells, secrete bone matrix on the implant and become osteocytes embedded in the secreted bone matrix. The deposition of bone matrix is followed by an intermittent mineralization of interfacial matrix creating a cement line. The process is called *de novo* bone formation creating direct bone-implant surface contact. De novo bone formation will continue until the contiguous implant surface is covered by bone. Finally, immature woven bone will be re-modelled into a lamellar bone[25-27].

Effects of estrogen and estrogen deficiency on growth and differentiation of MSCs and osteoblasts

Estrogen plays an important role in the skeletal growth and maintenance. Estrogen maintains bone mass in woman by suppressing bone resorption. A decreasing of estrogen levels in menopause women accelerates bone loss by increasing bone remodeling intensity of basic multicellular units (BMUs). Bone remodeling of BMUs becomes more negative as estrogen deficiency increase the lifespan of osteoclasts and decrease lifespan of osteoblasts[28]. Effects of estrogen on bone resorption activity of osteoclasts might be a major cause of the increasing of bone resorption and the decreasing of bone formation in osteoporosis[29].

Estrogen plays a significant role in maintaining bone density in women. Administration of estrogen at the early onset of osteoporosis is able to increase bone mass of affected women in a dose dependent manner[30, 31]. Estrogen suppresses adipogenic and enhances osteoblastic differentiation of precursor cells[32] and enhances bone formation by activating BMP-2 gene expression in mouse MSCs[33, 34]. Estrogen maintains bone mass in postmenopausal women by restoring the balance between osteoblastic and adipogenic differentiation of bone marrow stromal cells thus influencing a balance between bone and fat formation[35]. Estrogen is essential to positive effects of mechanical stimuli on bone remodeling[36]. These evidences underline important roles of estrogen on bone formation and remodeling in estrogen deprived menopause condition.

A supporting role of estrogen on bone formation is clearly demonstrated. Estrogen replacement therapy reduces the risk of osteoporotic fractures by suppressing bone resorption and stimulating bone formation capacity of osteoblasts[37, 38]. Observations in ovarectomized rats support clinical observations that estrogen replacement therapy increases bone to implant contact (BIC) in ovarectomized rats[39]. Giro and coworkers[39] report that bone to implant contact and mineral apposition rate in ovarectomized rats are not significantly different from a normal control group[39]. A key mechanism by which estrogen may affect bone remodeling is through the regulation of osteoblasts. Estrogen may extend the life span of osteoblasts by inhibiting apoptosis of osteoblasts thus enables the bone formation process[40]. Chen and coworker[41] report that 17β-estradiol enhances osteoblastic proliferation and alkaline phosphatase activity of chicken osteoblasts. Additionally, low

doses of 17β-estradiol increase proliferation of human mesenchymal stem ceslls (hMSCs) and osteoblasts[42].

Effects of estrogen deprived or osteoporosis on osteointegration

Osteoporosis might be considered as a risk factor in implant therapy. There are evidences demonstrate that alterations of bone turnover and microarchitectural deterioration of bone tissue of osteoporotic bone have potentially negative effects on optimal osseointegration and implant stability. Although, osteointegration is achievable in the osteoporosis model, bone mineral density (BMD) in bone marrow region of ovarectomized rats are significantly 30-40% lower than non-osteoporosis group[2]. In ovarectomized rabbits, removal torque of implants in osteoporotic tibia is significantly decreased[4]. Various methods have been applied to increase BMD surrounding implant and improve bone implant contact in osteoporotic bone such as transplanting MSCs[43] and delivering bFGF[44] into the implantation sites, implant surface modifications[45], systemic supplementation of antiosteoporotic agents such as strontium ranelate[46], zolodronic acid[47] and systemic and locally applied simvastatin[48, 49]. Based on clinical results, it is hypothesized that osteoporosis affects the maxilla or the mandible in the same manner as the other parts of the skeleton, and an alteration of bone metabolism may reduce the healing capacity of bone surrounding the dental implants, an a proper adjustment of the surgical technique and a longer healing period may be required to maximize osteointegration in osteoporotic bone[50].

Effects of titanium surface microtopography on growth and osteoblastic differentiation of osteoblasts

Surface microtopography regulates cell attachment, cell proliferation, extracellular matrix synthesis and osteoblastic differentiation[51, 52]. Oseoblasts exhibit the decreasing of cell attachment and proliferation but increasing of osteoblastic differentiation markers on rough titanium surface. As a result, rough surface promotes osteogenic differentiation[5, 53, 54] and promotes bone implant contact[55, 56].

Surface microtopography and hydrophilic properties regulate stem cell fate and osteoblastic differentiation. Despite decreasing of cell attachment on hydrophobic sandblasted and

acid-etched titanium implants, the surface concurrently enhances osteoblastic differentiation. Morphological change during cell attachment on different substrate architectures might initiate the activation of integrin binding system leading to modulation of cell growth and differentiation[57, 58]. Mechanism mediating the cellular response to surface topography needs to be further investigated.

Sandblasted and acid-etched titanium surface

There are several methods to improve implant surface microtopography, structure and chemistry, morphology and structure aiming to improve the bio-mechanical properties and enhance osseointegration of implants such removal of surface contamination and improving of wear and corrosion resistance. In the 1990s, mechanical (blasting) and chemical (acid etching) were applied to create macro- and micro-textures on the titanium surfaces. As a result, sandblasted and acid-etched (SLA) titanium surface is constituted with uniformly scattered gaps and holes[59].

To fabricate SLA titanium surface, titanium surface is blasted with a large grit of 250-500 μm in diameter and by hydrochloric/sulfuric acid etching, which results in an identical macro- and microstructures on the treated surface. After that sandblasted and acid-etched titanium surface are rinsed with water and air dried. The macrostructure offers the ideal topography for attachment of bone cells with a diameter of 20–40 μm. The microstructure is optimal for the attachment of the "arms" (filopodia) of the bone cells to the surface[60]. Surface roughness of SLA is shown to have an effect on the proliferation, differentiation, and protein synthesis of human osteoblast-like cells[6]. Surface roughness of SLA titanium surface promotes early differentiation, production of prostaglandin E2 of MG63 osteoblast cell line, bone formation and implant integration[7]. A higher removal torque of SLA titanium implant in miniature pigs is reported[8].

However, SLA titanium surface is hydrophobic. A direct air contact of a dry SLA surface creates a passive layer of hydrocarbon depositing on an active layer of hydrocarbon on SLA titanium surface. As a result, passive layer of hydrocarbon on SLA surface prevents interactive contact between cells and an active hydrocarbon layer and decreasing cell adhesion on the surface[61].

Aim of the study

Aim of the study was to investigate effects of titanium surface microtopographies, smooth and sandblasted and acid-etched (SLA) titanium surfaces, on growth and osteogenic differentiation of estrogen-deprived human bone marrow stromal cells (ED-hBMSCs).

Hypotheses

It was hypothesized that

1. Estrogen-deprived cell culture would decrease growth and osteogenic differentiation of hBMSCs.
2. Sandblasted and acid-etched titanium surfaces would be able to enhance osteogenic differentiation of hBMSCs in conventional osteogenc media and
3. Stimulating effects of SLA titanium surface would be able to enhance osteogenic differentiation of ED-hBMSCs on the titanium surface.

Objectives of the experiment

1. To establish estrogen-deprived cell culture model
2. To observe effects of ED-cell culture on cell viability, attachment and morphologies of hBMSCs on the titanium surfaces
3. To investigate growth and osteogenic differentiation of hBMSCs by performing cell viability assay and measuring levels of alkaline phosphatase activity and calcium contents of hBMSCs, respectively, on cell culture plates and smooth and SLA titanium surfaces in conventional and estrogen-deprived osteogenic media

Chapter 2

Materials and methods

Outline of the study

Human bone marrow stromal cells (hBMSCs) at passages 3-5 in growth medium (FBS-growth medium) were seeded on 24-well cell culture plate and smooth and sandblasted and acid-etched titanium disks in non-treated 24-well cell culture plates. For cell adhesion and cell viability staining and assay, hBMSCs were seeded at $1x10^4$ cells/cm^2 or $2x10^4$ cells/disk and $2x10^4$ cells/cm^2 or $4x10^4$ cells/disk for the osteogenic differentiation study. Seeded cells were incubated in humidified incubator with 5% CO_2 at 37ºC in growth medium for 24 hr. After that growth medium was changed to estrogen-deprived (ED) growth medium and cells of all groups were cultured in ED-growth medium for 24 hr. Sequentially, cells were cultured in osteogenic medium (OS) either conventional OS with FBS (FBS-OS) or ED-OS media for 21 days to induce osteogenic differentiation (Fig. 1 and Table 1).

For investigations on effects of titanium surfaces and ED-condition on cell adhesion and morphology, at 3 hr. after cell seeding culture medium was changed to either FBS or ED-growth media for 24 hr. Then vital CellTracker™ Green staining was performed and examined under confocal laser scanning microscope (CLSM). *For cell viability assay*, after 24 hr. in ED-growth medium, hBMSCs were cultured in either FBS- or ED-growth media according to groups of the study for 21 days. After that cell viability assay was performed every 7 day (Fig. 1). *For osteogenic differentation study*, following 24 hr. culture in ED-growth medium, hBMSCs were cultured in either FBS- or ED-osteogenic media to induce osteogenic differentiaion for 21 days. Growth and cell viability during osteogenic induction were examined by performing vital CellTracker™ Green staining and examining cell attachment and morphology under scanning electron microscope on days 7, 14 and 21. Alkaline phosphatase activity and levels of calcium contents were measured to determine osteogenic differentiation. Results were derived from 2 independent experiments. The investigations at each time point were performed in 4-5 consecutive samples (Fig. 1 and Table 2).

Groups of study

The study was categorized into 6 groups, ***Control groups***, Groups A - C, cell culture in osteogenic medium supplemented with fetal bovine serum (FBS-OS), Groups A: on cell culture plates and Group B: on smooth and Group C: on SLA titanium disks, and ***Experimental groups***, Groups D-F, cell culture in estrogen deprived osteogenic medium (ED-OS), Group D: on cell culture plates, Group E: on smooth and Group F: on SLA titanium disks (Table 1).

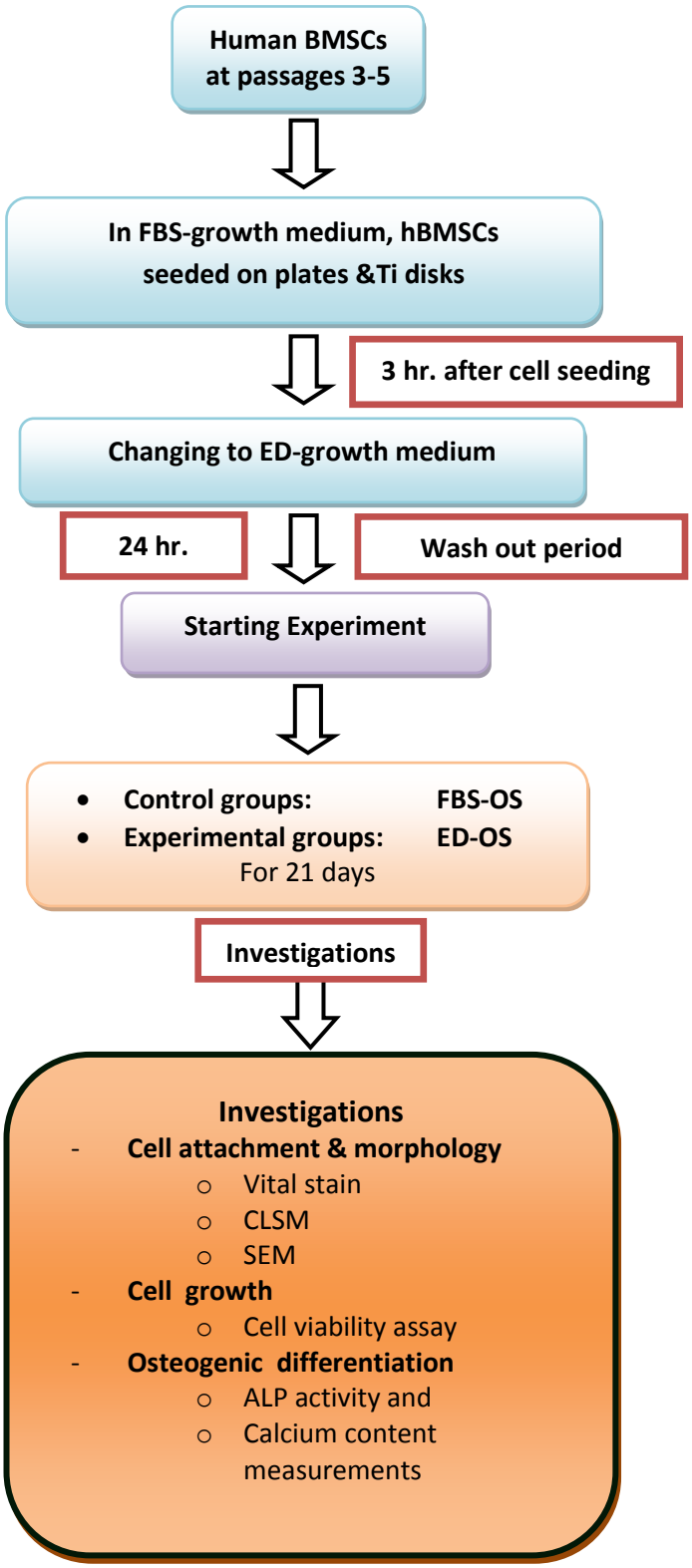


Fig. 1 Outline of the study

Table 1 Groups of study

Groups of study		Descriptions
Control groups	**A**	FBS-OS-Plate
	B	FBS-OS-SM
	C	FBS-OS- SLA
Experimental groups	**D**	ED-OS- Plate
	E	ED-OS- SM
	F	ED-OS- SLA

Note: FBS-OS is an abbreviation for osteogenic medium containing FBS, ED-OS for estrogen deprived osteogenic medium containing charcoal stripped FBS, Plate, for cell culture plates and SM, for smooth and SLA, for sandblasted and acid-etched titanium surfaces.

Table 2 Summary of the investigation procedures

Investigations	Procedures	Investigating time
Cell adhesion	Vital stain	At 3 hours after cell seeding
	CLSM	
Cell attachment and morphology	Vital –dead stain	At 24 hr and On days 7, 14 and 21
	CLSM	
	SEM	On days 7 and 21
Cell growth	Cell viability assay	On days 2, 7, 14 and 21
Osteogenic differentiation	ALP activity analysis	On days 7 and 21
	Calcium content assay	On day 21

Note: CLSM is an abbreviation for confocal laser scanning microscope, SEM, scanning electron microscope, and ALP, alkaline phosphatase.

