Effect of Novel Glass- Ionomer Cements on Human Osteoblasts

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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.

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ชื่อวิทยานิพนธ์ ผลของกลาสไอโอโนเมอร์ซีเมนต์ชนิดใหม่ต่อเซลล์สร้างกระดูกมนุษย์
ผู้เขียน นางสาวจิรพร แสงสุวรรณ
สาขาวิชา ชีววิทยาโมเลกุล และชีวสารสนเทศ
ปีการศึกษา 2557

บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาผลชีวภาพบางประการของวัสดุกลาสไอโอโนเมอร์ซีเมนต์(GI) สูตรต่างๆ ต่อเซลล์สร้างกระดูกมนุษย์ที่มีการเติมโปรตีนและไม่เติมโปรตีน *Pmer*-TCTP

วิธีการทดลอง: ชิ้นทดสอบกลาสไอโอโนเมอร์ซีเมนต์สูตรใหม่ (BIO-GI)เตรียมโดย ผสมไคโตซานร้อยละ 15 ตามสัดส่วนน้ำหนักและ อัลบลูมินร้อยละ 5 ลงในส่วนผงของกลาสไอโอโนเมอร์ซีเมนต์และโปรตีน *Pmer*-TCTP โดยองค์ประกอบส่วนน้ำคงเดิม แบ่งกลุ่มตามองค์ประกอบเป็น 4 กลุ่ม ดังนี้ กลุ่มที่ 1: GI กลุ่มที่ 2: GI+TCTP กลุ่มที่ 3: BIO-GI กลุ่มที่ 4: BIO-GI+TCTP ทดสอบกับเซลล์สร้างกระดูก โดยศึกษาการคงคุณสมบัติของโปรตีน *Pmer*-TCTP ที่ปลดปล่อยออกมาจากชิ้นงาน ด้วยการทดสอบเวสเทิร์น บลอท (western blotting) ดูการเพิ่มจำนวนของเซลล์ ตรวจหาอัคคาไลน์ฟอสฟาเตสแอคติวิตี้ (alkaline phosphatase activity) ตรวจดูการสร้างแร่ธาตุ และดูการแสดงออกของยีน BMP2และOPNในเชิงปริมาณ และนำชิ้นทดสอบไปศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอน

ผลการทดลอง: เมื่อเซลล์สร้างกระดูกได้รับโปรตีน *Pmer*-TCTP พบว่าความมีชีวิตของเซลล์เพิ่มขึ้นที่ระดับความเข้มข้น (1-100 ng/ml) โดยแปรผันตามความเข้มข้นของโปรตีน *Pmer*-TCTP และเวลาที่ใช้เพาะเลี้ยงเซลล์ ชิ้นงาน GI และ BIO-GI ที่เติม*Pmer*-TCTP สามารถเพิ่มจำนวนเซลล์ขึ้นอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ค่าอัคคาไลน์ฟอสฟาเตสแอคติวิตีของซลล์ในกลุ่ม BIO-GI+TCTP ที่เพาะเลี้ยงเป็นเวลา 7วัน มีค่าสูงกว่ากลุ่มอื่นๆอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ผลการสะสมแร่ธาตุเรียงจากมากไปน้อยได้ดังนี้ ชิ้นทดสอบ BIO-GI+TCTP >GI+TCTP> BIO-GI> GI การศึกษาด้วยภาพจุลทรรศน์อิเล็กตรอนแบบส่องกราด แสดงให้เห็นเป็นก้อนกลมซึ่งอาจจะเป็นโปรตีน *Pmer*-TCTP ภายในชิ้นงาน กลุ่มที่มีการเติม *Pmer*-TCTP ค่อยๆมีการแสดงของยีน BMP-2, OPN เพิ่มขึ้นหลังจากเลี้ยงเซลล์บนชิ้นงาน 1 วัน และมีการแสดงออกลดลงหลังจากเพราะเลี้ยงเป็นเวลา 7 วัน

สรุปผลการทดลอง: ฤทธิ์ทางชีวภาพของโปรตีน *Pmer*-TCTP ที่เติมลงไปในกลาสไอโอโนเมอร์ซีเมนต์ไม่ได้ถูกทำลายไปและเพิ่มความมีชีวิต เพิ่มจำนวนของเซลล์รวมทั้งสนับสนุนการแปรสภาพ (differentiation) และสะสมแร่ธาตุของเซลล์สร้างกระดูกมนุษย์

คำสำคัญ: โปรตีน *Pmer*-TCTP, กลาสไอโอโนเมอร์ซีเมนต์, เซลล์สร้างกระดูกมนุษย์, ไคโตซาน

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ABSTRACT

Objective: The purposes of this study were to evaluate the some biological properties of different formula of glass ionomer cements (GIs) with and without *Pmer*-TCTP of GIs on human osteoblasts.

Methods: Conventional glass ionomer cement (GI) has been modified to BIO-GI by adding 15% chitosan (weight/weight), 5%BSA and *Pmer*-TCTP to the GI powder while the liquid components had no change. Four groups of the specimen due to different compositions were assigned as: GI, GI+TCTP, BIO-GI, BIO-GI+TCTP. *Pmer*-TCTP released from GIs was detected by western blottingHuman osteoblasts (NHOst cells) were seeded on specimens directly before these assays: BrdU assay, alkaline phosphatase activity, Alizarin red S (ARS) assay and quantitative real-time PCR (qPCR). Specimens were examined under scanning electron microscope (SEM).

Results: The viability of NHost cells was significantly increased after *Pmer*-TCTP (1-100 ng/ml) treatment in a dose- and time-dependent manner. The cells proliferation of NHost cells was significantly increased in TCTP supplemented in the specimen at 100 ng/ specimen ($p < 0.05$). The mean of alkaline phosphatase activity in the BIO-GI+TCTP at 7 days was significantly higher than other groups ($p < 0.05$). The percentages of mineralization were reported respectively from the highest to the least, BIO-GI+TCTP > GI+TCTP> BIO-GI> GI. The crosssection SEM micrographs of the specimens showed some spherical particleswithin the voids that possibly are globular protein *Pmer*-TCTP. The fold change of BMP-2, OPN gene expression of NHost cells cultured on *Pmer*-TCTP supplemented GIs increased gradually up- regulation after day 1 and down- regulation on day 7.

Conclusion: The biological properties of *Pmer*-TCTP released from the GIs were not denatured by the matrix formation and data demonstrated that *Pmer*-TCTP increased the viability, proliferation and differentiation and mineralization in human osteoblasts.

Keywords: *Pmer*-TCTP, Glass ionomer cement, Human osteoblasts, chitosan

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Jiraporn Sangsuwan

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LISTS OF ABBREVIATIONS

α-MEM	Alpha modification of Eagle's medium
ß	Beta
ANOVA	One-way analysis of variance
ALP	Alkaline phosphatase
bp	Basepair
BMP-2	Bone morphogenic protein 2
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CS	Chitosan
ºC	Degree celcius
DMSO	Dimethyl sulfonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bivin serum
G	Gram
GIC	Glass ionomer cement
HEPE	(N-(2-Hydroxyethyl) piperazine-N'(2- ethanesulfonic acid))
H	Hours
dNTP	Deoxyribonucleoside triphosphate
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertaini
µl	Microlitre
µg	Microgram
µM	Micromolar

LISTS OF ABBREVIATIONS (CONTINUED)

mg	Milligram
min	Minute(s)
ml	Milliliter
mM	Millimolar
%	Percent
M	Molar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide
OD	Optical density
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline- Triton X-100
PCR	Polymerase Chain Reaction
pH	-Log hydrogen ion concentration
rpm	Round per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TEMED	N, N, N, N'-tetrametyl-ethylenediamine
Tris-HCl	Tris (hydroxymethyl) aminoethane hydrochloric acid
Trypsin-EDTA	Trypsin-ethylenediamine tetraacetic acid
U	unit (s)
v/v	Volume/volume
w/v	Weight/volume
WR	Working reagent

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CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONAL

Tissue engineering has regeneration of functional as a promising approach for used an implant materials in the damaged bone and regeneration of tissues/organs lost or damaged as a result from trauma, injury, bone pathology disease or aging. The development and popularization of a shortage of living tissues and organs available for transplantation.[6,9] The most important advances in the field of biomaterials over the past few year have been in bioactive biomaterials. Currently, materials have been developed for bioactivity through biological recognition, including incorporation of adhesion factors, incorporation of growth factors, polyanionic sizes and endless quantity that mimic the electrostatics of biological regulatory polysaccharides involved in cell proliferation and cell migration. [6, 7, 9]

Glsss ionomer cements (GIC) have been extensively used in dentistry and medical applications.[17] GIC due have to their excellent biocompatibility in dental beneficial properties included adhesion to untreated tooth mineral, ion leachability, antibacterial properties, capacity to bone, enamel, dentine and meterials.[17, 21] GIC this biomaterial has to prolong the release of fluoride.[17] In addition, to increase the property of sustained release of protein such as growth promoting factors and bioactive molecules including vital pulp therapy and bone tissue engineering.[32]

Chitosan is a product of chitin, a hight molecular weight natural polymer. Chitosan is a co-polymer of glucosamine and N-acetyl-D-glucosamine. Chitosan has been widely used as scaffold in bone tissue engineering. Advantageous properties of chitosan are its non-toxicity, biodegradability, osteoconductive, availability and biocompatibility.[11, 13] It is a biodegradable polymer used in various

biomedical and pharmaceutical application due to its biocompatibility, the slow release of active molecules and used in controlled drug release.[13]

Translationally controlled tumor protein (TCTP) is a highly conserved protein, and the levels of TCTP are regulated by a various signals, such as growth signal, cytokines various stress conditions and anti-apoptotic. However TCTP can up- or down-regulate.[3, 4] Currently, TCTP is in important cellular processes, include cell growth, cell proliferation and cell cycle progression, and can protect cells from heat shock, oxidative, Ca^{2+}, heavy metals and apoptosis due to its biological properties.[34]

In this study, the biological properties of *Pmer*-TCTP released from GIs have been developed to incorporate bioactivity through growth factor and biological molecules therefore this study is to evaluate the role and impact of biomaterials in bone healing/regeneration application.

LITERATURE REVIEW

1. Bone cell biology

Bone is a dynamic biological tissue that skeleton is a formidable mass of living tissue occupying about 9% of the body by bulk and no less than 17% by weight.[6, 12] The stability and immutability of dry bones and their persistence over the centuries, and even millions of years after the soft tissues have turned to dust, gines us a false idea of bone during life. Bone is a connective tissue that consists principally of a mineralized extracellular matrix plus the specialized cells, osteoblasts, osteocytes and osteoclasts. The structural component of the organic phase is collagen type I (fibrous) which comprises about 90% of the bone protein the in-organic phase is mainly tiny crystals of the alkaline mineral hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$[6, 12, 22].

Long bones distinctive of the body's extremities, exhibit a number of commom rudiment structure features. The central region of the lone bone is the most clearly tubular. At one or commonly both ends, the diaphysis flares outward and assumes a predominantly cancellous internal structure. This region (metaphysis) (Fig.1) functions to transfer loads from weight-bearing joint surfaces to the diaphysis. [9] Eventually, at the end of a region know as an epiphysis, which exhibits a cancellous internal structure and comprises the bony substructure of the joint surface. Prior to full skeletal maturity the epiphysis is separated from the metaphysis by a cartilaginous plate called the growth plate or physis in bones[7, 9] with complex articulation or bones with multiple protuberances there might several separate epiphyses, each with its growth plate.

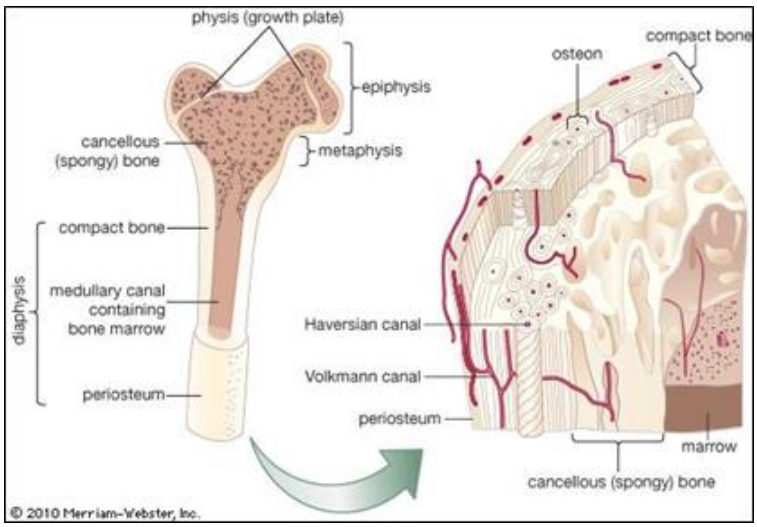


Figure 1. Internal structure of a human long bone, with a magnified cross section of the interior. (Chen *et al*., 2007)

1.1 Osteoprogenitor cells

The osteoprogenitor cells of osteogenic differentiation involves the expression of alkaline phosphatase, the synthesis and deposition of collagen type I, bone matrix protein (osteonectin, osteopontin) and glycosaminoglycans (decorin, biglycan) followed by the expression of osteocalcin and bone sialoprotein at the onset of mineralization.[22] At the end of bone formation, most osteoblasts become flattened lining cells, some become osteocytes and other undergo apoptosis.[30,32] After appropriate stimuli of differentiation of mesenchymal stem cells, in this case towards the osteoblastic pathway, the cell are referred to as committed osteoblastic, or osteoprogenitor cells. These cells are in the first stage of bone cell recruitment. The later stages include preosteoblastes and transitory osteoblasts, both are capable of division. Finally, the functional bone-forming osteoblasts that are post-mitotic and secretion of mineralize matrix.[30]

1.2 Osteoblasts

The bone forming cells, secrete the organic component of the bone matrix and regulates in subsequent mineralization. They are found on all bone surfaces and are enlarged and active at all sites of bone growth and repair. Mature osteoblasts always act in groups of 100-400 cells, and they can be active for up to some 12 weeks.[38,45] These cells are characterized at the light microscopic level by a Golgi apparatus in between basophilic cytoplasm, a basally located and round nucleus. The fate of an osteoblast is to become either an osteocyte approximate 10-20% of the osteoblast covered by its own produced matrix, a flattened bone-lining cell.[48] There cell is genetic indicating that the early commitment for mesenchymal stem cells into osteoblasts requires expression of the transcription factor core binding factor A1 (Cbfa1) which also called Runx2. Cbf a1 is a member of the runthomology family of transcription factor, which regulates several osteoblast phenotypic genes that carry consensus sequences in their promotor regions, such as genes encoding the (α1) collagen chin, bone siloprotein, transforming growth factor-β (TGF-β), osteopontin and osteocalcin this transcription factor is an important modulator of osteoblast commitment and it required for maintainace of osteoblast differentiation.[48, 50]

1.3 Osteocytes

As soon as the osteoblast are found within the bone matrix and they function to maintain the surrounded bone tissue by the mineralized bone matrix, itbbecome as osteocyte, dealing with the metabolic requirements, waste products, mineral homeostasis etc. The space in which the osteocyte is located is referred to as a lacuna. The osteocyte is astellate-shape cell[49], characterized by numerous cytoplasmic procrsses that make contact with neighboring osteocytes within the bone and presumable[38] also with lining the cells at the bone surface.

Age and activity regulate the morphological appearance of the osteocytes.[49,53] In a young osteocyte, the cytoplasmic volume is somewhat decreased in comparison to the osteoblast. Also the organelles involved in protein systhesis, i.e. the enoplasmic

recticulum and the Golgi complex, display decreased volumes.[49] The cell and organelle reduction increases with increasing age, and glycogen is accumulated in granules within the cytoplasm.

1.4 Osteoclasts

Osteoclasts are derived from a type of bone marrow cell.[48] These cell are multinucleate cells which vary greatly in size. They are involved in the mobilisation of calcium and the destruction of the bone matrix. Osteoclasts are found on bone surfaces and are important in the normal growth, mineralized tissue, maintenance and repair of bone. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone. The ruffled border is surrounded by the clear zone[53], which serves for the attached of osteoblasts to the bone surface at sites of active bone resorption. Osteoclasts are differentiated from hemopoietic cells of the monocyte/macrophage lineage under the control of bone microenvironments[48,53]. Osteoblast regulate osteoclast differentiation providing the microenviroment similar to bone, recent discovery of new members of the TNF receptor-ligand family, terms osteoprotegerin (OPG) clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells.[52,53]

1.5 Bone resorption and remodeling

Renewal in tissues such as muscle occurs largely at a molecular level, renewal of bone occurs at a tissue level and is similar to the remodeling of buildings in that local removal[52] (resorption) (Fig. 2) of old bone into new bone deposition. Remodeling phase is most vigorous during the years of active growth, when deposition predominates over resorption. After that remodeling gradually declines, in humans until about 35 age [48,52], after that its rate remains unchanged or increases negligible. The fourth decade on, resorption exceeds formation, resulting in an approximate 9 percent loss in bone mass per decade, equivalent to a daily loss of 15 to 29 mg of calcium.[52,53]

Except for the moreover of the ossification mechanisms inside cartilage, growth and development relate to exactly the same type of remodeling as that in the adult skeleton. Addition both equire continuous, proably irreversible differentiation of osteoblasts and osteoclasts, the former from circulating monocytes in the blood and the later from the undifferentiated bone mesenchyme. The life span of osteoclasts is from a few hours to a few days, while osteoblasts is a few day to at most a few weeks.

