Chapter 2

Materials and Methods

1. Scope of study

This study is an *in vitro* study using cell culture models. Mouse calvarial osteoblast cell lines, MC3T3-E1, were seeded on a *smooth surface* (acid prickle) titanium disk and cultivated in a mineralized culture medium for 21 days. Specific and non-specific COX-2 inhibitors NSAIDs were added into the culture medium according to groups of study. The culture medium was changed every two days.

Based on the natural growth curve of cells on titanium disks (Fig.10), the experiments were performed in three different growth phases, the *static*, *log* and *plateau* phases. Each experimental phase, groups of the study were categorized into 5 groups, A – E. Group A: cells on a titanium disk treated with non specific COX-2 inhibitors, indomethacin, $0.1x10^{-6}$ M; Group B: cells on a titanium disk treated with specific COX-2 inhibitors, celecoxib, $1.5x10^{-6}$ M; Group C: cells on a titanium disk treated with celecoxib, $3x10^{-6}$ M; Group D: cells on a titanium disk treated with celecoxib, $9x10^{-6}$ M, and Group E: cells on a culture disk without medication treatment (Table 6).

To determine effects of the treated NSAIDs on attachment, growth and differentiation of cells, attachment of cells on titanium disks, cell vitality and expressions of ALP activity and osteocalcin were investigated. Levels of PGE_2 in a culture medium were measured to demonstrate inhibitory effects of the treated NSAIDs on PGE_2 production of cells.

The investigations in each experimental phase were performed when cells were incubated in a *serum free culture medium treated with NSAIDs* for 1, 3 and 5 days, on triplicate samples (Groups A-E, n=15). The results within each group and among groups at different investigation times were compared. Significant differences were set at $p< 0.05$.

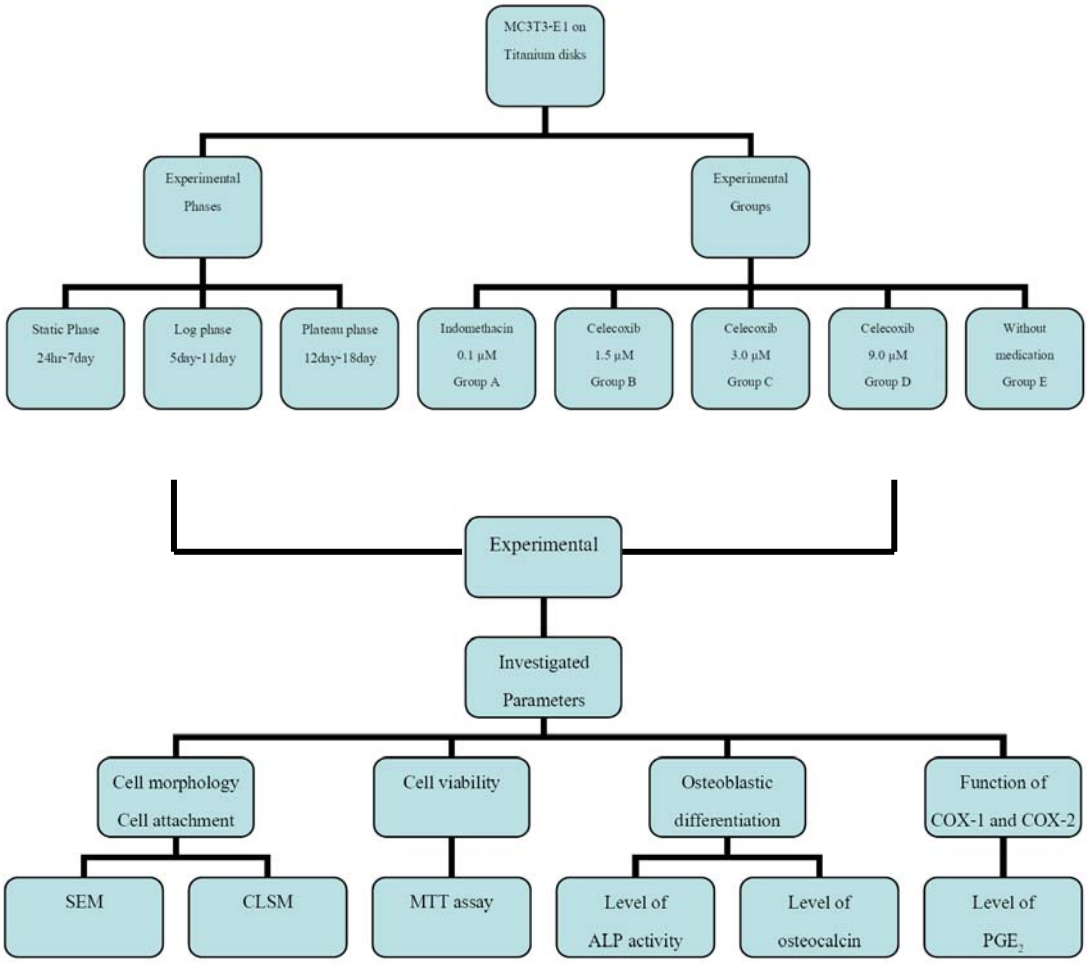


Fig. 7 Scope of study

Table 6 Groups of study

Groups of study	Descriptions
A	Cells on titanium disk + non specific COX inhibitors, indomethacin, $0.1x10^{-6}$ M
B	Cells on titanium disk + selective COX-2 inhibitors, celecoxib, $1.5x10^{-6}$ M
C	Cells on titanium disk + selective COX-2 inhibitors, celecoxib, $3x10^{-6}$ M
D	Cells on titanium disk + selective COX-2 inhibitors, celecoxib, $9x10^{-6}$ M
E	Cells on titanium disk + DMSO only