Cell culture model

Human bone marrow Cell culture

Under permission from an Ethical Committee of Songklanagarind Hospital and patient written informed consent, human bone marrow stromal cells (hBMSCs) were harvested from healthy adult patients (age 19-45 years) undergoing orthopedic surgery at Prince of Songanakarind Hospital. The procedure in brief, hBMSCs were collected in growth medium containing 100 unit/ml heparin (Paisley, UK), in a ratio 1:1 of bone marrow to collecting medium. After that the mixture was centrifuged (Labofuge400 R, Heraeus, USA) at 1000 rpm (170 g) for 15 minutes, then buffy coat in the middle layer between clear plasma and red blood cells was collected and seeded on cell culture plates in growth medium as previously described [62]. Human BMSCs were expanded in growth medium and passaged at 80% confluence for 3-4 passages. Human MSCs at passage 4-5 were used in the analyses (Figure 2&3)

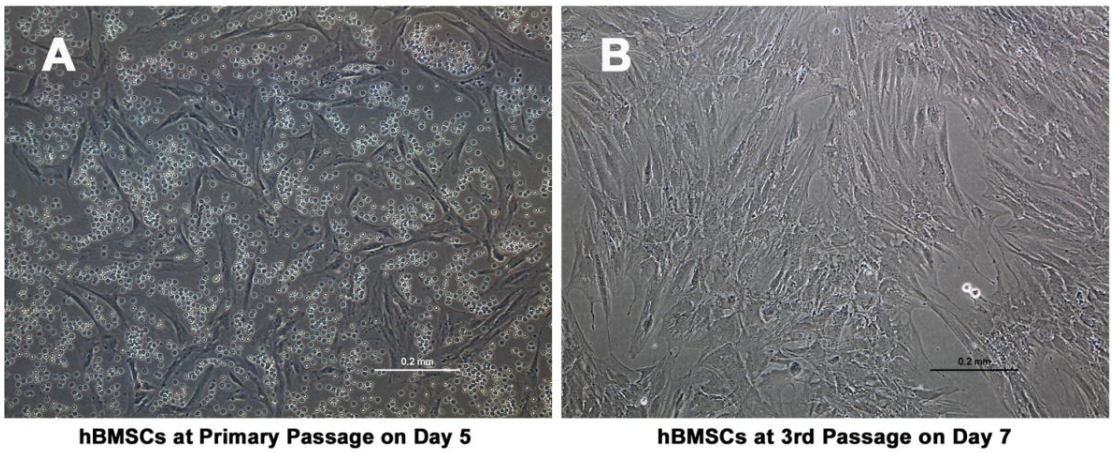


Fig. 2 Images of hBMSCs at primary and third passages under inverted microscope, (A) Demonstrating small individual spindle-shaped cells attaching on cell culture plate and floating blood cells on day 5 after bone marrow cell seeding and (B) Exhibiting uniform fibroblast-like cells of hBMSCs at passage 3 at 80% confluence and being ready for cell seeding.

Culture Media

Growth medium

Growth medium (FBS-growth) comprised of DMEM-F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.5% fungizone (all from Gibco/Invitrogen, USA)[63].

Osteogenic medium

Osteogenic medium (FBS-OS) was growth medium supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM Dexamethasone (all from Sigma, USA)[63].

Estrogen-deprived culture media

For estrogen-deprived (ED) culture media, DMEM-F12 with phenol red and fetal bovine serum were replaced by phenol red free DMEM-F12 and charcoal stripped FBS (all from Gibco/Invitrogen).

Estrogen-deprived growth medium (ED-FBS) comprised of phenol red free DMEM-F12 (Gibco/Invitrogen), 10% charcoal stripped FBS (Gibco/Invitrogen), 1% penicillin/streptomycin and 0.1% fungizone (Gibco/Invitrogen)[64, 65].

Estrogen-deprived osteogenic medium (ED-OS), ED-FBS supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM Dexamethasone (all from Sigma)[66].

Preparation of titanium disks for cell seeding

Titanium disks with smooth and sandblasted and acid-etched surfaces, 15 mm in diameter with 1 mm thickness, were kindly provided by Straumann (Straumann, Waldenburg, Switzerland). Prior to cell seeding, titanium disks were prepared as described by Lohmann and co-workers[67]. Procedures in brief, titanium disks were sonicated (Cavitator® ME11, Mettler Electronics Corp, USA) in 300 ml of 70% ethyl alcohol for 15 min, then rinsed twice in 500 ml of double distilled water for 15 minutes. Subsequently they were neutralized in 300 ml of 5% sodium bicarbonate for 15 min and rinsed three times in 300 ml of deionized water, 5 min per time. After that, disks were air dried at 80ºC and sterilized by autoclaving at 121ºC for 20 minutes (Hiclave™ HB-50, Hirayama Manufactoring, Japan). Then, sterile disks were kept in a clean and dry place until used.

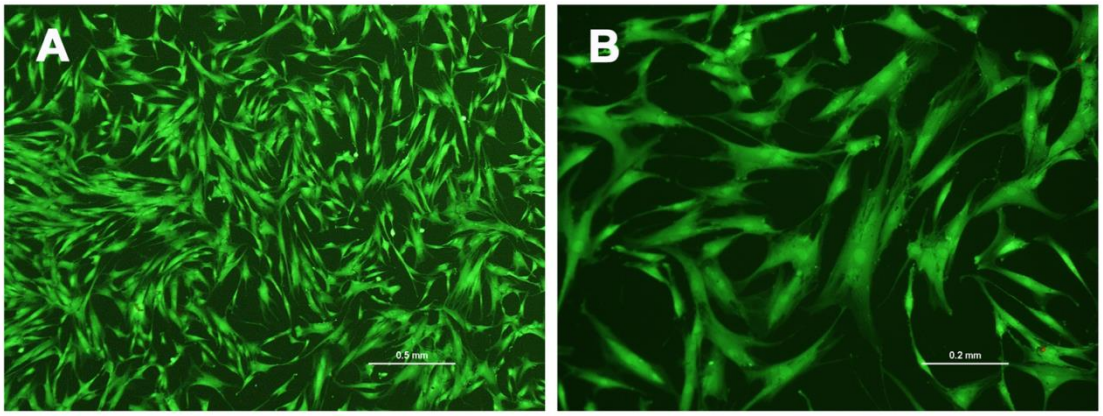


Fig. 3 Confocal laser scanning microscope images of vital cell staining (CellTracker™ Green, Molecular Probes, USA) of hBMSCs at passage 4 on cell culture plate in growth medium on day 7. Images demonstrated viability of spindle-shape and fibroblast-like cells creating intercellular contact at 70% confluence which were ready for cell seeding.

Cell seeding

Titanium disks, size 15x1 mm (Straumann) were placed in non-treated 24-well cell culture plate (Costar, USA), one well for one disk for cell seeding and culture. Human BMSCs were seeded on 24-well cell culture plate (Costar, USA) and titanium disks, smooth and SLA titanium surfaces and cultured in a humidified incubator with 5% CO_2 at 37ºC.

For cell growth and attachment study, hBMSCs in 300 µl were seeded at $1x10^4$ cells/cm^2 and $2x10^4$ cells/cm^2 for osteogenic differentiation study. After cell seeding, cells were cultured in a minimum growth medium (300 µl) for 3 hr. Then, for an investigation on cell adhesion at 24 hr. after cell seeding, seeded cells were cultured in either FBS- or ED-growth media for 24 hr. before examining under CLSM. For cell viability assay and osteogenic differentiation study, seeded cells were cultured in FBS-growth medium for 24 hr. after cell seeding, followed by ED-growth medium for 24 hr. Subsequently, cells were cultured either FBS-or ED-growth media for cell viability assay and FBS-OS or ED-OS for ostogenic differentiation study for 21 days according to groups of the study. (n=3-5, Mean±SD) (Fig. 4).

Investigations

Titanium disks, smooth and SLA titanium disks, were examined under SEM. *Vital cell staining* was performed to examine cell viability, growth, adhesion, attachment and morphology on titanium surfaces at 24 hr. after cell seeding and every 7 day in osteogenic medium, under a fluorescence microscope, confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM). *Live-dead cell staining* was performed to demonstrate effects of titanium surface and culture medium on cell viability and cell dead on days 7, 14 and 21 in osteogenic medium. *Cell viability assay* was conducted to establish growth curves of cells on different surfaces in conventional and ED cell cultures. To determine osteogenic differentiation potential, levels of alkaline phosphatase activity and calcium contents were measured on days 7 and 21 and day 21, respectively. The investigations were performed on 4-5 samples per group at each investigation time (n=4, mean±SD) (Table 2).

Investigating procedures

Fluorescence vital cell tracking

Cell attachment and viability on the discs were examined using CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate) (Molecular Probes/Invitrogen, USA). CellTracker™ Green dye contain a chloromethyl group that reacts with intracellular glutathione yielding a nonfluorescent product that when interact with intracellular esterase yielding green fluorescence (Product insert, CellTracker™ Green, Molecular Probes). The procedure in brief; the disks were washed twice with PBS. Then cells on disks were incubated in 5 μM CellTracker™ Green in serum free culture medium for 30 minutes at 37ºC in a humidified 5% CO_2 incubator. After that the disks were rinsed twice with phosphate buffer solution (PBS), fixed in 10% buffered formaldehyde and examined under fluorescence microscope (Ti-S100, Nikon, Japan) and confocal laser scanning microscope (CLSM) (FV300, Olympus, Japan) (Fig.5). Vital cell staining was performed at 24 hr. after cell seeding and then on days 7, 14 and 21 in osteogenic medium to observe cell viability, growth, adhesion, attachment and morphology (n=2).

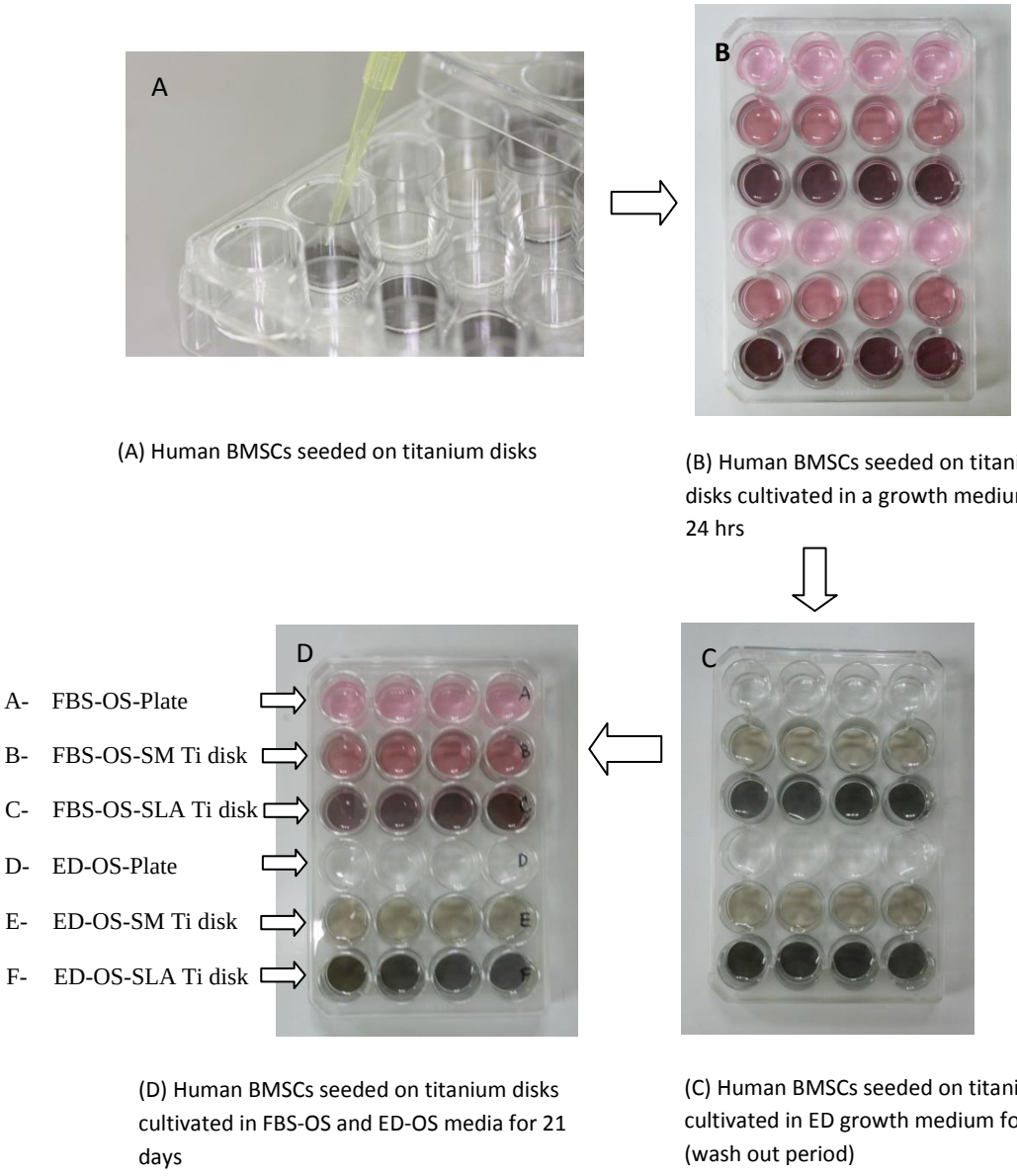


Fig. 4 Demonstrating cell seeding on Ti disks and cell culture scheme for osteogenic differentiation study, (A) Cell seeding on Ti disks and (B) At 24 hr. after cell seeding in growth medium, (C) Subsequently, cell seeding in ED-growth medium for 24 hr. wash out period and (D) Then, cells were cultured in conventional (FBS-OS) and estrogen-deprived osteogenic media (ED-OS) for the osteogenic differentiation study.

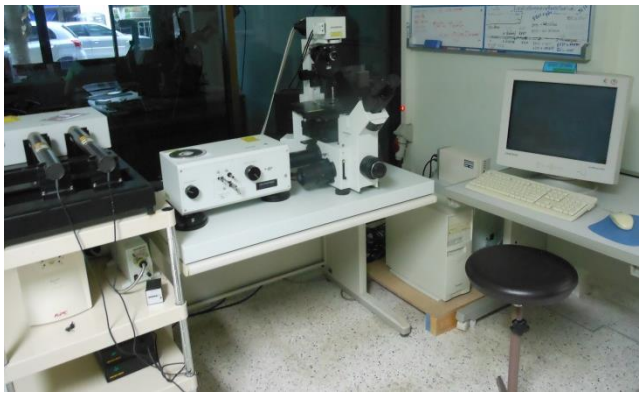
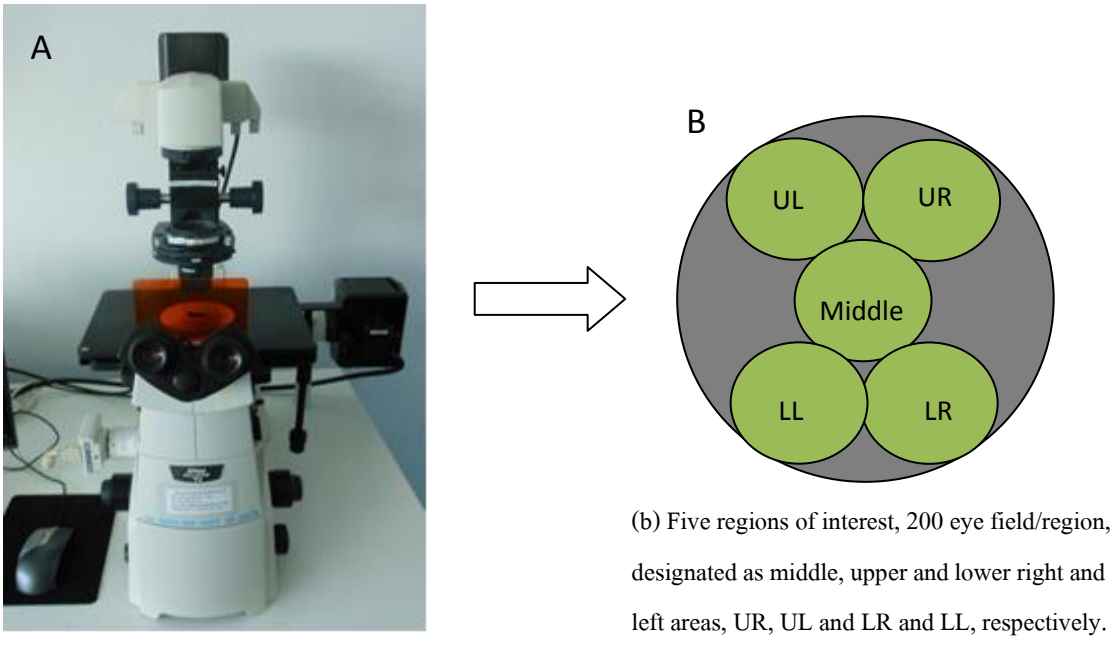


Fig. 5 Demonstrating confocal laser scanning microscope (CLSM) (FV300, Olympus, Japan) used to examine fluorescence live-dead staining of cells on titanium disks.