Bone resorption is produced by clusters of osteoclasts that either erode free surfaces or from cutting cones that tunnel through compact bone and create the cylindrical cavities that may be subsequently filled by osteons. Osteoclastic cells secrete hydrogen ions and enzymes onto the bone surface, dissolution the mineral and digesting the matrix at virtually the same moment.[59] The process is associated with locally augmented blood flow and with a greater surface acidity than elsewhere in bone, notwithstanding the fact that fact that the process of dissolution apatite consumes hydrogen ions. Resorption is often a much more rapid process than formation.[59]

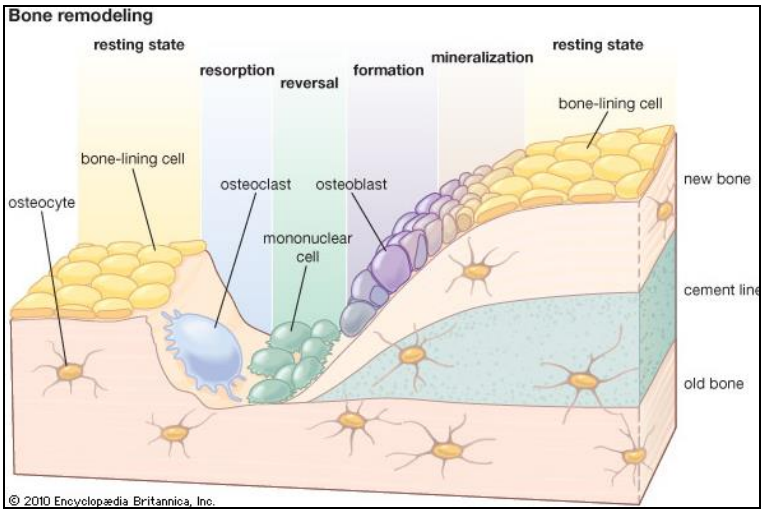


Figure 2. Bone remodeling (Walsh *et al*., 2003)

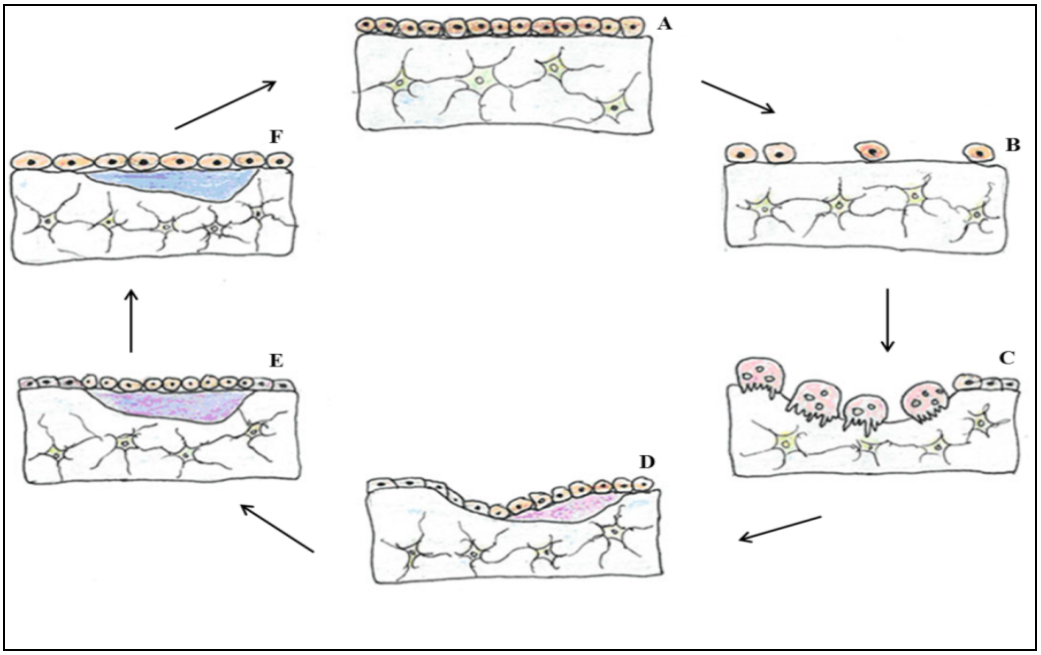


Figure 3. Bone remodeling: (A) quiescent phase where flat bone lining cells are seen lining the endosteal membrane (B) activation phase characterized by cell retraction with resultant membrane resorption (C) activated osteoclasts resorbing the underlying bone (D) formation phase where the osteoclasts are replaced by osteoblasts with underlying new osteoid matrix (E) mineralization of osteoid matrix (F) formation of bone structure unit with progression to quiescent phase (Chrischilles *et al*., 1991).

1.7 Regulation of bone metabolism

Osteoblasts express receptors to many bone active agents such as PTH, parathyroid hormone- related protein (PTHrP), gonadal, vitamin D, adrenal steroids, and certain cytokines and growth factors. The major product of osteoblasts is type 1 collagen, which, along with other proteins forms the organic osteoid matrix that is mineralized to hydroxyapatite .[59]

1. Parathyroid Hormone (PTH). is an 84-amino acid peptide synthesis by two pairs of parathyroid glands , and has a catabolic effect on bone,both through increase bone resorption and through inhibit bone formation . The mature PTH is packaged into dense secretory granules for regulated secretion. The major regulatory signal for PTH secretion is serum calcium. Serum calcium inversely affects PTH secretion, with the steep portion of the sigmoidal response curve corresponding to the normal range of both. The parathyroid gland senses the concentration of extracellular ionized calcium through a cell-surface calcium-sensing receptor (CSR) for which calcium is an agonist. The same sensor also regulates the responses to calcium of thyroid C cells, which secrete calcitonin in direct relationship to extracellular calcium; the distal nephron of the kidney, where calcium excretion is regulated; the placenta, where fetal-maternal calcium fluxes occur; and the brain and GI tract, where its function is unknown; and the bone cells. Serum phosphate has an inverse effect on calcium concentration, and ambient phosphate directly increases 1,25-dihydroxyvitamin D_3 on the kidney to stimulate resorption of calcium. PTH has a direct on both the activation of already osteoclasts and on the development of new osteoclasts (Sugimoto *et al*., 1993). But the resoption of bone is dependent on the presence of osteoblasts, since mature osteoclasts do not have PTH receptors themselves. The decrease in bone formation due to PTH is mediated through direct action of PTH on the osteoblast. Thus, serum phosphate may directly and indirectly regulate PTH expression.
2. Calcitonin (CT). is a 32-amino acid peptide whose main effect is to inhibit osteoclast-mediated boneresorption. Calcitonin is secreted by parafollicular C cells or extrafollicular of the thyroid and other neuroendocrine cells. In contrast to PTH, hypercalcemia increases secretion of hypocalcemia-inducing calcitonin, while hypocalcemia inhibits secretion. It inhibits resorption of bone, increases calcium, phosphorus excretion, and decreases the blood levels of calcium and phosphorus .[46, 59]
3. Vitamin D**.** is a secosterol hormone that is present in an endogenous (vitamin D_3) and exogenous (vitamin D_2). The endogenous form of vitamin

D_3 (cholecalciferol), is synthesized in the skin under ultraviolet radiation from the cholesterol metabolite 7-dehydrocholesterol (Deftos *et al*.,1998). The exogenous form of vitamin D_2 (ergocalciferol) is produced by ultraviolet irradiation of the plant sterol ergosterol and is available through the diet. Vitamin D is by itself an inactive substance that must be hydroxylated in the liver and in the kidneys to from 1,25-dihydroxyvitamin D, also called calcitriol. 1,25-dihydroxyvitamin D is able to induce bone resoption in *vitro* and in *vivo*. Vitamin D promotes the mineralization of osteoid and causes bone resorption by mature osteoclasts, but this effect is indirect, requiring cell recruitment and interaction with osteoblasts and the fusion of monocytic precursors to osteoclasts. Vitamin D also regulates the expression of several bone proteins, notably osteocalcin. [46] It promotes the transcription of osteocalcin and has bidirectional effects on type I collagen and alkaline phosphatase gene transcription.

4. Estrogen. Also exert multiple effects on osteoblasts through receptor α and β (Spelsberg et al., 1999). The role of estrogen receptors in the control of osteogenesis is not fully understood, as the deletion of these genes in mice induces complex effects on bone mass.[59]
5. Other Hormones. In addition other hormones play an important role in calcium and skeletal metabolism. Glucocorticoids are deleterious to all skeletal functions. Insulin, androgens, growth hormone, and thyroid hormones promote skeletal growth and maturation. Excess production of the latter can cause hypercalcemia. [46,59] While in adults bone metabolism is basically limited to bone maintenance, the most obvious feature of bone metabolism in children and adolescents is increase in bone size in all three dimensions.

1.8 Tissue engineering of bones

Tissue engineering utilizes both the eneineering and life science disciplines to either maintain existing tissue structure or enable tissue growth

Lee and Mooney, 2001 The term tissue engineering has implied to some combination of cells, the 3D scaffold materials and bioactive peptides that can modulate cellular activities, be delivered into the desired site in a patient's body and direct new tissue formation into scaffolds.

Langer, 1990 Delivery systems for protein therapeutics that promote tissue regeneration. Rrcently, with the advancement of material science in a drug controlled release, it is possible to increase the efficacy of these drugs.

2. Chitosan

Chitosan is a natural saccharide that result from the partial deacetylated of chitin, the second most abundant natural polysaccharide and is commonly obtained found in the exoskeletons from shrimp shells and crab of crustacean the cuticles of insects and the cell walls of fungi *mycelia*.[1,2] Most chitin applications are based on its deacetylated from chitosan.

2.1 Structure of chitosan

Chitosan is a copolymer *N*-glucosamine and *N*-acetyl-glucosamine unit which are linked in β (1-4) manner. Chitin can be degraded by chitinase. Its immunogenicity is exceptionally low, in spite of the presence of nitrogen. It's a highly insoluble material resembling cellulose in its solubility and low chemical recativity. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamidi group. Like cellulose it functions naturally as a structural polysaccharide[1,2]. Chitin is a wihite, hard, inelastic, nitrogenous polysaccharide and the major source of source of surface pollution in coastal areas.

Chitosan is a copolymer *N*-glucosamine and *N*-acetyl-glucosamine unit. derivative of chitin, although this *N*-deacetylation is almost never complete. A shrimp nomenclature with respect to the degree of N-deacetylation has not been defined between chitin and chitosan. The structures of cellulose, chitin and chitosan are show (Fig 4.) This poly *N*-acetyl glucosamine exhibite structure similarity to

cellulose differing only in the replacement of C-2 hydroxyl residues by acetamide group.[2] Depending on the polysaccharide source and isolation conditions, chitin could have a different degree of acetylation. Chitosan is produced by chitin deacetylation with concentrated alkali solutions at elevated temperatures. The degree of deacetylation and the molecular weight, ranging from 30 kDa to well above 1,000 kDa of chitosan[2,11] can be controlled by the temperature of the process.

Furthermore chitosan is a biopolymer that is easy to develop in various designs, i.e., film[11], sponges[11], scaffolds and hydrogels[11], an essential fact for the preparation of a great diversity of wound dressings and tissue-engineering materials. Regarding targeted functionalization chitosan bears two type of reactive group that can be modified the free amino groups on the deacetylated units and the hydroxyl groups on the C3 and C6 carbons on both acetylated and deacetylated units. This allows its functionalization with a variety of (bio) active molecules or the use of crosslinking reagents for blending with other biopolymers[2].

Chitosan is a positively charged polymer and has high charge density in solution. The charge density allows chitosan to form insoluble ionic complexes with a wide variety of water-soluble anionic polymers. It is insoluble in water but soluble in organic acid at pH 6.5. In diluted acid (pH < 6) free amino group are protonated and the molecule becomes fully soluble below ~ pH 5.[11] The pH dependent solubility of chitosan provides a convenient mechanism for processing under mild condition. Chitosan is biodegradable, degraded by lysozyme-mediated hydrolysis process targeting acetylated residues. Biodegreding process of chitosan in aqueous medium is sloe, even if lysozyme is present. There are evidences that some proteolytic enzymes show low levels of activity with chitosan. The degradation products are chitosan oligosaccharides of variable length. The degradation rate is inversely related to the degree of crystallinity which is controlled mainly by the degree of deacetylation. Highly deacetylated chitosan (i.g. > 85%) show higher levels of crystallinity and exhibits lower degradation rates.[24, 35] Chitosan may last several months *in vivo*.[35] The minimum crystallinity of chitosan exhibits intermediate levels of deacetylation and higher degradation rates. In fact, the low degradation rate of highly deacetylated chitosan implants is believed to be due to the inability of

hydrolytic enzymes to penetrate the crystalline. So chitosan sponge is expected to remain stable in shape and size during cell culture.[28]

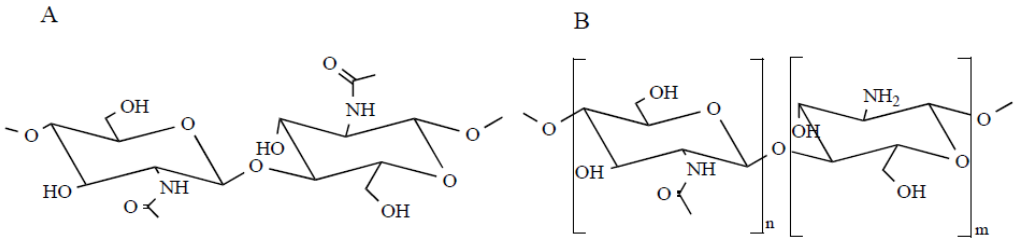


Figure 4. (A) Chemical structure of chitin **(B)** chemical structure of chitosan (where m>60) (Aranaz *et al*., 2010).

Chitosan is currently receiving a great deal of attention for medical and pharmaceutical application. The main reasons forchitosan are undoubtedly its appealing intrinsic properties of chitosan include biocompatibility, biodegradability, non-toxicity, adsorption properties, film-forming ability, bioadhesivity, anti-microbial activity against fungi, bacteria, viruses and drug delivery system, in addition to its haemostatic effect. [2,11]

2.2 Biomedical application of chitosan

Chitosan have been used in a wide variety of biomedical application including surgical thread, bone healing promote material wound dressing, sustained release drug carrier systems and tissue engineering (Ma et al., 2001).

1. Chitosan and wound healing**.** The effect of chitosan on wound healing has been evaluated by several ivestigators in animal study models. Chitin and chitosan improved wound healing in animal, accelerated increasing of tensile strength of wounds and promoted growth of granulation tissue with abundant neovasculature. Chitosan scaffold enhanced sythesisnof collagen

in the first few days of wound healing and induce the production of type III and IV collagen in tendon. Chitosan inhibited contraction of fibroblast-populated collagen lattice (FPLC). An inhibition effect was dependent on the level of deacetylation of the polymer. The higher the level of deacetylatation of the polymer was the lower of the level of contraction. High decetylated chitosans were more biologically active than chitin and low deacetylated chitosan. It was useful as a potential anti-scarring agent in wound healing therapy.

2. Chitosan and biocompatibility. Chitosan is essential to improve biocompatibility and physical properties of chitin and chitosan in the tissues because they are not strural component in human tissues. Mori and coworkers examined the effects of chotin and its derivatives on the proliferation of fibroblasts. The results showed that chitosan had no effect on proliferation and did not directly accelerate ECM production of fibroblast *in vitro*. The ECM product could be increased by and addition of transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF) into the culture system.[31]
3. Chitosan and three dimensional scaffolds. Chitosan has a hight potential as a biomaterial in tissue engineering. It has both reactive amino and hydroxyl groups that can be chemically modified and is relatively easy to manipulate for different pore structures.[35] Chitin gel underwent lyophilization process produced porous matrices with pore sizes ranging from 100 to 500 μm.[31] Varying of pore size and porous structure of matrix were dependent on the varying of freezing temperature and cooling rates. Mouse and human fibroblast cell culture exposed to these chitin matrices were found to be growing and proliferating indicating the feasibility of using these porous chitin matrices for cell transplantation applications to regenerate tissues.

