Effect of *Penaeus Merguiensis*-Translationally Controlled Tumor Protein on *TPT1* Expression of Heat-Treated Human Pulp Cells

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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 ผลของ *Penaeus merguiensis*-translationally controlled tumor protein ต่อการแสดงออกของยีน *TPT1* ในเซลล์เนื้อเยื่อในโพรงประสาทฟันมนุษย์เมื่อได้รับความร้อน

ผู้เขียน นาย คณัสนันท์ จิรโชติกูล

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ปีการศึกษา 2556

บทคัดย่อ

วัตถุประสงค์: ในขบวนการบูรณฟันด้วยการกรอฟันส่วนที่ผุออกจะส่งผลให้เกิดความร้อนขึ้น ซึ่งหากอุณหภูมิในโพรงประสาทฟันสูงขึ้นจากปกติ ประมาณ 5.5°C อาจทำให้เซลล์เนื้อเยื่อในโพรงประสาทฟันมนุษย์เสียหายและตายได้ อย่างไรก็ตามเซลล์จะตอบสนองด้วยการผลิต heat shock proteins (HSPs) ขึ้นมาปกป้องเซลล์ เมื่อไม่นานมานี้มีงานวิจัยหลายชิ้น ค้นพบว่า โปรตีน Translationally controlled tumor protein (TCTP) มีความสำคัญในขบวนการต่างๆของเซลล์ ทั้งในการแบ่งเซลล์ และ ปกป้องเซลล์จากสภาวะกดดันต่างๆ เช่น ขาดแคลนอาหาร อยู่ในสภาวะที่มีโลหะความเข้มข้นสูง สภาวะที่มี Ca^{2+} ความเข้มข้นสูงภายในเซลล์ และ สัญญาณ proapoptotic/cytotoxic ต่างๆ เป็นต้น ซึ่งมีงานวิจัยน้อยชิ้นมากที่สนใจศึกษา ผลของสภาวะกดดันเซลล์จากความร้อนต่อการตอบสนองของ โปรตีน TCTP ดังนั้น งานวิจัยนี่จึงต้องการศึกษาผลของสภาวะกดดันเซลล์จากความร้อนและการเติมโปรตีน TCTP จากภายนอก ต่อเซลล์เนื้อเยื่อในโพรงประสาทฟันมนุษย์ และ ต่อการตอบสนองของ TCTP ภายในเซลล์

วิธีการทดลอง: นำเซลล์เนื้อเยื่อในโพรงประสาทฟันมนุษย์จากคนไข้สามคน มาทดลองเลี้ยงภายใต้ สภาวะปกติ และสภาวะกดดันเซลล์จากความร้อน ที่ 43°C เป็นเวลา 45 นาที จากนั้นเซลล์ทั้งสองกลุ่มจะแบ่งเป็นสองกลุ่มย่อย ทั้งที่เติม และไม่เติม โปรตีน TCTP จากนั้นเซลล์ทั้ง 4 กลุ่มจะนำไปทดสอบความมีชีวิตของเซลล์ด้วยวิธี MTT assay ดูรูปแบบการตายของเซลล์โดยย้อมนิวเคลียสด้วยสี Hoechst 33258 ตรวจสอบการทำงานของเอนไซม์ caspase-3 และ ดูการแสดงออกของ TCTP ทั้งในระดับยีน ด้วยวิธี Quantitative PCR และโปรตีนด้วยวิธี western blotting

ผลการทดลอง: พบว่าโปรตีน TCTP ที่ความเข้มข้นต่ำ สามารถกระตุ้นการเจริญของเซลล์ได้ และชักนำให้มีการแสดงออกของยีน *TPT1* และโปรตีน TCTP เพิ่มขึ้น ในสภาวะกดดันเซลล์จากความร้อน ส่งผลให้เซลล์มีการตายแบบ อะพอปโทซิส กระตุ้นการทำงานของ เอนไซม์ caspase-3 และ กระตุ้นให้มีการแสดงออกของ ยีน *TPT1* มากขึ้น แต่กลับลดการแสดงออกของโปรตีน TCTP ภายในเซลล์ จากการเติมโปรตีน TCTP ให้กับเซลล์หลังจากผ่านสภาวะกดดันเซลล์จากความร้อน พบว่า ช่วยปกป้องเซลล์จากการตายได้เล็กน้อย และลดการแสดงออกของโปรตีน TCTP ภายในเซลล์มากขึ้น

สรุปผลการทดลอง: โปรตีน TCTP มี คุณสมบัติในการกระตุ้นการเจริญของเซลล์ และมีผลช่วยปกป้องเซลล์เมื่อได้รับภยันตรายจากความร้อนได้เล็กน้อย ซึ่งความร้อนดังกล่าวจะส่งผลให้เซลล์เกิดการตายแบบ อะพอปโทซิส

คำสำคัญ: เซลล์เนื้อเยื่อในโพรงประสาทฟันมนุษย์ อะพอปโทซิส โปรตีน TCTP สภาวะกดดันเซลล์จากความร้อน

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Major Program Molecular Biology and Bioinformatics

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ABSTRACT

Objective: During restorative procedures in carious teeth, cavity preparation can produce heat. The intrapulpal temperature rise of 5.5°C can be cause of damage to dental pulp and induced to apoptosis. However, pulp cells may survive such injuries. This may be due to the increased synthesis of heat shock proteins (HSPs). Several recent studies showed that TCTP plays an important role in cell cycle progression, early development, and protection against diverse cell stresses such as starvation, heavy metals, calcium and proapoptotic/cytotoxic signals. Less attention has been paid to the effect of heat stress on TCTP expression. Therefore, this study aimed to investigate the effect of heat stress and TCTP on human dental pulp cells (HDPCs).

Methods: HDPCs obtained from 3 patients were cultured in either normal condition or heat stress at 43^{o}C for 45 min and with or without added TCTP in the culture medium. The cell viability was determined by MTT assay. The nuclei of cells were stained with Hoechst 33258 dye and caspase-3 was measured. The expression of TCTP was studied at gene revel by Quantitative PCR and protein revel using western blotting.

Results: TCTP at low concentration can promote HDPCs proliferation and induced the up-regulated of both *TPT1* gene and TCTP protein. The heat stress affected to induce cells dead by apoptosis, activated caspase-3 and resulting in up-regulation of *TPT1* gene but down-regulation of TCTP protein. The addition of TCTP in heat-treated cells slightly protected cell death and down-regulation of TCTP protein.

Conclusion: TCTP can promote HDPCs proliferation and slightly protect them from heat stress effect that induced cells dead by apoptosis.

Keywords: HDPCs, Apoptosis, Heat stress, *TPT1*, TCTP

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Finally, I especially grateful to my family for their love and encouragement throughout my life also support and patience during the long process of my education. The usefulness of this thesis, I dedicate to my father, my mother, my brother and my sister.

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LISTS OF ABBREVIATIONS

ANOVA	=	One-way analysis of variance
˚C	=	Degree celsius
Caspases	=	Cysteinyl aspartate specific proteases
g	=	Gram
h	=	Hour (s)
HDPCs	=	Human dental pulp cells
HEPES	=	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
mg	=	Milligram
min	=	Minute (s)
ml	=	Milliliter
mM	=	Millimolar
ng	=	Nanogram
OD	=	Optical density
SD	=	Standard deviation
Trypsin-EDTA	=	Trypsin-ethylenediamine tetraacetic acid
α-MEM	=	Alpha modified Eagle's medium
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
µM	=	Micromolar
%	=	Percentage

CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

The translationally controlled tumour protein (TCTP) was discovered about 30 years ago. It is a highly conserved protein and abundantly expressed in all eukaryotes. This protein have been reported in various cellular functions and molecular interactions. TCTP is related to growth-promoting, acts as heat shock protein (HSPs) and anti-apoptotic properties. Its expression levels are up regulated in response to various cellular stimuli and stresses. During restorative procedures in carious teeth, cavity preparation can produce heat that induce death signals and lead to apoptosis. The intrapulpal temperature rise of 5.5°C can cause damage to dental pulp and induced to apoptosis. However, pulp cells may survive such injuries suggests that pulp cells have a recovery defense system. This may be due to the increased synthesis of HSPs. Several recent studies showed that TCTP plays an important role in cell cycle progression, malignant transformation, early development, protection against diverse cell stresses such as starvation, heavy metals, calcium or proapoptotic/cytotoxic signals. Less attention has been paid to the effect of heat stress on TCTP expression. Therefore, this study aimed to investigate the effect of heat stress and TCTP on human dental pulp cells.

LITERATURE REVIEW

1.1 The Translationally Controlled Tumour Protein (TCTP)

1.1.1 History of TCTP's discovery

The first publication of the translationally controlled tumour protein (TCTP) date back since 1981. This protein was discovered by three independent groups that interesting in translational regulated-proteins. First Thomas-group, they identify protein name 'Q23', the protein has molecular weight about 23 kDa which are up-regulated to response of serum-stimulated mitogenic in Swiss 3T3 cells, mouse fibroblasts (1). In 1982, Yenofsky- group that discover abundantly mRNAs that are represent in untranslated messenger ribonucleoprotein (mRNP) particles in mouse sarcoma ascites cells coding for polypeptide they designated 'P21'(2) and found in mouse erythroleukemia cells (3). In 1989, Bohm-group publish protein name 'P23' that found to be preferentially synthesized in the exponentially growing Ehrlich ascites tumor cells (4). cDNA sequencing of 21 kDa protein in the mouse erythroleukemia cells (5) and sequencing of P23 cDNA indicate that P23 (4) was identical with a 21 kDa protein of mouse erythroleukemia cells (2, 5). cDNA sequences of this protein in human mammary carcinoma (6) were published and it was called 'translationally controlled tumour protein (TCTP)'. Recently TCTP was quite increased the attention from several researchers in various biologically in a wide range of cell process. So that novel function of TCTP was revealed, TCTP displayed an extracellular function as a histamine release factor, led the authors to suggest names as 'histamine releasing factor (HRF) (7). In 2001, Li's group discover that TCTP was a novel anti-apoptotic protein involved in cell survival and apoptosis regulation. These findings cause other names 'fortilin' to this protein (8). It is also known under several different names. Many name relate for only specific function but unspecific name 'TCTP' is still broadly used.

1.1.2 Gene and mRNA structure

TCTP in the human encoded by a *TPT1* gene (locus ID7178) at a chromosomal localization of *TPT1* gene which has been determined in the 13q14 region consists of 4,211 base pairs (9, 10). The *TPT1* gene contains two highly conserved CRE sites between -50 and -89 in close vicinity to a TATA-box at -30 (10) and several promoter elements, in reporter gene assays show this region indicate a strong promoter activity comparable to viral promoters (11). *TPT1* consist of 5 introns and 6 exons. This gene generates 2 difference type of mRNAs indicate in TCTP mRNA1 (843 nucleotides) and TCTP mRNA2 (1,163 nucleotides) that same coding and 5´ untranslated regions (5´ UTRs) but unlike only in the length of their 3´ UTRs (Figure 1) because there use of two alternative polyadenylation signals. The expression of both types of these mRNAs shown in all mammalian tissues were investigated but their quantity and ratio of expression was different. The shorter mRNA usually being more abundant than tong mRNA at ratio about 3:1 or 6:1 or 25:1 in different tissues (9). The 5´UTR is CG-rich about 80%, TCTP mRNA has been shown a highly structured RNA that is able to activate the dsRNA-dependent protein kinase PKR (12). The 3´UTR present AU-rich regions and AUUUA elements (13).

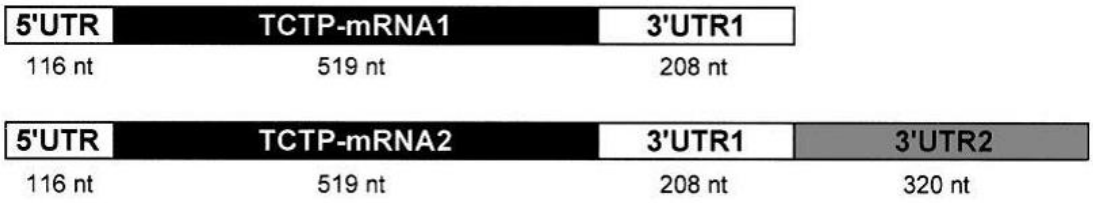

Figure 1. cDNA recombinants representing TCTP mRNAs 1 and 2 were isolated from a rabbit (9).

1.1.3 Protein structure and conservation

TCTP is a highly conserved multifunctional protein over a long term of evolution. It is widely expressed in all eukaryotic organisms. Since the discovery of TCTP, its expression level was investigated in more than 500 tissues and cell types. TCTP is highly expressed in mitotically active tissues as skeletal muscle and low

expression in non-mitotic tissue as brain (9). TCTP no similarity to other proteins that discover before so it was listed as a ‘family’ on its own. TCTP is a 23 kDa protein in human, 21 kDa in mouse and about 26 kDa of the banana prawn

Human TCTP consist of 172 aa, The C-terminal self-interaction domain from aa 126 to 172. The polo-like kinase interaction domain from aa 107 to 172. The tubulin binding domain from aa 79 to 123. The Ca^{2+} binding region from aa 81 to 112. The triad binding surface consisting of Glu12, Leu74, and Glu134 for binding the Rab G protein-like translation elongation factor eEF1A. Arg21 critical for the interaction between TCTP and the Bcl-xL homolog Mcl-1. Bcl-xL interaction region was localized to the TCTP N-terminal 1-40 aa region (14).

The 3D solution structure of TCTP in the fission yeast shown this molecule consists of three domains, the core β-sheet domain, an α-helical domain and a flexible loop structure were shown in Figure 2 (15).