Live and dead cell staining

To determine numbers of cell dead, cells were incubated in a mixture of 0.5 mg/ml propidium iodide and 5 μM CellTracker™ Green (Molecular Probes) in serum free culture medium for 30 minutes in a humidified incubator with 5% CO_2 at 37ºC. After that the disks were rinsed twice with PBS, fixed in 10% buffered formaldehyde and examined under CLSM (Fig. 5) and fluorescence microscope (Ti-S100, Nikon) (Fig. 6A). Subsequently, numbers of positive red stain nuclei were counted from 5 regions of interest designated as 200 eye field/region. Five designated areas were designed at the middle and other four areas on the upper and lower right and left corners of the disk (Fig. 6B). Then average numbers from the 5 areas were reported as numbers of dead cells on each disk. After that mean numbers of dead cells from days 7, 14 and 21 in FBS-and ED-OS media were calculated and reported (n=3, Mean±SD) (Fig. 6).



(b) Five regions of interest, 200 eye field/region, designated as middle, upper and lower right and left areas, UR, UL and LR and LL, respectively.

Fig. 6 Demonstrating setting of live-dead cell staining examination, (A) Fluorescence microscope (Ti-S100, Nikon) and (B) Five regions of interest of a 200 eye fields.

Confocal laser scanning microscope

Vital staining using CellTracker™ Green and Propidium iodide was observed by scanning confocal laser scanning microscope (CLSM, FV300, Olympus, Japan) at the Scientific Equipment Center, Prince of Songkla University. Following cell staining, cells were fixed in 10% buffered formaldehyde and protected from light. Then cells were examined at the excitation/emission 490/520 nm wavelengths for green color and images were captured using Fluoview Application Software (Olympus, Japan). Numbers of samples were 2 samples per group at each investigation time[68] (Fig. 5).

Scanning electron microscope

Cell morphology and attachment on titanium disks were assessed optically by scanning electron microscope (SEM) (5800LV, JEOL, Japan). At each investigation time, culture medium was removed and the disks were washed twice by PBS to remove unattached cells on the surfaces. Then attached cells were fixed in 4% glutaraldehyde and 10% formaldehyde. After that they were dehydrated in ethanol series of 30-100%, dried, gold sputter-coated (SPI Module™

Sputter Coater, SPI, USA) and examined and imaged (SEM, 5800LV, JEOL)[69]. Numbers of samples were 2 samples per group at each investigation time (Fig. 7).

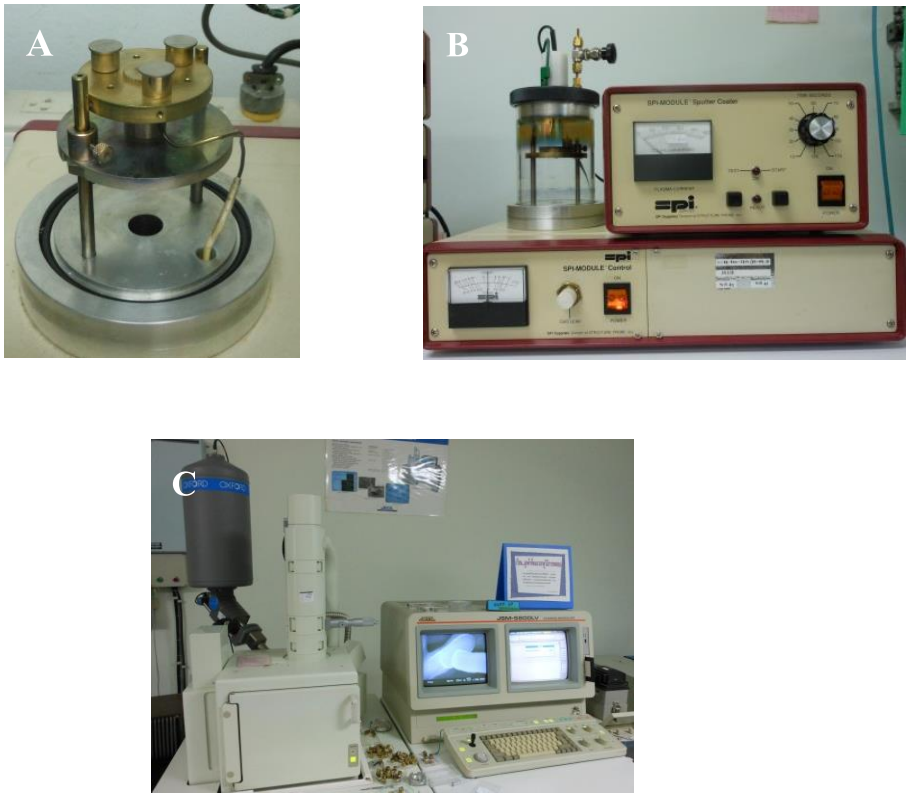


Fig. 7 Demonstrating scanning electron microscope (SEM) sample preparation equipment and SEM machine, (A) Metal stub for positioning titanium disks for sputter coating, (B) Sputter coating equipment (SPI Module™ Sputter Coater, SPI, USA) used to sputter-coat gold-palladium particles on the samples and (C) examining and image capturing microscope, SEM (5800LV, JEOL)

Cell viability assay

Growth curve was established using cell viability assay. Cell viability was measured as an indicator of cell numbers and CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA) was used. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay was a colorimetric assay for measuring the activity of cellular enzymes that reduce the yellow tetrazolium dye to purple formazan in living cells. An increase in an amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture (Product insert, CellTiter 96® Aqueous One Solution, Promega). Cells were incubated in 20% of

the assay in ED-growth medium for 2 hr., then the formazan dye was quantified at 440 nm absorbance in duplicate using a microplate reader (Multiskan GO, Thermo Scientific, Finland) (Fig.8). Cell viability was determined on days 2, 7, 14 and 21 in osteogenic media[70]. Numbers of samples are 4 samples per group at each investigation time (n=4, Mean±SD).

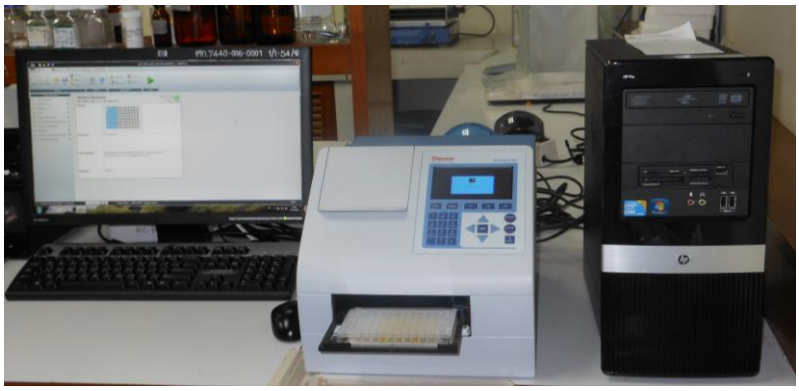


Fig. 8 An image of microplate reader (Multiskan GO, Thermo Sciencific, Finland)

Alkaline phosphatase activity and calcium content measurements

Human BMSCs on cell culture plates and titanium disks in osteogenic medium on day 7, 14 and 21 were lyzed in Ripa buffer (Sigma) to obtain total protein solution and cell pellets. Amount of total protein contents and levels of alkaline phosphatase (ALP) activity in the protein lysis solution were measured, and pellets from the same samples were kept for calcium content assay. Then, ALP activity and calcium content levels were neutralized by amount of total protein contents from the same sample and reported per mg protein.

Cell lysis for protein solution

Cells were lysed using Ripa buffer (Sigma). RIPA Buffer composes of 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. The procedure in brief:

At each investigation time, cells on cell culture plates and titanium disks were washed twice in PBS. Then 100 µl of ice cold RIPA buffer was added into each well and immediately stored at -70ºC. After that the frozen samples were undergone 2 cycles of freezing and thawing for 1 hr. at -70ºC and 1 hr. at RT. Then thawed samples were placed on ice and cell layers were scraped using disposable cell scrapers (SPL LifeSciences., Korea), transferred to 1.5

ml microcentrifuge tubes, vigorously vortexed and kept on ice for 1.30 hr. Subsequently, they were centrifuged (Labofuge 400R, Heraeus, USA) at 12000 rpm (2500 g) at 4ºC for 15 min then supernatant was transferred into new 1.5 ml tubes as protein lysis solution for measuring protein contents and ALP activity. Cell pellets were stored at -70ºC for a calcium content analysis[69].

Measuring protein concentrations

The quantification of amount of protein in cell lysate was performed based on Lawry's assay using Bio-rad® DC™ Protein assay kit (Bio-Rad, USA) following manufacturer's instruction. The reactions were read at 650 nm absorbance in duplicate using a microplate reader (Multiskan GO) (Fig.8) and optical density was extrapolated with standard curve of a serial dilution of bovine serum albumin protein standard (Bio-Rad). Protein concentations were reported as mg protein/ml. A total cellular protein analysis was performed on the same samples as the ALP activity and calcium content measuring assays[69].

Measuring levels of alkaline phosphatase activity

The assay was aimed to measure alkaline phosphatase (ALP) activity of protein extract solution obtained from RIPA buffer cell lysate solution mentioned in a previous section. This assay measures ALP activity by monitoring the color change as colorless para-nitrophenol phosphate (pNPP) is cleaved to derive p-nitrophenol (p-nitrophenol, a yellowish solution) and phosphate. Color intensity was read at 405 nm absorbance in duplicate using a microplate reader (Multiskan GO) and optical density (OD) was extrapolated with a standard curve of serial dilutions of p-nitrophenol (Sigma). Then ALP activity was normalized by the amount of total protein contents of the same sample and reported as nano-Molar per milligram protein (nM/mg protein). Alkaline phosphatase activity was measured on days 7 and 21[71]. Numbers of samples were 4 samples per group at each investigation time (n=4, Mean±SD).

Measuring levels of calcium content in extracellular matrix

Demineralization of calcium contents in cell pellets

Following cell lysis for protein solution, cell pellets were washed in PBS. After that 150 µl of 0.5 M HCL in PBS was added into each pellet in 1.5 ml tubes, continuously shook overnight at RT on a horizontal shaker (HS260B, IKA® Werke, Germany), then centrifuged (Labofuge 400R) at 12000 rpm for 10 minutes. The supernatant was then collected and kept as demineralized calcium solution at -20ºC for the analysis of calcium contents[69].

Measuring of calcium contents in demineralized calcium solution

A quantification of calcium contents in the demineralized solution supernatant was analyzed using Calcium Colorimetric Assay kit (Biovision Inc., USA) following a manufacturer's instruction. Calcium Colorimetric Assay kit utilizes the chromogenic complex formed between calcium ions and o-cresolphthalein. The solutions were read at 575 nm absorbance in duplicate using a microplate reader (Multiskan GO). Optical density was extrapolated with a standard curve of a serial dilution of calcium standard solutions. Then calcium content levels were normalized by the amount of total protein contents of the same samples and reported as nano-Gram calcium per milligram protein (ng/mg protein). A calcium analysis was performed on the same sample as the ALP activity on day 21[69]. Numbers of samples were 4 samples per group at each investigation time (n=4, Mean±SD).

Data analysis

Sample size determination

Sample size was estimated by employing an estimate sample size for two-sample comparison of means method[72]. A computation was performed by using reference data from De Oliva's and coworkers[73]. It was found that appropriate sample size for the study was 4 samples for each group (Table 3).

Statistical analysis

The data were tested for normal distribution and homogeneity of variances then differences among groups at each time point were analyzed using one-way analysis of variance (ANOVA). When there were statistically differences, a multiple comparison test was then performed with either the Tukey HSD or Dunnette T3 methods as appropriate. If the data distribution was not normal, the Kruskal-Wallis analysis was used. Then if a difference was statistically significant, a MANN-Whitney test was performed. Significant differences were set at $p<0.05$. Data were reported as Mean±SD.

Table 3 Demonstrating sample size determination method using estimating power for two-sample comparison of means [72]

ALP activity (µmol/mg protein) at day 4 [73]		P value
Control	29.8 ± 10.1	0.01
Growth factor + proteins	9.6 ± 2.1	0.01

To Estimated power for two-sample comparison of means, the following formula was used

$$n/group = 2(Z_{\alpha/2} + Z_{\beta})^2 \sigma^2 / (\Delta)^2$$

n/group = Sample size per group, α = α error, β = β error, Δ = Mean difference and σ^2 = Pooled variance

$$\text{Pooled variance} = (n_1\text{-}1)\, sd_1^{\,2} + (n_2\text{-}1)\, sd_2^{\,2} / [(n_1+n_2)\text{-}2]$$

Assumptions:

$\alpha = 0.05$ (Two-sided) $\beta = 0.2$

$Z_{\alpha/2} = Z_{0.05/2} = 1 \quad .96$ $Z_{\beta} = Z_{0.2} = 0.84$

$n_1 = 5$ $n_2 = 5$

$sd_1 = 2.1$ $sd_2 = 10.1$

$\sigma^2 = 53.21$ $\Delta = 20.2$

$$n/group = \frac{2\,(1.96 + 0.84)^2 (53.21)}{(20.2)^2} = 2.044$$

Sample size for each group: 4 disks

Chapter3

Results

Titanium Surface Microtopography

Smooth and sandblasted and acid-etched (SLA) titanium surfaces exhibited different surface microtopographies. Surface of smooth titanium surface was uniformly covered with flatten-large and shallow pits (Fig. 9A). SLA titanium surface was rough with multi levels macro pits having diameters larger than 10 μm forming primary roughness, and micro pits creating secondary roughness within primary roughness of macro pits (Fig. 9B).

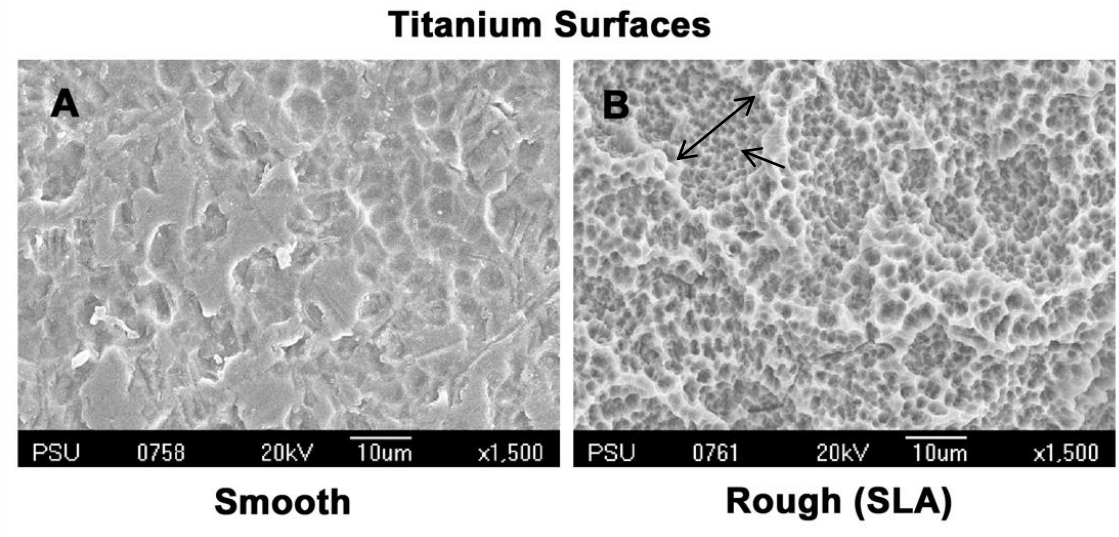


Fig. 9 Scanning electron microscope images of titanium surfaces, (A) smooth and (B) rough,sandblasted and acid-etched (SLA) titanium surfaces. Double end arrow represents diameter of one of macro pits and arrow points at micro pits on base of a macro pit.