Ma *et al*., 2001 investigated the utility of bilayer chitosan scaffolds to support growth and differentiation of human neonatal dermal fibroblasts. They found that chitosan scaffold remained stable in shape and size during the cell culture period.[31]

Fakhry et al., 2004 studied the effect of chitosan on MC3T3-E1 osteoblast and 3T3 fibroblast cell attachment and suggested that chitosan supported the initial attachment and spreading of osteoblasts preferentially over fibroblasts and that manipulation of the biopolymer could alter the level of attachment and spreading of cells.

4. Chitosan and osteoblasts. Chitosan is osteoconductive promoting new bone formation. Muzzarelli and coworkers applied chitosanin human periodontal defacts and found that chitosan decreased fibroplasia and enhanced cell proliferation and tissue organization of periodontal soft tissues. Chitosan powers accelerated healing of periodontal pockets and large intrabony defects of extracted socket. Methylpyrrolidinone chitosan promoted mineralization of bone osteoid formation in bone defect of rabbits. Porous chondroitin-4-sulfate (CS)-chitosan sponges incorporated with platelet-derived growth factor-BB induced the increasing of fetal ral calvarial osteoblasts migration and proliferation as compared with chitosan sponge alone and could gradually release of PDGF-BB from CS-chitosan sponge resulting in significant enhancement of osteoblast migration and proliferation.[35,56]

Ohara *et al*., 2004 study detected the expression of osteoblasttic genes of osteoblasts *in vitro* using a low concentrstion of water soluble chitosan oligomer and found that chitosan oligomer modulated the activity of osteoblastic cells in mRNA levels and chitosan controlled the genes concerning cell proliferation and differtiation.

Ho *et al*., 2004 They found that the scaffolds supported attachment spreading of cytoplasmic process demonstrating the potential applicability of the scaffolds in tissue engineering.

2.3 Chitosan in combination with other materials

Chitosan has been used in combination with other material including, inorganic ceramics and bioactive polymer such as chitosan ascorbate gel, Glass ionomer cement, tropocollagen, sodium alginate combination of chitosan with other

biomaterials was expected to enhance cell adhesion, differentiation, and tissue compatibility while maintaining their original physical properties and enhancing bone growth and tissue regenerative efficacy.[40,44,56]

Kawakami and coworkers reported that the chitosan-bonded hydroxyapatite paste enhanced osteoconduction when applied to the tibia of adult Japanese white rabbits as compared to controls.[39]

Lee and coworkers., 2000 developed chitosan/tricalcium phosphate (TCP) for tissue engineering of fetal rat calvarial MCT3T3-E1 Osteoblasts. The author found that chitosan/TCP supported proliferation and differentiation of osteoblasts on the sponges, as it was indicateded by the hight ALP activities of cells and deposition of mineralized matrices on the sponges. The results suggested that the chitosan/TCP can be used as a biodegradable matrix for tissue engineering.[43]

3.Glass-ionomer cement (GIC)

Glass ionomer cements success and used in pediatric materials widely by Wilson and Kent developed in 1969.[17] Their usefulness in pediatric restorative dentistry is preferential relative to other materials because of due to fluoride release, chemical adhesion to tooth structure, and availability to use in a variety of clinical scenarios. The first practical glass-ionomer cement was introduced to the market in early 1972, and used to glass ionomer cement in dental restoration materials in the present.[17,18]

3.1 The Glass-ionomer cement component

Glass ionomer cement are derived from the neutralization reaction between a basic powerder of aluminium silicate glass, also containing calcium alumino-silicaye glass, fluoride, silica (SiO_2), alumina (Al_2O_2), aluminium phosphate ($AlPo_4$), calcium fluoride (CaF_2), cryolite ($Na_3Al_2F_6$) and Aluminium triphosphate.[18,21] Appropiate mixture of ingredients in the temperature rang 1100 to 1500 ºC. After fusion, the glass melt is shock-cooled, either by pouring it onto a cool

metal plate and then into cool water. The immediate product is a coarse frit, and this usually ground further, typically by dry milling in a ball mill. Particle size requirements for the final glass power are that they shoud be less than 45 μm for a filling grade cement and less than 15 μm for a fine-grained luting cement. Foe the reduced acidiy of polyacrylic acid compared with phosphoric with phosphoric acid. This was not strictly necessary once the rate-modifying effects of tartaric acid were discovered, since this is sufficiently strong an acid to react satisfactorily with the older glass formulation. However, most glass-ionomer still employ the glasses of greater basicity first described by Wilson and his co-workers.[18]

3.2 Setting reaction consideration

The setting reaction of the glass ionomer cement is complex and may very with composition. It has been represented wiilson and MCLean as an acid-base reaction between the polyacid liquid and the glass in which calcium ion and Aluminium ion are released by attack on the surface of the glass particles and ultimately cross-linking of the polyacid chain into a network. This ion release is facilitated by tartaric acid that readily forms complexes with these ions which form a molecular structure with the polyacrylate chain.[18,21]

Initial setting (gelation) is now regarded as due to chain entanylement as well as weak ionic cross-linking which corresponds with the viscoelastic behavior of the freshly set material. That, contrary to previous belief, there is no sequence tial release of Ca^{2+} and Al^{3+} ions. Instead, these and other species are liberated together with differential rates of reaction in matrix formation. As the cement matures over the first 24 h, progressive cross-linking occurs possibly with hydrated Al ions since the sensitivity to moisture of the set cement decreases and the percentage of bound water and glass transition temperature increase . The Þnal set structure has been presented as a complex composite of the original glass particles sheathed by a siliceous hydrogel and bonded together by a matrix phase consisting of hydrated fluoridated calcium and aluminium polyacrylates.[18,21,]

3.3 Glass ionomer properties

Glass ionomer cement were originally developed for use as restorative dental meterials.[41] In this role beneficial properties included adhesion to untreated tooth mineral, and the release of fluoride ions that were thought to confer resistance against dental caries, can influence their behavior in a biological environment. Development of these bone cements will also be addressed. Conclude with the research currently being employed to potimise the biocompatibility of these important biomaterials.[29,41]

4. Translational Control tumor protein (TCTP)

The translational Control tumor protein (TCTP) or fortilin was discovered about 30 years ago.[3] TCTP was first identified as a growth-related protein in Ehrlich ascites tumor cell line, erythroleukemia cells and variety of cell.[3,4] TCTP by three groups interested in translationally regulated genes. TCTP, also know as IgE-dependent histamine releasing factor (HRF) is a 23 kDa protein (P23) in humans, 21 kDa protein (P21) in mouse homologue and Q23, respectively. The cDNA sequences of the mouse and the human protein were published in the late eighties. The name translationally controlled tumour protein was coined based on the fact that the first cDNA seqences was cloned from a human mammary tumour. The addition, observation that TCTP is regulated at the translational level. Elucidation of the primary sequence didnt reveal any similarity with other protein families. Only the recent determination of the solution structure of the fission yeast protein in dicated similarity with a small chaperone family. In addition, TCTP displays growth-promoting and antiapoptotic properties. These findings led the authors to use other more unspecific names “fortilin” or “TCTP”.[4]

TCTP is ubiquitously expressed in all eukaryotic organisms and in tissues[4] and cell types investigated so far. However expression levels very widely, depending on the cells or tissue type and on the developmental stage. TCTP is expression in mitotically active tissues, where as expression levels are low in postmitotic tissue like brain. In numerous experimental settings and biological systems, it was established that TCTP levels are highly regulated in response to a wide range of extracellular signals and cellular condition (Fi stress g. 5). Typically, growth signals and cytokins have been reported to rapidly induce TCTP systhesis. Various stress condition, such as start vation heat shock, oxidative, toxins or drugs, calcium stress, heavy metals proapoptotic signals result in ither down-or up regulation of TCTP levels[16].

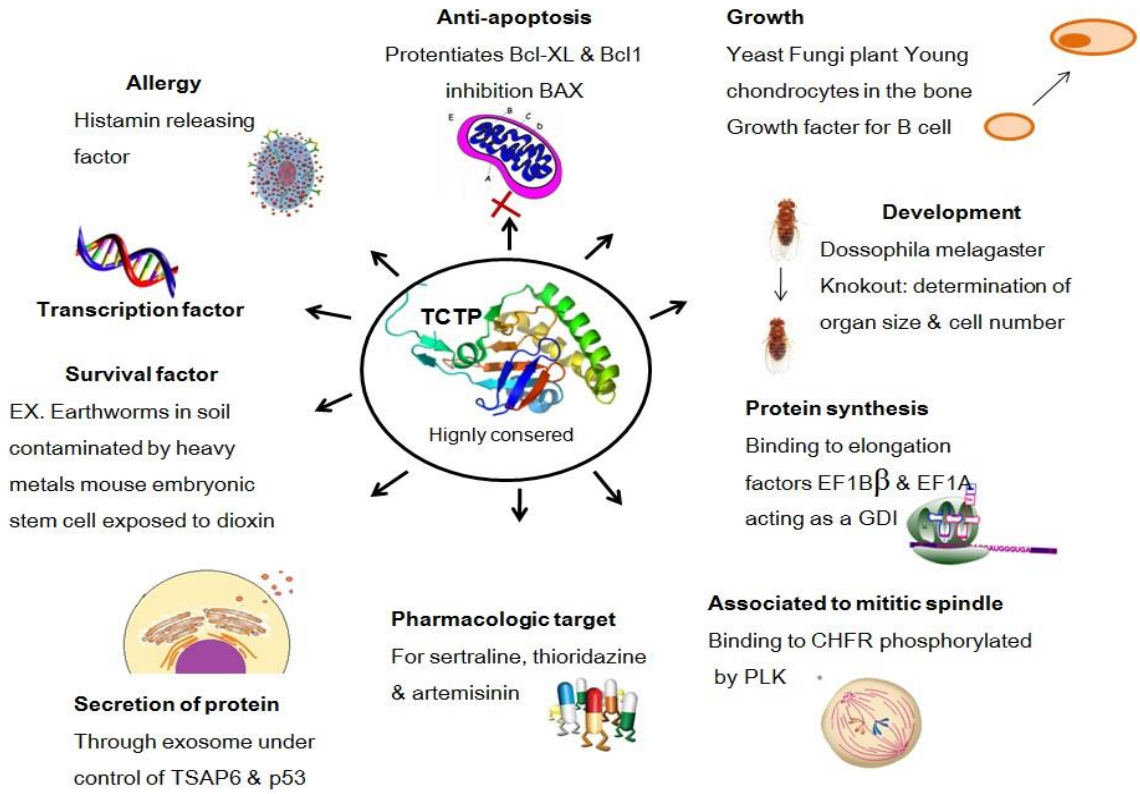


Figure 5. Regulation and functional importance of TCTP. Extracellular signals and conditions that result in regulation of TCTP levels and the cellular and extracellular importance of the protein *(Nature Reviews Cancer* 9., 2009).

4.1 Protein structure and conservation

The translational controlled tumor protein (TCTP) is a highly conserved protein that is found in normal cells line including mammals, higher plants. Sequence aligment of TCTP sequences from more than 30 different. Sequence alignment of TCTP sequences from more than 30 different species reveals a high degree of conservation over a long period of evolutionwe aligned TCTP sequences from five species representing one kingdom each. Nine of the approximately 169 residues are completely conserved and six additional ones are only mismatched in one sequence, making up a total of nearly 9% absolutely conserved amino acids.[5] The invariant residues are largely clustered on one side of the β-stranded coredomain, flexible loop and α-helical domain indicating that this side is important for molecular interactions. The fold of this domain displays significant similarity to that of the Mss4 and Dss4 proteins, two small chaperones reported to bind to the nucleotide-free form of Rab proteins.[16] TCTP is therefore now grouped in one protein family together with Mss4/Dss4 protein. The other major domains, the helical domain and flexible loop (Fig. 6), are specific for TCTP. The middle of the loop contains a highly conserved area, listed in the prosite database as TCTP. The only molecular functions of the TCTP protein mapped so far are the tubulin-binding region[4] and the Ca^{2+} binding area[4,16]. Both coincide with the helical domain, which also represents the most basic part of the molecule.[5,16]

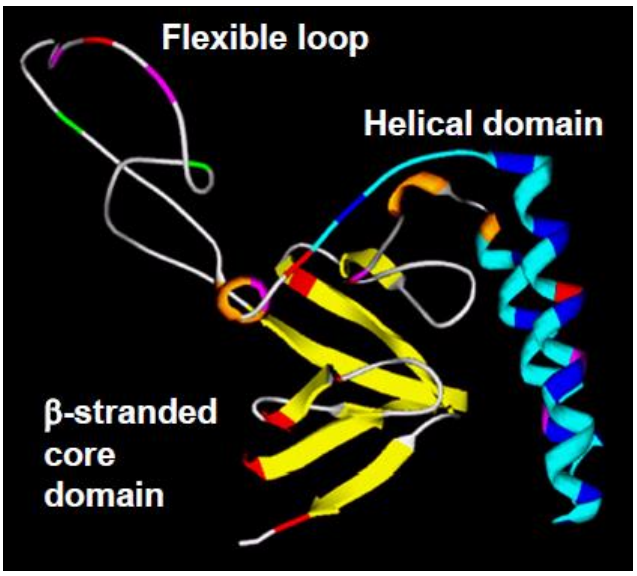


Figure 6. Three-dimensional structure of the TCTP protein. The domain structure is indicated. The β-stranded core domain is shown in yellow, and the α-helical domain region is marked in blue (Bangrak et al., 2004).

4.2 TCTP Protect cells from death

TCTP protect cells death by path directly reduce cellular Stress. TCTP expression increases in response to a variety of cell stresses and stimuli, and in some cases, TCTP could directly reduce stress, protecting cells from death (Fig. 7). The first case we describe deals with its protection of cells from heat shock induced cell death[34]. TCTP is markedly up regulated in a variety of cells following thermal shock. Recent studies demonstrated that TCTP is a heat shock protein and serves as a molecular chaperone. TCTP binds to denatured proteins, refolds them, and also interacts with native proteins and protects them from denaturation.

TCTP Inhibits apoptosis/cytotoxic signals. cellular stresses induce apoptosis via mitochondrial pathway by TCTP is able to inhibit of apoptosis by regulating the release signal pathways (Fig. 6).[34] TCTP protects cells from apoptosis triggered by treatment or serum deprivation with etoposide, taxol, or 5-fluorouracil. Intiation mitochondria contain pro-apoptotic proteins such as apoptosis inducing

factor (AIF), cytochrome C and DIABLO/Smac. In the course of apoptosis, these proteins are released from mitochondria following the formation of the permeability transition pore in the membrane by the action of proapoptotic Bcl-2 family proteins such as Bax and BH3. Other Bcl-2 family members such as Bcl-2, Bcl-xL, and MCL1 are known to suppress apoptosis by binding and inactivating the proapoptotic proteins.[34]

Oxidative stress-induced cell death. Depending regulation of hydrogen peroxide such as reaction oxygen species (ROS), hydroxyl radicals, and superoxide are generated in cells in the course of normal metabolism, including electron transport and various oxidase reactions. Oxidative stress induced enhanced in cells by ROS with high TCTP levels depending on the severity of the cells stress In addition oxidative stress caused down regulation of TCTP following by cells death. [55]

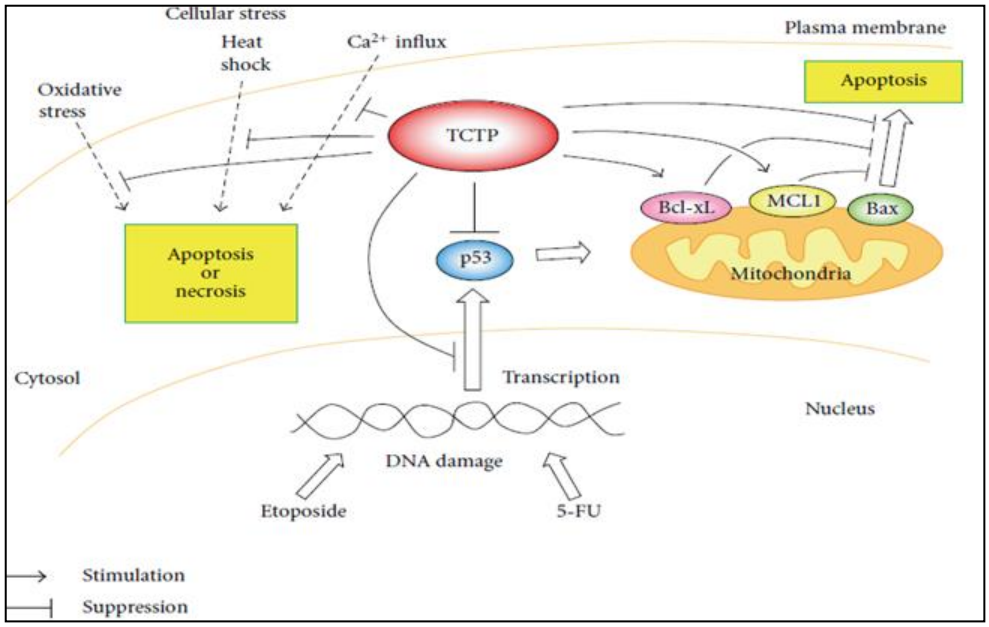


Figure 7. TCTP protects cells fromcell death. TCTP inhibits cell death induced by oxidative stress, heat shock, or influx of Ca^{2+}. In addition, TCTP can protect cells from apoptosis triggered by treatment with genotoxic reagent such as etoposide and 5-fluorouracil. TCTP inhibits apoptosis by stabilizing antiapoptotic Bcl-2 family proteins, MCL1 and Bcl-xL and by inhibiting activation of proapoptotic Bcl-2 family protein) (Michyo *et al*., 2012).