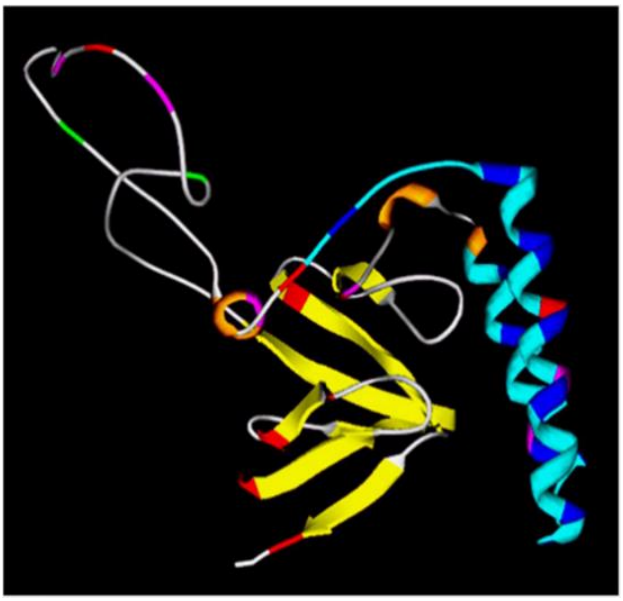

Figure 2. Three-dimensional structure of the TCTP protein. The ribbon structure of fission yeast TCTP as originally published (15) is shown (PDB: 1H6Q). The domain structure is indicated. The β-stranded core domain is shown in yellow, and the α-helical domain region is marked in blue. The most conserved residues are labelled in red or magenta. Green indicates the positions of the serine residues phosphorylated by the mitotic kinase Plk-1.

1.1.4 The functions of TCTP

1.1.4.1 TCTP's property in cells

TCTP play a role as a multifunctional protein and cytoprotective function in cell. TCTP expression is associated with a several biological activities such as the cell cycle that conserved mitotic growth integrator in animals and plants (16), anti-apoptosis, cytoskeleton, protein synthesis, immune response, development, cancer, tumor reversion and its crucial role in development.

The α-helical domain of TCTP structure interaction with the mitotic spindle for microtubule stabilization in a cell cycle-dependent manner and predominantly to the poles but is detached from the spindle during metaphase to anaphase transition (17, 18). There are two phosphorylation sites for the mitotic polo-like kinase (Plk-1) in the flexible loop of the TCTP structure (19), phosphorylation of TCTP by Plk-1 case detachment of TCTP from the spindle microtubules that might be important for the metaphase-anaphase transition. However, the overexpression of TCTP actually slows cell cycle progression (17) and a recent study showed that overexpression of TCTP induces mitotic defects and chromosome miss-segregation in hepatocellular carcinoma development (20), so it appears that mass induction of TCTP expression levels is essential for progression through mitosis (21). The association of TCTP with filamentous actin (F-actin) structures was observed for an involvement in cell shape regulation and integrated with cytoskeletal interactions both in interphase and mitosis (22). The overexpression of TCTP activation of several growth signal pathways comprise tyrosine phosphorylation of the EGF receptor, Ras/Raf/ERK pathway, phosphorylation of PLC-ɣ pathway and the PI3K/Akt pathways (23) and inhibit Na,K-ATPase thought binding to the cytoplasmic domain of Na,K-ATPase results in Src release from Na,K-ATPase a subunit and Src activation of EGFR, PI3K/Akt and its downstream signaling pathways to cell survival and motility (23, 24).

TCTP has been associated with cancer since its discovery in tumor cells (1), it express in differing levels all normal cell types and tissues (9, 25) but high expression in cancer cells (21, 25) related to cancer relating to growth behavior, anti-

apoptotic properties and tumor-regeneration, moreover TCTP induces a faster mitotic exit and chromosome miss-segregation result in chromosome instability (20). Inhibition of TCTP expression by siRNA results in suppression of the malignant phenotype (26), Down-regulation of TCTP was found in the tumour cells reverting back from the malignant to the normal phenotype (tumour reversion) and the knockdown expression of TCTP in various malignant cell lines increased revertant cells was raised up to 30% (27). The gene silencing of expression of TCTP was associated with reduced cell viability (28) and increased sensitivity to oxidative stress in cancer cells (29).

In addition, TCTP mRNA has been shown a highly structured RNA that is able to interact and activate the dsRNA-dependent protein kinase PKR (12), activation process of PKR might remove free TCTP mRNA that would otherwise be available for translation. Also, a higher level of PKR might lower TCTP protein levels. PKR activates P53 that directly represses TCTP transcription. The antagonistic control between level of both P53 and TCTP might determine which pathway to choose, cell cycle arrest or apoptosis (30).

1.1.4.2 Anti-apoptotic properties of TCTP

Anti-apoptotic function of TCTP was first demonstrated in 2001 (8). They discovered that the overexpression of TCTP can protect HeLa cells from etoposide-induced apoptosis and coined the new TCTP's name 'fortilin' after that several papers has reports that TCTP response to protect cells form various stresses. The high Ca^{2+} levels in the cytosol could attack and injure mitochondrial membranes lead to the release of pro-apoptotic molecules, cytochrome c and AIF (apoptosis-inducing factor) than go to programmed cell death (31). Many groups have demonstrated Ca^{2+}-binding of TCTP, the region from aa 81 to 112 (32), the residues E58 and E60 that located in the floppy loop (31), the residues N131, Q132 and D150, located in the β-stranded core domain close to the connection with the α-helical domain (33) are involved in Ca^{2+}-binding. These reported on the binding activity of TCTP to Ca^{2+} is required for TCTP to binding and sequestering an intracellular Ca^{2+} scavenger to protect cells against Ca^{2+}-dependent apoptosis. TCTP act as anti-oxidative stress prevents hydrogen peroxide-induced cell death (29, 34) and its

functions as an antioxidant protein in the filarial parasites (35). The antagonism between TCTP and P53 was determined, P53 is a specific transcription factor nuclear protein that functions as a regulator of transcription to mediate many of its downstream effects by the activation or repression of target genes to inhibit cell division and induce apoptosis. These essential for prevent the tumor development (36). A negative feedback loop between P53 and TCTP. TCTP inhibits MDM2 auto-ubiquitination and promotes MDM2-mediated ubiquitination and degradation of P53 lead to cell survival. In addition, P53 directly represses TCTP transcription (37). Bax is pro-apoptotic protein, which have been previously implicated in increasing the mitochondrial membrane permeability during apoptosis. TCTP has been shown to binds to MCL1 and Bcl-xL, they are anti-apoptotic members of the Bcl-2 family. TCTP antagonizes apoptosis by interact with MCL1 and Bcl-xL to inserting into the mitochondrial membrane and inhibiting Bax dimerization shown in Figure 3 (38). The knocking down of TCTP expression by small interfering RNA (siRNA) increased apoptosis of cancer cells (28) and the knockout of TCTP gene leads to increased spontaneous apoptosis during embryogenesis and causes lethal in mice (38, 39).

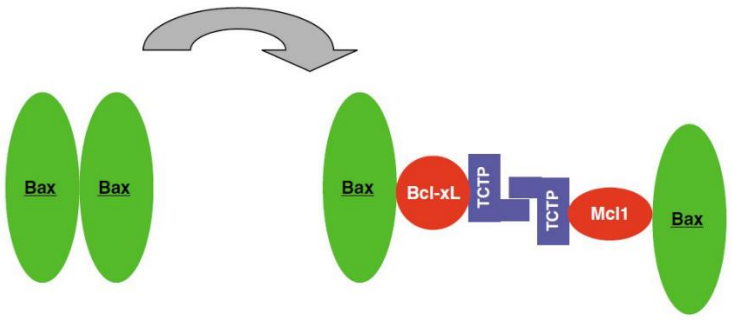

Figure 3. TCTP antagonizes apoptosis by inserting into the mitochondrial membrane and inhibiting Bax dimerization (38).

1.1.4.3 Heat shock protein-like activity of TCTP

TCTP highly expresses in heat stress conditions was found in parasitic organisms, such as *Trichinella* (40, 41) and *Schistosoma* (42). In the cabbage plants, silencing of the TCTP gene using RNAi led to reduced vegetative growth rate and decreased tolerance of cold, high temperature and salt stresses (43). The NMR

structure analysis also revealed the similarity of the highly conserved β-sheet domain of TCTP with guanine nucleotide-free chaperones, Mss4/Dss4 proteins family which bind to the GDP/GTP-free form of Rab proteins (15). Human TCTP acts as heat shock protein (HSPs) that up-regulate in cells following heat stress conditions, its binds to denatured proteins, refolds them, and also interacts with native proteins and protects them from denaturation in heat shock (42).

1.1.5 Translationally controlled tumor protein of the banana prawn (*Pmer*-TCTP)

The banana prawn (*Penaeus merguiensis* de Man) also known as the White Prawn or White Shrimp. It is one of several prawn species. Banana prawns are large, with an average weight of 17-18 per pound. Their body is pale yellow or translucent and speckled with reddish brown dots and the molecular mass of the native *Pmer*-TCTP was about 26 kDa. This work we used *Pmer*-recombinant TCTP. Sequencing of *Pmer*-recombinant TCTP was found to be 98% similar to *Penaeus monodon* TCTP (*Pm*-TCTP), 44% to human TCTP and 42% to mouse TCTP. The expressed *Pmer*-recombinant TCTP in *Escherichia coli* produced a protein of about 19.2 kDa on SDS-PAGE (44).

Figure 4. Banana prawn.
(http://fish.gov.au/reports/crustaceans/prawns/Pages/white_banana_prawn.aspx)

1.2 Pulp and human dental pulp cells (HDPCs)

Structures of the teeth consider a two types, the external structure and internal structure. The external structure is divided into two parts: the crown and root with the neck of teeth as a basis for discrimination. The internal structure of the tooth is composed of four parts (Figure 5).

1. Enamel is the most solid part of the tooth. Outer covering of the tooth along. Most of the area is thick and gradually thin into the neck area of the tooth. This act like armor to help protect the layer of dentin tissue and teeth. Basically, there is a white enamel.
2. Dentine is the next to enamel with yellow color. Dentine is more solid than bone but softer than enamel.
3. Cementum is the outer covering of the root tooth with the light yellow color and opaque.
4. Dental pulp tissue is a soft tissue. Dental pulp is made of ecto-mesenchymal components with neural crest-derived cells (45). Pulp tissue consisting of blood vessels and nerve that pass into the pulp through the opening of root tip. These organs within the central tooth called pulp cavity serve food to nourish the tooth and get a sense of the tooth to the brain. The cells in pulp tissue refer to HDPCs consist of odontoblasts, fibroblasts, undifferentiated mesenchymal cells, immunocompetent cells, neuronal and vascular networks (46)

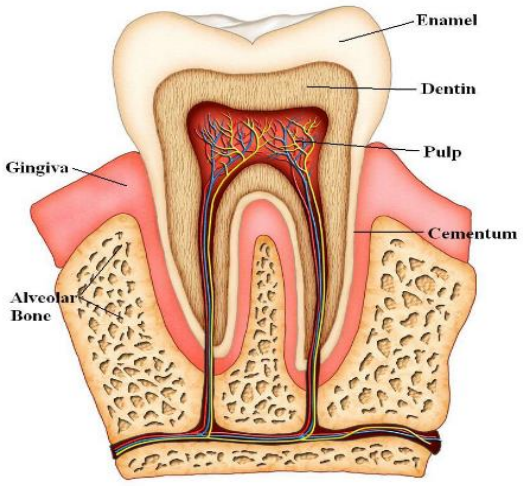

Figure 5. The cross section of tooth indicate dental pulp tissue in the central tooth. (http://culpepperdds.wordpress.com/for-patients/basic-tooth-anatomy/)

1.3 Cellular heat stress responses

The cells can respond to stress in several ways ranging from the activation of survival pathways to the activation of cell death pathways that resulting in eliminates damaged cells. However the stressed-cells mount a protective or destructive stress response depends to a large extent on the nature and duration of the stress as well as the cell type. The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from the insult. However, if the noxious stimulus is unresolved, then cells activate death signaling pathways (47).

Reactive oxygen species (ROS) is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen that can cause damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates, and lipids. The heat stress can induce ROS was indicated in several papers (48-50), increases Ca^{2+} via activation of the reversed mode of Na^{+}/Ca^{2+} exchangers that resulting in Ca^{2+} attack and injure mitochondrial membranes lead to the release of pro-apoptotic molecules (31, 51), activation of NF-kB that lead to inflammation (49, 51) and heat stress induced P53 translocate to mitochondria effect to induce early apoptosis through activating mitochondrial pathway with changes in mitochondrial membrane potential resulting in releasing of cytochrome c, so activate of caspase-3 and caspase-9 (48). Heat stress involve to down-regulate of anti-apoptotic protein, Bcl-2 (50) and repression of splicing of mRNA precursors (52). The cellular consequences of these stresses is protein damage leading to proteins denature.

The responses of cells to protect, which ensures the cell's survival by increase the expression of chaperone proteins that help to refolding of denatured proteins and alleviate protein aggregation. This confers a transient protection, leading to a state that is known as thermotolerance (53). In heat stress, the general protein transcription and translation are halted. However, the heat shock factors (HSFs), transcription factors that activated under these stress. Transcriptional activators in the inducible expression of genes encoding heat shock proteins (HSPs) (54). HSPs achieve heat stress effects directly, their capacity as molecular chaperones, inducible

HSPs bind to and aid the refolding of denatured proteins, thereby preventing protein aggregation (55). HSPs can inhibit apoptosis and caspase activation (56, 57). One of HSPs, HSP27 can also interact with other proteins that regulate cell survival, Akt and may also act in stabilizing Akt in heat stress (58).

1.3.1 Heat stress on dental pulp cells

During restorative procedures in carious teeth, cavity preparation can produce heat. The thermal of pulp chamber during cavity preparation varies over a wide range of temperatures (Table 1). Heat stress able to induce death signals and lead to apoptosis (59). The intrapulpal temperature rise of 5.5 °C can be cause of damage to dental pulp and induced to apoptosis (60). When pulp cells in a state of heat stress, the stress can stimulate pro-inflammatory cytokines, reactive oxygen species (ROS) and interleukin-8 (IL-8). They cause cells inflammation (49). Moreover, heat stress decrease activity of alkaline phosphatase and damage intercellular communication which reduce interaction between cells. However, pulp cells may survive such injuries. The response of the pulp to restorative procedures, may be due to the increased synthesis of HSPs (61).