1.1 Groups of study

There were five groups (A-E) in the study. In Group A, a culture medium was treated with non specific COX-1 and COX-2 inhibitor, indomethacin, $1x10^{-7}$ M (0.1 μM); Group B: specific COX-2 inhibitors, celecoxib $1.5x10^{-6}$ M (1.5 μM); Group C: celecoxib $3.0x10^{-6}$ M (3.0 μM); Group D: celecoxib 9.0 $x10^{-6}$ M (9.0 μM), and Group E: a serum free culture medium in DMSO only (Fig 8, Table 6). Cell seeding on a culture plate was also done for a system control.

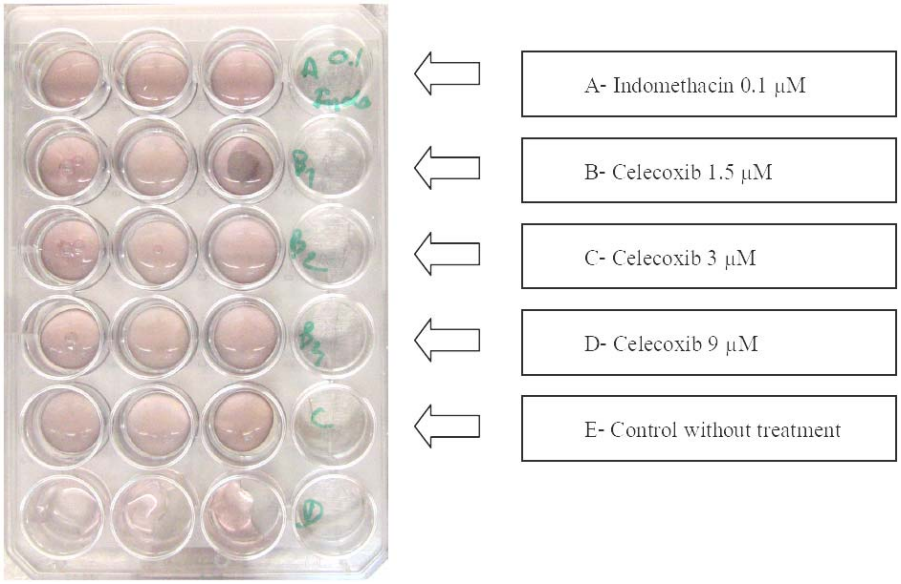


Fig. 8 MC3T3-E1 seeded on titanium disks, cultivated in a culture medium, treated with medications according to the group of study, (n=3)

2. Materials preparation

2.1 Material and reagent preparation procedures

2.1.1 Preparation of titanium disks for cell seeding

Commercially pure titanium disks with acid pricked surface, 15 mm in diameter with 1 mm thickness were kindly provided by Straumann (Straumann, Waldenburg, Switzerland). Disks were prepared for cell seeding according to Lohmann and co-workers [95]. Procedures in brief were as follows.

Prior to cell seeding, 15 titanium disks were sonicated (Cavitator® ME11, Mettler Electronics Corp.) in 300 ml of 70% Ethyl alcohol for 15 minutes, then they were 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 Corp.) (Fig.9). Disks were kept in aclean and dry place until used.