OBJECTIVE

The purposes of this study were to evaluate the biological properties of *Pmer*-TCTP that release from GIs on Human osteoblasts and to compare the biological properties with and without *Pmer*-TCTP of GIs incorporate. The objectives of this study are listed as follows:

1. The effects of *Pmer*-TCTP on cell viability in cultured human osteoblasts (NHost cells).
2. The effects of *Pmer*-TCTP supplement GIs on cells proliferation of NHost cells.
3. The effects of *Pmer*-TCTP supplement GIs on cells differentiation and mineralization of NHost cells.
4. The effects of *Pmer*-TCTP supplement GIs on gene expression of NHost cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals for cell culture

Chemical name	Company
Alpha-minimum essential medium (α-MEM)	GIBCO^{R}, Invitrogen Corporation, USA
Antimycrotic- Antibiotic	Sigma, Life Science, USA
Ascorbic acid	Sigma, Life Science, USA
Dimethyl sulfoxide (DMSO)	Sigma, Life Science, USA
Fetal bovine serum (FBS)	Biochrom AG, Germany
L-Glutamine	GIBCO^{R}, Invitrogen Corporation, USA
Penicillin- Streptomycin	GIBCO^{R}, Invitrogen Corporation, USA
Sodiumbicarbonate	Merck Inc Darmstadt, Germany
0.5%Trypsin-EDTA	GIBCO^{R}, Invitrogen Corporation, USA
β-glycerophosphate	Sigma, Life Science, USA

2.1.2 Chemicals for analysis

Chemical name	Company
Absolute ethanol	Merck Inc Darmstadt, Germany
Acrylamide	Fluka-chemika, Switzerland
Alizarin Red	Sigma, Life Science, USA
Ammonium persulfate	Fluka-chemika, Switzerland
Acetic acid	J.T. Baker
Brilliant albulue R	Sigma, Life Science, USA
Bromophenol blue	Fluka-chemika, Switzerland
Calcium chloride dehydrate	Merck Inc Darmstadt, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT assay)	Sigma, Life Science, USA
Dithiothreitol (DTT)	Merck Inc Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck Inc Darmstadt, Germany
10X Trysin-EDTA	GIBCO^{R}, Invitrogen Corporation, USA
Folmaldehyde	Merck Inc Darmstadt, Germany
Glycerol	Fisher
Glycine	Merck Inc Darmstadt, Germany
Hexamethyldisilazame HMPS	Sigma, Life Science, USA
Hexadecylpyridinium chloride monohydrate	Sigma, Life Science, USA
Hydrocloric acid	Merck Inc Darmstadt, Germany
Magnesium sulfate hydrate	Merck Inc Darmstadt, Germany
Magnesium chloride-hexahydrat reinst	Merck Inc Darmstadt, Germany
Methanol	Merck Inc Darmstadt, Germany
N, N′-methylene-bis-acrylamide	Bio-rad,USA
Diamine (TEMED)	
polyethylene glycol-8000	Merck Inc Darmstadt, Germany

Chemicals for analysis (Cont.)

Chemical name	Company
Potassium acetate	Merck Inc Darmstadt, Germany
Potassium chloride	Fluka
Potassium dihydrogenphosphate	Merck Inc Darmstadt, Germany
Potassium sulphate	Merck Inc Darmstadt, Germany
Sodium acetate	Merck Inc Darmstadt, Germany
Sodium chloride	LAB-SCAN
Sodium dihydrgen orthophosphate	Merck Inc Darmstadt, Germany
Sodium hydrogen carbonate	Merck Inc Darmstadt, Germany
Sodium hydroxide pellets	Merck Inc Darmstadt, Germany
Sulfuric acid	LAB-SCAN
Tween-20	Merck Inc Darmstadt, Germany

2.1.3 Molecular biology grade

Chemical name	Company
Agarose	BioExpress - Kayasville, USA
Ampicilin	Sigma, Life Science, USA
Ethidium bromide solution	Promega -Madison, USA
Isopropyl-β-D-thiogalacto-pyranositol (IPTG)	Qiagen - Hilden, Germany
Optical Cap 8X Strip QTY.120	Qiagen - Hilden, Germany
QIA shredder ™	Qiagen - Hilden, Germany
Random Primers	Invitrogen Life Technologies, USA
RNaseOUTTM Recombinant RNase	Invitrogen Life Technologies, USA
SuperScriptTM III Reverse Transcriptase	Invitrogen Life Technologies, USA
Clear blue X – ray film	Thermo Scientific, USA
100 bp ladder	Qiagen - Hilden, Germany

2.1.4 Instruments and special equipments

2.1.4.1 Instruments

Instrument	Company
Autoclave	Tomy SS-325, US
Auto pipette	Accu-jet^{R} pro, BrandTech Scientific, Germany
Cell counter	Need, Fuji Nishi ind, Japan
Centrifuge	Savant, Speed fuge HSC 15 R, USA
Centrifuge (Micro)	Hettich Mikro 220 Centrifuge, UK
CO_2 incubator	HERA cell 240, Heraeus, Germany
ELISA	Becthai Bangkok
Freeze Dryer	Alpha 2-4 LSC
Light microscope	Nikon TMS, Japan
Microflow advance biosafety cabinet-class II	Astec air BHA 48, England
Real-time PCR	Light Cycler® Nano, Roche
PCR	TouchDown Thermal Cycling system
pH meter	Orion Research
Refrigerator -20 ºC	SANYO
Refrigerator -20 ºC	Dometic
Spectrophotometer	Shimadzu UV-120
Water bath	Memmert, Technical science and service, Germany
Weight two positions	OHAUS, NJ USA
Weight five positions	Sartorius MC 210s

2.1.4.2 Special equipments

Instrument	Company
75 cm^2 cell culture flasks	Nunclon™, Nunc, Denmark
50 ml centrifuge tube	Corning Incorporation, USA
12 ml centrifuge tube	Corning Incorporation, USA
24 well cell culture plates	Nunclon™, Nunc, Denmark
96 well cell culture plates	Nunclon™, Nunc, Denmark
Clear blue X – ray film	Thermo Scientific, USA
Microcentrifuge tube 1.5 ml (Eppendorf)	Bioline, UK
Millipore filter 0.22 µm.	Millipore corporation, USA
Microscope glass slide	Sail Brand, China
Nitrocellulose membrane	Bio-RAD, USA
Pipette tip 20, 200, 1000 µl.	Bioline, UK

2.1.3 Bacterial strains

Escherichia coli stain BL21 Genotype: F´, *omp*T, *hsd*S_B,(r_B´, m_B´), *gal*, *dcm* was purchased from Invitrogen, Netherlands.

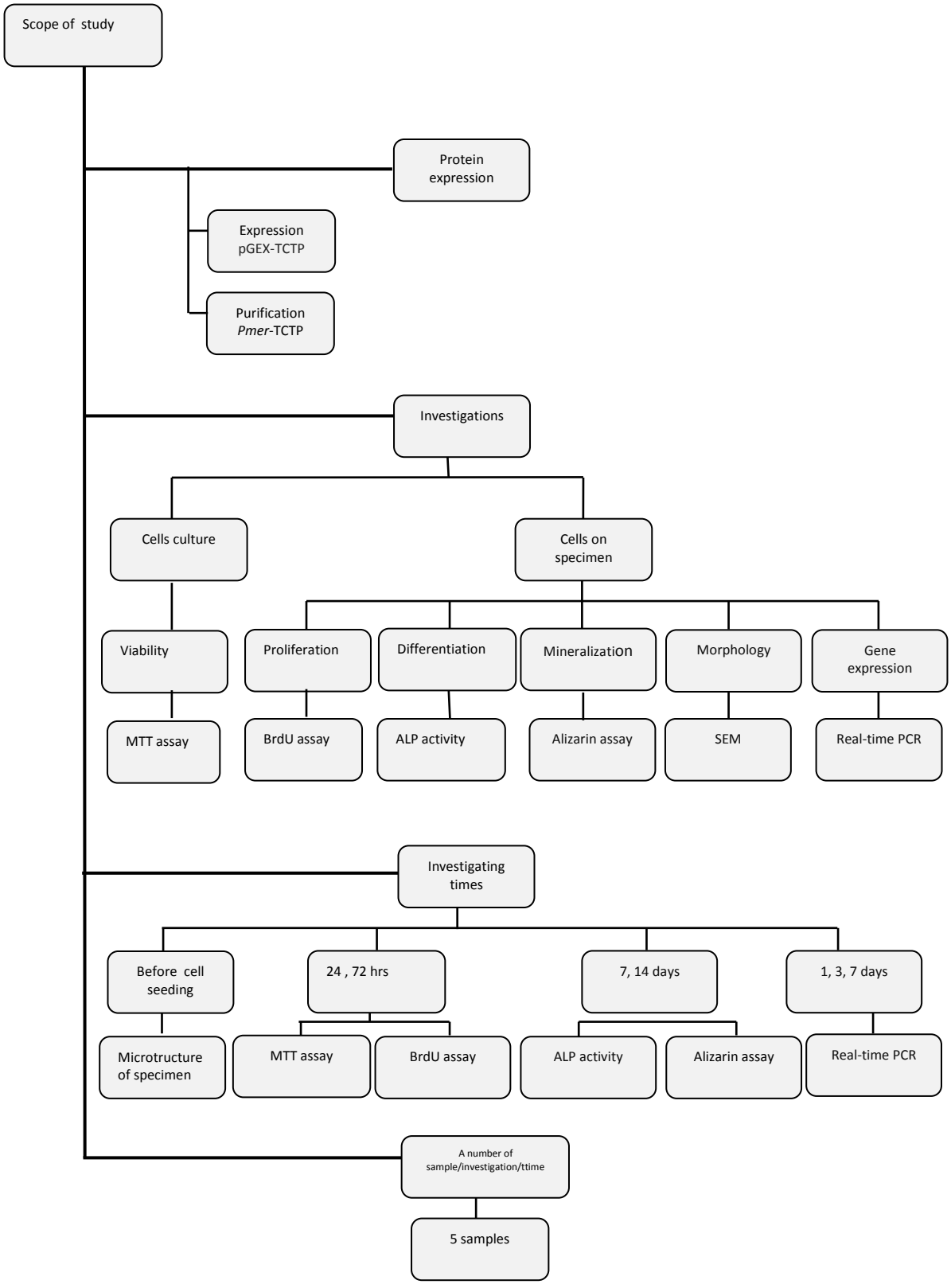
Escherichia coli stain BL21 recombinant DNA pGEX-TCTP was assist from Assoc. Prof. Dr. Wilaiwan Chotigeat (The Center for Genomics and Bioinformatics Research) Science in Molecular biology and bioinformatics Prince of Songkla University.

2.1.4 Cell line

The NHost cell were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in growth medium Alpha-modified Eagle's medium (α-MEM) supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin and 100 mg/ml Antibiotic-Antimycotic.

2.1.5 Oligonucleotide primer (Invitrogen Life Technologies, USA)

The nucleotide primer for qPCR, as shown in Table 3, were purchased from Invitrogen Life Technologies, USA.


Scope of study
Protein expression
Expression pGEX-TCTP
Purification *Pmer*-TCTP
Investigations
Cells culture
Cells on specimen
Viability
Proliferation
Differentiation
Mineralization
Morphology
Gene expression
MTT assay
BrdU assay
ALP activity
Alizarin assay
SEM
Real-time PCR
Investigating times
Before cell seeding
24 , 72 hrs
7, 14 days
1, 3, 7 days
Microtructure of specimen
MTT assay
BrdU assay
ALP activity
Alizarin assay
Real-time PCR
A number of sample/investigation/ttime
5 samples

METHODS

2.2.1 Expression and purification of translationally controlled tumor protein (TCTP)

The full-length cDNA encoding TCTP protein of translationally controlled tumor protein (TCTP gene) derived from *Penaeus merguiensis* was cloned and expression in bacterial Escherichia coli (*E.coli*) stain BL21. The stimulate GST-TCTP from recombinant DNA pGEX-TCTP host (a gift from Assoc Prof. Wilaiwan Chotigeat). The *E.coli* stain BL21 harboring pGEX-TCTP was inoculated into 100 ml of LB medium containing 100 µg/ml ampicillin at 37°C for 16-18 h by shaking 170 rpm/min (Wanachottrakul N., 2011). The overnight culture was inoculated in 100 ml of the culture medium and was diluted to 1000 ml of 2XYT medium containing 100 µg/ml ampicillin for 50-60 min until at $OD._{600}$ reached 0.3- 0.6. The culture was induced by 1 mM IPTG (isopropyl β-D-thiogalactopyronositol) and incubated 37°C in shaking incubator at 170 rpm/min for 3 h, the cells were harvested by centrifugation at 4,000×g ,4°C for 20 min. The pellet was suspended in 10 ml of ice-cold lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Tris-HCl, pH 8.0) containing lysozyme final concentration 1mg/ml which placed on ice for 30 min and sonicated 10 sec before incubated on ice for at least 15 sec repeated for six times and after that centrifuged at 10,000×g , 4°C for 20 min. The soluble protein was purified by using Glutathione Sepharose 4 Fast Flow (GE Healthcare Bio Science, Piscataway, NJ, USA) was examined by SDS-PAGE. GST- *Pmer*-TCTP protein is purified till a single band was detected by SDS-PAGE. After that thrombin was used for cleavage of GST-tagged protein by adding 10µl of thrombin and diluted with PBS to1ml/1ml of Glutathione Sepharose 4 Fast Flow. After incubate at 24°C for 16 h and kept at room temperature for 20 min, the soluble protein in supernatant was collected. *Pmer*-TCTP protein was separated by thrombin and flow through amicon ultra 30K device before concentrated by amicon ultra 10K device. The purified TCTP protein with molecular mass about 19.2 kDa was examined by 12% SDS-PAGE and Western blotting. Protein concentration was determined by a BCA protein assay kit (Pierce

Biotechnology, Inc., Rockford. IL. USA) in 96-well plate. The absorbance at OD 562 nm was used to determine protein concentration of the sample by calculating from the protein standard plot.

2.2.2 Osteoblast cell culture

Normal Human Osteoblast cells (NHost cells) from human long bones (femur) were purchased from Cambrex (Lonza Walkersville, Inc., USA). The NHost cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Alpha-modified Eagle's medium (α-MEM) supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin and 100 mg/ml Antibiotic-Antimycotic (Invitrogen Corporation, Grand Island, NY) (Nikom J., 2013). The culture medium was replenished every 2-3 days. After reaching 90% confluence, cells were trypsinized using 0.01% trypsin with EDTA. In all experiments, NHost cells passage between two and six were used for the study. Cells were stored in complete media with 10% of dimethyl sulfoxide (DMSO) in Cryo-freezing container at -80°C overnight before transfer to liquid nitrogen tank until use.

2.2.3 Specimen preparation

Glass ionomer cement (GI) used in this study was a conventional material 3MTM (3M ESPE KetacTM Molar Easymix, st Paul, MN, USA). The powder (batch no. 56633) was composed of Al-Ca-La fluorosilicate glass and 5% copolymer acid (acrylic and maleic acid), the liquid was composed of polyalkenoic acid, tartaric acid and water. The new formulation of GI in this study was assigned as BIO-GI. This BIO-GI was composed of GI powder incorporated with 15% of chitosan (by weight) and 5% (by weight) of bovine serum albumin (BSA). Chitosan used in this study had a molecular weight of about 62 kDa and 89.1% degree of deacetylation (TMECO, Thailand). The liquid part of BIO-GI and GI had the same compositions. The groups of different GIs and their compositions were described in Table 1. Two

sizes of disk specimens prepared by Teflon molds (7 mm diameter and 1 mm thickness for cell proliferative assay, 12 mm diameter and 1 mm thickness for alkaline phosphatase and Alizarin assay and 34 mm diameter and 0.5 mm thickness for Real time Polymerase Chain Reaction (qPCR)) were used. The GI cements were hand-mixed following the manufacturer's instruction. One powder scoop was mixed with one drop of liquid using a stainless steel spatula and then packed into split ring Teflon molds at a room temperature of 22±3ºC. TCTP was added at this time for the two groups with TCTP supplement (GI+TCTP and BIO-GI+TCTP). The uniform mixing cement was then transferred into the ring Teflon molds. A polythene sheet and glass slide was then placed over the filled mold after which light hand pressure was applied. Specimens were retained in the mold during storage in an incubator at 37ºC for 1 h. After storage in an incubator, the specimens were removed from their molds and ready for further experiments.