Table 1. Temperature increase in the pulp during cavity preparation (60).

Currently available therapies	**Corresponding intrapulpal temperature rise (°C)**
Laser assisted tooth ablation	2.3–24.7
Laser assisted caries prevention	1.2–4.0
Polymerization of dental restorative materials (DRMs)	2.9–7.8
HSHPs cavity preparation (without water, high load)	16.4–19.7
HSHPs cavity preparation (without water, low load)	7.1–9.5
HSHPs cavity preparation (with water, high load)	2.2–5.9
HSHPs cavity preparation (with water, low load)	-1.8 - 5.0

1.4 Apoptosis

1.4.1 The programmed cell death

When cells in multicellular organisms that are infected or damaged or that have simply reached the end of their functional life span often enter a program of controlled cell suicide called programmed cell death. Moreover, cells death plays a crucially important in development into adulthood of animal to eliminate unwanted cell for example cell death keeps our hands from webbed forming (Figure 6).

Apoptosis (from the Greek, meaning "falling off;' and used in a classic Greek poem to refer to leaves falling from a tree) is one form of programmed cell death (62). The programmed cell death in animals usually occurs from apoptosis. The term of apoptosis was first describe in 1972 (63).

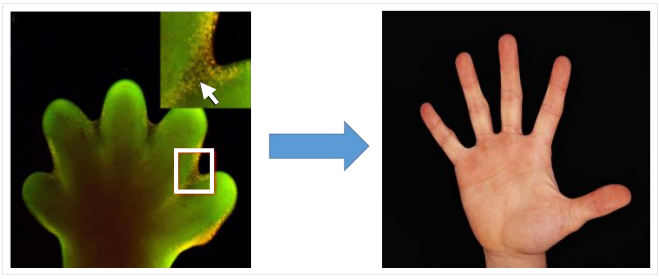

Figure 6. Cell death keeps our hands from webbed forming. The white arrow indicate apoptotic cells during development of finger. (http://php.med.unsw.edu.au/embryology/index.php?title=Musculoskeletal_System_-_Limb_Development and http://eofdreams.com/photo/hand/02/)

1.4.2 Morphology of Apoptosis

During the early of apoptotic process, cell shrinkage, round and pyknosis (chromatin condensation) are appear. Shrinking cells are smaller in size, the cytoplasm is condensed, and organelles are more tightly packed in cells. The irreversibility chromatin condensation in the nucleus of a cell that cause pyknosis, or karyopyknosis. This is the most characteristic feature of apoptosis. Then followed by karyorrhexis (fragmentation of the nucleus), plasma membrane blebbing and

separation of cell fragments into apoptotic bodies that consist of cytoplasm with tight pack of organelles with or without a nuclear fragment during a process called "budding."(Figure 7) all of this are pack within an intact plasma membrane. Afterward these bodies are phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within its phagolysosomes. Apoptotic process is no inflammatory reaction that unlike necrosis (Table 2) because: (1) process of apoptosis do not release their intracellular into the surround tissue; (2) they are quickly phagocytosed by surrounding cells and (3) the engulfing cells do not produce anti-inflammatory cytokines (64)

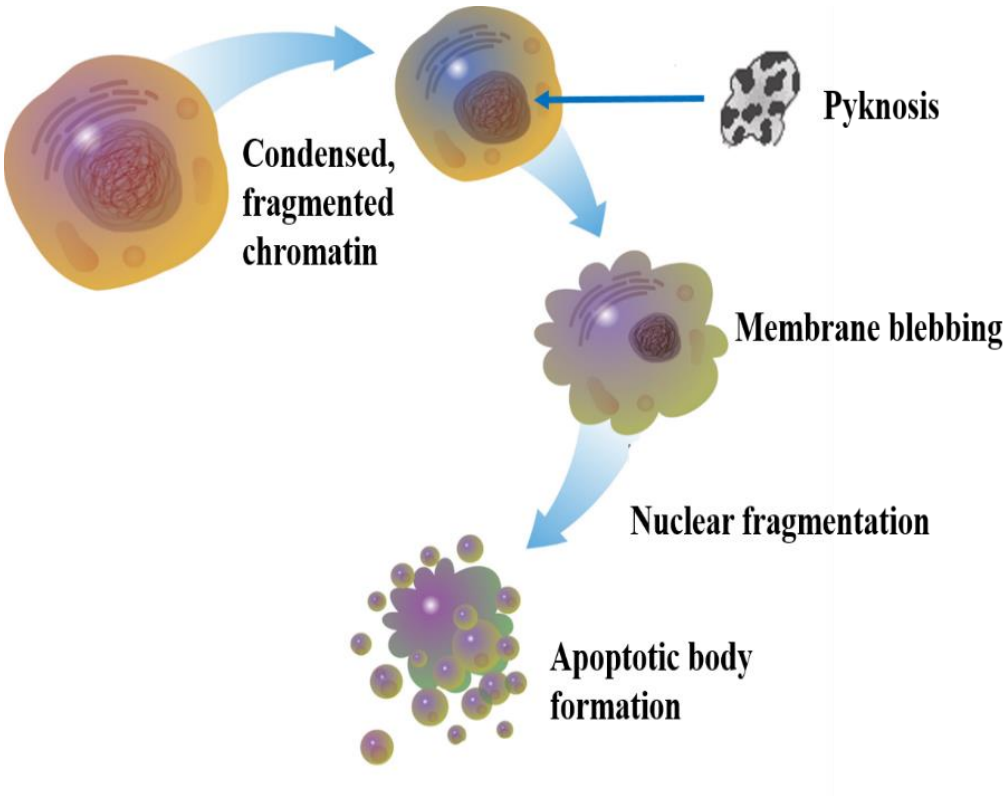

Figure 7. A morphology of apoptosis.
(http://www.genome.gov/dmd/img.cfm?node=Photos/Graphics&id=85266)

Table 2. Comparison of morphological features of two different ways for cells to die.

	APOPTOSIS	**NECROSIS**
ATP required	Yes	No
Cellular pattern	Chromatin condensation, single-cell death, shrinkage	Swelling, organelle disruption, tissue death
DNA breakdown	Nucleosome-sized fragments	Random fragments
Plasma membrane	Blebbed (apoptotic bodies)	Bursts
Reaction in tissue	No inflammation	Inflammation

1.4.3 Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and involve in energy-dependent cascade of molecular pathway. Recent research indicates that there are three main of apoptotic pathways: (1) the extrinsic (death receptor pathway); (2) the intrinsic (mitochondrial pathway) and (3) perforin/granzyme (T-cell mediated cytotoxicity). However three of apoptotic pathways converge on the same terminal (execution pathway) via cleavage of pro-caspase-3 to activate caspase-3 cause endonuclease activation to degradation of DNA, protease activation to degradation of nuclear and cytoskeletal proteins, cytomorphological changes, chromatin and cytoplasm condense, nuclear fragmentation and finally formation of apoptotic bodies indicate in Figure 8. Moreover phosphatidylserine flips to the outer leaflet in early apoptotic process for phagocytic cell receptors than uptake by phagocytic cells.

1.4.3.1 The Extrinsic Pathway

In the case depicted in the figure, the stimulus for apoptosis is carried by an extracellular messenger protein called tumor necrosis factor (TNF), which was named for its ability to kill tumor cells. TNF is produced by certain cells of the immune system in response to adverse conditions, such as exposure to ionizing radiation, elevated temperature, viral infection, or toxic chemical agents such as those used in cancer chemotherapy. The first messengers, TNF evokes its response by binding to a transmembrane receptor, TNFR1. TNFR1 is a member of a family of related "death receptors" that turns on the apoptotic process. The cytoplasmic domain of each TNF receptor subunit contains a segment of about 80 amino acids called a "death domain" that mediates protein–protein interactions. Binding of TNF to the death receptors produces a change in conformation of the receptor's death domain, which leads to binding of the adapter protein TRADD with recruitment of the adapter protein FADD and RIP. FADD then associates with procaspase-8. Death-inducing signaling complex (DISC) is formed, resulting in the activation of procaspase-8. Caspase-8 is described as an initiator caspase because it initiates apoptosis by cleaving and activating downstream, or executioner caspases. Finally caspase-3 is activated resulting in undergoing execution pathway cause apoptotic formation as described above.

1.4.3.2 Intrinsic Pathway

Internal stimuli, such as irreparable genetic damage, lack of oxygen (hypoxia), extremely high concentrations of cytosolic Ca^{2+} that attack and injure mitochondrial membranes (31), viral infection, or severe oxidative stress trigger apoptosis by the intrinsic pathway that non-receptor mediated stimuli. Its produce intracellular signals that act directly on targets within the cell.

All of these stimuli cause changes in the inner mitochondrial membrane via induced members of the Bcl-2 family proteins, pro-apoptotic protein such as Bax, Bak, Bid, Bad, Puma, and Bim. In a healthy cell, pro-apoptotic protein are either absent or strongly inhibited, and the anti-apoptotic Bcl-2 proteins are able to

restrain pro-apoptotic protein. The stress that pro-apoptotic proteins are expressed or activated, thereby shifting the balance in the direction of apoptosis. In these circumstances, the restraining effects of the anti-apoptotic Bcl-2 proteins are overridden, and certain pro-apoptotic members of the Bcl-2 family, such as Bax, are free to translocate from the cytosol to the mitochondrial membrane that Bax molecules undergo a change in conformation that causes them to insert into the mitochondrial membrane and assemble into a multisubunit, protein-lined channel. Once formed, this channel dramatically increases the permeability of the mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore and promotes the release of certain mitochondrial proteins. The first most notably cytochrome c. Cytochrome c binds and activates apoptotic protease activating factor-1 (Apaf-1) to form an apoptosome that activate caspase-9 which described as an initiator caspase because it initiates apoptosis by cleaving and activating downstream, or executioner caspases. Finally caspase-3 is activated resulting in undergoing execution pathway cause apoptotic formation as described above.

1.4.3.3 Perforin/granzyme Pathway

The cytotoxic T cells are able to destroy target cells usually abnormal cells via this pathway, its stores a pore-forming protein called perforin (which is homologous to the complement component C9) in secretory vesicles and is released by local exocytosis at the point of contact with the target cell. Perforin then polymerizes in the target cell plasma membrane to form transmembrane channels. The secretory vesicles also contain serine proteases, which are thought to enter the target cell cytosol through the perforin channels. The serine proteases, usually granzyme A and granzyme B. One of the proteases, called granzyme B, activates a pro-apoptotic Bcl-2 protein called Bid by producing a truncated form of the protein called tBid; tBid then releases cytochrome c from mitochondria at the same time granzyme B activate caspase-10 in the end to activate caspase-3 resulting in undergoing execution pathway and cause apoptotic formation as described above. The granzyme A activation of caspase-independent pathway via break down the SET complex results to releasing of NM23-H1 to damage DNA undergoing apoptosis (64).

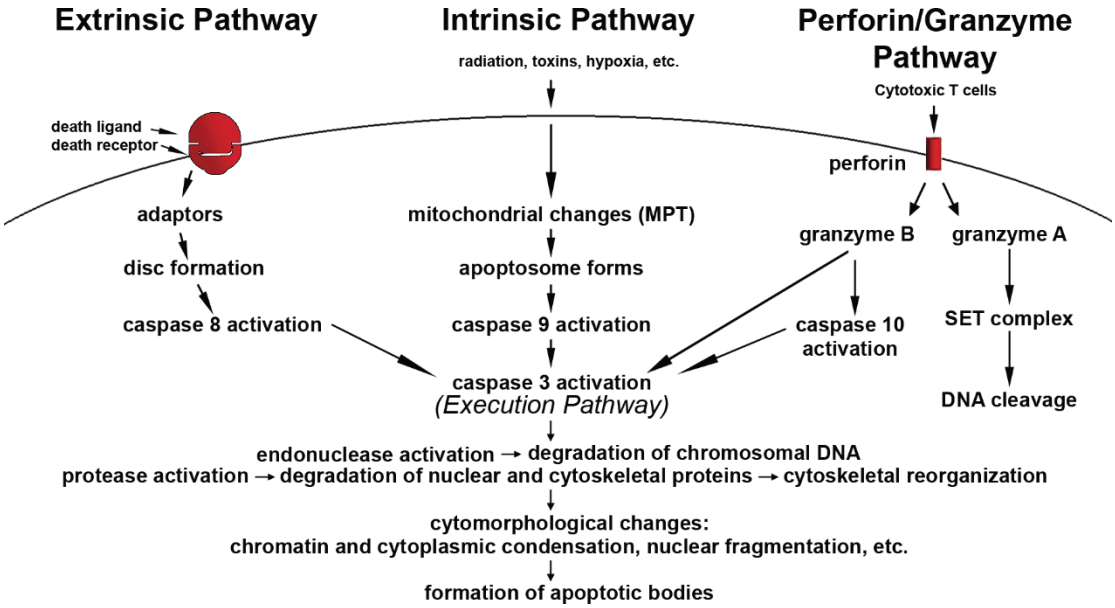

Figure 8. Presentation of three pathway of apoptotic mechanisms (64).

OBJECTIVE

There are little information on the responses of dental pulp cells after direct heat stimulation. In addition no information on expression of TCTP on human dental pulp cells (HDPCs).