Influences of titanium surface microtopography and estrogen-deprived cell culture on cell shape and attachment on titanium surfaces

Rough titanium surface and estrogen-deprived cell culture decreased cell attachment and size.

Following an initial 3 hr. cell adhesion on titanium surfaces, culture medium was changed to conventional (FBS) and estrogen-deprived (ED) culture media to determine effects of ED condition on initial cell attachment. Vital staining clearly showed good viability of cells in FBS and ED growth media at 24 hr. after cell seeding (Fig. 10). Cells on smooth surface flatten themselves on the surface by spreading out theirs cytoplasm directly on a smooth substrate, creating large cell-substrate contact area (Fig10A-D). On rough surface of SLA titanium surface, cells extended their cytoplasmic process across the rough surface creating multiple small focal contact points and stellate shaped cells (Fig10E-H).

Effects of ED-cell culture on cell attachment and size were most obvious on SLA titanium surface. Estrogen-deprived condition decreased attachment of cells on the surface resulting in a smaller cell size in ED- than FBS- growth media (Fig. 10). By comparing size and shape of cells on Fig10A&B and C&D and E&F and G&H, the images demonstrated that ED-condition reduced spreading of cell cytoplasm on substrates creating smaller cell size and forming smaller cell-substrate contact areas in ED- than FBS-growth mediums. However, based on image magnifications, a direct comparison on size and shape of cells could be compared only between Fig10D&H only.

Effects of surface microtopography on cell shape were clearly shown on day 7 in estrogen deprived medium. On smooth surface cell cytoplasm was spread out surrounding large nuclei forming a polyhedral cell sheet (Fig. 11A). On SLA titanium surface, ED-hBMSCs spread their cytoplasmic membrane across rough surface and extended cytoplasmic processes to form intercellular network of stellate shaped cells (Fig. 11B).

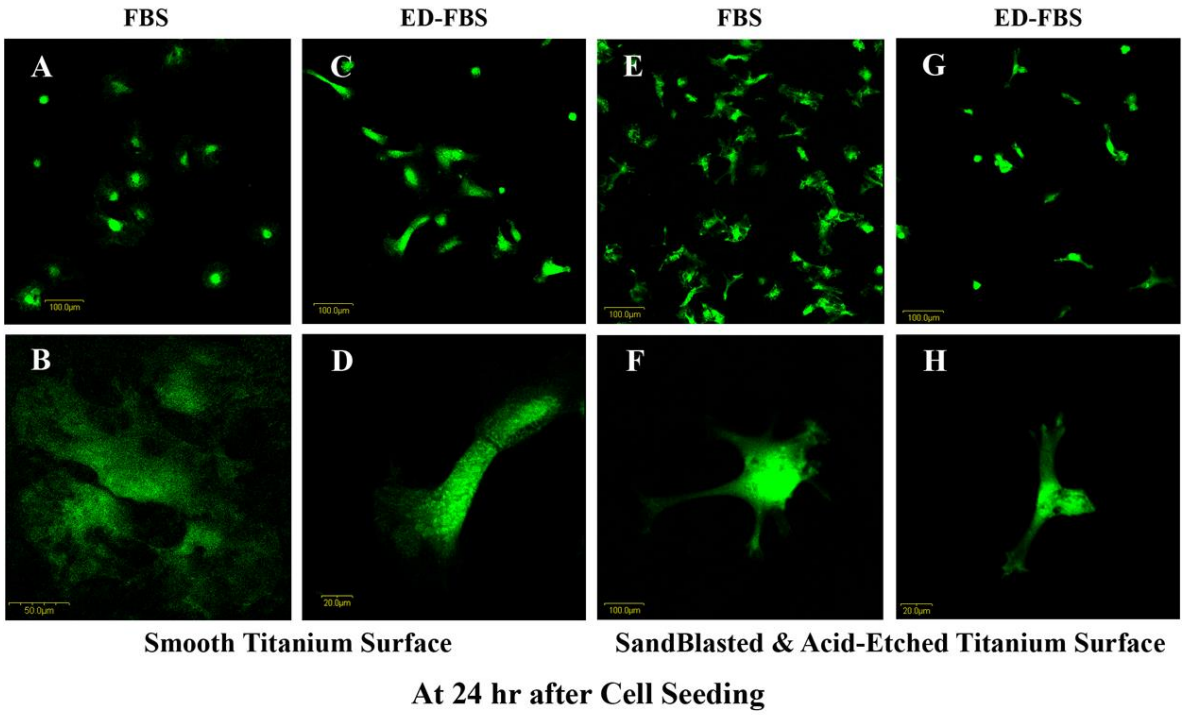


Fig. 10 Confocal laser scanning microscope images exhibiting green fluorescence vital cell staining of hBMSCs on titanium surfaces at 24 hr. after cell seeding, (A-D) on smooth and (E-H) on rough, sandblasted and acid-etched (SLA) titanium surfaces and (A&B); and (E&F) hBMSCs in conventional (FBS) and (B&D) and (G&H) in estrogen-deprived (ED) growth mediums.

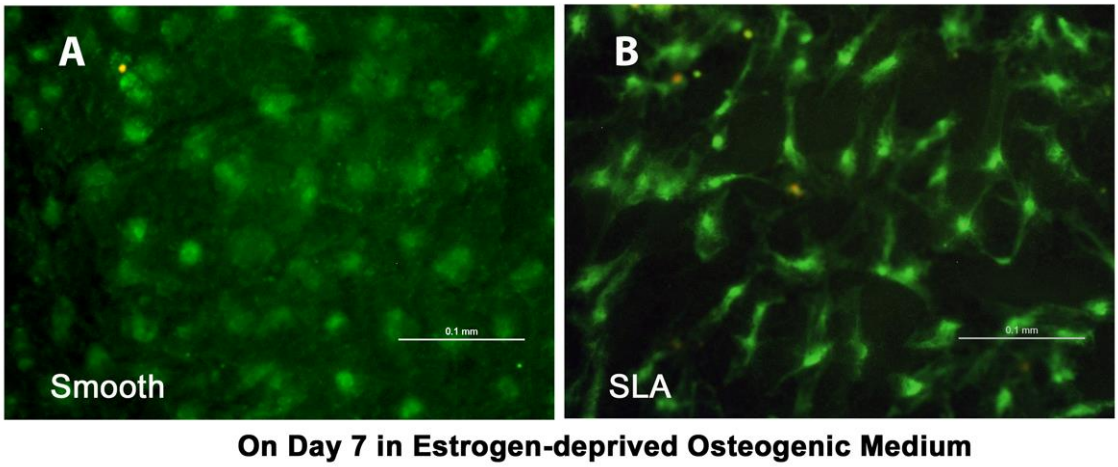


Fig. 11 Fluorescence microscope images of green fluorescence vital cell staining of estrogen-deprived (ED) hBMSCs in ED cell culture on day 7, (A) on smooth (Smooth) and (B) sandblasted and acid-etched (SLA) titanium surfaces.

Effects of titanium surfaces and estrogen-deprived cell culture on growth and osteogenic differentiation of hBMSCs

Cell seeding for osteogenic differentiation study

For osteogenic differentiation study, hBMSCs were seeded at high cell density, $2x10^4$cells/cm^2. At 24 hr. after cells seeding in FBS-growth medium, vital cell staining demonstrated high cell density that was evenly distributed covering 80-90 % of the surface areas on smooth (90% coverage) and SLA (80% coverage) titanium surfaces. Cells on cell culture plate and smooth titanium surface were spindle-shaped, while on SLA, stellate-like cells (Fig.12).

Rough titanium surface jeopardized poor cell growth in ED-OS medium.

Different growth of cells on different surfaces and culture media could be seen on culture-days 7, 14 and 21. Increasing of cell density suggested increasing of cell numbers and cell layers with cell culture-time. Human BMSCs in conventional osteogenic medium (FBS-OS) were increased in size and numbers compared to those in estrogen-deprived osteogenic mediums (ED-OS) medium. Cell density in FBS-OS was continually higher than cells in ED-OS, as it could be seen that cell density in ED-OS medium was lower than FBS-OS. As stated in a previous section, hBMSCs on smooth and SLA surfaces exhibited different cell shapes of flatten polyhedral and stellate-like cells on smooth and SLA surfaces, respectively, and ED-cell culture tended to decreased cell size and cell numbers (Fig.13).

Influences of titanium surfaces and estrogen-deprived cell culture on cell growth were supported by cell viability assay, which demonstrated different cell growth on smooth and SLA titanium surfaces in FBS- and ED-OS media. When cells were seeded at $1x10^4$ cells/cm^2 for cell viability assay, growth of cells in all groups was significantly increased from days 2 - 7, excepting on SLA in ED-OS medium (ED-SLA). As a control group, representing growth pattern of hBMSCs in FBS-OS medium, hBMSCs on cell culture plate in FBS-OS medium exhibited highest levels of cell growth and was significantly higher than other groups on day 7 (days 2 & 7, $p<0.05$) before entering static cell growth or plateau phase on days 14 and 21 ($p>0.05$). On titanium surfaces, smooth and SLA, cell growth continued to increase and reached the highest level on day 14 (days 2 & 14, $p<0.05$) before decreasing on day 21 (days 14 & 21, $p<0.05$). In ED-OS, cell growth on cell culture plate and smooth titanium surface reached the highest level on day 7 (days 2 & 7, $p<0.05$) before continually decreased on days 14 and 21 (days7 & 21, $p<0.05$). Throughout cell culture in FBS-OS, growth patterns and levels of cell growth on smooth and SLA surfaces were similar, and on days 14 and 21 cell growth levels on titanium surfaces in FBS-OS were significantly higher than all groups, in ED-OS on cell culture plates and titanium surfaces ($p<0.05$). The lowest level of cell growth was found on SLA titanium surface in ED-OS medium throughout cell culture ($p<0.05$) (Fig.14). Furthermore, low levels of cell viability on SLA titanium surface in ED-OS medium was supported by a higher number of cells dead in ED-OS than FBS-OS, particularly on SLA titanium surface (Fig.15).

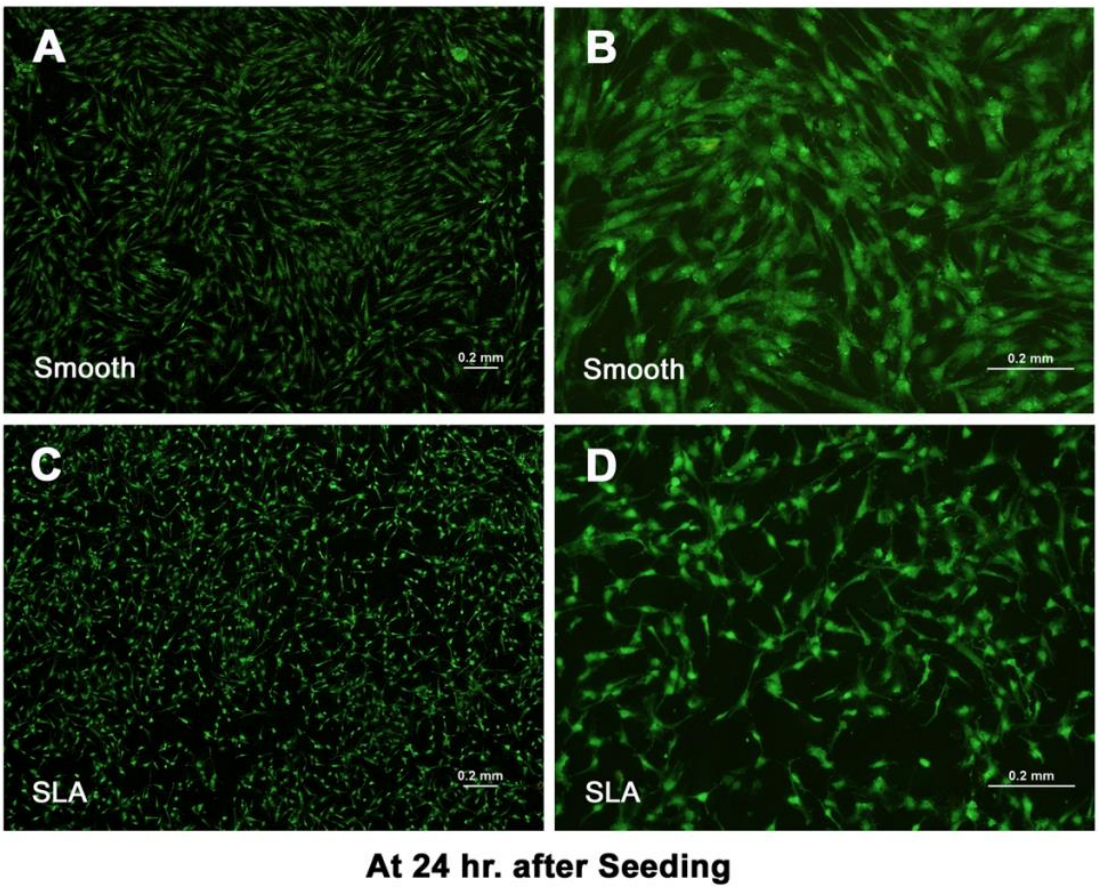


Fig. 12 Fluorescence microscope images of green fluorescence vital cell staining of hBMSCs at 24 hr. after cell seeding for osteogenic differentiation study at $2x10^4$ cells/cm^2 in conventional growth medium supplemented with 10% fetal bovine serum, (A&B) on smooth (Smooth) and (C&D) rough, sandblasted and acid-etched (SLA) titanium surfaces.

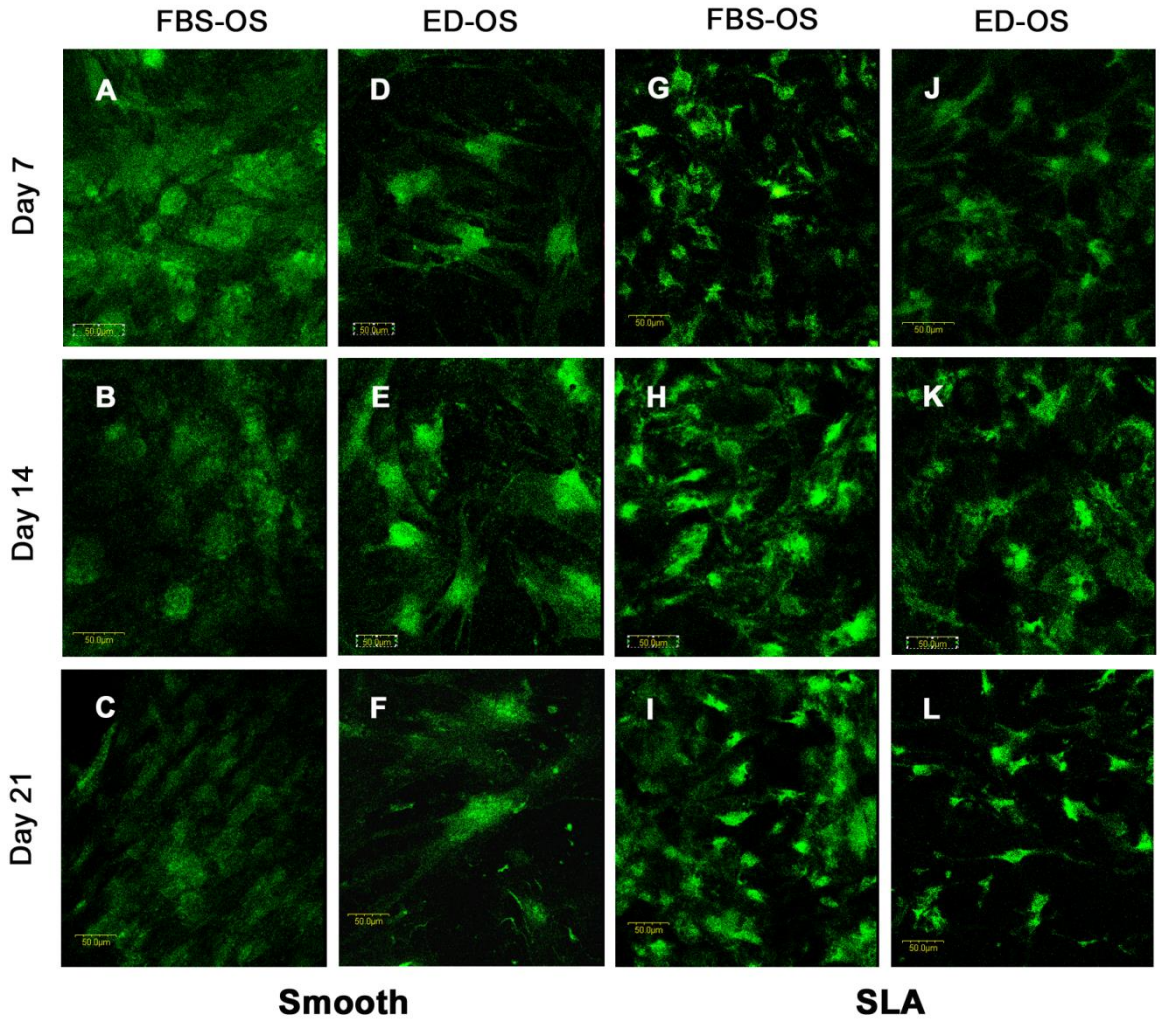


Fig. 13 Confocal laser scanning microscope images of green fluorescence vital cell staining of hBMSCs on titanium surfaces for osteogenic differentiation on days 7, 14 and 21 in conventional (FBS-OS) and estrogen-deprived osteogenic (ED-OS) mediums, (A-F) on smooth (Smooth) and (G-L) rough, sandblasted and acid-etched (SLA), titanium surfaces; (A, D, G, J) on days7, (B, E, H, K) day 14 and (C, F, I, L) day 21.