Table 1. Compositions of the powder of different groups of GIs specimens.

Group of specimens	**Powder compositions**
GI	- calcium fluoroaluminosilicate glass - 5% copolymer acid (acrylic and maleic acid)
GI+TCTP	- calcium fluoroaluminosilicate glass - 5% copolymer acid (acrylic and maleic acid) - added TCTP during mixing the cement
BIO-GI	- calcium fluoroaluminosilicate glass, - 5% copolymer acid (acrylic and maleic acid) - 15% chitosan - 5% BSA
BIO-GI+TCTP	- calcium fluoroaluminosilicate glass, - 5% copolymer acid (acrylic and maleic acid) - 15% chitosan - 5% BSA - added TCTP during mixing the cement

Table 2. Amount of TCTP added in GIs specimens.

Size Teflon mold	Amount of TCTP added
7 mm diameter and 1 mm thickness	100 ng in a specimen size for cells proliferative assay
12 mm diameter and 1 mm thickness	200 ng in a specimen size for alkaline phosphatase and Alizarin assay
34 mm diameter and 0.5 mm thickness	700 ng in a specimen size for Real time Polymerase Chain Reaction (qPCR)

2.2.4 Cell viability assay

The effect of TCTP at various concentrations at two time periods (24 and 72 h) on cell viability was determined by 3- (4, 5- dimethylthiazol-2-yl)-2, 5-diphenylterrazolium bromide or MTT assay. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. NHost cells at 5×10^3 cells/well was seeded in 96-well plates at 37 °C in a humidified atmosphere containing 5% CO2. After 24 h, the culture medium was changed with fresh medium mixed with TCTP at various concentrations from 0.01µg/ml to 15µg/ml for 24 and 72 h. The control group was normal culture medium without TCTP and each group had 6 replications. After that, the medium was removed, 200 µl for fresh medium containing 10 mM HEPES pH 7.4 added each well, and 50 µl MTT solution at 5 mg/ml in phosphate buffer solution (PBS) at pH 7.4) were added to each well and incubated in the dark for 3 h at 37 °C in a humidified atmosphere containing 5% CO2. The medium and MTT were then carefully removed next 200 µl of dimethyl sulfoxide (DMSO) and 25 µl of Sorensen's glycine buffer (0.1 M glycine plus 0.1 M NaCl equilibrated to pH 10.5 with 0.1 M NaOH) were added for dissolution the formazan crystals. The optical density of formazan production was measured at 570 nm using ELISA reader. The optical density (OD) values corrected for blank (medium as culture) of the

experimental groups were divided by OD of the control and expressed as a percentage of viable cells.

2.2.5 Cell Proliferation

The effect of GIs and TCTP on osteoblast cells were determined by cell proliferation assay using a commercial kit, Cell Proliferation ELISA, BrdU (colorimetric) (Roche, Mannheim, Germany) This assay designed to quantitate cell proliferation based on the measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis in proliferation cell. The experiment was divided into six groups, each group composed of 6 replicates: Four groups were cells seeded on different specimens including GI, Bio-GI, GI+TCTP and Bio-GI+TCTP, TCTP at 100ng/specimen was supplemented in each group of the specimen during mixing the powder and liquid components. NHost cells was seeded at 7×10^3 cells directly on each specimen (size 12 mm diameter and 1 mm thickness) which was placed on 96-well culture plate and fed with 200 µl of normal culture medium. The other two groups were cells cultured at the same density on the same culture plate in normal medium with and without 1ng/ml of TCTP, respectively. The culture plates were kept at two time periods, 24 h and 72 h before the BrdU assay was performed. The assay was followed the manufacturer's instruction. Briefly, the culture medium was removed and refreshed with 100 µl/well normal medium pre-mixed with BrdU labeling solution (at the ratio 1:100 by volume) and left for 2 h at 37 °C. Then removed the labeling medium and added 200 µl/well FixDenat after that incubated for 30 min at 25 °C. Remove FixDenat solution thoroughly and added 100 µl/well anti-BrdU-POD working solution, and then incubated 90 min at 25 °C. Antibody conjugate was removed and washed three times with 200 µl/well with PBS at pH 7.4 and add 100 µl/well substrate solution until color development was sufficient for photometric detection (5-10 min). Then, added 25 µl 1M H_2SO_4 to each well and incubated 1 min on the shaker at 300 rpm. The absorbance of the sample was measured at 450 nm against its own blank. The blank of each group was the similar specimen or the

culture plate without seeded cells but was treated the same procedure as the groups that had seeded cell

2.2.6 Western blotting

The released TCTP from GI+ TCTP and BioGI+ TCTP was examined by western blot assay. TCTP at 100ng/specimen was supplemented in each group of the specimen during mixing the powder and liquid components that has been described above while the negative control groups were BIO-GI and GI without TCTP. Each specimen was placed in plastic tube with 1ml of PBS. The PBS with 1 ng/ml of TCTP in a plastic tube was used as a positive control. All tubes were kept for 1 h in shaking incubator at 37ºC and 170 rpm. Then the immersed PBS was frozen dry (Alpha 2-4 LSC) and were resolved in 10 µl PBS before analyzed with western blot and the amount of protein was measured using BCA protein assay kit (Pierce, Rockford, IL, USA). Briefly, equal amount of protein from each sample was mixed with Laemmli sample buffer with reducing agent and subjected to electrophoresis on a 12% polyacrylamide gel along with pre-stained high molecular weight standards (Bio-Rad, Hercules, CA, USA). The proteins were transferred to nitrocellulose (Pierce, Rockford, IL, USA) using a trans-blot cell (Gibco BRL, Carlsbad, CA, USA) at 80 mA overnight. The nitrocellulose was incubated in 5% nonfat milk (Difco, Sparks, MD, USA) for 1 h and incubated with primary monoclonal antibody of mouse anti-Penaeus merguiensis TCTP (Abmart, Shanghai, China) diluted at 1:1,000 with 5% skim milk in TBS-T (0.5% Tween® 20, 154 mM NaCl, 48 mM Tris-base, 40 mM Tris-base) for 1 h and washed 15 min each for six times with TBS-T buffer. The membrane was incubated with secondary antibody of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson Immunoresearch, USA) diluted at 1:50,000 with 5% skim milk in TBS-T for 1 h on and washed six times with TBS-T buffer. Bound antibodies were detected by using an equal volume of Supersignal West Pico stable/peroxide solution with Supersignal West Pico luminol/enhancer solution (1:1) for 5 min (SuperSignal Wignal West Pico Chemiluminescent Substrate, PIRCE) The excess reagent was drained off and the membrane was wrapped in Saranwrap. After

that the membrane was placed in a film cassette containing autoradiography film (X-ray film from Thermo Scientific, USA), and exposed the radiation for 2 min. The X-ray film was developed using developing machine (Konica, USA).

2.2.7 Alkaline phosphatase (ALP) activity assay

The effect of GIs specimens on osteoblast differentiation was determined by ALP activity assay. There were 4 groups of the specimens (see Table. 1 and 2) TCTP at 200ng/specimen was supplemented in the group with TCTP during mixing the powder and liquid components and the control group was cells cultured without the specimens (n = 5 in each group). NHost cells at 4×10^4 cells were seeded on each specimen that had been placed on 24-well culture plate with normal culture media supplemented with 50 µg/mL of ascorbic acid. The medium was changed every 2 day for 7 and 14 days before the assay. The ALP activity was measured using *p*-nitrophenol phosphate as a substrate as previously described. Cells were rinsed twice with PBS pH 7.4 and scraped in 0.2 ml of alkaline lysis buffer (2 mM MgCl2, 10 mM Tris-HCl, 0.1% Triton-X 100, pH 10). Centrifuged at 1,000×g at 4 °C for 5 min, the supernatant was collected and analyzed for the total protein and ALP activity. For ALP activity, 5 µl of each sample was incubated in 50 µl of buffer containing 2 mg/ml p-nitrophenol phosphate in 0.1 M 2-amino-2-methyl-1-propanol (AMP), 2 mM MgCl2, pH 10.5 as a substrate. After 30 min at 37°C to stop the reaction, 0.8 ml 50 mM NaOH was added to each well and the absorbance was measured at 405 nm its own blank. The total protein of sample was investigated by using BCA kit (bicinchoninic acid). Protein assay was read at 570 nm (Pierce Biotechnology, Holmdel, NJ). ALP activity was calculated as nanomolar of p-nitrophenol per µg of total protein and then adjusted to the percentage compared to the control which was cells cultured without any specimen

2.2.8 Scaning slectron microscopy (SEM)

The NHost cells (5×10^3) were culture in specimens seeded in 96-well plates for 24 h that processed for scaning electron microscope (SEM). After the removal of the medium, cells were rinsed twice with PBS pH 7.4 and fixed with 2% glutaraldehyde in 0.01M phosphate buffer pH 7.4 for 1 h at room temperature. After being rinsed several time in the same buffer, the cell were post-fixed in 1% osmium tetroxide for 1 h at 4 °C and dehydrated in a graded ethanol-water series of 50%, 60%, 70%, 80%, 90%, and 100%, each step taking for 15 minutes (Oliva A., 1996) and two time at each level. The specimens were then immersed in 100% hexamethyldisilazane for 10 minutes and air dried in an exhaust hood at room temperature. Next, processed specimens were mounted on stuffs, coatd with gold and the examined with a scanning electron microscope. Images recorded at 150X-4,000X magnification.

2.2.9 Alizarin red S (ARS) assay

Alizarin red staining (ARS) assay (Gregory A., 2004). was used to determine mineralization. There were four groups of the specimens (see Table. 1 and 2.) TCTP at 200ng/specimen was supplemented in each group of the specimen during mixing the powder and liquid components and the control group was cells cultured without the specimens (n = 5 in each group) was cells cultured in inductive medium which composed of 10 mM β-glycerophosphate, 100 units/ml penicillin, 100 mg/ml streptomycin and 100 mg/ml antibiotic-antimycotic in α-MEM with 20% fetal calf serum. NHost cells (4×10^4 cells/well) were seeded on specimens (12 mm diameter and 1 mm thickness) that had been placed on 24-well plate. The cells were fed with 500 µl/well of inductive medium at 37 °C in a humidified atmosphere containing 5% CO2. After the first 24 h, the medium was refreshed and then the medium was replaced every 2 day. After 7 and 14 days the medium was removed and cells were washed with PBS pH 7.4 and fixed in 10% (v/v) formaldehyde (Sigma, Life Science, USA) at room temperature for 15 min and washed twice with 500 µl/well of 40 mM

ARS pH 4.1 in ultrapure water at room temperature for 30 min. After remove of ARS the plate washed four times with ultrapure water while shaking for 5 min, dry extraction and then stored at -20 °C. Quantification of staining 100 µl of 10% (v/v) acetic acid was added to each well and incubated at room temperature for 30 min with shaking. The scraped from the plate with a cell scraper and transferred to 1.5 ml tube added 100 µl mineral oil (Sigma, Life Science, USA), heated to exactly 85 °C for 10 min and transferred to ice for 5 min. After that was then centrifuged at 20,000×g for 15 min, Then 40 µl of 10% (v/v) ammonium hydroxide. Measure the absorbance of the sample in an ELISA reader at 405 nm against its own blank.

2.2.10 Real time Polymerase Chain Reaction (qPCR)

Real-time PCR was used to confirm the differentiation and mineralization expression of the selected sequences. The primer (5′- and 3-′) were designed from the select sequences and synthesized by Invitrogen Life Technologies, USA as shown in Table 3. This experiment was divided to three groups (two groups: Gi and BioGi with TCTP at 700 ng/specimen) in normal medium consisting of 100 units/ml penicillin, 100 mg/ml streptomycin and 100 mg/ml antibiotic-antimycotic in α-MEM with 20% fetal calf serum (FBS). NHost cells (1×10^7 cells/well) were culture in specimens (34 mm diameter and 0.5 mm thickness) seeded in contact grid plate. The cells were culture in 3,000 µl/well of medium at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation for 24 h, the medium was refreshed and each specimen. The medium was changed every 2 day. After 24, 72 h. and 7 days the medium was removed and all cells in each well were washed with PBS pH 7.4.

Table 3. Primer used for qPCR (Invitrogen Life Technologies, USA)

Gene	Sequence (5'→ 3')	GenBank accession no.
OPN	F:ACACATATTGATGGCCGAAGGTGA R:TGTGAGGTGATGTCCTCGTCTGT	(NM_00582)
BMP-2	F:GCTTCCGCCTGTTTGTGTTTG R:AAGAGACATGTGAGGATTAGCAGGT	(NM_007553.2)
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: ATGGTGGTGAAGACGCCAGT	(NM_002046)

2.2.10.1 RNA extraction

NHost cells were culture on specimens that extracted and purified of total RNA by RNeasy® Plus Micro kits (Qiagen, USA). NHost cells were harvested by washing the cells 2-3 times with PBS that treated with 0.1% DEPC and added 350 µl of buffer RLT into the sample tube following shacked and pipetted to mix, and ensured that no cell clumps were visible after that pipetted the cells lysate directly into a QIA shredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at 10,000 x g. Next 350 µl of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting to precipitates RNA before transferred to an RNeasy spin column placed in a 2 ml collection tube next centrifuged at 8,000 x g for 15 s than discarded the flow-through. 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged at 8,000 x g for 15 s to wash the spin column membrane than discarded the flow-through. 500 µl of Buffer RPE was added to the RNeasy spin column after centrifuge at 8,000 x g for 15 s to wash the spin column membrane discarded the flow-through and added 500 µl Buffer RPE to the RNeasy spin column next centrifuged for 1 min at 8,000 x g to ensure that no ethanol is carried over during RNA elution. The RNeasy spin column was placed in a new 2 ml collection tube next to centrifuged at 10,000 x g for 1 min placed the RNeasy spin column in a new 1.5 ml collection tube. Finally 30 µl of sterile DEPC treated milli Q water was added directly

to the spin column membrane at central and centrifuged for 1 min at 8000 x g to elute the RNA. The RNA quality was measured by spectrophotometer at OD260/OD280 and 1.2% agarose gel electrophoresis. The total RNA was stored at–80°C or used for cDNA synthesis, immediately.

2.2.10.2 First strand cDNA synthesis

Total RNA 1µl was used as template in RT-PCR reaction mixture according to the manufacturer's instruction The PCR reaction was performed in a total volume of 50 µl (Total RNA 4µg, 50 µM Oligo-dT, 25 mM $MgCl_2$, 10x RT buffer, 0.1 M DTT, RNase^{TM} and SuperScript^{TM} III reverse transcriptase, Invitrogen, USA). At first, the template-primer mixture was denatured by heating the tube at 65 ºC for 5 min in a thermo cycler. Then, adding the remaining components of the mixture and followed profile of the reaction was applied at 50 ºC for 30 min followed by inactivation of reverse transcriptase at 85 ºC for 5 min with subsequently added 1µl of RNase inhibitor at 37 ºC for 20 min. cDNA products was fractionated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining by UV transillumination. The cDNA products were calculated by measuring OD at 260 nm. cDNA was stored at -20 ºC for further qPCR analysis.

2.2.10.3 qPCR detection of OPN and BMP-2 gene

SYBR Green PCR master mix (Roche, Mannheim, Germany) was used for qPCR analysis, the primer used for qPCR was shown in Table 3. The qPCR mix consisted of 2 µl cDNA, 0.8 µl of forward and reverse primer, 10 µl of SYBR Green and 6.6 µl of nuclease-free water, and the negative control was distilled water that instead of cDNA sample. The reaction was performed manually in triplicate for each sample, at a final volume of 20 µl. The thermal profile for amplification of the investigated gene was followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing at 58 ºC for 30 s and elongation at 72 ºC for 30 s. After the end of the last

cycle, the final quantification was reported as a quantity after normalization to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative values were analyzed using comparative cycle threshold (CT) method (ΔΔCT method).

2.2.11 Statistics analysis

The result was analyzed for normality testing using Shapiro and Wilk method. The descriptive data was expressed as mean ± standard deviation (SD). T-test was used in the analysis of one or two data sets. Comparisons of the data more than two groups were performed by one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey's post hoc test. *P*-values less than 0.05 will be considered as statistically significant.

CHAPTER 3

RESULTS

3.1 Expression and purification of translationally controlled tumor protein (TCTP)

Translationally controlled tumor protein (TCTP) derived from *Penaeus merguiensis* was cloned and expressed in bacterial Escherichia coli (*E.coli*) stain BL21. The GST-TCTP protein was generated from recombinant DNA pGEX-TCTP in *E. coli* strain BL21 using GST protein as a control. The recombinant DNA in this pGEX were coded to GST-TCTP protein with a molecular weight of 48 kDa (Fig. 8), which composed of the GST protein with a molecular weight of 29 kDa and the TCTP protein of approximately 19.2 kDa (Fig. 8). When the GST-TCTP protein was extract and purified with Glutathione-S-transferase bead (GST-bead). The supernatant solution was measured protein (BCA assay) and detection protein with 12% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) the TCTP protein has its molecular weight approximately 19.2 kDa (was shown in Fig. 9) and was further investigated by Western blot analysis as shown in Fig. 9.