This study aimed to investigate:

1. The effect of heat stress on cells death and expression of *TPT1* gene and TCTP protein on HDPCs
2. The effect of *Penaeus merguiensis*-translationally controlled tumor protein (*Pmer*-TCTP) on *TPT1* gene and TCTP protein expression in normal and heat-treated cells.
3. The effect of *Pmer*-TCTP on the survival of the heat-treated HDPCs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

2.1.1.1 Reagents for primary cell culture

Reagent name	Source
Alpha-minimum essential medium (α-MEM)	GIBCO^{R}, Invitrogen Corporation, USA
Fetal bovine serum	Biochrom AG, Germany
Penicillin- Streptomycin	GIBCO^{R}, Invitrogen Corporation, USA
Fungizone	GIBCO^{R}, Invitrogen Corporation, USA
L-Glutamine	GIBCO^{R}, Invitrogen Corporation, USA
Ascorbic acid	Sigma-aldrich, USA
Sodiumbicarbonate	Merck Inc Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Sigma-aldrich, USA
0.5%Trypsin-EDTA	GIBCO^{R}, Invitrogen Corporation, USA

2.1.1.2 Reagents for analysis

Reagent name	Source
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)	Sigma-aldrich, USA
Caspase 3 Assay Kit, Colorimetric	Sigma-aldrich, USA
Hoechst 33258 dye DNA staining	Sigma-aldrich, USA

RNeasy Mini Kit	Qiagen, Crawley, UK
QIAshredder	Qiagen, Crawley, UK
SuperScript. III First-Strand Synthesis System	Invitrogen Corporation, NY
FastStart Universal SYBR Green Master	Roche, Mannheim, Germany
BCA kit	Pierce, Rockford, USA
VECTA STAIN® ABC-AmP™, Wes tern Blotting Immunodetection Kit	Burlingame, CA

2.1.2 Instruments and special equipments

2.1.2.1 Instruments

Instrument	Company
Microflow advance biosafety cabinet-class II	Astec air BHA 48, UK
CO_2 incubator	HERA cell 240, Heraeus, Germany
Centrifuge	Savant, Speed fuge HSC 15 R, USA
Centrifuge (Micro)	Hettich Mikro 220 Centrifuge, UK
Light microscope	Nikon TMS, Japan
Fluorescence microscope	Olympus corporation DP71, Japan
Microplate Reader	Titertek Multiskan^{R} PLUS, USA
Autoclave	Tomy SS-325, USA
Hemocytometer	Laboroptik. Germany
Auto pipette	Accu-jet^{R} pro, BrandTech Scientific, Germany
Water bath	Memmert, Technical science and service, Germany

2.1.2.2 Special equipments

Special equipments	Company
6 well cell culture plates	Nunclon^{TM}, Nunc, Denmark
24 well cell culture plates	Nunclon^{TM}, Nunc, Denmark
96 well cell culture plates	Nunclon^{TM}, Nunc, Denmark
25 cm^2 cell culture flasks	Nunclon^{TM}, Nunc, Denmark
75 cm^2 cell culture flasks	Nunclon^{TM}, Nunc, Denmark
50 ml centrifuge tube	Corning Incorporation, USA
15 ml centrifuge tube	Corning Incorporation, USA
Microcentrifuge tube 1.5 ml	Bioline, UK
Millipore filter 0.45 µm.	Millipore corporation, USA
Filter Pipette Tips 10, 20, 200, 1000 µl.	Neptune corporation, Mexico
Pipette tip 20, 200, 1000 µl.	Corning Incorporation, USA
Microscope glass slide	Sail Brand, China
Round 12 mm dia. cover glass for Growth	Fisherbrand™, USA
Amicon ultra 10K device	Millipore corporation, USA
Amicon ultra 30K device	Millipore corporation, USA

2.2 Methods

2.2.1 Expression and purification of *Penaeus merguiensis*-translationally controlled tumor protein (*Pmer*-TCTP) in *Escherichia coli*

The full-length cDNA encoding TCTP protein of *Penaeus merguiensis* was tagged with a glutathione-S-transferase (GST) fusion system into pGEX-4T-1 vector (Amersham, Thailand) and transformed into bacterial *Escherichia coli* (*E.coli*) strain BL21 host (a gift from Assoc Prof. Wilaiwan Chotigeat). Expression of GST-*Pmer*-TCTP protein, the *E.coli* strain BL21 was inoculated into 50 ml of LB medium containing 100 µg/ml ampicillin for overnight in a shaking incubator at 37°C, 180 rpm. The inoculum was diluted in 500 ml (1:10) with preheat 2 x YT medium containing 100 µg/ml ampicillin. When the cell density reached 0.5, at OD 600 the cultures were induced by adding final concentration 1 mM IPTG and incubate at 37°C for 3 h. Afterthat, the cells were harvested by centrifugation at 4,000 x g and 4°C for 20 min. The pellet was suspended in 10 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Tris–HCl, pH 8.0) and sonicated at 200-300W for 10 s and rise 1-2 min/time (10-15 times) on ice (until solution was clear). Then, centrifuge for 20 min at 10,000 x g, 4°C. Before, collecting the supernatant (soluble protein) and analyzed by 12% SDS-PAGE. The soluble protein was purified by adding 10 ml of soluble protein to 5 ml of the prepared Glutathione Sepharose 4 Fast Flow (GE Healthcare Bio-Science, Piscataway, NJ, USA) and incubated for 1 h at room temperature with gentle agitation. Sediment the medium by staying still for 25 min. Carefully decant the supernatant and washed the Glutathione Sepharose 4 Fast Flow by adding 45 ml of 4°C Phosphate-buffered saline (PBS). Inverting the tube to mix and gentle agitation on a shaker for 5-10 min. Sediment the medium by staying for 25 min and repeat the washing process again about 5 times, 5 µl of the purified protein by this Glutathione Sepharose 4 Fast Flow was examined by SDS-PAGE. The washing process can stop and the protein store at 4°C and continue again tomorrow. To wash (12-15 times) until GST- *Pmer*-TCTP protein is purify as only a single band can be detected by SDS-PAGE. After that thrombin was used for cleavage of GST-tagged protein by adding 35 µl of thrombin and dilute with PBS to1ml/1ml of Glutathione Sepharose 4 Fast Flow. After incubate at 24°C for 16 h and kept at room temperature at 25 min,

the soluble protein in supernatant was collected. *Pmer*-TCTP protein was separated from thrombin by flow through amicon ultra 30K device than concentrate by amicon ultra 10K device. The purified *Pmer*-TCTP protein was dissolved in supernatant, its molecular mass about 19.2 kDa was analyzed by 12% SDS-PAGE and western blotting. Protein concentration was determined by a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.2.2 Primary cultures of human dental pulp cells

Human dental pulp cells (HDPCs) were isolated from adults premolars (n= 3) of S1 (23 years, woman), S2 (20 years, woman) and S3 (18 years, woman) at the Dental Hospital, Faculty of Dentistry, Prince of Songkla University, with consent forms approved by the Research Ethics Committee, Faculty of Dentistry, Prince of Songkla University. The isolation method followed as previously described (65). Briefly, the tooth surfaces of the freshly extracted teeth were cleaned with 70% ethanol and pound at the cement-enamel junction using a sterile fissure bur to reveal the pulp chamber. Then, gently separated the pulp tissue from the pulp chamber and root. Pulp tissue was minced into small pieces (1-2 mm^2) and digested in a pre-warm solution of 3 mg/mL collagenase type I (GIBCO-Invitrogen, Carlsbad, CA) and 4 mg/mL dispase (GIBCO-Invitrogen) for 45 minutes at 37°C. After spindown, supernatant was collected and centrifuged for 5 minutes at 600 x g. The supernatant was removed by gentle aspiration and suspended the cell pellets in complete medium which composed of alpha modified Eagle's medium (α-MEM), supplemented with 20% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 100 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, MO, USA), 100 µM L-glutamate, 2.5 µg/ml fungizone, 100 µg/ml streptomycin and 100 units/ml penicillin in 25-cm^2 culture flasks at a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced every 3 days. After approximately 15-20 days, cells grow to 70% confluence. Subculture was performed by withdrawn the culture medium and rinsed two times with PBS than were detached with 2 ml of 0.05% Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) at 37°C for 2-3 minutes than stop the reaction by adding 2 ml of complete media and pipet mixing to obtain single-cell

suspensions. Then cells were seeded in 75-cm^2 culture flasks with complete media (10% FBS) and were defined first passage cells line. Cells during passage 3 to 5 were used in all experiments. Store cells 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 Heat stress

The cultured cells were seeded at a density of 1.5 x 10^5 cells in 35 mm cell culture dish and maintained in humidified atmosphere of 5% CO_2 at 37°C for 24 h in prior the heat stress. For rapid heat stress, culture medium was replaced to the medium which was preheated at 43°C than the culture dishes were placed on the preheated blocks at 43°C, 5% CO_2 and kept in the incubator at 43°C for 45 minutes (59, 66). After heat stress, the medium was changed to medium with or without *Pmer*-TCTP that preheated to 37°C followed by incubation at 37°C (time point 0). The samples were harvested at specific time points (0 min to 72h post-heating).

2.2.4 Cell viability (MTT assay)

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay for assessing cell viability to measure the number of cells growth and survival. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. HDPCs at a density of 4 x 10^3 cells/well were cultured in 96 well plates at a humidified atmosphere of 5% CO_2 at 37°C for 24 h in prior the heat stress. In non-heat-treated group, the cells were cultured at 37°C. For heat-treated group, heat stress was induced by incubating the cell at 43°C for 45 min after that expose to variation of concentration of *Pmer*-TCTP and incubated for 24 and 72 h in humidified atmosphere of 5% CO_2 at 37°C. After which, the medium was removed than 200 µl of fresh medium containing 10 mM HEPES (pH 7.4) and 50 µl MTT solution (5 mg/ml in PBS) were added to each well than incubated in the dark for 4 h at 37°C. 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 (OD) of formazan production was measured at 570 nm using ELISA reader. The OD values corrected for a blank (medium only) of the experimental groups were divided. The mean values obtained from 6 wells for each condition were computed and plotted. Cell viability, which represented in the percentage. % viable cells was defined as (OD_{570} experimental group/OD_{570} control group) x 100%. Triplicate experiment were performed.

2.2.5 Hoechst 33258 dye DNA staining

HDPCs were seeded on 12 mm dia. cover glass for growth (Fisherbrand™) at 1.5 x 10^5 cells in 35 mm cell culture dish for 24 h in prior the heat stress. For heat-treated group, HDPCs were exposed to heat stress at 43°C for 45 min. After heat stress, the medium was replaced by the medium with or without *Pmer*-TCTP which preheated at 37°C followed by incubation at 37°C (time point 0). Samples were stained at the specific time points (0, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h post-heating). As non-heat-treated group, HDPCs were cultured at 37°C for 24 h. The medium was replaced by the medium with or without *Pmer*-TCTP which preheated at 37°C and harvested at the specific time points (0, 6 h, 24 h, 48 h and 72 h). For staining process, the medium was aspirated and washed cells once with 1ml of PBS. The cells were fixed in 500 µl of a solution of 3.7% of formaldehyde in PBS at room temperature (RT) for 15 min. The permeabilization step was performed by withdrawn the fix solution and added 500µl of -20°C of 100% methanol at RT for 20 min. Afterthat, the cells were rinsed once with 1 ml of PBS next 0.125 µg/ml of Hoechst (dilute fresh with PBS) was added and incubated for 15-20 min at RT in the dark place than rinsed 3 times with PBS before placed cover glass on the slide. Nuclear morphology was examined under a fluorescent microscope at 340-380 nm.

2.2.6 Caspase-3 Assay

The heat stress was induced by incubating the cells for 45 min at 43°C. After heat stress for 6 h, the induced cells were detached with 0.05% trypsin-ethylenediamine tetraacetic acid (Trypsin-EDTA). After centrifugation at 600 x g for 5 minutes at 4°C, removed the supernatant by gentle aspiration and washed the cell pellets once with 1 ml of PBS than centrifuged at 600 x g for 5 minutes at 4°C next supernatant was completely removed by gentle aspiration. The cell pellets were suspended in 1x lysis buffer at a concentration of 100 µl per 1 x 10^7 cells and incubated on ice for 20 minutes after that the lysed cells was centrifuged at 18,000 x g for 15 minutes at 4°C. The supernatants was transferred to new tubes. For analyzation, 5 µl of cell lysate was placed in the appropriate 96 well plate. 85µl of 1x Assay Buffer was added and started the reaction by added 10 µl of 2 mM caspase-3 substrate (Ac-DEVD-pNA) than mixed gently by shaking-avoid forming bubbles in the wells. The plate was incubated at 37°C in the dark for overnight. Absorbance at 405 nm was read with ELISA reader.

2.2.7 Purification of total RNA from HDPCs by RNeasy Mini Kit (Qiagen, Crawley, UK)

HDPCs at a density of 1.5 x 10^5 cells were seeded in 35 mm cell culture dish and cultured at a humidified atmosphere of 5% CO_2 at 37°C for 24 h in prior the heat stress. For heat-treated group, heat stress was induced by incubated the cells at 43°C for 45 min. After the heat stress, medium was changed to medium with or without *Pmer*-TCTP which preheated at 37°C followed by incubation at 37°C (time point 0). The samples were harvested at the specific time points (0, 6 h, 24 h, 48 h and 72 h post-heating). As non-heat-treated group, HDPCs were cultured at 37°C for 24 h. The medium was changed to medium with or without *Pmer*-TCTP and harvest at the specific time points (0, 6 h, 24 h, 48 h and 72 h). HDPCs were harvested by washing the cells 3 times with PBS that treated with 0.1% DEPC and added the 350 µl of Buffer RLT following shacked and pipetted to mix, and ensure that no cell clumps were visible after that pipetted the cells lysate directly into a QIAshredder

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 2 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 40 µl of RNase-free 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 total RNA was stored at– 80°C or used for RT-PCR immediately.

2.2.8 cDNA synthesis using SuperScript. III First-Strand Synthesis System (Invitrogen Corporation, NY, USA)

The each reaction of cDNA synthesis was added 4 µl of total RNA, 1 µl of 50 µM oligo $(dT)_{20}$, 1 µl of 10 mM dNTP mix, and added DEPC-treated water 4 µl filliped to mix than incubated at 65°C for 5 min after that placed on icebox (-20°C) for 2 min. Following 10 µl of cDNA Synthesis Mix was added, which composed of, 2 µl of 10 x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl). The mixture was gently mixed after assembled by brief centrifugation incubated for 50 min at 50°C than incubated at 85°C for 5 min next immediately chilled on icebox (-20°C). The reactions was collected by brief centrifugation and 1 µl of RNase H was added finally incubation for 20 min at 37°C. cDNA synthesis was stored at -20°C or used for qPCR immediately.