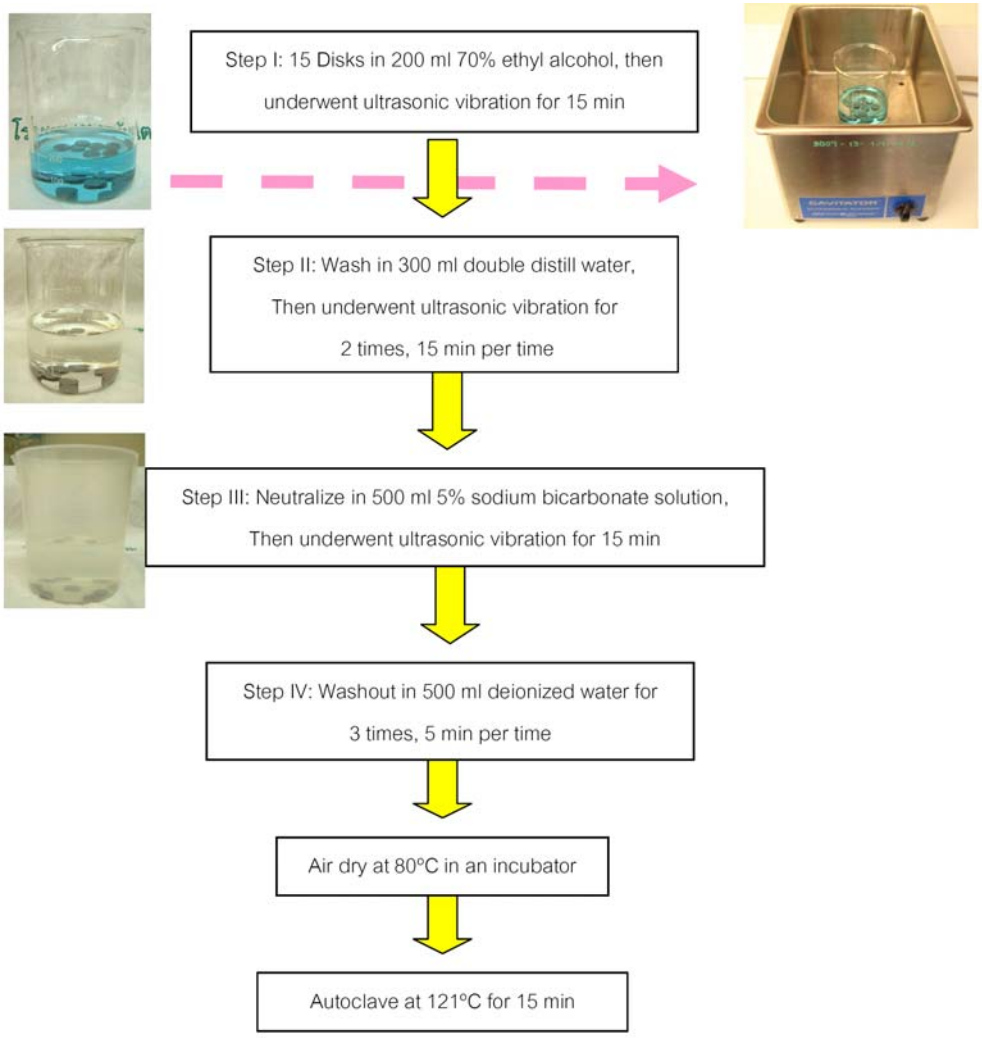


Fig. 9 Flow chart of preparation of titanium disks for cell seeding

2.2 Preparation of stock solutions for treated medications

Stock solutions of celecoxib 5,000 µM and 10,000 µM and indomethacin 5,000 µM in DMSO were prepared and kept at -20ºC until used.

Celecoxib stock solution was prepared by dissolving cerebrex in DMSO[28]. One capsule of cerebrex contains 200 mg of celecoxib and 75 mg of other inactive ingredients such as

croscarmellose sodium, edible inks, gelatin, lactose monohydrate, magnesium stearate, povidone, sodium lauryl sulfate and titanium dioxide, for a total weight of 275 mg per capsule.

To prepare 10 ml of 5,000 µM and 10,000 µM celecoxib stock solutions, 15.92 and 31.84 mg celecoxib (MW. 318.38) or 21.89 and 43.77 mg of celebrex powder was dissolved in each 10 ml DMSO, respectively.

To prepare 10 ml of 5,000 µM indomethacin stock solutions, 5 mg indomethacin (MW. 357.79, Sigma, USA) was dissolved in 10 ml DMSO using multiple pipetting to ensure a complete dissolution.

When diluting stock solution, 300- 1,000 µl of stock solution was used. Either indomethacin or celecoxib stock solutions were diluted into 1,000 µl DMSO to obtain desi10red concentrations for treatment. Then a total volume of 10 µl of the diluted stock solution was added into 10 ml of serum free α-MEM culture medium to achieve designated concentrations of medications in culture mediums according to the study groups. In a control group, Group E, 10 µl of DMSO without medication was added in to a serum free α-MEM culture medium. A concentration of DMSO in a culture medium was set not to be higher than 0.157% vol/vol and a treatment was done immediately just before use [96]. A list of concentrations of stock solutions and volume of treated medications are demonstrated in Table 7.

Table 7 A list of concentrations of stock solution, volume of treated medications

Stock solutions	Concentrations of stock solutions (µM)	Concentrations of ***diluted*** stock solution (µM)	Preparation of 1000 µl of ***diluted*** stock solutions		Concentrations of treated medications in culture medium (10 µl diluted stock : 10 ml culture medium)
			Volume of stock solution	Volume of DMSO	
Indomethacin	100 µM	0.1	1000 µl	-	0.1 µM
Celecoxib	5,000 µM	1.5	300 µl	700 µl	1.5 µM
	10,000 µM	3	300 µl	700 µl	3 µM
	10,000 µM	9	900 µl	100 µl	9 µM
Control group	without treatment	-	-	1000 µl	0 µM

2.3 Osteogenic cell culture of MC3T3-E1

A fetal mouse calvarial osteoblast cell line, MC3T3-E1 (ATCC, USA), was passaged every 2-3 days into passages 6-8. MC3T3-E1 cells were cultivated in a complete culture medium consisting of an alpha-minimum essential culture medium (α-MEM) treated with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% Fungizone, 50 µg/ml ascorbic acid and incubated in 5% CO_2 at 37ºC in 95% relative atmospheric humidity. The culture medium was changed every 2 days[59].

MC3T3-E1 cells in passages 6-8, $4x10^4$ cells in 200 µl of culture medium, were statically seeded onto each 15 mm. in diameter titanium disk in 24-well culture plates. Cells were allowed to attach on disks in a minimal culture medium for 3 hours in 5% CO_2 at 37ºC in 95% relative atmospheric humidity. Subsequently, 1.5 ml of a complete culture medium was added into each well. On the plastic surface of a 24-well cell culture plate, MC3T3-E1 cells in passages 6-8, $4x10^4$ cells in 1.5 ml of complete culture mediums were added into each well.