Growth of cells was decreased on sandblasted and acid-etched titanium surface in estrogen-deprived-cell culture.

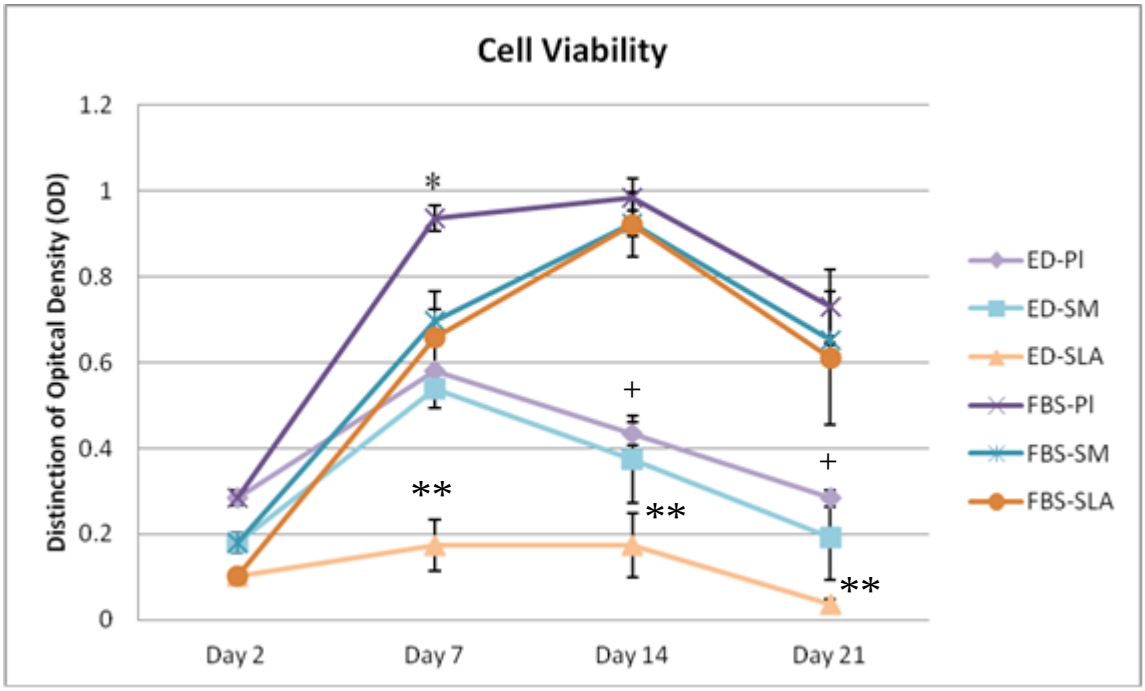


Fig. 14 Cell viability assay demonstrating growth curve of hBMSCs on titanium surfaces in conventional and estrogen-deprived growth mediums for 21 days. Abbreviations ED represents estrogen-deprived and FBS, conventional growth media; Pl, cell culture plates and SM, smooth and SLA, sandblasted and acid-etched titanium surfaces. Symbols * represents the highest level, +, lower of ED-Pl and ED-SM than groups in FBS and higher than ED-SLA and **, the lowest level at the investigated time at $p<0.05$ (n=4, Mean±SD).

Estrogen-deprived cell culture increased numbers of cell dead

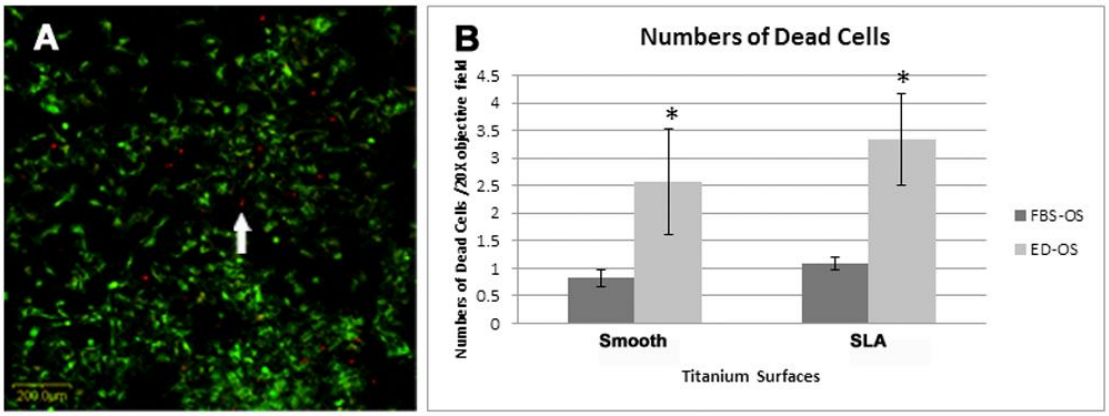


Fig. 15 Demonstrating effects of titanium surfaces and estrogen-deprived cell culture on numbers of dead cells, (A) A representative image of live-dead cell staining (CellTrackerTM Green and Propidium iodide (red, arrow) of hBMSCs on sandblasted and acid-etched titanium surface and (B) Average numbers of cell dead per 200 magnification eye field of inverted fluorescence microscope. Abbreviations Smooth represents smooth and SLA, sandblasted and acid-etched titanium surfaces, and FBS-OS, conventional and ED-OS, estrogen-deprived osteogenic media.

Scanning electron microscope images demonstrating influences of titanium surface microtopography and estrogen-deprived condition on cell attachment, growth and shapes

Rough titanium surface decreased cell-surface contact areas and further aggravated adverse effects of estrogen-deprived condition on cell attachment and growth.

On smooth titanium surface, hBMSCs spread out their cytoplasm on smooth surface forming cell sheet covering cell culture surface on day 7 (Fig. 16) and formed multiple cell layers on day 21 (Fig. 18). Different cell shape and attachment on rough, SLA, titanium surfaces were clearly shown. On SLA surface, hBMSCs shark their cytoplasm and extended their cytoplasmic processes across macro pits to attach on micro pits on rough surfaces and formed star-like cells (Fig. 17&19). Size and cell density in ED-OS were smaller and lower than FBS-OS on both titanium surfaces. Different cell-surface contacts between cells on smooth (Fig. 16 & 18) and SLA (Fig. 17 & 19) titanium surfaces were clearly shown on high magnification SEM images. On SLA titanium surface, hBMSCs extended their cytoplasmic processes to anchor on macro pit and extended multiple small cytoplasmic processes on micro-pits creating multiple focal point contacts (Fig. 20).

It could be seen that FBS-OS promoted cell attachment on titanium surfaces. On smooth titanium surface in FBS-OS, cell sheet was denser and thicker than ED-OS (Fig. 16&18). Effects of estrogen-deprived cell culture on cell attachment and growth were highlighted on SLA titanium surface. On SLA in FBS-OS, hBMSCs were able to extend their cytoplasm across rough surface creating larger cell size on day 7 (Fig. 17A&B) and forming cell sheet on day 21 (Fig. 19A&B), while in ED-OS medium cell shrank and demonstrated lower cell growth and cell density (Fig. 18 and 19C&D). Inhibitory effects of ED-OS on cell growth were highlighted on SLA in ED-OS on day 21. Cell growth on SLA in ED-OS was markedly limited (Fig. 19C&D).

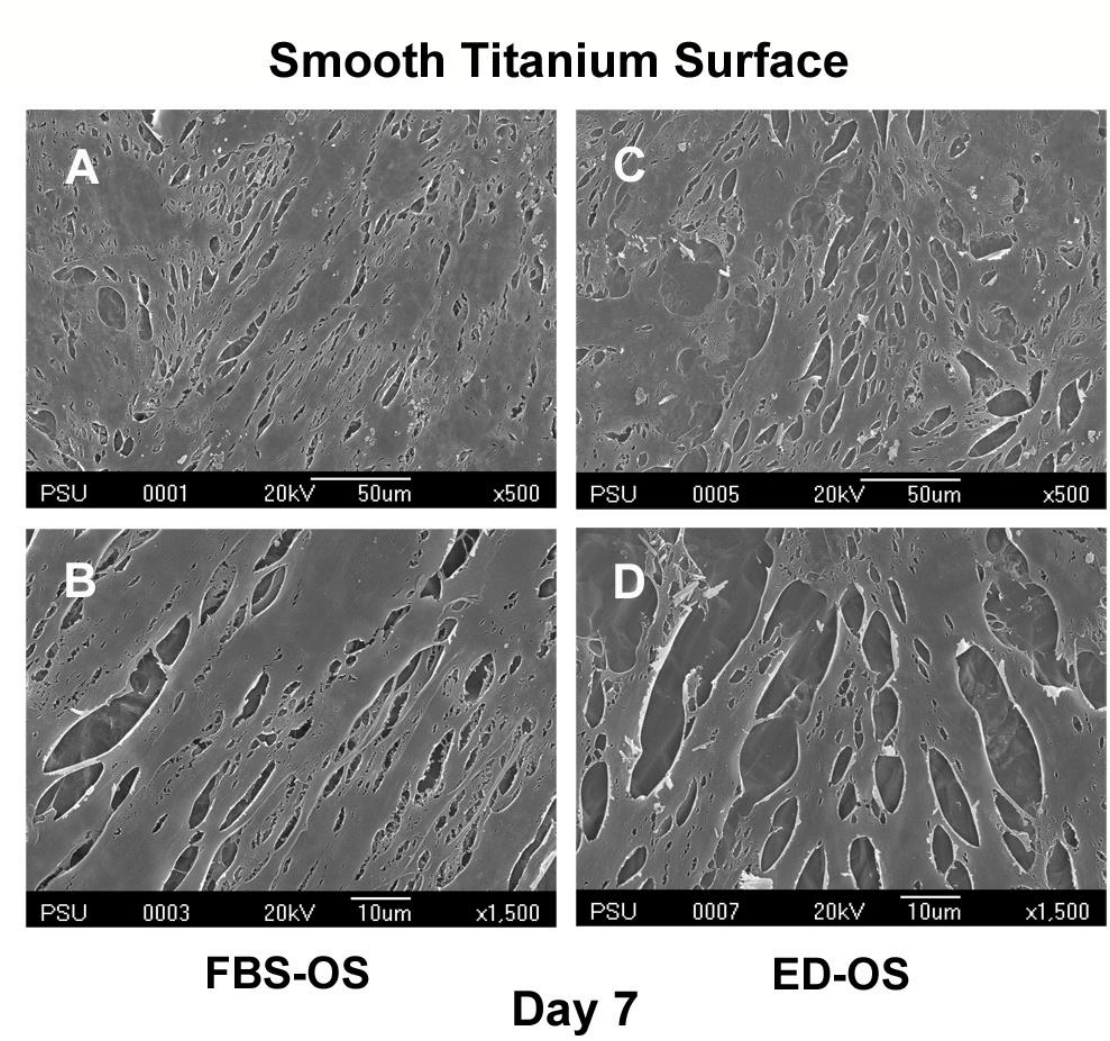


Fig.16 Scanning electron microscope images of hBMSCs on *smooth titanium surfaces* in conventional (FBS-OS) and estrogen-deprived osteogenic media (ED-OS) on day 7, (A&B) in FBS-OS and (C&D) in ED-OS media, (A&B) Human BMSCs spread out on smooth surface and forming cell sheet and (C&D) cell sheet was thinner in ED-OS mediums.

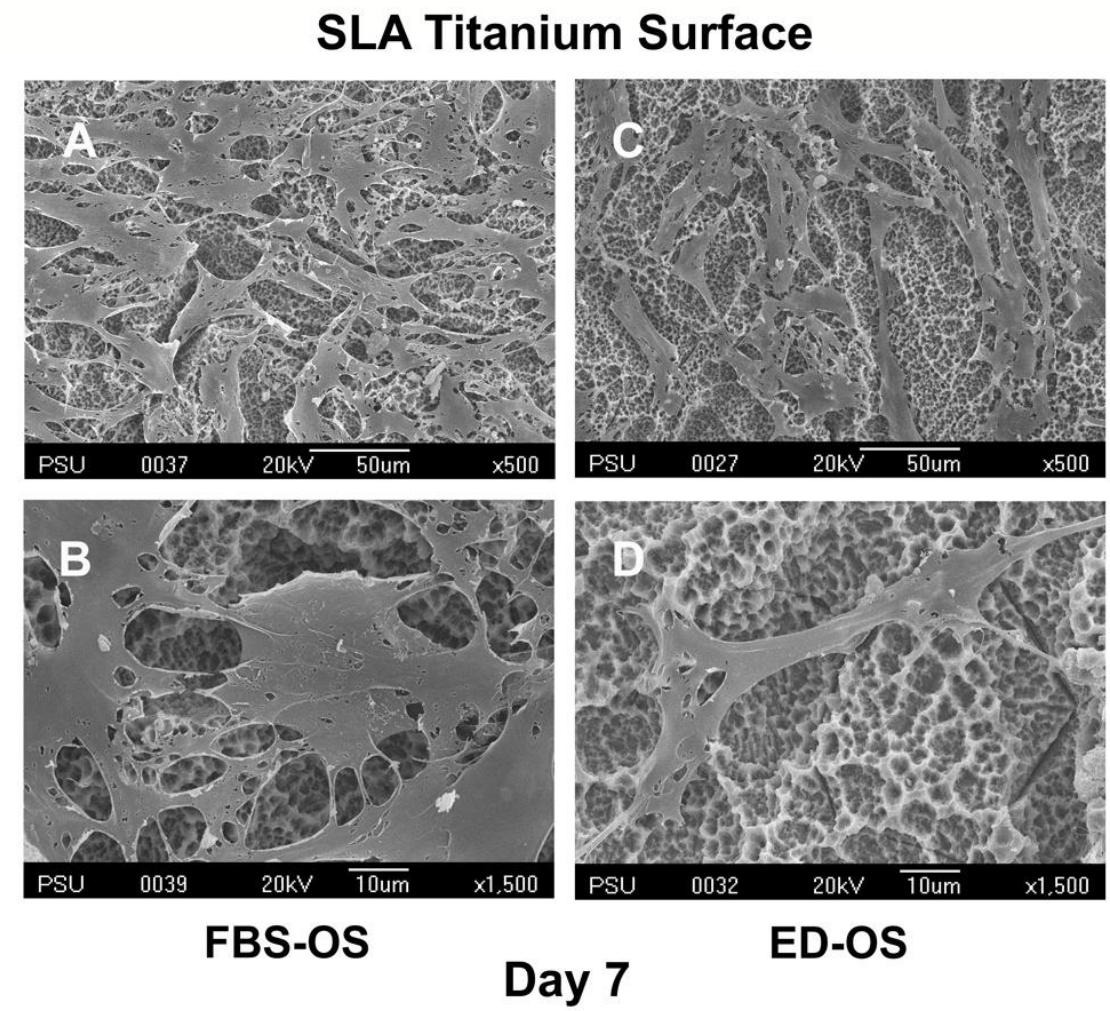


Fig. 17 Scanning electron microscope images of hBMSCs on *rough, sandblasted-acid etched (SLA) titanium surface* in conventional (FBS-OS) and estrogen-deprived osteogenic media (ED-OS) on day 7, (A&C) in FBS-OS and (D&F) ED-OS media. (A&B) On SLA titanium surface in FBS-OS medium, cell spread out and extended their cytoplasmic process to attach across rough surfaces, forming star-like cells and intercellular network but (C&D) in ED-OS medium, cell shrank into a smaller size with fewer numbers of cytoplasmic processes and markedly low cell density on the rough surface.