2.2.9 qPCR by FastStart Universal SYBR Green Master (Roche, Mannheim, Germany).

The each reaction of qPCR reaction was started with reaction volumes: 20 µl of master mix containing 10 µl of FastStart Universal SYBR Green Master (ROX); 1x final concentration (Final conc), 0.8 µl of 7.5 µM forward primer (final conc; 300nM), 0.8 µl of 7.5 µM reverse primer (final conc; 300nM), 7.6 µl of DI water and 0.8 µl of cDNA template (cDNA of control at 24 h dilute 1:1, 48 h dilute 1:2, 72 h dilute 1:3 with DI water before used in qPCR reaction). The primer sequence was used that indicate in table 3. The solution was carefully mixed then spindowned to avoid air bubble. Reactions were carried out at 95 °C for 10 minutes followed by 45 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 30 s. For data analysis, Expression values of *TPT1* genes were normalized with the expression of GAPDH. Relative values were analyzed using comparative cycle threshold (CT) method (ΔΔCT method).

Table 3. Real-time PCR primers (67).

Primer	Sequence	Length (bp)
GAPDH; Forward	GCTCATTTCCTGGTATGACAACG	213
Reverse	AGGGGTCTACATGGCAACTG	
TPT1 ; Forward	AAATGTTAACAAATGTGGCAATTAT	164
Reverse	AACAATGCCTCCACTCCAAA	

2.2.10 Western blotting for TCTP and β-actin Expression

HDPCs were seeded at 1.5 x 10^5 cells in 35 mm cell culture dish in a humidified atmosphere of 5% CO_2 at 37ºC for 24 h in prior the heat stress. For heat-treated group, HDPCs were treated in heat stress at 43°C for 45 min. After the heat stress, the medium was changed to medium with or without *Pmer*-TCTP which preheated at 37°C followed by incubation at 37°C (time point 0). Samples were harvested at the specific time points (0, 30 min and 24 h post-heating). As non-heat-treated group, HDPCs were cultured at 37°C for 24 h. The medium was changed to medium with or without *Pmer*-TCTP and harvest at the specific time points (0, 30 min and 24 h post-heating). The intracellular TCTP harvesting was performed by washed the cells 3 times with PBS and lysed with 100 µl of 1 x RLB (Promega Corporation, Madison, WI, USA). After centrifugation, the supernatant was collected and the total protein of each sample was determined with BCA kit (Pierce, Rockford, USA). The total protein of 20 µg per sample was separated by 12% SDS-PAGE and then the proteins were transferred to a nitrocellulose membrane and blocked with 5 ml of 1x casein solution for 15 min than incubated the membrane in 5 ml of the Rabbit anti-human TCTP antibody diluted at 1:600 with TBS (Tris buffered saline) with 0.05% Tween 20 and 5% skim milk (TBST with 5% skim milk) for 1 h. Then the membrane was washed 3 times with 5 ml of 1x casein (5 min/time) at room temperature with gentle agitation and incubated with 5 ml of Anti-Rabbit IgG (VECTA STAIN® ABC-AmP™, Western Blotting Immunodetection Kit) diluted at 1:1000 with 1x casein for 30 minutes. After that The membrane was washed 3 times with 5 ml of 1x casein (5 min/time) before incubated with 5 ml of VECTASTAIN® ABC-AmP™ Reagent for 15 min than washed 3 times with 5 ml of 1x casein (5 min/time). Therefrom the membrane was equilibrated by 10 ml of 0.1 M Tris buffer, pH 9.5 for 5 min and removed excess buffer by touched the edge of membrane to absorbent paper than 1 ml of DuoLux™ Substrate was added onto the membrane surface incubated for 5 minutes next immerged membrane in 10 ml of 0.1 M Tris buffer, pH 9.5 by dipped up and down 3 times (a second/time) by gentle agitation and immediately removed excess buffer. The membrane was placed in clear plastic wrap and exposed to x-ray film for 30 seconds. For β-actin, blocked with 15 ml of 5% skim

milk in TBST overnight. Membrane was then incubated with 10 ml of Rabbit anti-human β-actin antibody at 1:1,000 dilution with 5% skim milk in TBST for 1 h (Cell signaling Technology, Danvers, USA). After washed 5 times with 15 ml TBST (15 min/times) incubated the membrane in 10 ml of Goat anti–rabbit IgG conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Baltimore USA) at 1:5,000 dilution with 5% skim milk in TBST for 1 h than washed 6 times with 15 ml TBST (10 min/times). The membrane was incubated with 1 ml of ECL plus western blotting detection reagent for 5 min after removed excess buffer and placed the membrane in clear plastic wrap than exposed the membrane to x-ray film for 30 seconds. The immunoreactive bands intensity were measured by using Image J. The band of β-actin was used as loading control and internal control for normalization.

2.2.11 Statistical Analysis

Data are mean ± standard deviation (SD) of, at least, 3 independent experiments. T-test was used in the analysis of one or two data sets. Comparisons of the data between groups were performed by one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey's post hoc test. A p value less than or equal to 0.05 was considered significant.

CHAPTER 3

RESULTS

3.1 Expression and purification of *Penaeus merguiensis*-translationally controlled tumor protein (*Pmer*-TCTP) in *Escherichia coli*

E.coli strain BL21 host was induced by IPTG and GST- *Pmer*-TCTP protein that was about 48 kDa was extracted than purification using Glutathione Sepharose 4 Fast Flow. *Pmer*-TCTP was cut from GST (29 kDa) - Glutathione Sepharose 4 Fast Flow bead by thrombin. Figure 9 showed the purified *Pmer*-TCTP protein was concentrated, its molecular mass about 19.2 kDa which shown in lane 5 was analyzed by 12% SDS-PAGE and protein concentration was determined by a BCA protein assay kit was 1011.111 µg/ml in PBS solution (Appendix A)

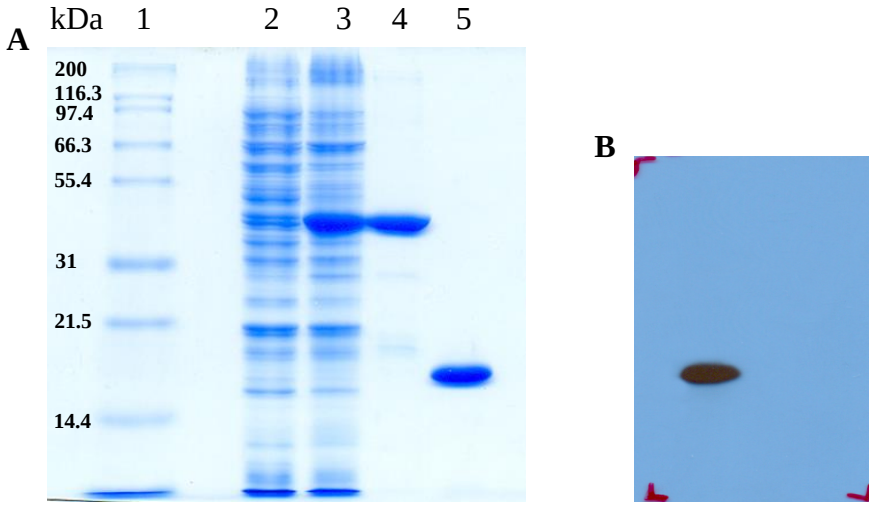

Figure 9. Expression and purification of *Pmer*-TCTP. (A) Lane 1 represents protein markers 12 of mysosin, β-galactosidase, phosphorylase b, BSA, glutamic dehydrogenase, carbonic anhydrase, trypsin inhibitor, and lysozyme. Lane 2: proteins from non-induced *E.coli* strain BL21 host. Lane 3: proteins from GST-*Pmer*-TCTP in E.coli strain BL21 host induced by IPTG. Lane 4: proteins from purification of GST-*Pmer*-TCTP using Glutathione Sepharose 4 Fast Flow. Lane 5: purified *Pmer*-TCTP after GST-cleavage by thrombin. (B) Western Blotting of *Pmer*-TCTP.

3.2 Primary cultures of human dental pulp cells (HDPCs)

HDPCs were isolated by gently separation the pulpal tissue from the pulp chambers and root canals. Pulp tissue has the same shape as the tooth (Figure 10). The tissue was minced into small pieces and digested in the solution of 3 mg/mL collagenase type I and 4 mg/mL dispase. Cells were cultured in α-MEM, supplemented with 20% FBS, 100 µM L-ascorbic acid 2-phosphate, 100 µM L-glutamate, 2.5 µg/ml fungizone, 100 µg/ml streptomycin and 100 units/ml penicillin at a humidified atmosphere of 5% CO_2 at 37°C (Figure 11).

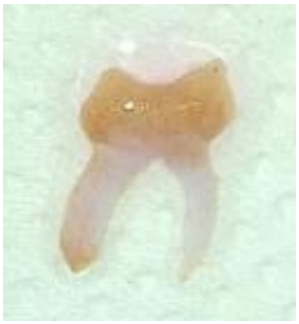

Figure 10. The pulp tissue isolated from premolars.

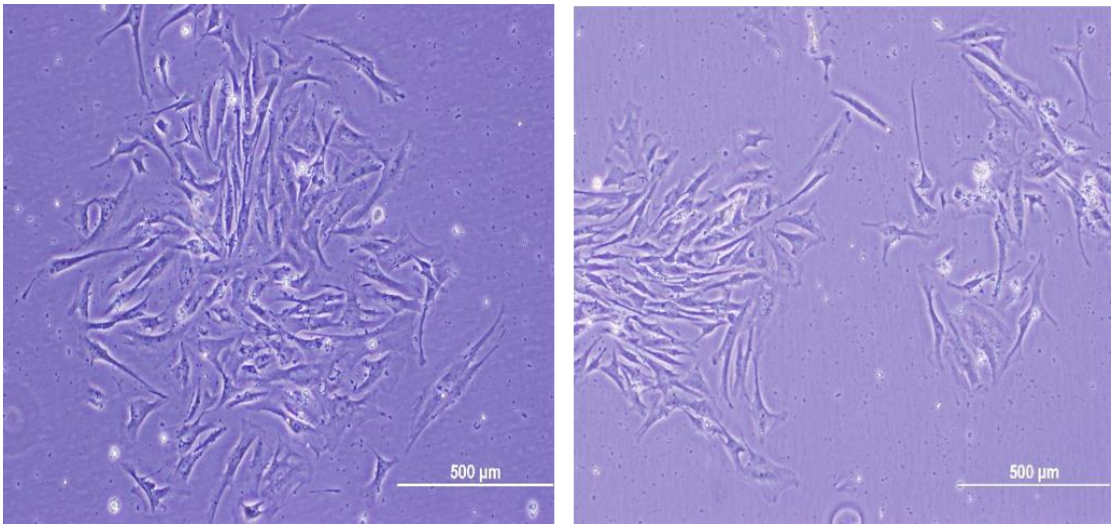

Figure 11. HDPCs after cultured for 5 days. Cells were examined under a phase contrast microscope at 100 x.

3.3 Cell viability (MTT assay)

In all subjects, it was clearly showed that heat stress was significantly ($P< 0.05$, two-way ANOVA) reduced the viability of cells in all groups with and without added *Pmer*-TCTP of both 24 h and 72 h at all concentrations of *Pmer*-TCTP. The percentages of viable cells at 72 h post-heating had significantly higher than 24 h at all concentrations of *Pmer*-TCTP ($P < 0.05$). Figure 12 indicated that the non-heat-treated cells with added *Pmer*-TCTP gave slightly higher percentages of viable cells than non-heat-treated cells without added *Pmer*-TCTP at both 24 h and 72 h. Heat-treated cells with added *Pmer*-TCTP had slightly higher recovery effect than heat-treated cells without added *Pmer*-TCTP at 72 h post-heating.

However, the effects of heat stress and *Pmer*-TCTP on cell viability were slightly different in each subject. In S1, *Pmer*-TCTP at 0.5 ng/ml and 1 ng/ml gave significantly ($P<0.05$) higher optical density and percentages of viable cells (promote 20 % and 11 % of the percentages of viable cells) than other concentrations in non-heat-treated cells and *Pmer*-TCTP at 0.5 ng/ml gave significantly ($P<0.05$) higher viable cells in heated cells which can recover 12 % of the percentages of viable cells compared to the heated cells without *Pmer*-TCTP at 72 h (Figure 13). In S2, there was no significant different of viable cells in all concentrations of *Pmer*-TCTP in both heat and non-heat-treated cells, *Pmer*-TCTP at 0.5 ng/ml gave highest viable cells (promote 10 % of the percentages of viable cells) in non-heat-treated cells at 72 h. However heat-treated cells with added *Pmer*-TCTP demonstrated slightly more recovery (9 % of the percentages of viable cells) than heated cells without added *Pmer*-TCTP at 72 h (Figure 14). While in S3, *Pmer*-TCTP at 1 ng/ml resulted in recovery 5 % of % viable cells in heat-treated cells compared to heat-treated cells without added *Pmer*-TCTP at 24 h. *Pmer*-TCTP at 0.125 and 1 ng/ml gave significantly ($P<0.05$) viable cells (promote 9 % of the percentages of viable cells) than other concentrations in non-heat-treated cells groups at 72 h (Figure 15). So the following experiments *Pmer*-TCTP at 0.5 ng/ml was used to study in S1 and S2 and *Pmer*-TCTP at 1 ng/ml was used to study in S3.