2.4 Establishing growth curves of cells on a titanium surface in culture mediums with and without fetal bovine serum

2.4.1 Culture medium with fetal bovine serum

To establish natural growth curve of cells on titanium disks, osteoblasts, MC3T3-E1, $4x10^4$ cells in passage 6 were cultivated in a complete culture medium for 21 days. The culture medium was changed every 3 days. A cell viability test using an MTT assay was performed every 3 days after cell seeding to establish the natural growth curve of cells (Fig.10).

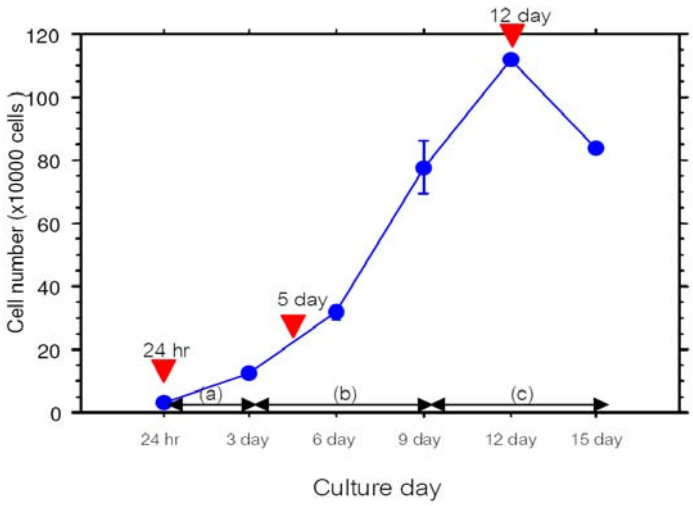


Fig. 10 Growth curve of MC3T3-E1 on titanium disks cultivated in culture medium treated with fetal bovine serum for 15 days, (a) growth of cells in *static* (b) *log* and (c) *plateau* phases

2.4.2 Serum free culture medium on titanium disks

To investigate effects of serum free culture medium on cell viability and determine a maximum culture time in serum free culture mediums for medication treatment. Osteoblasts $4x10^4$ cells in passage 6 were seeded on each titanium disk and were cultivated for 24 hours after cell seeding in a culture medium supplemented with 10% FBS. After that the culture medium was removed and cells were washed twice with PBS. Then 1.5 ml of culture medium without FBS was added into each well and cultivated for 15 days.

Growth of cells in the culture medium during 1 – 5 days of medication treatment was monitored in comparison to growth of cells in serum free culture medium without medication treatment.

Effects of serum free culture medium on attachment and morphology of cells on titanium disks in different stages of cell growth were investigated using scanning electron microscope (SEM).

2.5 Experimental phase

Investigations were performed in three different stages of cell growth which were categorized into experimental *static*, *log* and *plateau* phases according to growth curve of cells which started on days 1, 5 and 12 after cell seeding, respectively (Fig.10).

2.6 Medication treatment scheme

In each experimental phase, non-specific COX-1 and COX-2 inhibitors NSAIDs, indomethacin 0.1 μM and specific COX-2 inhibitors NSAIDS, celecoxib, 1.5, 3 and 9 μM, were added into a serum free culture medium for 1 – 5 days.

At the beginning of each phase the culture medium with fetal bovine serum was changed to a serum free culture medium. Cells were washed two times with 1.5 ml of phosphate buffer saline (PBS) and incubated in 1.5 ml of a serum free culture medium for 24 hrs. Then the culture medium was changed to a serum free culture medium with treated NSAIDs according to the groups of study and after that a fresh culture medium with treated NSAIDs was changed every 48 hrs. The investigations were performed after cells were incubated in culture mediums with treated NSAIDs for 1, 3 and 5 days (on days 3, 5 and 7 in a serum free culture medium), thus changing of the treated medium every 48 hrs allowed cells to be incubated in freshly treated NSAIDs for 24 hr before undergoing any investigations (Fig. 11).