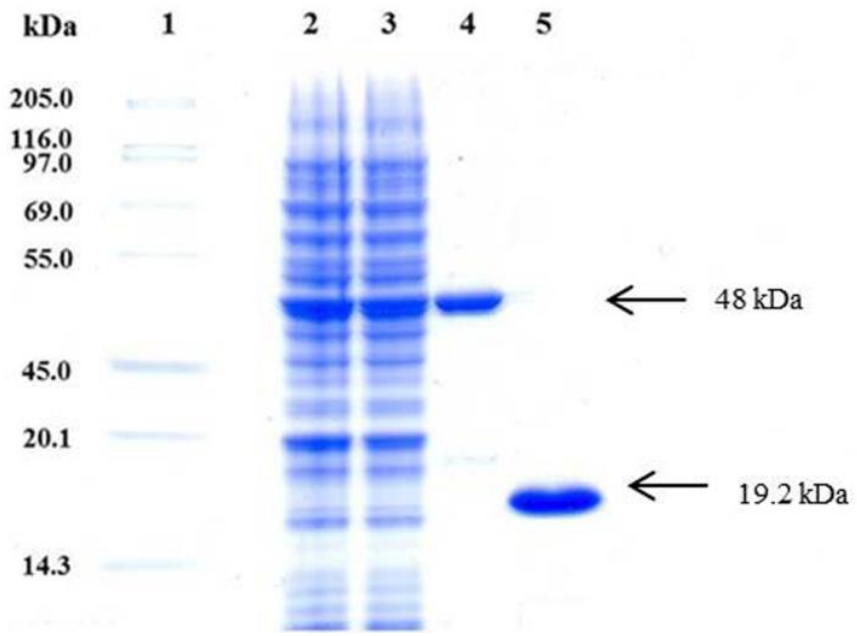


Figure 8. Expression and purification of protein *Pmer*-TCTP in *E. coli* strain BL21. Examined by 12% SDS-polyacrylamide gel electrophoresis. Lane 1: Standard molecular weight protein, lane 2: protein from induce insoluble GST- *Pmer*-TCTP, lane 3: protein from induce soluble GST- *Pmer*-TCTP, lane 4: GST- *Pmer*-TCTP using Glutathione Sepharose 4 Fast Flow, lane 5: purified *Pmer*-TCTP after GST-cleavage by thrombin. Protein was stained with 0.1% Coommasie Brilliant blue R250.

Figure 9. Western Blotting of *Pmer*-TCTP.

3.2 Effect of TCTP on Viability of NHost cells

NHost cells were exposed to various concentrations of *Pmer*-TCTP (Control, 1ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 15µg/ml) then cell viability was investigated by MTT assay.

In the present study, we demonstrated that *Pmer*-TCTP significantly increased cell viability, as measured by the activity of mitochondrial dehydrogenase on NHost cell in a dose- and time-dependent manner. In this experiment, the *Pmer*-TCTP treated on NHost cells from 1ng/ml-15 µg/ml for 24 and 72 hours. It was found that cells exposed to *Pmer*-TCTP had less viability than control (medium only) after culture for 24 hours. However, after 72 hours it was found that *Pmer*-TCTP at low concentration, especially 1ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml and 10 µg/ml had significantly higher cells viability compare to control (Fig.10) ($P<,0.05$, t-test) .. Higher concentrations of TCTP at 15 µg/ml had a significant inhibitory effect on cell viability. It was apparentthat after cells cultured for 72 hours, *Pmer*-TCTP at 1 ng/ml and 10 ng/ml gave significantly (*T-test*) higher optical density and percentages of viable cells (promote 7 % and 12 % compared to control).

Based from this result, it was decided that cells should exposed TCTP at concentration between 1-10 ng in order to promote cells growth and function for further studies.

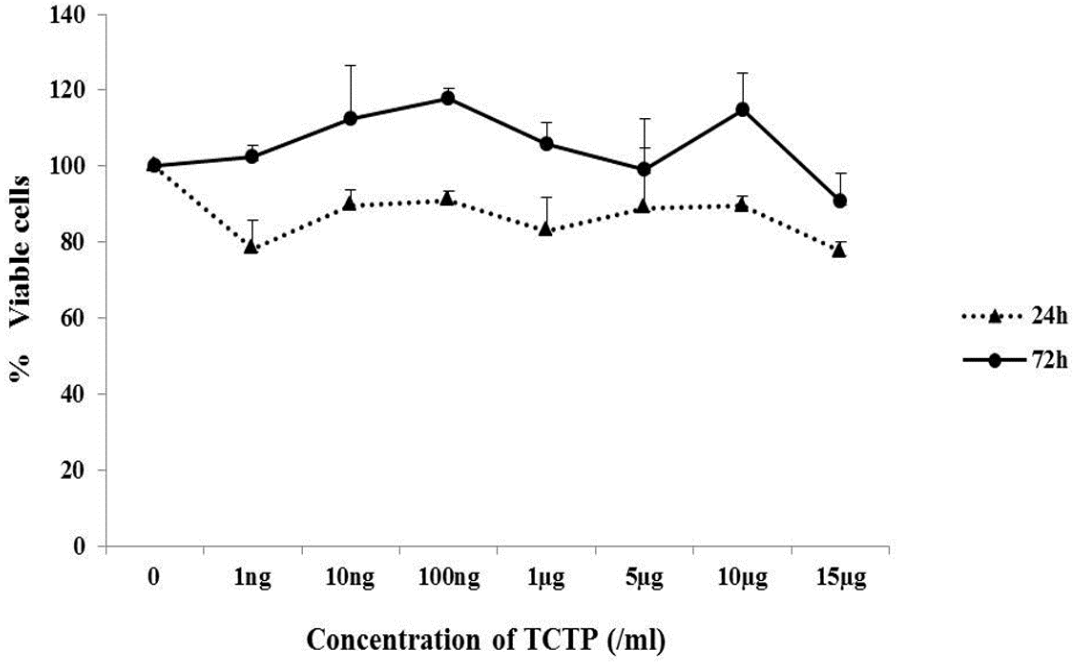


Figure 10. The effect of *Pmer*-TCTP on human osteoblasts viability cells were treated with various concentrations of *Pmer*-TCTP for 24 and 72 hour. Cell viability was investigated by MTT assay and indicated in average at OD_{570}. The percentage of viable cells compared to control (no *Pmer*-TCTP) has been shown. Data were represented as means ± standard deviation (SD), n = 6 in each group (*T-test*).

3.3 TCTP released from GIs supplemented TCTP

To determine the protein *Pmer*-TCTP release from specimen. Specimen with and without protein *Pmer*-TCTP was immersed in PBS for 24 hour then the protein release was investigated by western blotting analysis. The result of the released *Pmer*-TCTP band was shown Fig. 11.

Total protein *Pmer*-TCTP release from specimen when added *Pmer*-TCTP 100 ng per specimen the result showed that the protein release from group BIO-GI with added *Pmer*-TCTP release had higher quantity protein than group GI

with added *Pmer*-TCTP at 24 hour compared to control. In addition, protein *Pmer*-TCTP released from GI with added *Pmer*-TCTP was lower than to control. However, group GI and BioGI without *Pmer*-TCTP cannot detect the released protein because of without *Pmer*-TCTP.

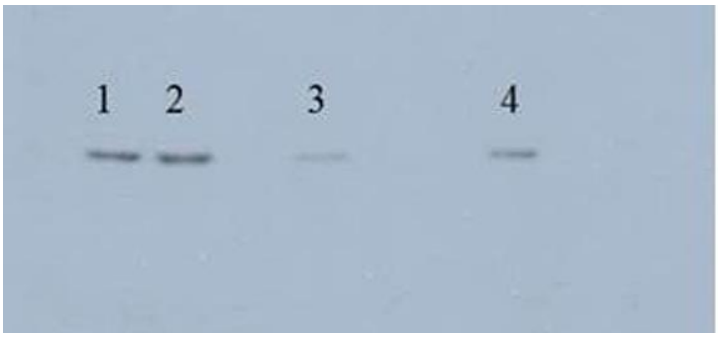


Figure 11. TCTP release from glass ionomer cement (GIs) with added *Pmer*-TCTP at 100 ng per specimen for 24 hour. Lane 1, 2: TCTP (control), Lane 3: *Pmer*-TCTP release from GI, Lane 4: TCTP release from BIO-GI was investigated by western blotting analysis.

3.4 Effect of GIs supplemented with TCTP on proliferation of NHost cells

The result of cell proliferation investigated by BrdU assay was shown in Fig 12. It was found that the specimens of BioGI added *Pmer*- TCTP at 1ng /specimen promoted cells proliferation higher than other groups ($P<0.05$).) and the percentages of proliferation cells were over 100% (Fig. 12) compared to control (medium only). In addition, group BioGI added *Pmer*- TCTP 10 ng per specimen promoted cells proliferation significantly less than group BioGI added *Pmer*- TCTP 1 ng per specimen($P<0.05$). However, it was noticed that group GI GI added *Pmer*-TCTP at 1, 10 ng and BIOGI is promote cells proliferation lower than control.

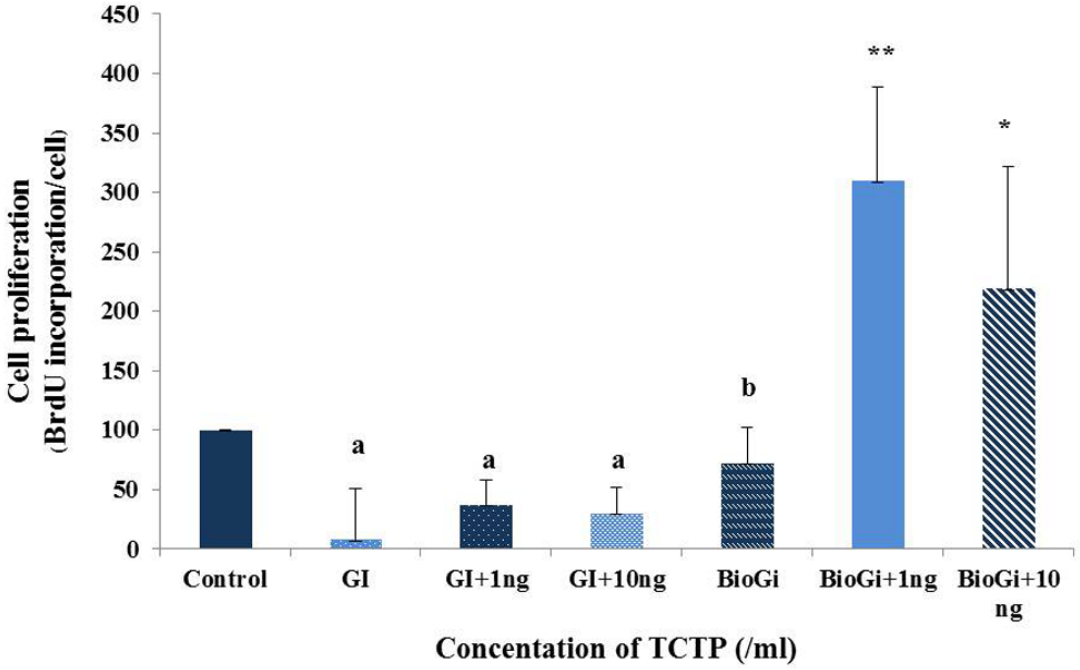


Figure 12. The cell Proliferative effect of TCTP on NHost cells, cells were cultured on different GIs specimens for 72 hour before BrdU assay. Data were represented as means ± standard deviation (SD) of percentages of cell populations compare to control. *,**, a, b denotes significant difference ($p < 0.05$, one-way ANOVA with Tukey multiple comparison test) (n=10 in each group).

3.5 Effect of GIs supplemented with *Pmer*-TCTP on alkaline phosphatase (ALP) activityof NHost cells

Levels of ALP activity of NHost cells after being cultured on GIs with added *Pmer*-TCTP for 7 and 14 day was investigated by ALP activity assay (Fig. 13).

The ALP activity of cells group BIO-Gi with and without *Pmer*-TCTP cultured 7 days was significantly ($P<0.05$) higher than the ALP activity of cells after cultured with the same group of the specimen for 14 days. The ALP activity of cells

cultured on BIO-GI added *Pmer*-TCTP for 7 days was significantly higher ($P < 0.05$) than the group BIO-GI without *Pmer*-TCTP . However, the ALP activites of cells on GI with and without TCTP were lower than other groups in both 7 and 14 days.

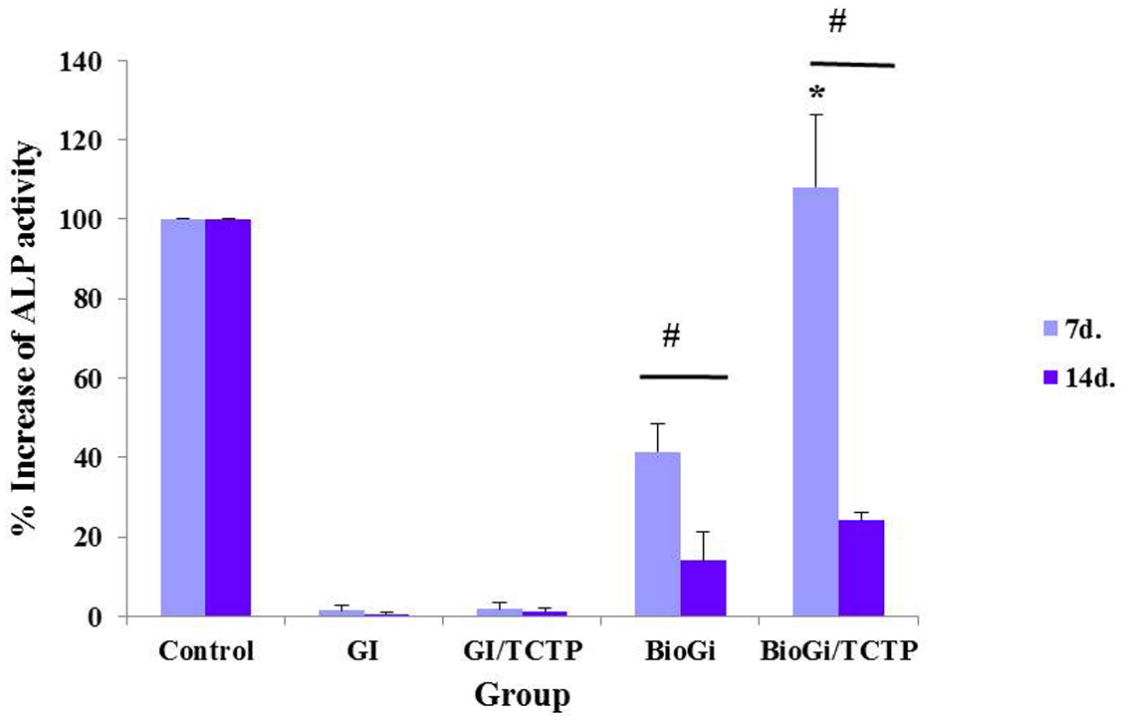


Figure 13. ALP activity was used to evaluate cell differentiation after cultured NHost cells cultured on with and without *Pmer*-TCTP supplemented GIs for 7 and 14 day. Data were represented as means ± standard deviation (SD) of cell populations. * Statistically significant difference compared with control group (culture medium) at $P<0.05$. # Statistically significant difference compared within group at $P<0.05$ (n=5 in each group) (two-way ANOVA with Tukey multiple comparison test).

3.6 Effect of GIs supplemented with TCTP supplement on mineralization of NHost cells.

The result of calcium deposition from NHost cells exposing with different GIs for 7 and 14 days was investigated by alizarin red s (ARS) assay as shown in Fig. 14.

It was found that level of calcium content group BIO-GI added *Pmer*-TCTP after cultured for 14 days was higher than the other specimen groups, but lower than control ($P < 0.05$). The mineralized area among group GI, GI added *Pmer*-TCTP, BIO-GI were relatively stable in a lower calcium deposition area compared to control.