However, the high concentration of *Pmer*-TCTP at 1-20 µg/ml gave slightly less viable cells than non-heat-treated cells without added *Pmer*-TCTP

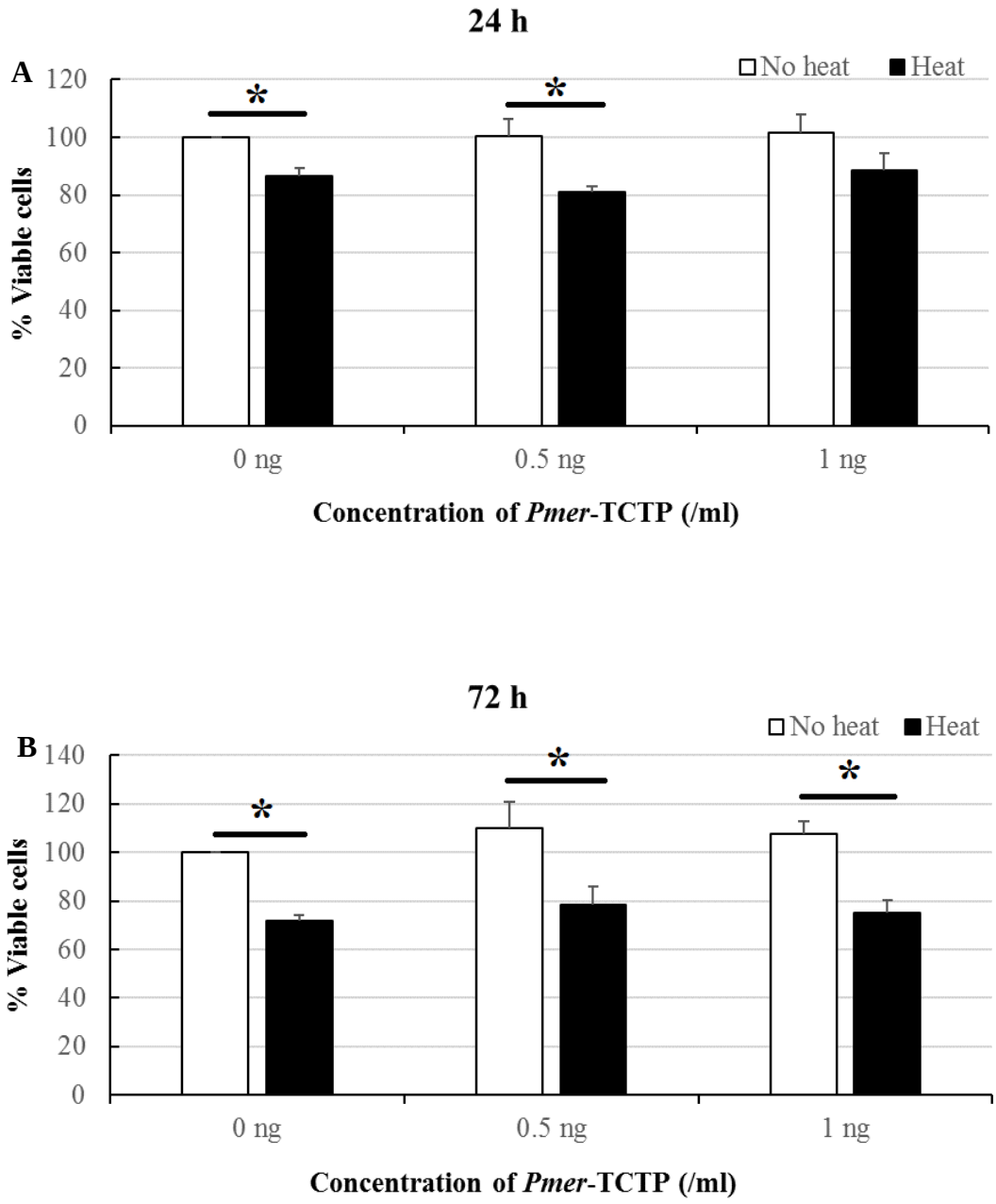

Figure 12. MTT assay of heat stress condition and *Pmer*-TCTP treated HDPCs from three subjects. (a) at 24h and (b) at 72h.

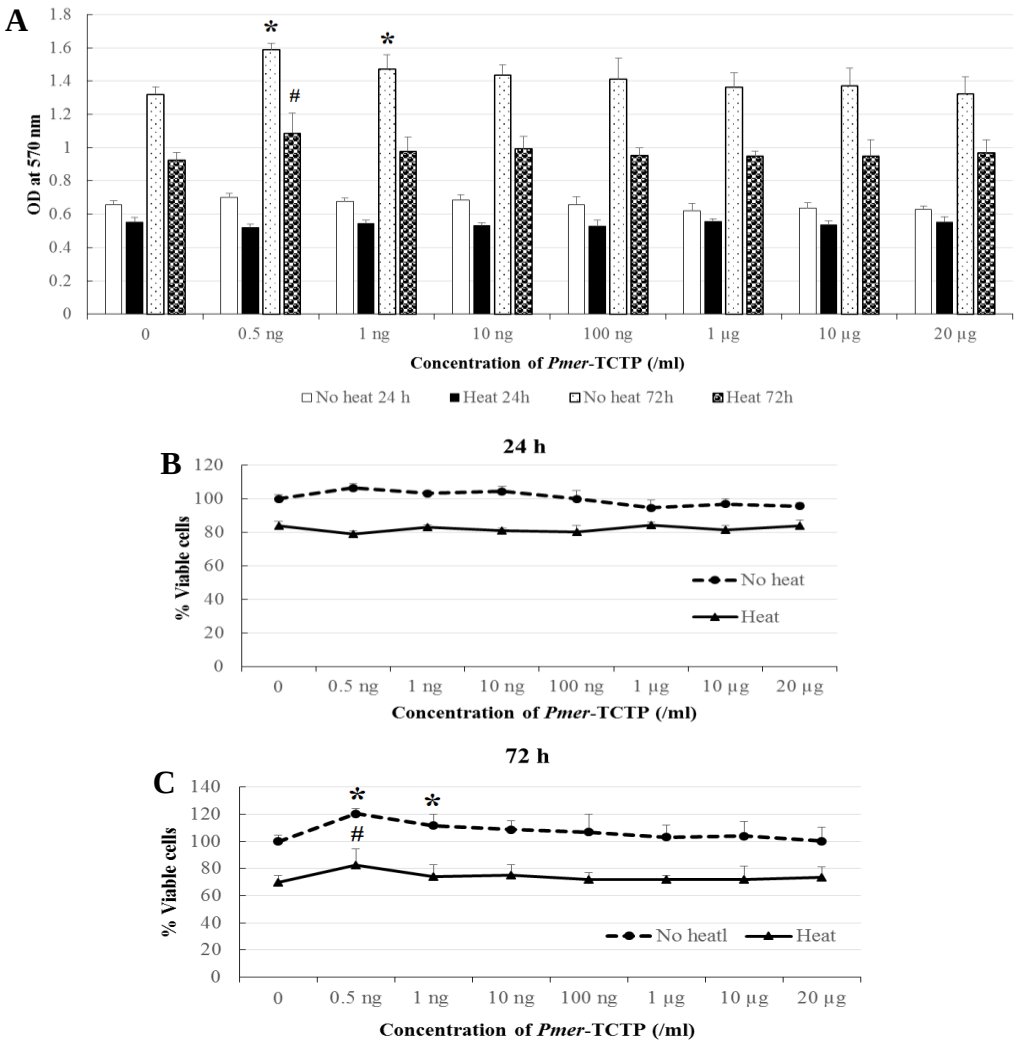

Figure 13. The effect of *Pmer*-TCTP on HDPCs from S1 in normal and heat stress condition. Cell viability was determined by MTT assay and indicated in average at OD_{570} (A) and % cell viability (B, C). HDPCs were cultured with or without *Pmer*-TCTP in the concentrations of 0.5 ng/ml up to 20 µg/ml for 24 and 72 h in normal and after heat stress. Data were represented as the values of means ± standard deviation (SD) (n=6)*Indicated significant difference compared within non-heat-treated group at 72 h with non-heat-treated and without added *Pmer*-TCTP group at 72 h and #Statistically significant difference compared within heat-treated group with heat-treated and without added *Pmer*-TCTP group at 72h. (P<0.05) (one-way ANOVA with Tukey multiple comparison test)

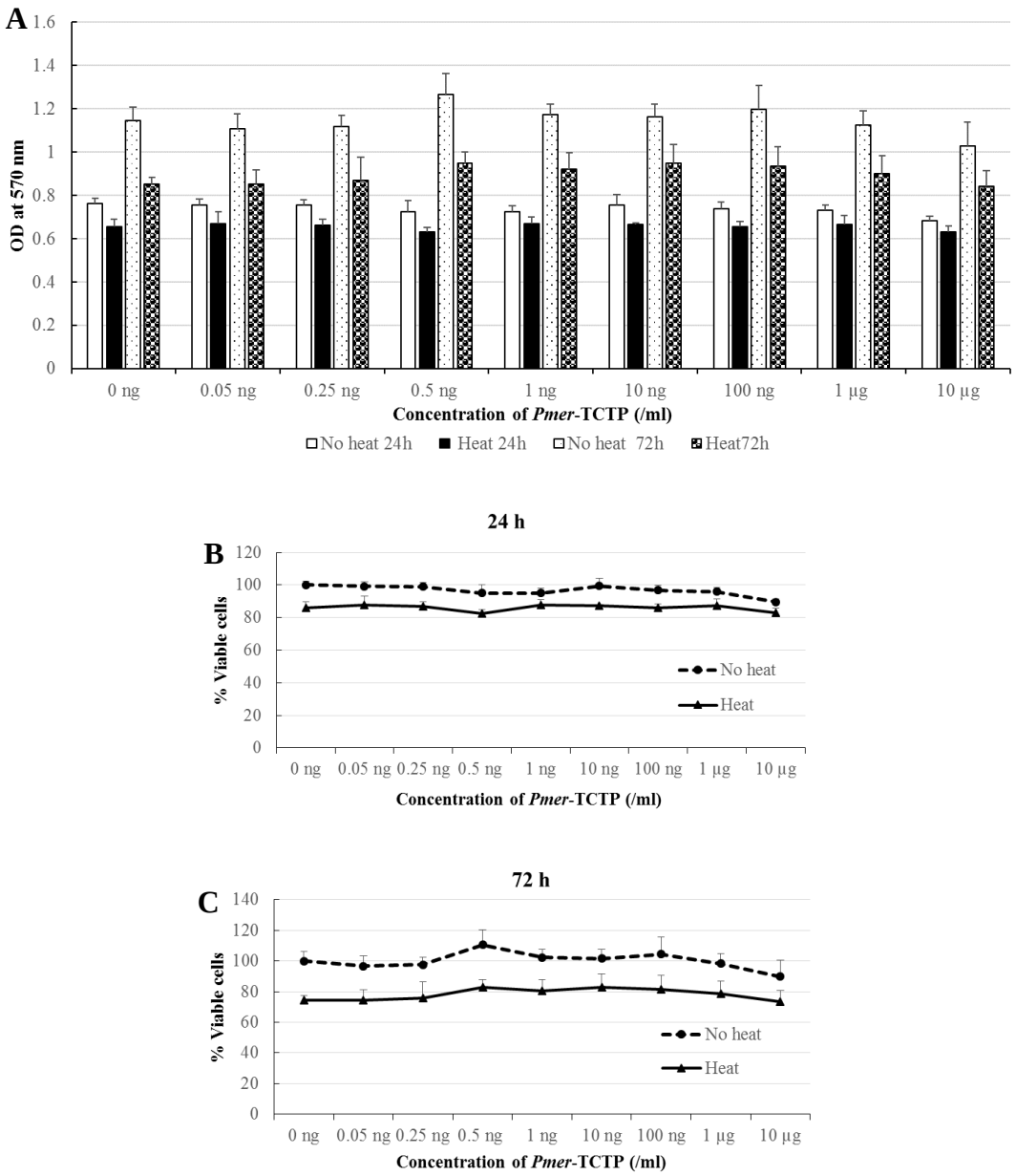

Figure 14. The effect of *Pmer*-TCTP on HDPCs from S2 in normal and heat stress condition. Cell viability was determined by MTT assay and reported as the absorbance at OD_{570} (A) and % cell viability (B, C). HDPCs were cultured with or without *Pmer*-TCTP in the concentrations of 0.05 ng/ml up to 10 µg/ml for 24 and 72 h in normal and after heat stress. Data were represented as the values of means ± standard deviation. (SD) (n=6)