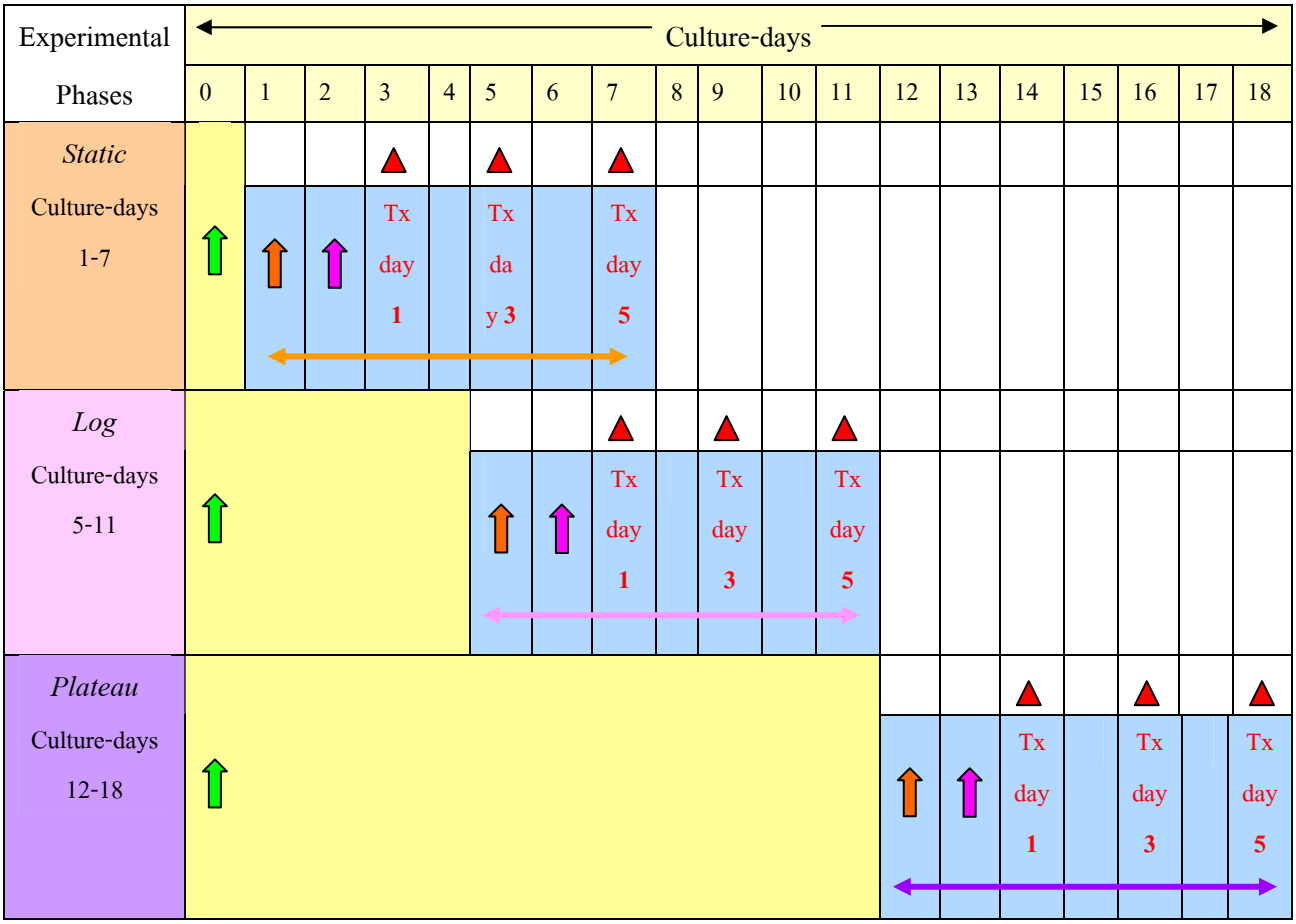


Fig. 11 An overview of experimental design (experimental phases categorization, NSAIDs treatment schemes and investigation time)

Note:

Cell culture on titanium disks in a culture medium supplemented with serum

NSAIDs treatment period in a serum free medium in each experimental phase, *static*, *log* and *plateau* phases

Starting of cell seeding on titanium disks in a culture medium supplemented with serum

Starting of serum free mediums of each experimental phase, *static* on culture-day 1, *log* on culture-day 5 and *plateau* phases on culture-day 12

Starting NSAIDs treatment

Investigation time, on treatment (Tx) days 1, 3 and 5 of each experimental phase

2.7 Investigation

2.7.1 Investigated parameters

Attachment of cells on titanium surfaces, cell growth, markers of osteoblastic differentiation, expression of ALP activity and osteocalcin, and secretion of PGE_2 in a culture medium were investigated. Attachment and morphology of cells on titanium disks were examined using SEM and CLSM. Cell viability was determined using MTT assay to determine growth of cells. Levels of ALP activity in cells and extracellular matrix and osteocalcin in culture medium were investigated using biochemical analysis and ELISA, respectively. Levels of PGE_2 in culture mediums were measured to demonstrate inhibitory effects of NSAIDs on the function of cyclooxygenase enzymes on PGE_2 production using ELISA.

2.7.2 Sets of investigations

Investigations were categorized into three sets of investigations, Set I: examining morphology and attachment of cells on titanium disks using SEM and CLSM, Set II: measuring cell viability, and Set III: measuring levels of ALP activity and protein contents in cells and extracellular matrix and levels of osteocalcin and PGE_2 in culture mediums. All investigated parameters were performed in all groups of study, except for assessment of attachment, and morphology of cells by CLSM were observed only in Groups A, C and E (Table 8).

Table 8 A summary of the investigation scheme

Sets of investigations	Investigations	Investigating times	Investigated Groups
I	SEM of cell morphology	On treatment day 5	Groups A, D and E
	CLSM of cell morphology	On treatment day 5	Groups A, D and E
II	MTT assay	On treatment days 1, 3 and 5	Groups A - E
III	ALP activity	On treatment days 1, 3 and 5	Groups A - E
	Osteocalcin in culture medium	On treatment day 5	Groups A - E
	PGE_2 content in culture medium	On treatment day 5	Groups A - E

2.8 Investigated times and numbers of samples

Cell viability and ALP activity were detected on treatment days 1, 3 and 5. Attachment and morphology of cells and levels of osteocalcin and PGE_2 in culture mediums were detected on treatment day 5 (Table 8,9). The investigations at each investigation time of each group of study were performed on triplet samples (n = 3) (Fig.11 and Table 8,9).