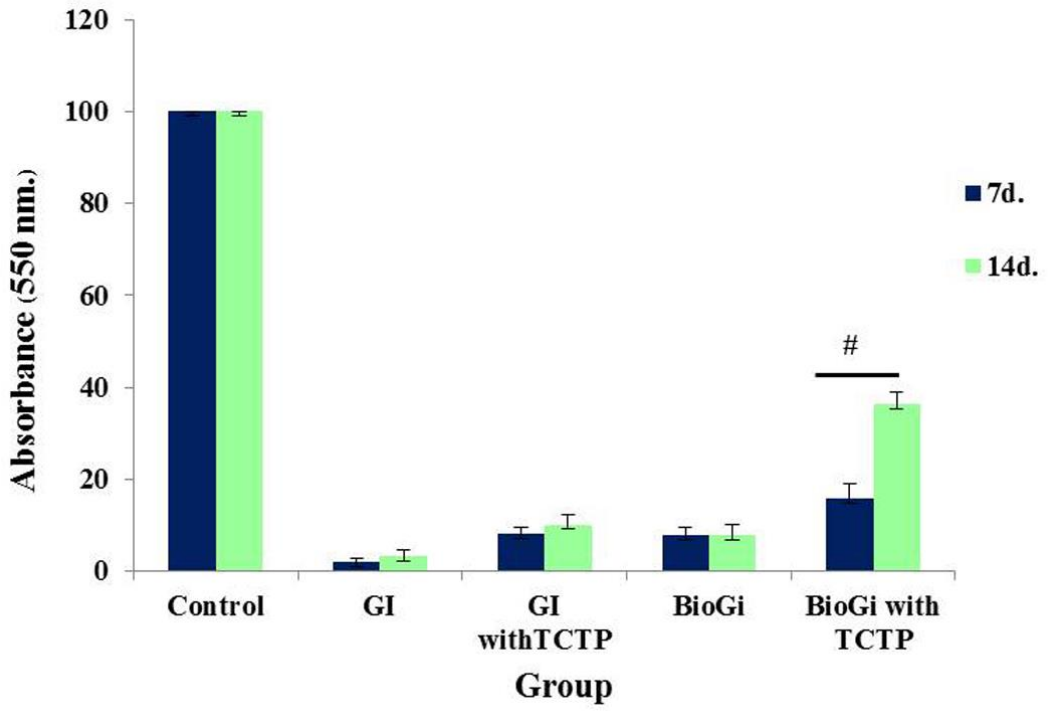


Figure 14. Cell mineralization was measured by alizarin red staing (ARS). NHost cells cultured on with and without *Pmer*-TCTP supplemented GIs for 7 and 14 day. Data were represented as means ± standard deviation (SD) of cell populations. # Statistically significant difference compared within group at $P<0.05$ (n=5 in each group) (two-way ANOVA with Tukey multiple comparison test).

3.7 Morphology

Scanning electron microscope (SEM) demonstrated microstructure of GIs before cell seeding and morphology and growth of cell on GIs (Figs. 15 and 16). NHOst cells were cultured for 24 hour on GIs SEM images of the surfaces and cross sections of the specimens.

It was found that the surfaces of all groups were relatively smooth and transverse fracture surface (was shown in Fig. 15 and 16). Cross section from scanning electron microscope (SEM) revealed porous material with gaps and cracks in several small positions. However, groups GI and BIO-GI added *Pmer*-TCTP proteins inserted in the specimen found some globules inserted within specimen.

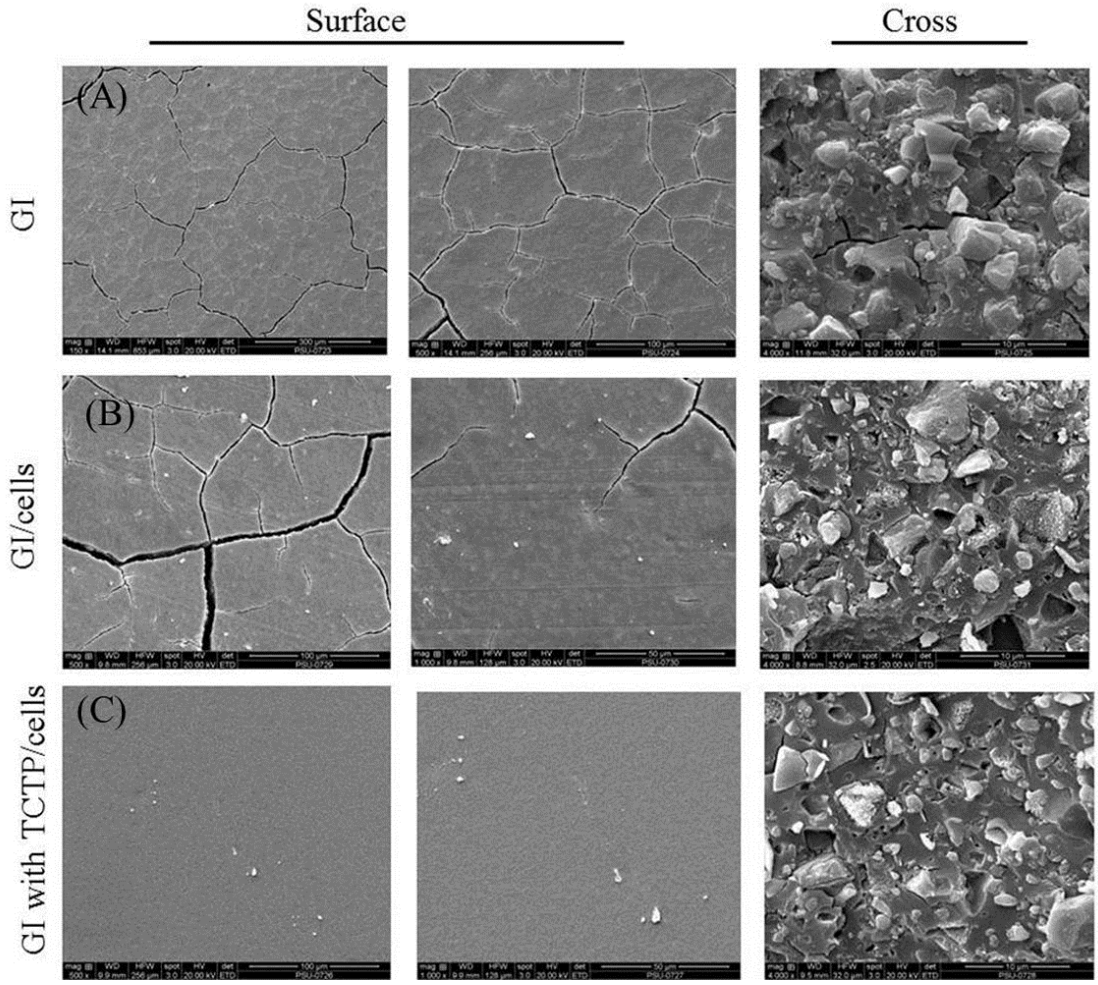


Figure 15. Morphology was observation by scanning electron microscope (SEM). NHOst cells cultured for 24 hour. on specimen surface and crosssection before cell seeding and morphology and growth of cell on GIs (A): GI, (B): GI/cells, (C): GI with TCTP/cells (Original magnification ×150, ×500 and ×4,000).

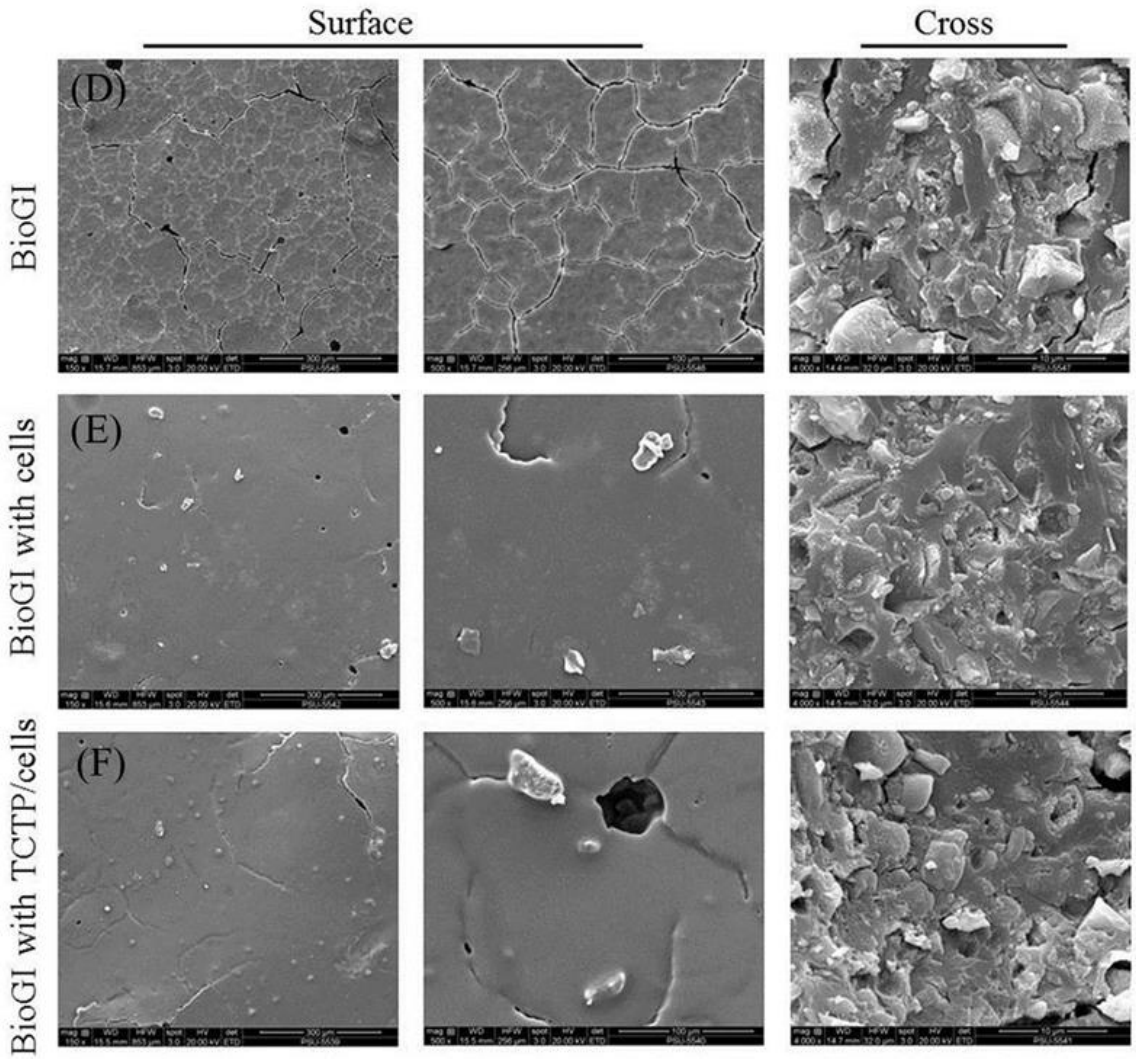


Figure 16. Morphology was observation by scanning electron microscope (SEM). NHOst cells cultured for 24 hour on specimen surface and crosssection before cell seeding and morphology and growth of cell on GIs (D): BioGI, (E): BIO-GI with cells and (F): BIO-GI with TCTP (Original magnification ×150, ×500 and ×4,000).

3.8 Effect of GIs supplemented with *Pmer*-TCTP on NHost cells gene expression

In this study, the expression of BMP-2, OPN of NHost cells after being cultured on different GIs for 1, 3 and 7 day was analyzed by quantitative real-time PCR (qPCR) assay.

The fold of BMP-2 gene expression was gradually different in each groups, its pattern was individual. The response of BMP-2 gene was reported in average of fold expression (Fig. 17). In BIO-GI with TCTP group, the BMP-2 gene expression increased gradually during culture. The expression of BMP-2 mRNA increased in the early phase of osteoblasts culture. A higher level of expression was observed in the *Pmer*-TCTP group on day 3 of the culture($P < 0.05$), later than the up-regulation of BMP-2 gene on day 3 , the expression of BMP-2 gene down-regulated on day 7. However, the down-regulation of BMP-2 gene was significantly indicated in GI and BIO-GI without added *Pmer*-TCTP on day 1 and day 7. Although a significant expression of BMP-2 gene was observed between GI and BIO-GI on day3, either with or without added *Pmer*-TCTP on day 1 and GI and GI added added *Pmer*-TCTP on day 7. In addition, *Pmer*-TCTP resulted in slightly up-regulated in BIO-GI with *Pmer*-TCTP cells immediately until day 3 compared with each time period of without added *Pmer*-TCTP group.

The expression of OPN gene was lower than control in all groups (Fig. 18). BIO-GI with and without *Pmer*-TCTP slightly up- regulation on day 1 until on day 3 and down-regulated on day 7($P < 0.05$). GI added *Pmer*-TCTP down-regulation on day 3 ($P < 0.05$) compare to control. However, the down-regulation of OPN gene was significantly indicated in BIO-GI without added *Pmer*-TCTP on bothday1 and day 3.

Although a significant expression of OPN gene was observed between GI and BIO-GI at 1 day either with or without added *Pmer*-TCTP on day 3 and GI and GI added *Pmer*-TCTP on day7.

The fold change of both BMP-2 and OPN gene expression of cells cultured on specimens with added *Pmer*-TCTP increased gradually up- regulation after day1 and reach the highest on day 3 then down- regulation on day 7.

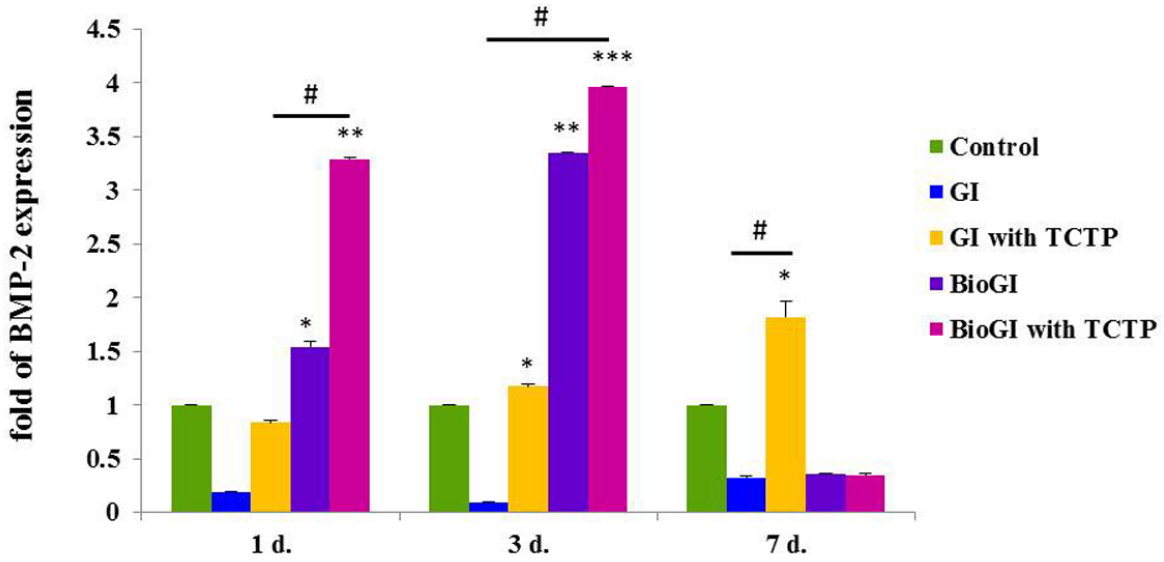


Figure 17. The fold change of BMP-2 expression of NHost cells cultured on GIs supplemented with and without *Pmer*-TCTP was determined using quantitative real-time PCR (qPCR). The cells were cultured for 24, 72 hours and 7 days (n=3). The values were normalized with GAPDH in $2^{-\Delta\Delta CT}$ method. Data are expressed as means ± standard deviation (n=3 in each group). *, **, *** Statistically significant difference compared with control group (culture medium) at $P<0.05$. # Statistically significant difference compared between group at $P<0.05$ (two-way ANOVA with Tukey multiple comparison test).