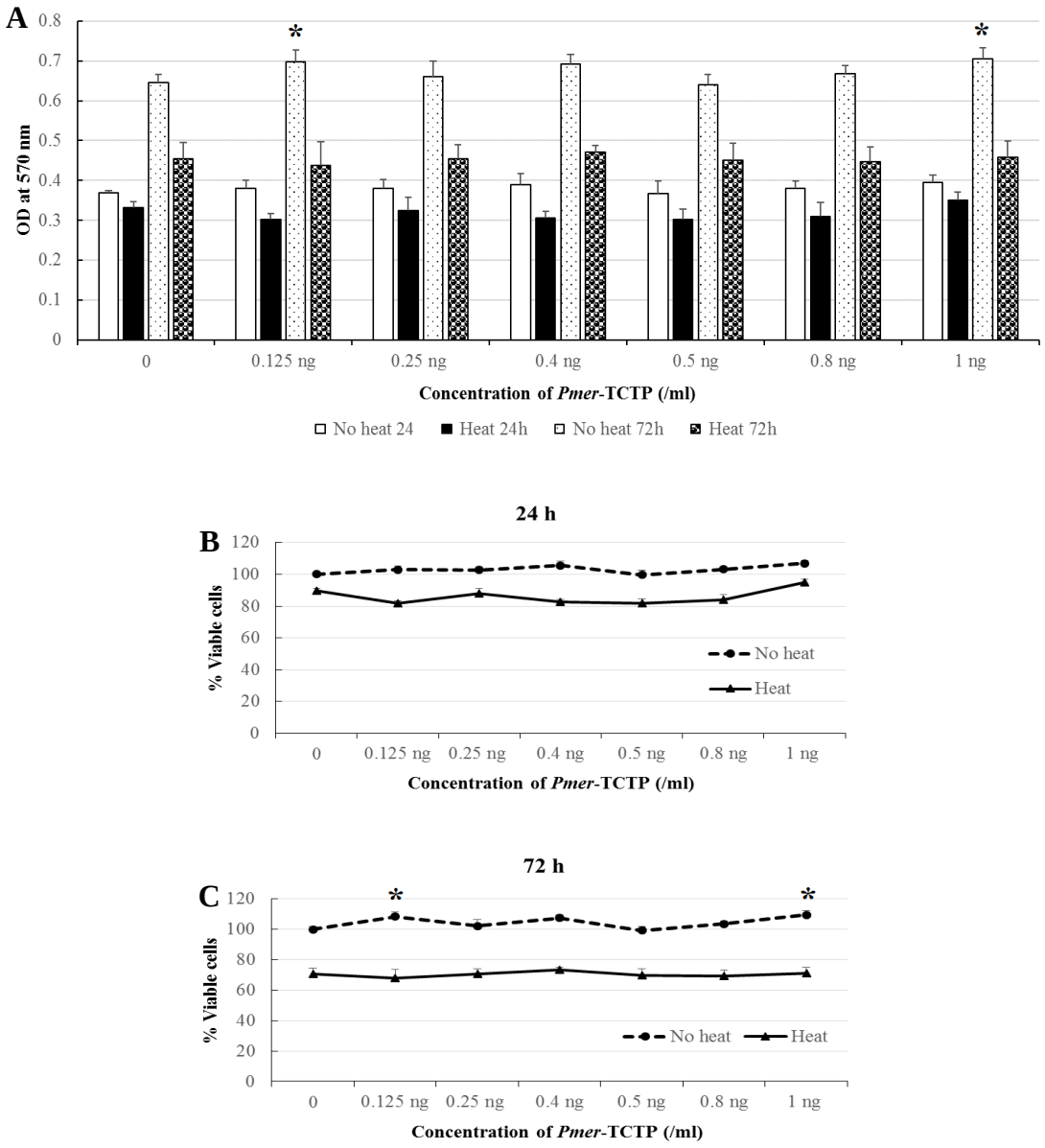

Figure 15. The effect of *Pmer*-TCTP on HDPCs from S3 in normal and heat stress condition. Cell viability was determined by MTT assay and reported as the absorbance at OD_{570} (A) and % cell viability (B, C). HDPCs were cultured with or without *Pmer*-TCTP in the concentrations of 0.125 ng/ml up to 1ng/ml for 24 and 72 h in normal and after heat stress. Data were represented as the values of means ± standard deviation (SD). (n=6) *Indicated significant difference compared within non-heat-treated group at 72 h with non-heat-treated and without added *Pmer*-TCTP group at 72 h. (P<0.05) (one-way ANOVA with Tukey multiple comparison test)

3.4 Cells morphology and DNA staining (Hoechst 33258 dye)

Nuclear staining by Hoechst 33258 was used to observe to the morphologic changes of cell nucleus in heat-treated cells, at various time periods. There was no morphological changes of nuclei in non-heat-treated cells at 0 min, 1 h, 12 h, 24 h, 48 h and 72h. But some slightly morphological changes of nuclei in some HDPCs cells were observed at time 0 and 1 h after heat stress treatment. Some heated cells were shrunk, turned round, and had relatively smaller volume than non-heated cells. Chromatin DNA was extensively condensed and DNA was fragmented (Pyknosis) begin observed at 3 h and 6 h. Finally, apoptotic body begin observed at 12 h after heat stress suggested that apoptosis was occurred in some heated cells (Figure 16). The result of 0, 12, 24 h, 48 h and 72 h after heat stress shown in Figure 17. Hoechst 33258 staining between living and dead cells was investigated in Figure 18.

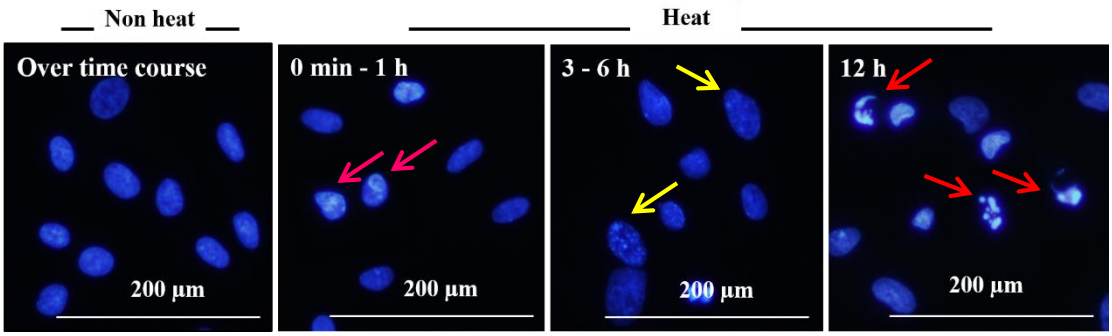

Figure 16. The morphological features of apoptotic cells following heat stress. For non-heated and heated HDPCs cell were observed by Hoechst 33258 staining. Chromatin condensation begin observed at 0 min (pink arrows). Pyknosis begin observed at 3 h (yellow arrows) and apoptotic body begin observed at 12 h (red arrows) after heat stress.

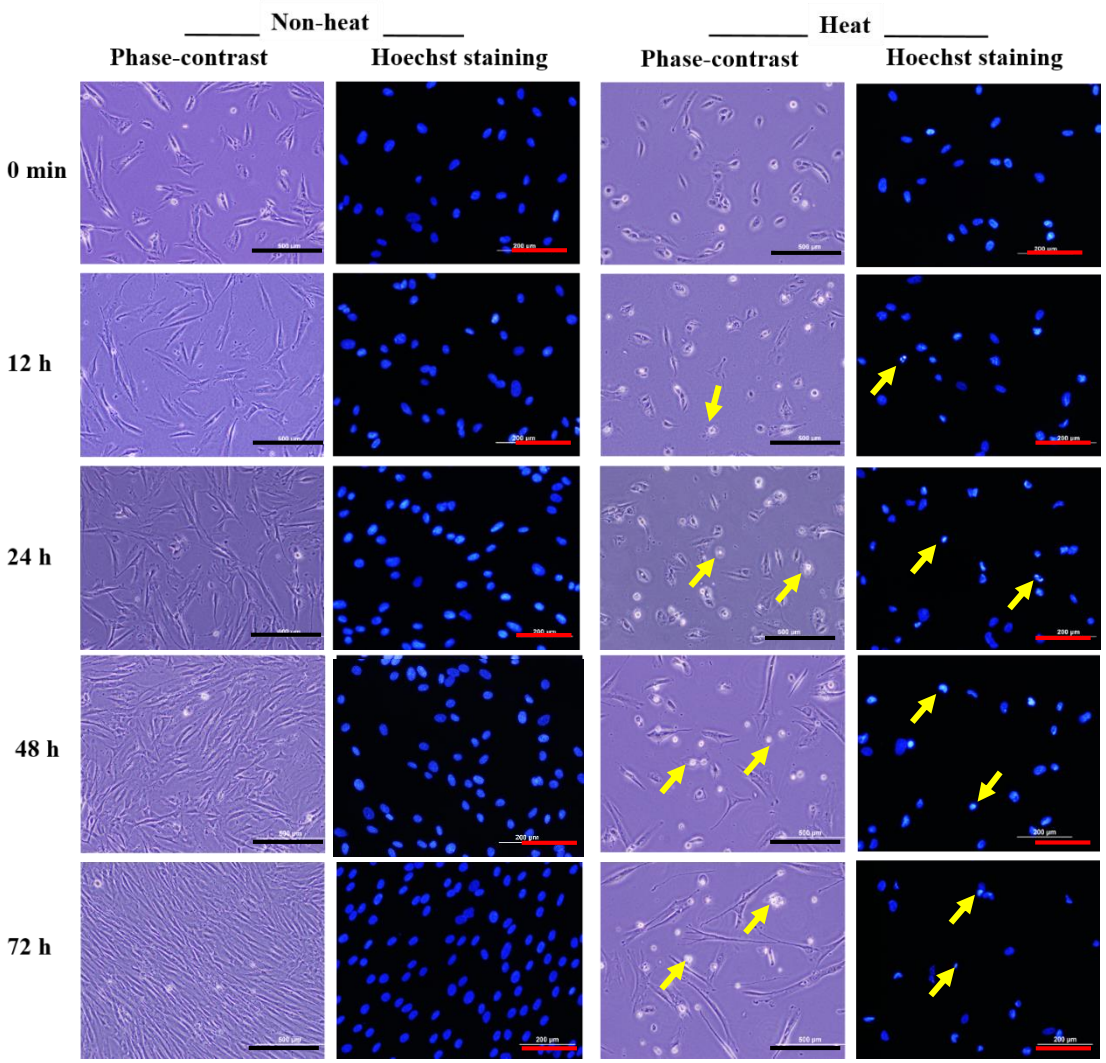

Figure 17. Observation of morphological features of apoptosis. For heat stress-induced apoptotic HDPCs cell by Hoechst 33258 staining under a fluorescence microscope and morphology of cells by phase contrast microscope. The staining showed shrunken nuclei and nuclear fragmentation in heat-treated cells and morphology of cells was shrunk and turned round immediately after heat stress compared with non-heat-treated cells, yellow indicated some of dead cells. Scale bars; —— = 500 µm and —— = 200 µm.

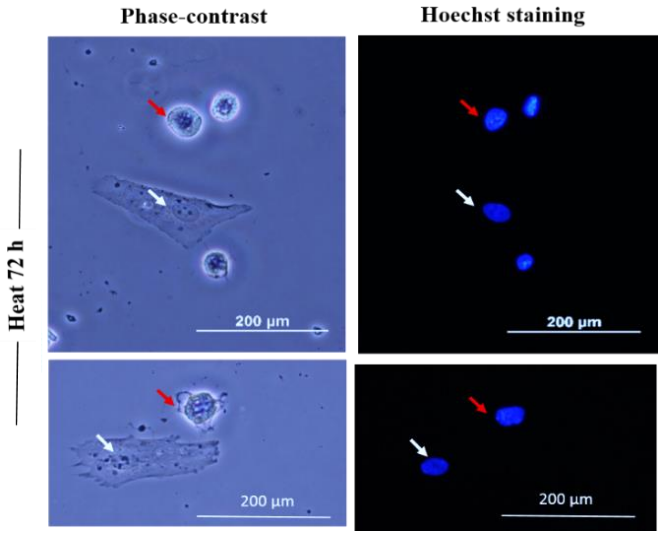

Figure 18. To indicate the likely of Hoechst 33258 staining to distinguish between dead cells and live cells. Red arrow indicated apoptotic cell death and white arrow indicated live cells.

3.5 Caspase-3 Assay

Caspase-3 activity was determined. In the heat-treated and heat-treated with *Pmer*-TCTP group, a statistically significant increase of caspase-3 activity was found, when compared to the control group ($P < 0.05$) (Figure 19).

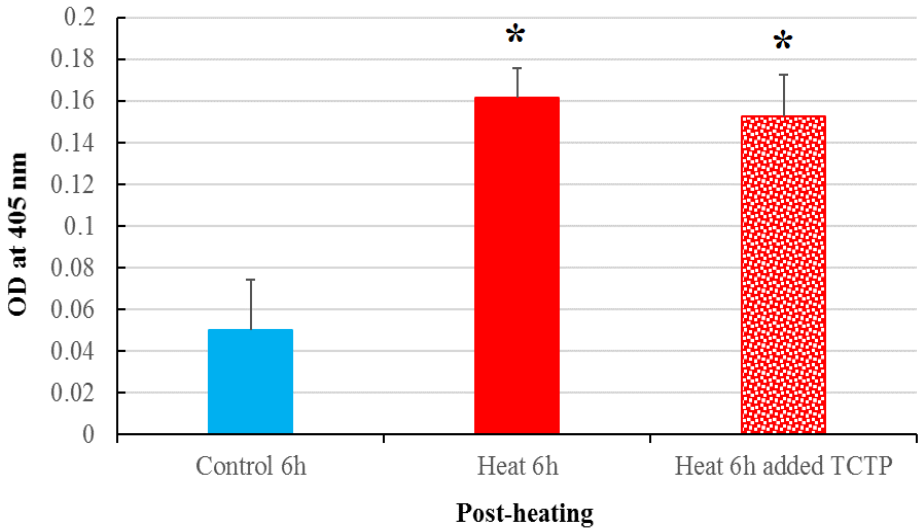

Figure 19. The activation of caspase-3 was involved in heat-induced apoptosis of HDPCs. *Indicated significant ($P<0.05$) (one-way ANOVA with Tukey multiple comparison test, n=3)

3.6 The expression of *TPT 1* gene against heat stress using qPCR

In order to examine responses of HDPCs against heat stress, the expression of *TPT1* was analyzed by qPCR. The fold of *TPT1* gene expression was slightly different in each subjects, its pattern was individual. The response of this gene in three subjects was shown in average of fold expression (Figure 20). *TPT1* gene was up-regulated in heat-treated cells and down-regulated in non-heat-treated cells at 24 and 48 h in all groups. A statistically significant decrease of *TPT1* expression at immediately and significant increase in heat-treated cells without added *Pmer*-TCTP at 24 h was found, when compared with non-heat-treated cells without added *Pmer*-TCTP at 0 min ($P < 0.05$). However, the down-regulation of *TPT1* gene was significantly indicated in non-heat-treated cells without added *Pmer*-TCTP at 24 h and 48 h. Although a significant expression of *TPT1* was observed between non-heat-treated cells and heat-treated cells at 24 h either with or without added *Pmer*-TCTP and at 48 h without added *Pmer*-TCTP. The addition of *Pmer*-TCTP resulted in slightly up-regulated in non-heat-treated cells at immediately until 72 h compared with each time period of non-heat-treated cells without added *Pmer*-TCTP.