Table 9 A summary of the investigation procedure

Investigations (n = 3)	Parameters	Timing / in each phase
Morphology	CLSM	Day 5
Growth	MTT assay	Day 1, 3 & 5
Differentiation	ALP activity	Day 1, 3 & 5
	Osteocalcin	Day 5
Function of COX	PGE_2	Day 5

3. Investigation procedures

3.1 Observing cells on titanium disks using a scanning electron microscope (SEM)

Cell morphology on the disk was examined using a scanning electron microscope (SEM) (JEOL, JSM-5800LV) at the Scientific Equipment Center, Prince of Songkla University. On the examination day, culture medium was removed and the disks were washed twice with PBS to remove unattached cells on the surfaces. Then the disks were fixed with 2.5% glutaraldehyde in PBS for 2 hours at RT, washed twice with PBS for 10 minutes each time and fixed with 1% osmium tetroxide for 1 hour at RT. After that, the disks were washed with distilled water three times and dehydrated with a graded ethanol series of 50%, 70%, 80% and 90% twice for 30 minutes in each step. Then they were dehydrated twice in 100% pure ethanol for 30 minutes each time and were put into baskets and fixed with absolute alcohol. After that they were critically point dried by a critical point dryer machine (Polaron, UK). Subsequently, the disks were sputtered and coated with gold-palladium, size 20 nm, using a SPI-Module™ Sputter coater (SPI,

USA) and were examined using SEM (JEOL, JSM-5800LV model, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of morphology and attachment of cells on to the disks[97] (Fig.12,13).

(a) Disks were attached on a stub

(c) SPI-Module™ Sputter coater (SPI, USA)

(b) Disk were sputtered coated with gold-palladium particles

Fig. 12 Demonstrating a gold-palladium coating procedure using a SPI-Module™ Sputter coater (SPI, USA)

JEOL, JSM-5800LV model, Japan

Fig. 13 Demonstrating examination of cells on titanium disks using SEM (JEOL, JSM-5800LV model, Japan)

3.2 Confocal laser scanning microscope (CLSM)

Attachment and morphology of cells on titanium disks were examined using Confocal laser scanning microscope (CLSM) (Olympus, FV300, Japan) at the Scientific Equipment Center, Prince of Songkla University. On an examination day, culture medium was removed and the disks were washed twice with PBS. Then cells on disks were incubated in 1.5 ml of 5 µg/ml fluorescein diacetate (FDA) in a serum free medium for 1.3 hrs at 37ºC in a 5% CO_2 incubator. After that the disks were rinsed twice with PBS and examined within 30 minutes under CLSM (Olympus, FV300 model, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of attachment and distribution of cells on the disks[98] (Fig.14).

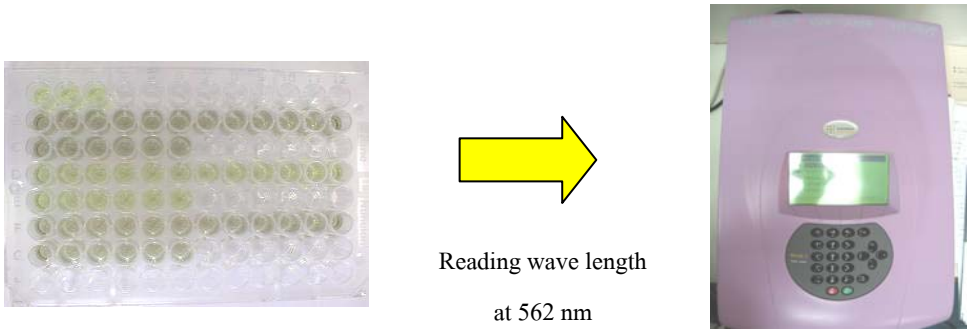
Fig. 14 Demonstrated examing of cells on a titanium disk using CLSM (Olympus FV300 Japan)

3.2.1 Cell viability assay

MTT assay is a calorimetric method determining the number of viable cells in proliferation or cytotoxicity assays. Metabolism of cells was measured in correlation to the number of cells. The principle of assay is based on bioreduction of mitochondria of viable cells in cleaving a tetrazolium compound into a color formazan dye. This quantity of formazan product is directly proportional to the number of living cells. Cell viability was determined using a CellTiter 96® Non-Radioactive Cell proliferation Assay (Promega, USA). Measurement procedure was performed according to the manufacturer's instruction.

The procedure in brief: seeded cells on titanium disks were incubated in 350 µl of complete culture medium containing 10% CellTiter 96® Solution for 2 hours in 5% CO_2 at 37ºC. Then an equal volume of solubilization solution/stop-mix was added into each well and left to stand overnight in an incubator at 37ºC with 5% CO_2. Then the incubated medium was read at 562 nm absorbance in triplicate using a microplate reader (Biotrak™ II microplate reader, UK) (Fig.15).

The cell number was determined from an established standard curve of optical density and cell numbers. A standard curve was developed from the assay of known numbers of MC3T3-E1 cells ranging from $1x10^4$ – $1x10^6$ cells/well performing in triplicate samples. A linear diagram was established from a best fit equation of reading optical densities and cell numbers with R^2 confidence levels of 0.995 (Exponential equation is $y = 0.0333x0.7133$) (modified from Rea *et al*., 2004)[99,100] (Fig.16).