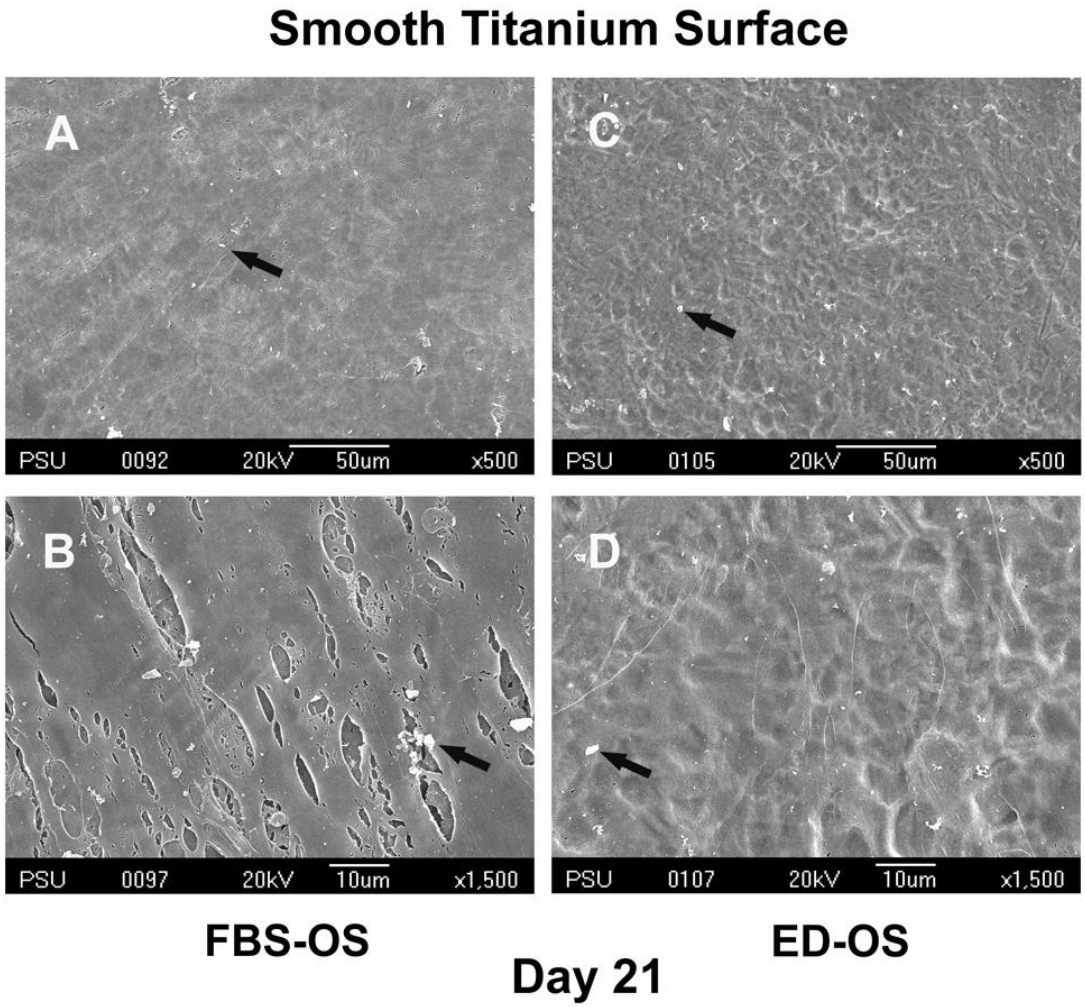


Fig. 18 Scanning electron microscope images of hBMSCs on smooth (Smooth) titanium surface in conventional (FBS-OS) and estrogen-deprived (ED-OS) osteogenic media on day 21, (A&B) in FBS-OS and (C&D) in ED-OS media. On day 21 (A&B) in FBS-OS medium, hBMSCs forming thicker multi-layers cell sheet with mineralized speckles localized on the surface of cell sheet (arrows) and (C&D) in ED-OS medium, cells forming thin cell sheet and surface texture of smooth titanium surface could be seen underneath the thin cell sheet and small and few mineralized speckles (arrows) could be seen on thin cell layer.

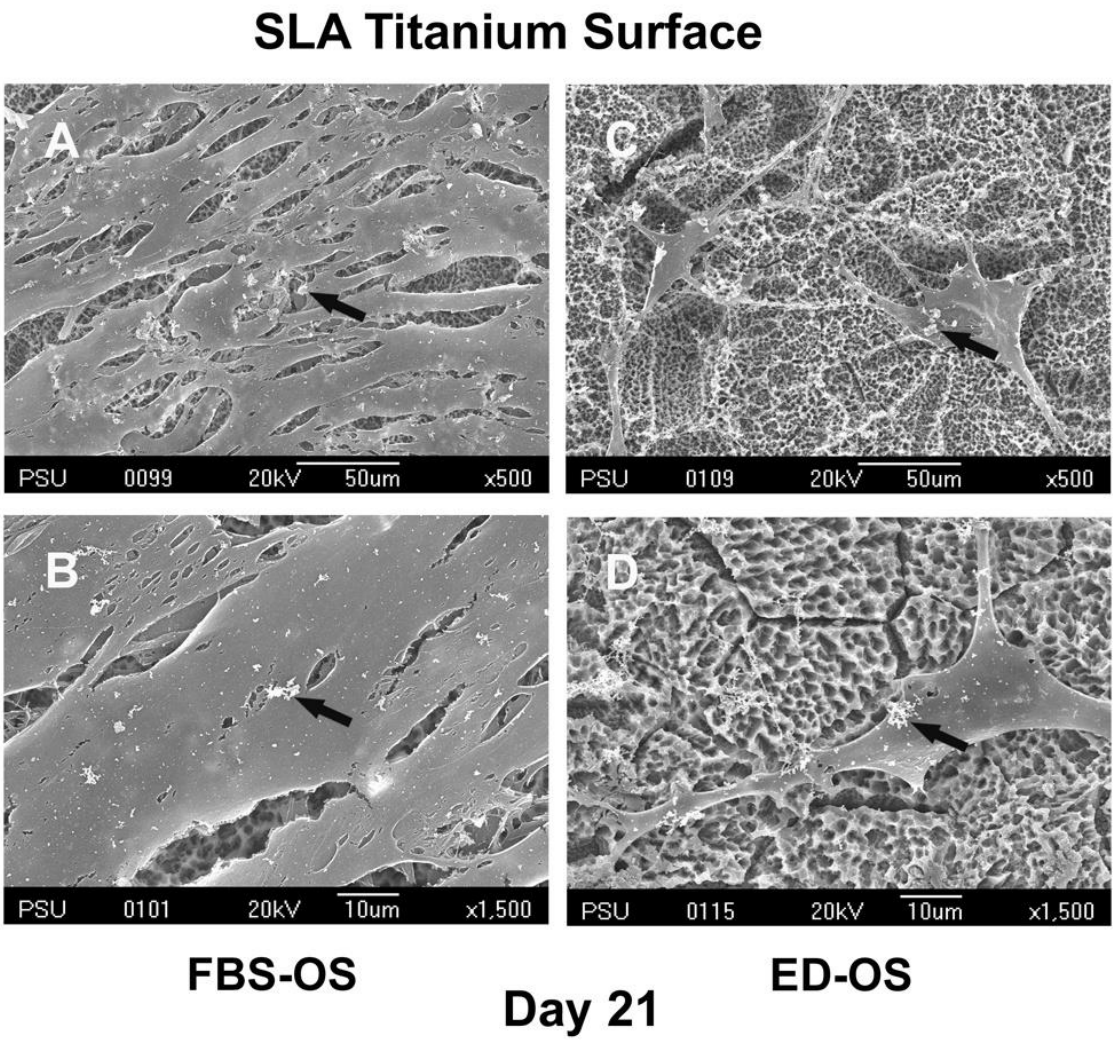


Fig. 19 Scanning electron microscope images of hBMSCs on *sandblasted-acid etched (SLA), titanium surfaces* in conventional (FBS-OS) and estrogen-deprived (ED-OS) osteogenic media on day 21, (A&B) in FBS-OS and (C&D) ED-OS media. On day 21 (A&B) hBMSCs in FBS-OS medium on SLA multiplied, established intercellular contact and formed loosen cell sheet partially covering the SLA surface. (C&D) On the contrary, hBMSCs in ED-OS medium demonstrated poor cell growth and low cell density. ED-hBMSCs could not form cell sheet, only extending their thin cytoplasmic processes to establish intercellular contact on the rough surface. Higher numbers of mineralized speckles could be seen on cell sheet in FBS-OS (A&B, arrows) than on individual cells in ED-OS media (C&D, arrows).

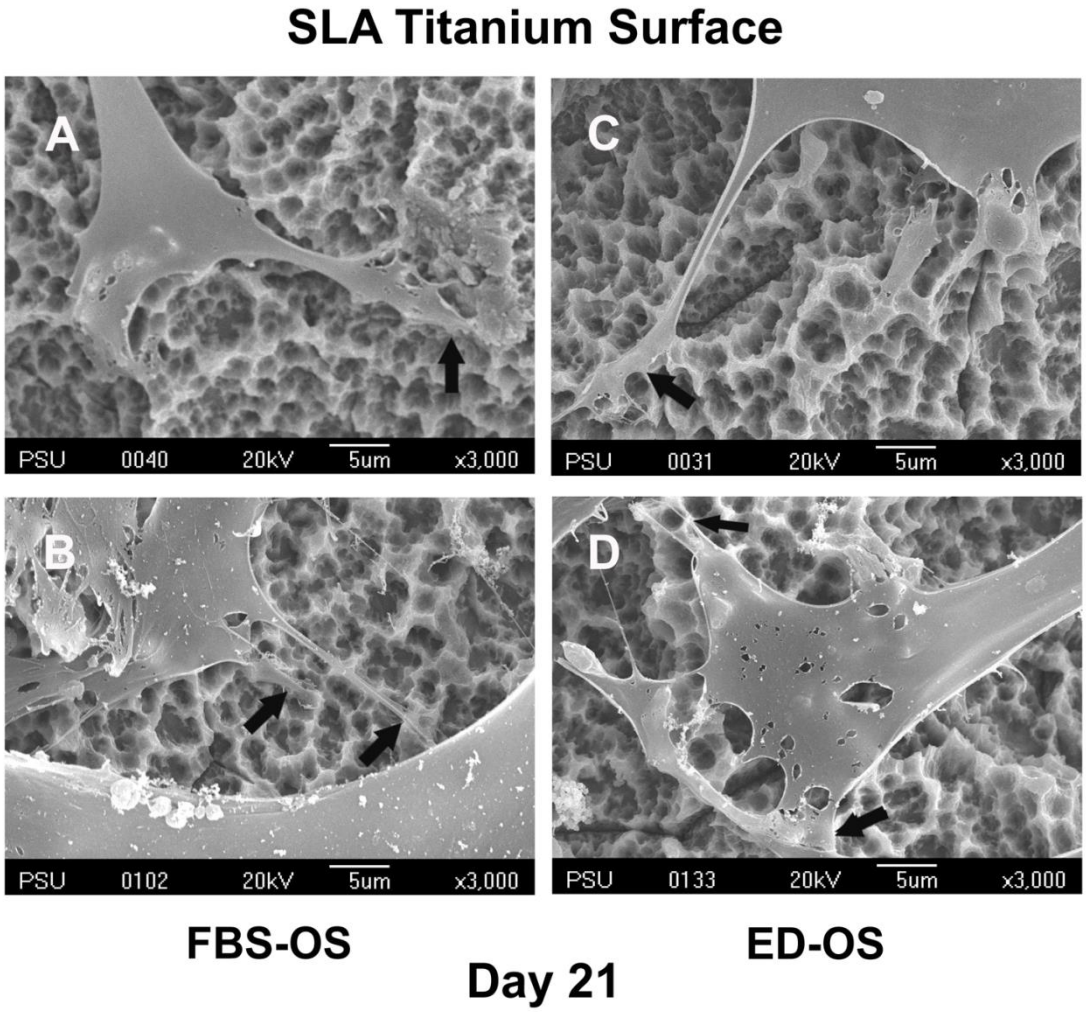


Fig. 20 Scanning electron microscope images demonstrating intercellular-surface contact of hBMSCs on *rough, sandblasted-acid etched (SLA) titanium surfaces* in conventional (FBS-OS) and estrogen-deprived osteogenic mediums (ED-OS) on day 21, (A&B) in FBS-OS and (C&D) ED-OS media. Human BMSCs established focal contact points on the rough surface (arrows) by extending cell body across macro pores while attaching cytoplasmic process on micro pores of the SLA surface. It could be noticed that hBMSCs in FBS-OS (A&B) established higher numbers of contact points than hBMSCs in ED-OS (C&D) media. Arrows indicate cell intercellular-surface contact of cytoplasmic processes on micro-pores of the SLA titanium surface.

Estrogen-deprived condition decreased osteogenic differentiation potential of hBMSCs and alleviated promoting effects of SLA surface on osteogenic differentiation of hBMSCs.

Sand blasted and acid-etched titanium surface promoted osteogenic differentiation potential of hBMSCs in FBS-OS by increasing levels of ALP activity and calcium contents..

Alkaline phosphatase activity

Estrogen-deprived cell culture decreased ALP activity of hBMSCs on cell culture plate and smooth and SLA titanium surfaces. In ED-OS, ALP activity levels were continually low on all surfaces and the differences among groups and between cell culture days 7 and 21 were minimal and not significant ($p>0.05$) and the activities were significantly lower than FBS-OS on day 21 ($p<0.05$). In FBS-OS medium, ALP activity levels of hBMSCs on all surfaces were significantly increased on day 21 ($p<0.05$). On day21, the highest level was found on SLA in FBS-OS ($p<0.05$) and the levels on cell culture plate and smooth titanium surface were not significantly different ($p>0.05$). ALP activity on SLA in FBS-OS on day 21 was higher than other groups at all investigation times ($p<0.05$) (Fig. 21).

Levels of calcium contents

It was clearly shown that FBS-OS medium promoted *in vitro* mineralization of hBMSCs and SLA titanium surface enhanced osteoblastic differentiation of hBMSCs in FBS-OS medium. Calcium contents on all surfaces in FBS-OS were significantly higher than ED-OS ($p<0.05$) and the level on SLA in FBS-OS was significantly higher than smooth titanium surface and cell culture plates ($p<0.05$). In ED-OS, calcium content levels were significantly lower than FBS-OS ($p<0.05$). The levels on titanium surfaces, smooth and SLA, were not significantly different ($p>0.05$) but they were significantly higher than on cell culture plate ($p<0.05$) (Fig. 22).