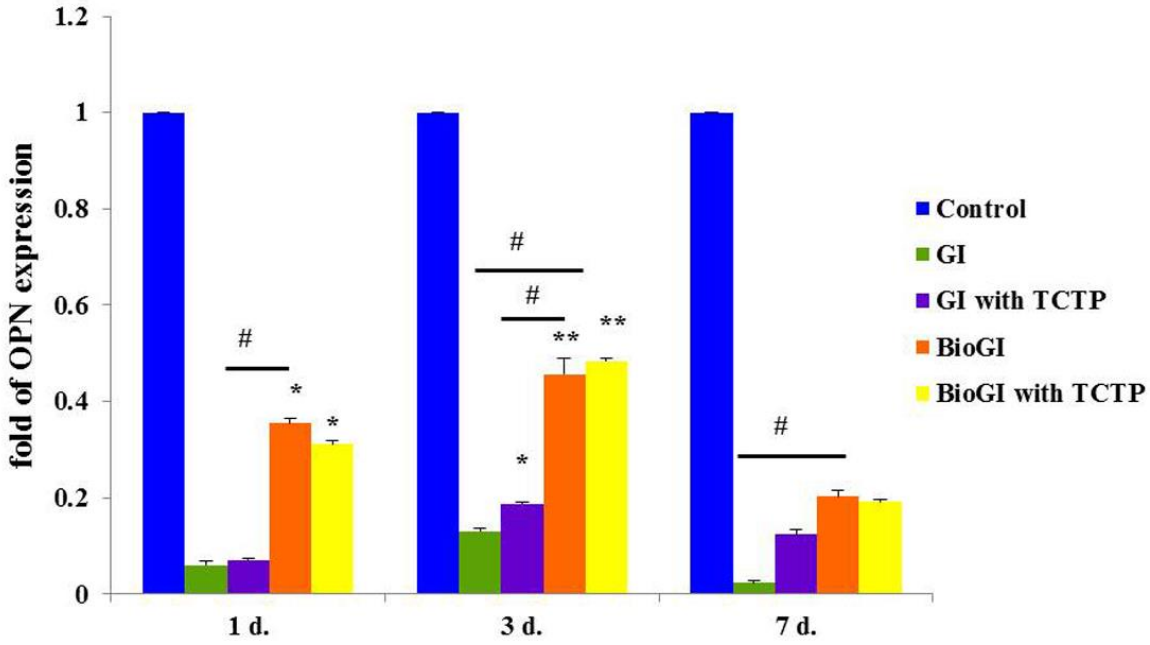


Figure 18. The fold change of OPN expression of NHost cells cultured on GIs supplemented with and without *Pme*r-TCTP was determined using quantitative real-time PCR (qPCR). The cells were cultured for 24, 72 hours and 7 days (n=3). The values were normalized with GAPDH in $2^{-\Delta\Delta CT}$ method. Data are expressed as means ± standard deviation (n=3 in each group). *, **, *** Statistically significant difference compared with control group (culture medium) at $P<0.05$. # Statistically significant difference compared between group at $P<0.05$ (two-way ANOVA with Tukey multiple comparison test).

CHAPTER 4

DISCUSSION

This study investigated *Pmer*-TCTP release from GIs on human osteoblasts (NHost cells). In addition, the bioactivity of the released substances requires further investigation.

Glass ionomer cement (GI) used in this study was a conventional material. GI has been modified by added incorporated with 15% of chitosan (by weight) and 5% of bovine serum albumin (BSA) (by weight). Chitosan used in this study had a molecular weight of about 62 kDa. They were mixed thoroughly into the powder of glass ionomer cement prior specimen preparation by *Pmer*- TCTP added one hundred nanograms per 0.1 gram of glass ionomer cement (by total weight) and if loaded in a 7 mm diameter and 1 mm thickness Teflon mold, the total TCTP will be about 1 ng per specimen.. We used this concentration of TCTP in the specimen by based on the result from MTT assay which was found that *Pmer*-TCTP treated cells at 1 and 10 ng/ml is not toxic to cells, in contrast, it can increase cells viability after exposure for 72 hours. From our previous study, we found that protein release from the specimen at the early phase was approximately 1/100 of the added protein. [41] It was found that protein release from group BIO-GI with added *Pmer*-TCTP released higher quantity of the protein than group GI with added *Pmer*-TCTP after 24 hours. Because chitosan and albumin added in glass ionomer cement can prolong and increase the release of protein which might due to the formation of polyelectric complexes between the anionic group of poly (acrylic acid) and the cationic group of chitosan that used to prolong drug delivery. In addition, chitosan is a naturally biodegradable polymer, non-toxic to cells and be biocompatible.[11] From our previous study, we found that chitosan added to GIC was able to prolong the release of BSA for 2 weeks and BSA by itself can promote release and was able to reduce the toxicity

of the material. This study we reduced the percentages of chitosan from 20% by weight to 15% to reduce the viscosity and enhance releasing property of the cement.[41]

TCTP is a highly conserved protein and has become broadly being concerned. TCTP can function as a growth signal and its expression associates with tumorigenessis.[4] Furthermore, TCTP is involved in important cellular processes, cell growth and cell cycle progression, and can protect cells from various stress conditions and apoptosis due to its biological properties.[4] The results from MTT assay, it was found that the addition of *Pmer*-TCTP at low concentration 1- 100 ng/ml can promote cells growth and function. From the previous study, TCTP can function as growth promoter and the hight levels of TCTP expression associates with tomorigenesis while P53 that prevent the growth and survival of potentially malignant cells. Repression between TCTP and P53 was reported that high concentration of *Pmer*-TCTP (10-20µg/ml) resulted to slightly depressed cell proliferative which may represent to the excess of TCTP may activate antagonism like P53 to balance cells proliferation.[53]

The result from BrdU assay suggested that the added *Pmer*-TCTP of GIs can promoted bone cells proliferation and BIO-GI may release higher amount of *Pmer*-TCTP than GI which corresponsed with the releasing experiment that detected by Western blot. However, it may require the proper amount because BIO-GI with 1ng/ml TCTP promote cell proliferation than BI-GI with 10ng/ml TCTP. IT also noticed that GI may release some toxic substances because the proliferation was the least and less than control. BIO-GI gave higher cell proliferation than GI which may be due to less cytotoxicity. From our previous study, chitosan did not increase the cytotoxicity of GI. The mechanism of chitosan to reduce cytoxicity is not known, whereas BSA itself has the property to bind toxic chemical. The addition of chitosan can reduce the percentages of DNA damage caused by 2-hydroxyethyl methacrylate and also can promote pulp cell regeneration in both *in vitro* and *in vivo*.[11] BIO-GI+TCTP gave the highest percentage of cell proliferation which may concluded that BIO-GI+TCTP have good cellular adaptability and support cells proliferation and a better prolonged released TCTP from the specimens.[41]

Pmer-TCTP added in BIO-GI demonstrated its capability to increase ALPase activity after 7 days and promote mineralization higher than other specimens after 14 days according to the ARS assay. This study we used *Pmer*-TCTP fron banana prawn which still affect on human osteoblasts (NHost cells). This result support its conserved protein can promote cell proliferation and differentiation.

The present study found the expression of BMP-2, OPN on GIs supplemented with and without *Pmer*-TCTP was not stable and gradually up-regulated at day 1, 3 and down-regulated on day7. The expression of BMP-2 mRNA increased in the early phase of osteoblasts culture. A higher level of expression was observed in BIO-GI+TCTP added *Pmer*-TCTP on day 3 of the culture ($P < 0.05$) according ALPase activity higher than in group BIO-GI+TCTP.

Osteopontin (OPN) is phosphorylated sialic acid rich non-collagenous bone matrix protein. OPN has been implicated as an important factor in bone remodeling, is a prominent component of the mineralized extracellular matrix of bone. OPN is characterized by the presence of a polyaspartic acid sequence and sites of serine/ threonine phosphorylation.[59] OPN are bone mineralization, regulation of immune cell function, inhibition of calcification, control of tumour cell phenotype and cell activation.[61] Bone morphogenetic protein 2 (BMP-2) is an implicated in a variety of functions, induce the formation of both cartilage and bone. Acts as a disulfide-linked homodimer and induces bone and cartilage formation. It is a candidate as a retinoid mediator. Plays a key role in osteoblast differentiation.[62]

The expression of OPN was down- regulated in all groups compare to control. In addition, the expression of OPN corresponse to Alizarin red staining because decrease in all groups when compare to control however, the expressions of OPN in BIO-GI and BIO-GI with TCTP were higher than GI which may confirmed about the higher cytotoxicity of GI than BIO-GI and BIO-GI with TCTP, respectively.

This preliminary study demonstrated that it was possible to develop a new glass ionomer cement that could provide sustained release of growth factor or prolong drug delivery and application for bone tissue engineering.

CHAPTER 5

CONCLUSION

This study evaluated the effect of novel glass ionomer cement supplemented with TCTP on human osteoblast cells. This novel glass ionomer cement or BIO-GI, is a new formula of the conventional GI that had added chitosan and albumin in order to prolong release of biological molecules. From the results of this study, we can conclude that:

1. Low concentration of translationally controlled tumor protein (*Pmer*-TCTP) from banana prawn (*Penaeus merguiensis*) at 1 ng/ml – 10 µg/ml in culture medium can promote bone cell viability and proliferation.
2. The BIO-GI supplemented with translationally controlled tumor protein from banana prawn (*Penaeus merguiensis*) at the proper concentration has an ability to promote bone cellsin proliferation, differentiation and mineralization.
3. This novel material showed a potential for the retained effect of added *Pmer*-TCTP longer than the GI.
4. BIO-GI added *Pmer*-TCTP to promote up-regulation of BMP-2 gene expression, but down-regulation of OPN gene expression.
5. However, this protein has the potential for further development of a novel biomaterial which has less toxicity including a new biomaterial which may induce regeneration of bone healing/ regeneration of tissue.

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APPENDIX

APPENDIX

MATERIALS

1.CELL CULTURE LABORATORY

1.1 Stock of α-MEM (Alpha-minimum essential medium) pH 7.2

α-MEM	13.9 g
$NaHCO_3$	2.2 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.2 with HCl. Add distilled water to 1000 ml. Sterilize the DMEM by filter 0.22 µm and store at 4 ^{o}C.

1.2 Nutrient Mix Media

stock α-MEM pH 7.3	156 ml
1% L-glutamin	2 ml
20% FBS	40 ml
1% Antibiotic-Antimycotic	2 ml

Adjust the volumn to 200 ml and Sterilize the DMEM by filter 0.22 µm and store at 4 ^{o}C.

1.3 L-ascorbic acid 100 mM

L-ascorbic acid	100 mg

Dissolve in 10 ml of distilled water and sterilized by filter with 0.22 µm membrane and store at 4 ^{o}C.

1.4 L-Glutamate stock 200 mM

1.5 Penicillin- streptomycin stock 10^4 unit/ml

1.6 Antimycrotic-Antibiotic

1.7 Phosphate-buffered saline (PBS) pH 7.4

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to 1000 ml. Sterilize the buffer by autoclaving and store at room temperature.

1.8 Prepare of 0.05 % Trypsin-EDTA

0.5 % Trypsin-EDTA

Dissolve 0.5 % Trypsin-EDTA in PBS pH 7.4 in ratio 1:10

2. MTT ASSAY

2.1 MTT solution

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide 50 mg

Dissolve in 10 ml of sterilized phosphate buffer saline (PBS) and sterilize by filter with 0.22 µm membrane

2.2 HEPES (pH 7.4) 10mM

HEPES	238 mg

Adjust the volume to 80 ml with distilled water before adjust pH to 7.4. After that, adjust volume to 100 ml with distilled water and then sterilize by filter with 0.2 µm membrane

2.3 Sorensen' glycine buffer

Glycine	750 mg
NaCl	585 mg

Adjust the volume to 80 ml with distilled water before adjust pH to 10.5. After that, adjust the volume to 100 ml with distilled water and then sterilize by filter with 0.22 µm membrane

3. Media and antibiotics for Bacterial culture

3.1 LB (Luria Bertaini) broth (Supplement with 100 µl/ml ampicillin)

Yeast extracts	5.0 g
Tryptone	5.0 g
Sodium chloride	2.5 g

Adjust the volume of the solution to 500 ml with distilled and sterilize by autoclaveing for 15 min at 15 psi. add 1 ml of ampicillin (100 mg/ml) into warm medium (50 ^{o}C)

3.2 2XYTA

Yeast extracts	5.0 g
Tryptone	8.0 g
Sodium chloride	2.5 g

Adjust the volume of the solution to 500 ml with distilled and sterilize by autoclaveing for 15 min at 15 psi.

3.3 Ampicillin Concentration 100 mg/ml

Ampicillin sodium salt	100 mg

Dissolve in 1000 ml with distilled water and store at -20 ^{o}C.

3.4 1 M IPTG

IPTG	2.38 g

Dissolve in 10 ml with distilled water and store at -20 ^{o}C.

3.5 Lysis buffer (Flegel pers. Comm., 1998)

Tris-HCl, pH 9.0	50 mM
EDTA	100 mM
NaCl	50 mM
20% SDS	100 ml

Adjust the volume of the solution to 500 ml with distilled and sterilize by autoclaveing for 15 min at 15 psi.

4. Solution for SDS-PAGE

4.1 30% Acrylamide-bisacrylamide

Acrylamide-bisacrylamide	29 g
N,N'-methylene-bis acrylamide	1 g

Dissolve in 50 ml with distilled water at 37 ^{o}C. Adjust the volume to 100 ml with distilled water, then sterilize by filter with 0.22 µm membrane and store at 4 ^{o}C.

4.2 10% sodium dodecyl sulphate (SDS)

SDS	10 g

Dissolve in 100 ml with distilled water and store the solution at room temperature.

4.3 10% Ammonium persulphate (APS)

APS	0.1 g

Dissolve in 1 ml with distilled water and store at -20 ^{o}C.

4.4 Separating gel

H_2O	1.7 ml
30% Acrylamide-bisacrylamide	2.0 ml
10% Ammonium persulphate	0.05 ml
1.5 M Tris-HCl pH 8.8	1.3 ml
10% SDS	0.05 ml
TMED	0.002 m

4.5 Stacking gel

H_2O	2.1 ml
30% Acrylamide-bisacrylamide	0.5 ml
10% Ammonium persulphate	0.03 ml
1.5 M Tris-HCl pH 6.8	0.38 ml
10% SDS	0.05 ml
TMED	0.003 ml

4.6 2X sample buffer

10 % SDS	4.0 ml
Glycerol	2.0 ml
1 M Tris –HCl (pH 6.8)	1.2 ml
1 M DTT	2 ml
Bromophenol blue	0.002 g

Adjust the volume of the solution to 10 ml and store at 4 ^{o}C.

4.7 Tris-glycine buffer

SDS	1.0 g
Glycine	14.42 g
Tris-base	4.0 g

Dissolve in 1000 ml with distilled water and and store the solution at room temperature.

4.8 Staining

Coomassie blue R-250	2 g
methanol	525 ml
Glacial acetic acid	75 ml

Dissolve in methanol and Glacial acetic acid. Adjust the volume to 1000 ml with distilled water and store at room temperature.

4.9 6M Guanidine hydrochloride

Coomassie blue R-250	57.32 g

Dissolve in 100 ml with distilled water and store at room temperature.

4.10 Destain I

50% Methanol	500 ml
7.5% Acetic acid	75 ml

Adjust the volume to 1000 ml with distilled water and store at room temperature.

4.11 Destain II

5% Methanol	50 ml
7.5% Acetic acid	75 ml

Adjust the volume to 1000 ml with distilled water and store at room temperature.

5. Solution for SEM

5.1 1M $Na_2H_2PO_4$

NaH_2PO_4	12 g

Dissolve in 500 ml with Ultrapure water and store at room temperature.

5.2 1M Na_2HPO_4

Na_2HPO_4	14.2 g

Dissolve in 500 ml with Ultrapure water and store at room temperature.

5.3 PB buffer

1M NaH_2PO_4	23 ml
1M Na_2HPO_4	77 ml

Adjust the pH 7.2-7.3 with NaOH and store at room temperature.

6. Solution for Western blot analysis

6.1 Electroblotting buffer

Glycine	7.9 g
Tris-base	5.8 g
Methanol	200 ml

Dissolve the ingredients in Ultrapure water and bring up to volumn 1000 ml with Ultrapure water.

6.2 5X TBS-T buffer

Tween® 20	5 ml
NaCl	45 g
Tris-HCl	31.75 g

Dissolve the ingredients in Ultrapure water and bring up to volumn 1000 ml with Ultrapure water

6.3 Blocking buffer

Low fat dry milk	5 g
1X TBS-T	100 ml

6.4 Washing buffer

Low fat dry milk	10 g
1X TBS-T	1000 ml

7. Solution for Alizarin red

7.1 40 mM Alizarin red solution

Alizarin	0.0685 g

Dissolve in 5 ml with Ultrapure water Adjust the pH 7.2-7.3 with 1% Ammonium.

7.2 1% Ammonium Hydroxide

37% Ammonium Hydroxide	0.033 ml

Adjust the volume to 10 ml with Uitrapure water and store at room temperature.

7.3 10% Formaldehyde in PBS

Formaldehyde	3.51 ml

Adjust the volume to 10 ml with PBS pH 7.4 and store at room temperature.

8. Solution for RNA

8.1 10 mM Tris-HCl pH 7.0

Tris-HCl	0.0631 g

Dissolve in 40 ml with Ultrapure water Adjust the pH 7.0 with NaOH.

8.2 10 mM Tris-HCl pH 7.5

Tris-HCl	0.0631 g

Dissolve in 40 ml with Ultrapure water Adjust the pH 7.5 with NaOH.

8.3 6X gel loading buffer

0.25% Bromophenol blue	0.05 g
0.25% Xylane Cyanol FF	0.05 g
30% Glycerol in water	6 ml

Adjust the volume to 20 ml with Ultrapure water and store at 4 ^{o}C.

8.4 1.2% Agarose

Agarose	0.3 g

Dissolve in 25 ml with 0.5X TAE buffer and Microwave for 1-2 min.

VITAE

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