MTT assay in 96-well plate

Biotrak™ II microplate reader

Fig. 15 MTT assay in a96 well plate was measured using a microplate reader under wave length at 562 nm (Biotrak™ II microplate reader, UK)

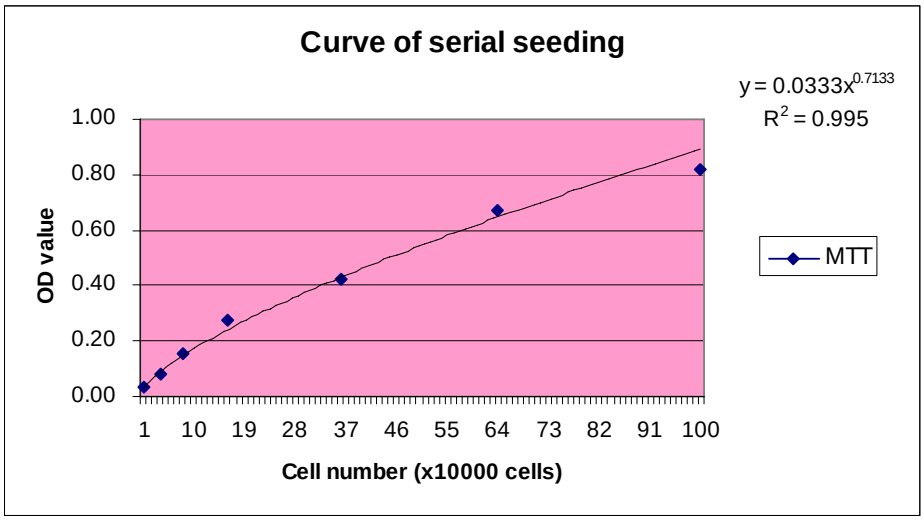


Fig. 16 An exponential diagram established from a best fit equation of optical density (OD value) and cell numbers

3.2.2 Measuring of alkaline phosphatase activity (ALP) in protein solution

3.2.2.1 Cell lyses

Cells were lysated using mechanical and chemical methods. Nonionic detergent, 1% Triton X-100 in phosphate buffer saline (PBS), a mild and nondenaturing detergent, was used to solubilize membrane proteins in a combination with vibration force to obtain protein solutions for ALP activity and protein content analyses.

The procedure in brief: 300 µl of 1% Triton X-100 in PBS were added into each well of the 24-well culture plates containing a titanium disk and kept at -20ºC. The disks underwent repeatedfreezing and thawing processes for two cycles. In one cycle, disks with cells were thawed at room temperature for 60 minutes and refrozen at -20ºC for 30 minutes. After two cycles, the plates were vibrated in an ultrasonic cleaner (Cavitator® ME11, Mettler electronics corp.) for 30 minutes, then cells were scraped from the disks using a rubber polish man (TRP AG, Germany). After that, solution and scrapped cells were transferred into 1.5 ml microcentrifuge tubes, vortexed vigorously for 30 seconds, incubated on ice for 1 hour and 30 minutes, re-vortexed vigorously for 30 seconds and centrifuged at 2000 g (Labofuge 400 R , Heraeus) at 4ºC for 10 minutes[101]. The supernatants were transferred into new microcentrifuge tubes and stored at -20ºC for analyses of ALP activity and protein content.

3.2.3 Measurement of ALP activity

The assay is aimed to measure alkaline phosphatase activity of protein extract solution obtained from detergent cell solubilization using 1% Triton X-100 in PBS. An assay for ALP activity is based on the hydrolysis of p-nitrophenyl phosphate by ALP yielding p-nitrophenol and inorganic phosphate. p-Nitrophenol converted into a yellow complex is readily measured at 400-420 nm.

The procedure in brief: cell lyses solutions were thawed on ice. Four hundred microliters of ALP lyses solution (2 mg p-Nitrophenyl phosphate in 1 ml 1.5mol/l concentrations 2-amino-2-methyl-1-propanolol) was pipetted into each tube containing 100 µl of cell lysate solution. The solution was thoroughly mixed on a vortexer and incubated at 37ºC for 1 hour. Then 400 µl of 0.05 N NaOH and 100 µl of distilled water were added into each tube to stop the reaction. The mixtures were vortexed for a thorough mixing of the solutions, after that they were pipetted into each well of 96-well plates 200 µl/well, in triplet manner. Levels of p-Nitrophenol in the solutions were measured by measuring light absorbance at 405 nm (Biotrak™ II microplate reader, UK).

The levels of intensity of yellow color are directly proportional to concentrations of p-Nitrophenol in standard solutions. The slope of absorbance used to calculate the ALP activity was based on values of a series of p-Nitrophenol standards. A stock solution of 10 µM p-nitrophenol is diluted with 0.02 N NaOH to obtain a series of p-nitrophenol standards ranging from 0.1-1 µM (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µM). The specific activity of ALP was calculated as units/mg protein[55].

3.2.4 Measurement of protein concentration

An analysis of protein concentration in cell lysate solution is performed according to Lowry's assay. A principle of an assay is based on the reaction of protein with copper in alkaline medium producing copper-treated protein. This reaction leads to a reduction of Folin reagent resulting in color development into a blue color with a range of absorbances at 405 - 750 nm. Levels of intensity of blue color are directly proportional to concentrations of protein standards in a standard curve. Measurements were performed according to the manufacturer's instruction (Bio-Rad, DC Protein Assay Instruction Manual).

The procedure in brief: a series of protein standard with a range of concentrations from 0.2-1.4 mg/ml (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/ml) were prepared by diluting 1.4

mg/ml bovine serum albumin in PBS with PBS. Sixty microlitres of protein solutions and standard solutions were pipetted into each tube containing 30 µl reagent A' (a mixture of 20 µl reagent S in 1 ml reagent A), then 240 µl reagent B were added into each tube and mixed gently. The mixtures were kept in darkness for 15 minutes, after that 200 µl of the mixtures were pipetted into 96-well culture plates in triplicate fashion. Levels of protein in the solutions were measured by measuring light absorbance at 620 nm (BiotrakTM II microplate reader, UK). Concentrations of protein were extrapolated from a standard curve of a series of protein standard (Bio-Rad, DC Protein Assay Instruction Manual). Concentrations of proteins in cell lysate solution were reported as mg/ml.