Estrogen-deprived cell culture decreased ALP activity of ED-hBMSCs

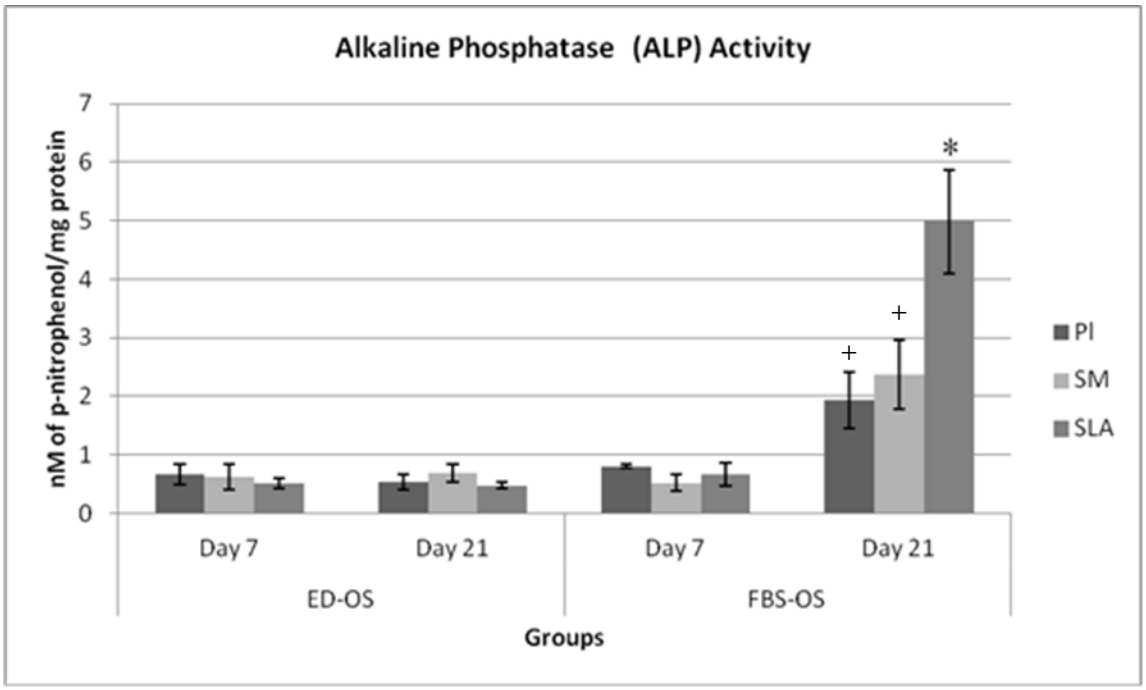


Fig. 21 Demonstrating alkaline phosphatase (ALP) activity of hBMSCs in conventional (FBS-OS) and estrogen-deprived osteogenic media (ED-OS) on cell culture plate (Pl) and smooth (SM) and sandblasted and acid-etched (SLA) titanium surfaces. Symbols * represents the highest level and +, significantly lower than SLA in FBS-OS on day 21 and higher than other groups on days 7 and 21 in ED-OS and FBS-OS media ($p<0.05$) (n=5, Mean±SD).

Estrogen-deprived cell culture decreased in vitro mineralization of ED-hBMSCs

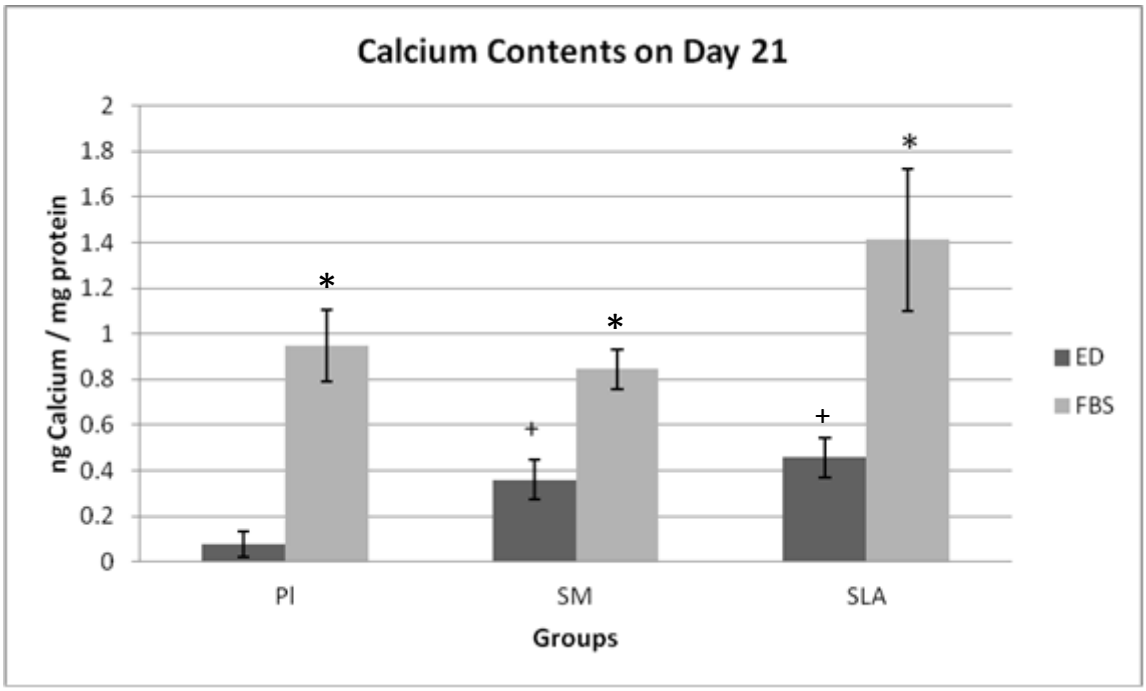


Fig. 22 Demonstrating calcium contents of hBMSCs on culture-day 21 in conventional (FBS-OS) and estrogen-deprived oseogenic mediums (ED-OS) on cell culture plate (Pl) and smooth (SM) and sandblasted and acid-etched (SLA) titanium surfaces. Symbol *, represents significantly higher than ED-OS and +, higher than cell culture plate of the same group ($p<0.05$) (n=5, Mean±SD).

Chapter 4

Discussion

Osteoporosis, one of the public health problems associated with aging, might be considered as a risk in implant therapy. To determine effects of osteoporotic on osteointegration of dental implant, the current study aimed to investigate effects of titanium surface microtopography, SLA titanium surface, on growth and osteogenic differentiation of estrogen-deprived hBMSCs (ED-hBMSCs). It was hypothesized that 1) estrogen-deprived cell culture would decreased growth and osteogenic differentiation of hBMSCs and (2) SLA titanium surface would be able to promote growth and osteogenic differentiation of ED-hBMSCs. The current study was separated into 2 parts, Part I: establishing a long term estrogen-deprived cell culture and Part II: investigating growth and osteogenic differentiation of estrogen-deprived hBMSCs on titanium surfaces.

Estrogen-deprived cell culture

In an effort to minimize adverse effects of estrogen-deprived in osteoporotic cases on bone regeneration and to improve osteointegration of dental implant, a long term estrogen-deprived (ED) cell culture model was established to mimic an estrogen deprived condition of hBMSCs in menopause osteoporotic cases and to investigate effects of ED cell culture on osteogenic differentiation potential of hBMSCs.

In the current study, estrogen deprived cell culture was established by utilizing charcoal stripped bovine serum and phenol red free culture medium. Phenol red was removed from cell culture media in order to avoid mimicking estrogenic activity of phenol red. It is reported that phenol red at concentrations of 15-45 μM which is used as a pH indicator in culture media can function as estrogen by binding to estrogen receptor and promotes proliferation of human cells expressing estrogen receptors[65]. Charcoal stripped bovine serum is a serum that lipophilic materials including growth factors and hormones such as estrogen have been absorbed with activated carbon resulting in lipid-like components free serum (Gibco/Invitrogen). Charcoal-stripped serum is useful for studying hormone, growth factors and cytokine responsive cell culture[64, 74, 75].

In the current study, charcoal-stripped bovine serum and phenol red free culture medium were used to remove estrogenic activity of fetal bovine serum and cell culture media to establish estrogen-free cell culture model. However, because of low levels or absence of multiple growth factors and hormones from charcoal stripped bovine serum, results of the study could be results of complex hormonal deprivation instead of estrogen-deprived effect alone, and the current study could be regarded as a platform to study effects of systemic hormones and growth factors on cell behaviors and intercellular-material interaction.

Effects of estrogen-deprived cell culture on growth of hBMSCs

It was clearly demonstrated in the current study that estrogen-deprived cell culture decreased cell size, cytoplasmic spreading on surfaces of substrates or cell attachment and cell growth. The findings support previous studies that estrogen deprived condition decreases cell growth of hBMSCs[34]. This is because serum is an essential factor influencing cellular proliferation and differentiation in cell culture[75]. Low levels of growth factors and lipophilic materials of ED-cell culture was a harsh condition for growth of cells particularly for 21 day cell culture. Estrogen-deprived cell culture decreased cell growth and cell viability demonstrated by a lower cell density (Fig. 13, & 16-19), levels of cell growth (Fig. 14) and higher numbers of dead cells (Fig. 15) on smooth and SLA titanium surfaces in ED-OS than FBS-OS. Live-dead cell staining and SEM images exhibited smaller cell size and lower cell density on SLA surface in ED-OS than FBS-OS on days 7 and 21 (Fig. 13-19). The findings suggested that decreasing of cell attachment and proliferation on SLA titanium surface[5, 53, 54] were aggravated by ED-condition.

To improve cell viability and differentiation potential, authors increased cell density from 1x104 cells/cm^2 to 2x104 cells/cm^2 (Fig.12). It was found that cells were able to reach confluence and grow in multilayer and mineralized ECM on culture-day 21 (Fig.13, 18&19). The improvement might be because high density cell seeding enhanced intercellular contact and promoted intercellular communication and paracrine and autocrine functions of cells which resulted in supporting growth and differentiation of hBMSCs in ED-cell culture[76]. Thus inter-cellular communication might have helped ED-hBMSCs to sustain low levels of growth throughout cell culture period and support osteoblastic differentiation of ED-hBMSCs.

It was observed that hBMSCs could not be acclimatized to ED-cell culture to generate long term ED-hBMSCs before cell seeding. Multiple passaging of ED-hBMSCs caused low cell attachment, slow growth and cell dead (data not shown). This is in contrast to studies on

breast cancer cell line, that cells are undergone multiple passages in ED-growth medium and long term ED- MCF-7 cell lines are generated[77, 78]. In the osteoblastic differentiation studies of human osteoblasts (hOBs), hOBs were cultured in estrogen-deprived for 48 hr. before starting the experiment to remove residual effects of estrogen from culture medium[79-81]. Consequently, in the current study, hBMSCs were expanded in growth medium instead of ED-growth medium and hBMSCs expanded in growth medium were seeded on titanium disks for the experiments. Subsequently, to minimize effects of different substrates and culture conditions on cell growth, equilibrate cell numbers, ensure similar cell density, acclimatize cells in ED-cell culture and remove residual effects of growth factors and hormones from growth mediums before initiating osteogenic induction, therefore, at 3 hr. after cell seeding hBMSCs on cell culture plates and titanium disks were cultured in ED-growth medium for 24 hr. before starting the osteogenic induction.

Limiting of cell growth and attachment on titanium surface in ED-cell culture in the current study could be considered as a limitation of the cell culture model that could not completely simulate clinical situation in skeletal defects. In clinical environment, titanium surface inserted in the osteoporotic bone will be covered with blood clot and body fluid that would be able to enhance cell attachment and growth on the titanium surfaces and alleviated direct effects of estrogen-deficiency on growth and differentiation on titanium surface and osteointegration[23, 24] and thus the effects of osteoporotic bone on osteointegation might be delay or obscured. Besides, the present culture model exhibited essential roles of hormones and growth factors on intercellular-material interaction. Taken together, the current cell culture model provided a well control experimental condition to investigate effects of estrogen-deprived condition on growth and osteogenic differentiaion of hBMSCs on the titanium surfaces.

Influences of titanium surface microtopography on cell shape and attachment

The findings in the current study that surface microtopography of smooth and SLA titanium surfaces influenced cell adhesion, attachment and shapes (Fig. 9) supports previous studies that surface microtopography regulates cell attachment, growth and differentiation[51, 52]. Osteoblasts exhibit a decreasing of cell attachment and proliferation but increasing of osteoblastic differentiation markers on rough titanium surface[5, 53, 54]. Promoting effects of rough titanium surface on osteogenic differentiation could be results of morphological change during cell attachment on different substrate architectures. This is because morphological change initiates

the activation of integrin binding system leading to modulation of cell growth and differentiation[57, 58]. Mechanism mediating the cellular response to the surface microtopography needs to be further investigated.

It was clearly exhibited in the current study that cell shapes, size and growth of hBMSCs on smooth and SLA titanium surfaces had been influenced by surface roughness since 24 hr. after cell seeding till day 21 in osteogenic medium (Fig. 12,13,16-19). Smooth titanium surface enhanced cell attachment by promoting cell spreading on smooth surface, while on SLA titanium surface, hBMSCs formed multiple small contact points on macro- and micro pores of the rough SLA surface (Fig. 16-17&20). Different cell attachment found in the current study could be explained by a previous report that surface microtopography of SLA titanium surface is optimal for the attachment of osteoblasts with a diameter of 20–40 μm on macro-pore and theirs filopodia to the micro pores[60]. Consequently, different contact features led to different cell shapes and thus differences of growth and differentiation on smooth and SLA titanium surfaces. On smooth surface, hBMSCs were relatively round in shape with spreading out of cytoplasm surrounding a round nuclei (Fig. 11A & 16), while on SLA surface cells extended multiple small cytoplasmic processes to attach on rough surface forming stellate-shaped cells (Fig. 11B & 17). Moreover, ALP activity and calcium content levels on SLA surface were higher than smooth titanium surfaces (Fig. 21&22). In summary, the findings underlined significant effects of the morphological change during cell attachment on different substrates' architectures on cell growth and differentiation that might be influenced by focal adhesion signal transduction and adhesion molecules [57, 58].

Combining effects of surface microtopography and estrogen-deprived cell culture on osteogenic differentiation of hBMSCs on titanium surfaces

Surface microtopography of titanium surface has been shown to be a major factor regulating cell response to biomaterials[51, 52]. It is reported that rough titanium surface promotes osteogenic differentiation and influences the differentiation potential of hBMSCs[82, 83]. Rough titanium surface increases alkaline phosphatase (ALP) activity, osteocalcin (OCN) production and secretion of osteogenic cytokines such as prostaglandin E2 and transforming growth factor-beta 1 (TGF-β1)[84-86]. In the current study, surface microtopography must have influenced cell functions since initial cell seeding, as different cell shapes on smooth and rough

surfaces had been shown since 24 hr. after cell seeding (Fig. 10&12) and continued throughout 21-day cell culture.

It should be noticed that effects of titanium surface microtopography on cell growth and differentiation were accentuated in hormonal-deprived cell culture medium. As it was shown in Fig. 14 that growth patterns and levels of hBMSCs on SLA titanium surface in FBS-OS medium were similar to smooth titanium surface, while in ED-OS cell culture, growth of cells SLA surface was continually lower than smooth titanium surface. On the other hand, hormonal-deprived cell culture alleviated effects of titanium surface microtopography on osteogenic differentiation of hBMSCs. As it could be seen that ALP activity and calcium contents on smooth and SLA titanium surfaces in ED-OS medium were not different throughout cell culture (Fig 21&22).