3.2.5 Measurement of osteocalcin in culture medium

A measurement of osteocalcin in a culture medium was performed using a Mouse osteocalcin EIA kit (BT-490, Biomedical Technologies Inc, USA). This sandwich EIA kit is highly specific for mouse osteocalcin recognizing both carboxylated and decarboxylated mouse osteocalcin. A polyclonal antibody directed against the N-terminus is bound to the polystyrene wells (plate coated with osteocalcin antibody). After incubation with a sample, a secondary antibody is applied. Detection is achieved by a third incubation using horseradish peroxidase-streptovidin conjugate and a subsequent enzyme assay. The osteocalcin concentration is proportional to color development. Standard solutions of highly purified rat osteocalcin are used to generate a standard curve.

The procedure in brief: cell culture medium without serum kept at -20ºC was thawed on ice and centrifuged at 16000 g for 10 min at 4ºC. The supernatants were used for the analysis. A series of standards were prepared as dilutional standard concentrations ranging from 1.56 - 50 ng/ml (1.56, 3.12, 6.25, 12.5, 25 and 50 ng/ml). Twenty five microliters of sample buffer, standard and samples were pipetted into a coated plate as designated followed by 100 µl of osteocalcin antiserum in all wells. A microplate was incubated in a humidified chamber at 4ºC for 18 hours. After an overnight incubation, the microplate was washed manually five times with a phosphate-saline wash buffer. Then 100 µl of Streptavidin–horseradish peroxidase reagent were added into each well, swirled and incubated at room temperature for 30 minutes. After that, a microplate was washed as stated above, then a mixture of 100 µl of 3,3',5,5'-tetramethyl benzidine (TMB solution) and Hydrogen peroxidase solution at 1:1 ratio was added into all wells and incubated at RT in darkness for 15 minutes followed by an addition of 100 µl of stop solution

in all wells. The mixture was swirled and measured absorbance at 450 nm within 15 minutes using a microplate reader (BiotrakTM II microplate reader, UK). Concentrations of ostreocalcin were extrapolated from a standard curve of serial dilution of highly purified mouse osteocalcin and reported as ng/mg protein (Mouse Osteocalcin EIA Kit Instruction Manual, Biomedical Technologies Inc.).

Osteoclacin is markedly unstable in vitro, rapidly degrading in sample at room temperature and at 4°C. It is recommended that samples are kept at 4°C immediately up on venepuncture serum or plasma separated as soon as possible, and sample frozen until assay. For short-term storage, 1 month freezing at-20°C will suffice, but for long-term storage it is advisable to store samples at -70°C. In this study the sample of osteoclacin measurement was immediately frozen at -20°C and for long term storage they are transferred to a freezer at -70°C[102].

3.3 Measurement of PGE_2 in culture medium

Prostaglandin E_2 in culture medium is measured using Prostaglandin E_2 High Sensitivity Immunoassay (DE2100, R&D Systems Inc., USA). The assay is based on the competitive binding technique in which PGE_2 present in a sample competes with a fixed amount of alkaline phosphatase-labeled PGE_2 for sites on a mouse monoclonal antibody. A microplate is coated with the goat anti-mouse antibody. During incubation the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove unbound samples and excess conjugate, a substrate solution is added to the wells to determine the bound enzyme activity. Immediately following color development, the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of PGE_2 in the sample.

The procedure in brief: serum free culture medium kept in -20ºC were thawed on ice and centrifuged at 11500 g for 5 min at 4ºC. The supernatants were used for the analysis. A series of standard dilution for concentrations ranging from 7.8-1000 pg/ml (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 pg/ml) were prepared by diluting, provided highly purified PGE_2. One hundred microliters of samples were added in to each well, followed by 50 µl of PGE_2 HS conjugate and 50 µl of PGE_2 HS Antibody Solution, respectively, and incubated in a humidified chamber for 18 hours at 4°C. After an incubation, the microplate was manually washed 5 times using 300 µl wash buffer per well per time. Then 200 µl of pNPP Substrate was added to all wells

and incubated for 1 hr at 37°C. After that 50 µl of Stop solution was added to all wells and measured absorbance immediately at 405 nm with wavelength correction set at 562 nm (Prostaglandin E2 High Sensitivity Immunoassay Instruction Manual).

4. Data analysis

Microstructure of disks and morphology of cells on disks were descriptively described. Parametric data of cell viability (MTT assay), ALP activity and levels of osteocalcin and PGE_2 of 3 consecutive samples were presented as average values with a standard deviation (Mean±SD).

The data was tested for normal distribution and homogeneity of variances. Differences among groups at each time point were analyzed using one-way analysis of variance (ANOVA). When a difference was statistically significant at $p < 0.05$, a multiple comparison test was then performed. If the variances of the data were homogenized, the Tukey HSD method was applied. Otherwise the Dunnette T3 method was used. Significant differences were set at a 95% confidence interval ($p<0.05$).

The analyses were performed on a personal computer using Commercial SPSS software (Version 11.0, Standard Software Package Inc., USA) for data analysis and Stat View for Windows (Version 4.5, USA) for graphic demonstrations.