Sandblasted and acid-etched titanium surface was used as rough titanium surface in the current study, because superior effects of the SLA titanium surface on osteogenic differentiation have been well established[60, 84]. Results of the current study were consistent with previous reports[67, 84, 86, 87] that SLA titanium surface promoted osteoblastic differentiation of hBMSCs indicated by increasing ALP activity and calcium content levels of hBMSCs in FBS-OS medium, but in estrogen-deprived cell culture osteogenic differentiation of hBMSCs was decreased both on smooth and rough titanium surfaces (Fig. 21 & 22). Based on, SEM images of hBMSC attachment on the SLA surface that hBMSCs extended small cell cytoplasmic processes to attach on uneven porous surfaces of SLA titanium surface (Fig. 20), it could be explained that surface microtopography of SLA titanium surface supported attachment of cells by promoting multiple contact points of cell cytoplasm and cytoplasmic processes on the macro and micro pores of the surface (Fig. 17, 19& 20)[55, 60] and thus the SLA surface microtopography is one of the contributing factors that promotes osteoblastic differentiation of hBMSCs on the SLA titanium surface[59].

However, it might be because systemic hormonal and growth factor components were essential to enable enhancing effects of SLA titanium surface on osteogenic differentiation was not found in ED-OS medium. The findings indicated that ED-cell culture alleviated promoting effects of SLA titanium surface on osteogenic differentiation of hBMSCs. Furthermore, markedly low ALP activity levels in ED-OS medium might have masked promoting effects of SLA surface on osteogenic differentiaton in ED-OS medium (Fig. 21). Additional

investigations on expression of osteoblast-related gene might be helpful in differentiating masked effects of SLA surface in this compromised cell culture condition.

As attachment to the ECM providing external signal regulating growth and survival of contact dependent cells[88] and estrogen regulates extracellular matrix synthesis[89], decreasing of cell growth, attachment and osteogenic differentiation in ED-hBMSCs might be results of a reduction of extracellular matrix (ECM) synthesis of hBMSCs in estrogen-deprived cell culture. It is reported that a decreasing of skin thickness and collagen contents of postmenopausal women can be reversed by estrogen replacement[89] and estrogen replacement decreases the increase of the matrix metalloproteinase 13 (MMP13, a collagease regulating ECM turnover) in mice[90]. ECM synthesis might be an essential factor facilitating cell attachment and growth and thus osteoblastic differentiation of cells on the SLA titanium surface. As a result, growth and osteogenic differentiaion on SLA titnaium surface were severely affected by ED-condition and SLA surface could not promote osteogenic differentiation as reported in previous studies [5, 53-55] and in FBS-OS in the current study (Fig 17, 19, 21&22). Investigations on ECM protein and adhesion molecules such as integrin and fibronectin should be further performed.

Effects of Genders of Donors on hBMSCs behaviors

Moreover, effects of sexual dimorphism on hBMSCs behaviors could contribute to response of cells to external stimuli such as titanium surfaces and hormonal-deprived condition. Differentiation potential of hBMSCs and response of cells to hormonal deprived condition could be regulated by sex of donors, male or female. As it is reported that growth, osteoblastic differentiation potential and bone formation capacity of BMSCs from female is lower than male mice [91]. In accordance with studies in mice, BMSCs of female rats exhibited lower osteoblastogenesis resulting in decreasing of osteoprogenitor cells and lowering of osteogenesis in female than male rats[92]. On titanium surfaces, responses of rat osteoblasts to different titanium surfaces and external hormonal stimuli such as estrogen and 1α,25(OH)2D3 were depended on genders of rats[93, 94]. Influence of genders on cell behaviors might be results of different endogenous estrogen and other hormone and cytokines of donors[94] and it is expected that optimal titanium surfaces and hormonal supplementation for men and women could be different. Effects of gender on hBMSC behaviors in the current study were minimized by using hBMSC-pool of different genders in the study.

Effects of estrogen-deprivation in vitro and implant survival in osteoporotic bone

Imbalance bone remodeling in post-menopausal osteoporosis cause deterioration of bone microarchitecture presented by thin trabecular bone, low bone mass and bone mineral density and adipocytic bone marrow[95]. It is expected that high bone turnover and decreasing osteogenesis would adversely affect osteointegration and implant survival [2, 4, 50]. The results support previous studies that decreasing of estrogen-levels decrease osteoblastic differentiation potential[34] and attachment and growth of hBMSCs on titanium surfaces[67, 96]. Additionally, the results pointed out that hormonal balance was an essential factor enabling intercellular-material interaction or regulating effects of titanium surfaces on cell behaviors. In contrast, the results are contradicted to clinical reports on implant survival that survival of dental implant is not effected by osteoporosis and up to now, osteoporosis is not a contra indication for dental implant placement[97]. This might be because impaired osteogenesis in osteoporotic bone prolongs secondary stability buildup time and increases dipping levels of primary strength during 2-4 week after implant insertion[60]. On the other hand, these defects could be minimized by applying good clinical care such as surgical techniques, longer non-loading time and good dental implant prosthesis[98]. It could be seen that effects of osteoporosis on osteointegration may be subtle and manageable, these factors might contribute to non-affected implant survival. However, based on previous reports and present study, implant placement in osteoporotic bone require close attention to ensure osteointegration and function of dental implant. Careful surgical technique, proper clinical management and implant dental prosthesis designs are recommended to accommodate compromised osteogenesis in an osteoporotic bone.

Limitations of the study

Estrogen deprived cell culture

Cell culture time of 21 days might be too long for the current ED-cell culture model. As it was stated earlier in the discussion that estrogen-deprived cell culture was a harsh condition to cell growth, particularly growth of cells on SLA surface and ALP activity in ED-OS culture and according to growth curve on Fig. 6, it might be better to reduce cell culture time to not longer than 14 days. However, a shorten cell culture time might effects investigations on osteoblastic differentiation in a terminal stage that usually require 21 day-cell culture in osteogenic medium to enable a detection of in vitro mineralization.

Adhesion and attachment of long term ED-hBMSCs on different titanium surfaces could not be performed, because hBMSCs could not tolerate multiple cell-passaging and culturing in estrogen-deprived culture medium, thus a long term Ed- hBMSC line could not be established. Instead, estrogen-deprived cell culture was established after cell seeding on titanium disks.

Vital cell staining and cell morphology examination on different surfaces

Vital cell staining was not sufficient to demonstrate cell morphologies and attachment. In the current study, vital cell staining using Tracker Green Vital Cell staining was used to determine not only cell viability but also cell shapes. Although vital cell staining was able to exhibit different cell shapes and sizes, additional immunohistochemical staining of cell cytoskeletal should be performed to clearly demonstrate cell cytosketon and thus cell shape and size and intercellular-substrate contact.

Limited investigating tools on osteoblastic differentiation

Additional investigations on osteogenic differentiation markers were required to differentiate effects of SLA on osteogenic differentiation of ED-hBMSCs, such as investigations on expression and synthesis of genes and proteins associated with osteogenic differentiation. This was because ALP activity levels in ED-OS medium were so low that it was difficult to measure and require higher protein concentration. Because of time and budget constrain, the additional analyses were not performed.

Requiring large numbers of disks and cells

Large numbers of titanium disks and hBMSCs were required for the experiments and analysis. Low levels of ALP levels and cell growth in ED-OS and a necessity to use high concentration protein lysis compelled a combining of 4 titanium disks for one sample or 16 disks per group for n=4 at each investigation time, and also an increasing of cell seeding density to 4x104 cells/disk. This resulted in a handle of large numbers of titanium disks and a demand for large numbers of cells at each round of the experiments. Titanium disks should have a larger diameter to fit in 6 well-cell cell culture plates instead of 24 well-cell culture plates as used in the study.

Prospective study

Increasing diameter of titanium disks

To reduce numbers of disks used for each experiment, increase amount of protein for protein lysis, and enhance analysis efficiency, size of titanium disks should be increased from a size of 24-well to be 6-well cell culture plates.

Adding investigations on genotypes and phenotypes of osteoblastic differentiation

As stated in the earlier section, quantitative real time polymerase chain reaction (qRT-PCR) and western blot analysis would be useful to determine expression and synthesis levels of genes and proteins associated with osteogenic differentiation, particularly in ED-condition. Calcein fluorescence staining should be performed to create visualization of in vitro mineralization on the titanium disks. The additional analyses might increase sensitivity of the investigations determining effects of SLA titanium surface on osteogenic differentiation in ED-condition.

Investigating mechanotransduction signals

As the results pointed out that attachment of osteoblasts to substrates initiated morphological change and intercellular-substrate interaction that might stimulate mechanotransduction signals and regulate growth and differentiation of cells on titanium surfaces, the interaction of mechanotransduction signals, through protein kinase C (PKC) and mitogen activated protein kinase (MAPK) cascades and mechano-responsive genes, c-fos and cox2 mediate osteointegraion[57, 99] should be investigated.

Supplementing osteogenic proteins or growth factors in estrogen-deprived cell culture

As it was shown that surface modification only could not restore suppressed osteogenic differentiation potential of ED-hBMSCs, it might be interesting to add active proteins such as bone morphogenetic protein-2, transforming growth factor-1 or simvastatin to enhance stimulating effects of rough titanium surface on osteogenic differentiation of hBMSCs in ED-cell culture.

Chapter 5

Conclusions

In conclusion, a long term estrogen-deprived cell culture model on titanium disks was established and a model provided a platform for studying effects of hormone and growth factor-deprivation on growth and differentiation of cells on titanium surfaces in vitro.

Titanium surface microtopography regulated growth and osteogenic differentiation of hBMSCs on the titanium surfaces. Cell adhesion, attachment, morphology, growth and osteogenic differentiation of hBMSCs were different on different cell culture surfaces. Stimulating effects of SLA on osteogenic differentiation was not found in hormonal-deprivation of charcoal-stripped cell culture. Sandblasted and acid-etched titanium surface could not promote osteogenic differentiation of ED-hBMSCs.

Charcoal-stripped cell culture decreased cell growth and osteogenic differentiation of hBMSCs. The results did not support a hypothesis that SLA titanium surface would be able to enhance osteogenic differentiation potential of estrogen-deprived-hBMSCs.

Inhibitory effects of charcoal-stripped cell culture on osteogenic differentiation of hBMSCs highlighted pivotal effects of hormones and growth factors on bone regeneration and osteointegration of dental implant.

The findings underlined a hypothesis that estrogen deficiency in postmenopausal osteoporosis cases could compromise osteointegration of the dental implants. A modification of titanium surface microtopography only might be inadequate to improve osteointegration in estrogen deficient osteoporotic bone.

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Appendix

Appendix

1. Equipment

1.1 Autoclave Hiclavetm,model HB-50, Hirayama Manufactoring Corp., Japan

1.2 Biohazard laminar airflow class II, astec microflow model ABS1200TCN, Bioquell lab, UK

1.3 Confocal laser scanning electron microscop,model FV300,Olympus,Japan

1.4 CO2incubator,seriesII water jacketed CO2 incubator model3111,thermo forma, USA

1.5 Digital camera,Nikon Coolpix model 4500, Nikon, Japan

1.6 Electric balance, adventurertm AR2140, Ohaus, USA

1.7 Hot air oven, model UM500 Memmert, USA

1.8 Inverted light microscope,elipse model TE2000-U, Nikon, Japan

1.9 Microplate reader (Multiskan GO, Thermo Scientific, Finland)

1.10 pH-meter,precisa model pH900,precisa instrument, Switzerland

1.11 Refrigerated Centrifuge, Labofuge model 400 R, Heraeus, Germany

1.12 Scanning electron microscope, JSM-5800LV, JEOL LTD, Japan

1.13 Sputter coater, SPI-ModuleTM, Model 11425, SPI supplies, USA

1.14. Ultrasonic cleaner, Cavitator Model ME11, Mettle Electronics Corp., USA

1.15 Water Bath, Model WB22, Memmert, Germany

2. Instrument

2.1 Automatic pipette, Portable Pipette-Aid Model XP, Drummond Scientific Company USA

2.2 Hemocytometer neubauer improved bright-line Dept 0.1 mm,model 717810,BRAND,Germany

2.3 Pipetter 20-microlitre, Pipetman Gilson®, France

2.4 Pipetter 200-microlitre, Pipetman Gilson®, France

2.5 Pipetter 1000-microlitre, Pipetman Gilson®, France

3. Materials

3.1 Tested material

3.1.1 titanium disks (Smooth and SLA), Straumann, Walden-burg, Switzerland

3.2 Disposable materials

3.2.1 Cell scraper 23 cm,SPL Life Sciences. Co. Ltd., Korea

3.2.2 Centrifuge tubes 15 ml, Corning, USA

3.2.3 Centrifuge tubes 50 ml, Corning, USA

3.2.4 Culture flask 75 square cm, Corning, USA

3.2.5 Culture plate, 6 well, Corning, USA

3.2.6 Culture plate, 24 well, Corning, USA

3.2.7 Culture plate, 96 well, Corning, USA

3.2.8 Disposable Pipette tips 10 µl, Treff AG, Switzerland

3.2.9 Disposable Pipette tips 200 µl, Treff AG, Switzerland

3.2.10 Disposable Pipette tips 1000 µl, Treff AG, Switzerland

3.2.11 Eppendorf centrifugation tube 1.5 ml, Eppendorf, Germany

3.2.12 Filter paper dia 110 mm, ALBET, Germany

3.2.13 Glass coverslips, BRAND, Germany

3.2.14 Glass Pasteur pipettes, BRAND, Germany

3.2.15 Microscope glass slides, BRAND, Germany

3.2.16 Sterile filter 0.2 µm, Sartorius, Germany

4. chemical and reagents

4.1 Alcohol 95%, LB Science, Thailand

4.2 Ascorbic Acid, Sigma, USA

4.3 Beta-glycerophosphate, Sigma, USA

4.4 CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA

4.5 CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate), Molecular Probes/Invitrogen, USA

4.6 Charcoal stripped FBS, Gibco, USA

4.7 Dexamethasone, Sigma, USA

4.8 Dimethylsulfoxide(DMSO), Analar®, BDHlaboratory supplies, England

4.9 Dulbecco's Modified Eagle Medium, Gibco, USA

4.10 Dulbecco's phosphate buffer saline, Gibco, USA

4.11 Ethanol absolute for analysis 500 ml, Sigma, USA

4.12 Fetal bovine serum, Biochrome AG, Germany

4.13 Fungizone , Amphotericin B, Gibco, USA

4.14 Hydrochloric Acid in 100 ml, Sigma, USA

4.15 Penicillin/Streptomycin, Gibco, USA

4.16 Phenol red free DMEM-F12, Gibco, USA

4.17 P-Nitrophenol standard solution 100ml, Sigma diagnostics®, USA

4.18 P-Nitrophenyl phosphate disodium salt, Calbiochem®, Germany

4.19 Protein standard II (Bovine Serum Albumin), Bio-Rad, USA

4.20 RC DC protien assay kit II, Bio-Rad, USA

4.21 Sodium Bicarbonate dry substance, Biochrom AG, Germany

4.22 Triton X-100, Sigma, USA

4.23 Trypsin/EDTA(0.5%/0.2%)in(10x)PBS without Ca2+,Mg2+, Biochom AG, Germany

5. Softwares

5.1 SPSS for windows, Version 16.0,Standard Software Package Inc., USA

Vitae

Name Miss Chonticha Chookiartsiri

Student ID 5510820025

Educational Attainment

Degree	Name of institutions	Year of Graduation
Doctor of Dental Surgery	Prince of Songkla University	2008
Higher Graduate Diploma in Clinical Science (Oral and Maxillofacial Surgery)	Prince of Songkla University	2012

List of proceeding

Oral Presentation

Chookiartsiri C, Pripatnanont P, Tangtrakulwanich B, Arpornmaeklong P **"**Effects of estrogen deprivation and titanium surfaces on osteogenic differentiation of human bone marrow stromal cells, an *in vitro* study" in The 12th Scientific Meeting of Dental Faculty Consortium Thailand (DFCT), July 1-3, Srinakharinwirot University, Nakorn Nayok, Thailand.