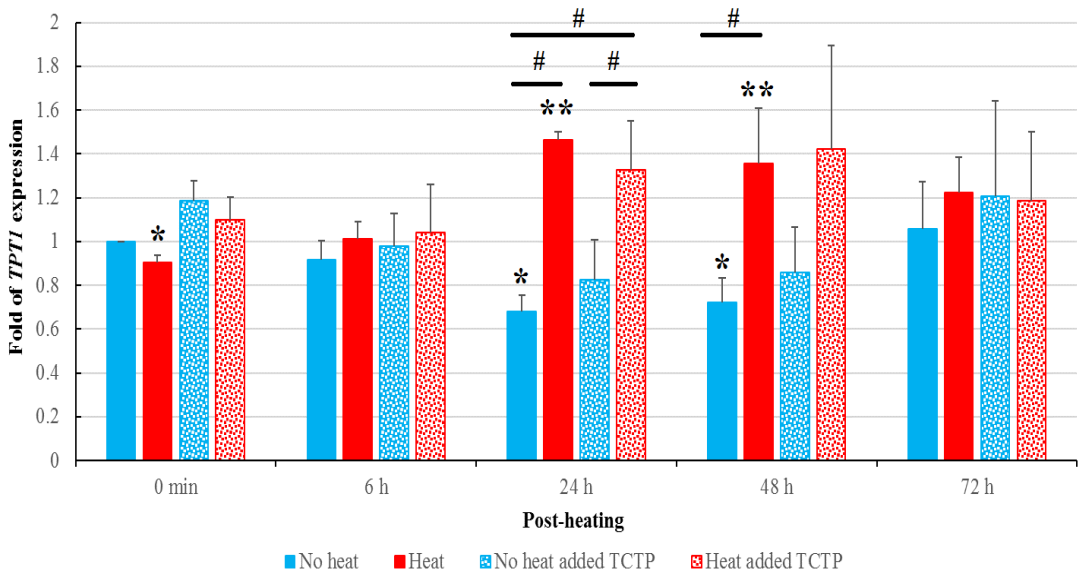

Figure 20. The fold change of *TPT1* expression in normal and heat-treated HDPCs with and without *Pmer*-TCTP compared with expression levels in non-heat-treated HDPCs without *Pmer*-TCTP at 0 min. The values were normalized with GAPDH in $2^{-\Delta\Delta CT}$ method. Data are expressed as means ± standard deviation (n=3). *, **Statistically significant difference compared with control group (No heat at 0 min) at $P<0.05$. #Statistically significant difference compared between group at $P<0.05$.

3.7 The expression of TCTP protein against heat stress using western blotting

To determine the effect of heat stress on HDPCs, the expression of TCTP protein was analyzed by western blotting. The expression was slightly different in each subjects, its pattern was individual. Densitometric analysis of TCTP protein band was shown in Figure 21.

In normal condition, the expression of human-TCTP in non-heat-treated cells without *Pmer*-TCTP was stable and slightly high express at 24 h. The non-heat-treated cells with added *Pmer*-TCTP had slight up-regulated of TCTP expressions at immediately and significantly increased at 30 min and at 24 h.

The stress on heat-treated cells either with or without added *Pmer*-TCTP resulted in down-regulated of TCTP expression and significantly decreased at immediately and 30 min in heat-treated cells with added *Pmer*-TCTP. The re-expression was found at 24 h. However expression of TCTP in heat-treated cells with added *Pmer*-TCTP still lower than expression of TCTP in heat-treated cells without added *Pmer*-TCTP over time course.

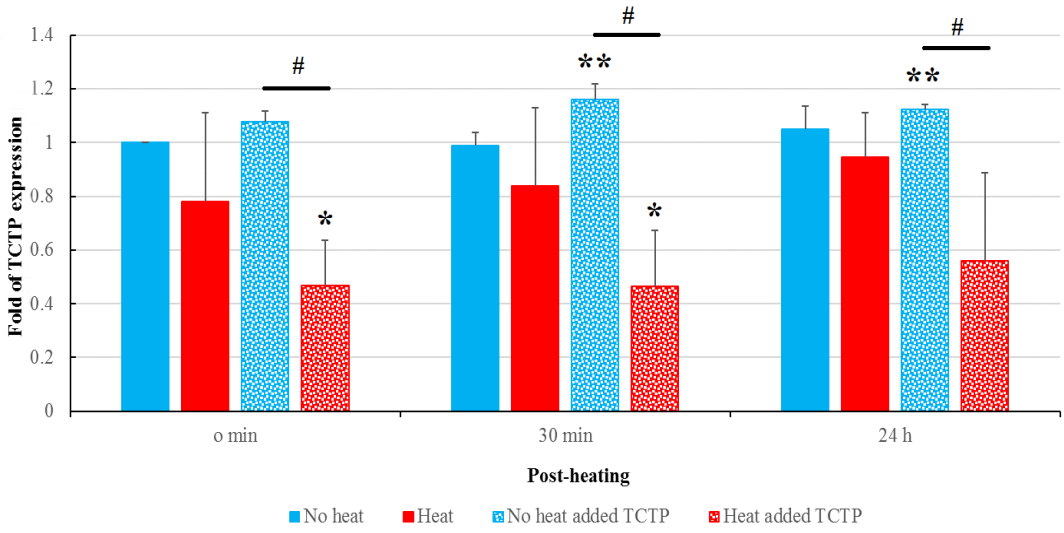

Figure 21. TCTP expression over time course by western blotting. Fold change relative to the control was determined from densitometric data after normalized with β-actin. Data are expressed as means ± standard deviation (n=3). *, **Statistically significant difference compared with control group (No heat at 0 min) at $P<0.05$ and #Statistically significant difference compared between group at $P<0.05$.

CHAPTER 4

DISCUSSION

4.1 The effect of heat stress and *Pmer*-TCTP on cells viability of HDPCs

The cells were used in this study, were isolated from freshly extracted premolars (n = 3) of humans. The isolation procedure followed as previously described (65). There was another researcher following the same method to obtained HDPCs and found that these cells can expression of mesenchymal stem cell markers and had multilineage differentiation property. That showed by example, Alizarin red staining indicated mineralization at 3 week after induced cultures (68). This report suggest that the cells were isolated with procedure followed as previously described that we used is HDPCs.

It is known that during restorative procedures in carious teeth, heat was the most severe stress on HDPCs and the intrapulpal temperature rise of 5.5°C can cause injury to dental pulp cells (60). This study clearly showed that heat stress at 43°C for 45 min that followed as previously described (59, 66) decreased HDPCs viability. The heat stress induced both apoptosis and necrosis (47, 69). Cell death from heat stress in this study may came from apoptosis which indicated from nuclear changing by Hoechst 33258 staining and the increasing of caspase-3 in heat treated cells which was confirmed that heat stress-induced apoptosis of pulp cells (59). These results also support other study that the heat stress induce apoptotic cells dead via caspase-3 (70). However, the effects of heat stress and *Pmer*-TCTP were slightly different in each subject. In addition, we found the same nuclei morphology between living and apoptotic cell death which remind the limitation to distinguish the dead cells by Hoechst 33258 staining.

TCTP has become broadly studied. It is a highly conserved multifunctional protein that related to growth-promoting and cytoprotective function in cell (13). The addition of *Pmer*-TCTP at low concentration (0.5-1 ng/ml) in non-heat-treated cells enhanced cell proliferation which corresponded to the previously

study (71). TCTP can function as growth promoter (72) and the high levels of TCTP expression associates with tumorigenesis (30). While P53 that prevent the growth and survival of potentially malignant cells (36) and the reciprocal repression between P53 and TCTP was reported (37). So we found that the high concentration of *Pmer*-TCTP (10-20 µg/ml) was resulted to slight depressed cell proliferative that may represented to the excess of TCTP which might activated antagonism like P53 to balance the cell proliferation process.

The addition of *Pmer*-TCTP in heat-treated cells had slight protective effect against heat stress. There was a study indicated that TCTP may acts as heat shock protein (HSPs) in cells following heat stress conditions. Either TCTP of *Schistosoma mansoni* parasites or human have the same property, binds to denatured proteins, refolds them, and also interacts with native proteins to protect them from denaturation in heat stress, prevents thermal aggregation and reactivates denatured protein (42).

We found the slight effect of *Pmer*-TCTP on the decreasing of caspase-3 activation but did not have statistically significant which may cause by the small sample size, there was a study indicated that the overexpression of TCTP in cells inhibited caspase-3 activity (8) and the addition of *Pmer*-TCTP had slight effect in increasing percentages of viable cells according to the result of MTT assay. The anti-apoptotic property of TCTP inhibited of caspase-3 activation (8) and a study reported that TCTP antagonizes apoptosis by interaction with MCL1 and Bcl-xL by inserting into the mitochondrial membrane and blocking Bax dimerization resulting in inhibition the pro-apoptotic proteins-released cytochrome c (38).

This study we used *Pmer*-TCTP from banana prawn which still has an effect on HDPCs. This result support that TCTP is a conserved protein which can promote cell proliferation and has a slight protective effect against heat stress.

4.2 The expression of *TPT1* gene and TCTP protein

The present study found the expression of *TPT1* in control group which had no heat and no added *Pmer*-TCTP was not stable and gradually decreased significantly at 24 h, 48 h and re-up-regulated to normal level at 72 h, however the

level of TCTP protein was nearly stable and slight up-regulated at 24 h. This result demonstrated that in normal condition, TCTP synthesis was regulated at gene and protein level. There was an evidence that TCTP synthesis is regulated at both the transcriptional and the translational level (21).

The addition of *Pmer*-TCTP caused slight up-regulated expression of *TPT1* gene and TCTP protein over the time course indicated that TCTP protein had positive feedback to up-regulated at both the transcriptional and the translational level. However, TCTP mRNA has been shown a negative feedback in the translational regulation by the TCTP mRNA-activated dsRNA-dependent protein kinase PKR resulting in removing of TCTP mRNA (12).

It is known that TCTP protein responses to various stress (21). The result of this study revealed the up-regulated about 2 fold of *TPT1* gene response to heat stress at 24 h and 48 h compared to control group at the same time period. However, the expression of TCTP protein was slight down-regulated. Demonstrating that in heat stress condition, TCTP synthesis was up-regulated at the transcriptional level (41-43). There was the study found that pulp cells were express HSP70 to protect and recovery viability of cells after heat stress (61). This suggest that heat shock protein play a main role as protective factors and recovery cells after heat stress (47, 53, 61)

The addition of *Pmer*-TCTP in heat-treated cells resulted slight up-regulated of *TPT1* expression at immediately and 6 h post-heating when compared to heat-treated cells without added *Pmer*-TCTP and resulted to more down-regulated of TCTP protein expression in heat-treated cells than heat-treated cells without added *Pmer*-TCTP over the time course. The present finding showed that the over expression of both *TPT1* gene and TCTP protein may not over the maximum level. For the effect of heat stress on TCTP protein, it was likely the cells may have to keep some level of TCTP protein. The addition of *Pmer*-TCTP resulted in down-regulation of TCTP synthesis to maintain the level of TCTP protein in cells. However, further study will be required for better understanding about the level of TCTP inside the cell and also its function whether in normal and injury condition.

CHAPTER 5

CONCLUSIONS

This study investigate the effect of heat stress and *Pmer*-TCTP on viability of HDPCs as well as the expression of TCTP that is a highly conserved multifunctional protein involving in several cell's process such as growth-promoting and cytoprotective function in cell. The present study, we can conclude that:

1. The expression of *TPT1* gene was not stable and gradually decreased at 24 h and 48 h and then re-expression to normal revel at 72 h.
2. Heat stress caused cells dead by apoptosis via caspase-3, induced the up-regulation of *TPT1* gene and resulting in down-regulation of TCTP protein.
3. *Pmer*-TCTP at low concentration (0.5-1 ng) can promote HDPCs proliferation and slightly protect cells from cell death after heat stress and the high concentration (10-20 µg) can slight depressed cell proliferative.
4. The addition of *Pmer*-TCTP resulted in slight induced the up-regulation of *TPT1* gene and slightly increase TCTP protein in normal condition. But resulted in reduction of TCTP protein expression in heat stress condition.

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APPENDICES

APPENDIX A

Table 4. Concentration of *Pmer*-TCTP calculated with standard curve in BCA protein assay kit

sample	OD			mean	sd	ug/ml dilution 1/5	ug/ml
	1	2	3				
***Pmer*-TCTP**	0.243	0.243	0.242	0.2426	0.0005	202.222	1011.111

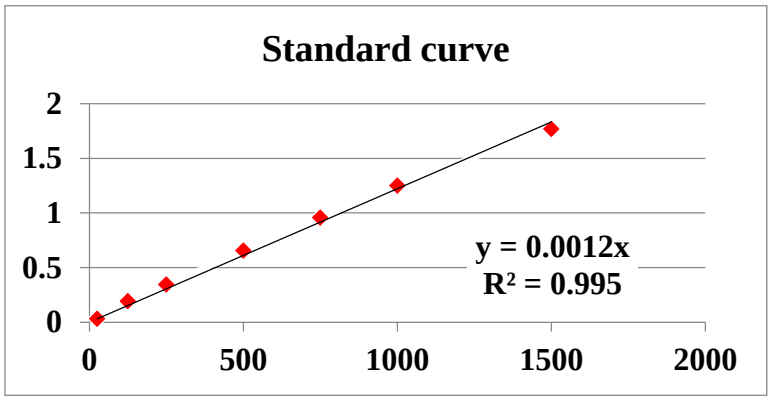

Figure 22. Standard curve in BCA protein assay kit that used on calculated concentration of concentrate purifying *Pmer*-TCTP.

APPENDIX B

MATERIALS

1. Expression and purification of *Pmer*-TCTP in Escherichia coli

1.1 LB (Luria-Bertani) broth 100 ml

Tryptone 1% (w/v)	1	g
Yeast extract 0.5% (w/v)	0.5	g
NaCl	0.5	g

Bring the volume of the solution to 100 ml with distilled water. Sterilize by autoclaving at 121°C for 15 min. Add 1 ml of amplicillin (100 mg/ml) to final concentration 100 µg/ml in to warm medium (50°C).

1.2 2xYT medium 1,000 ml

Tryptone	16	g
Yeast extract	10	g
NaCl	5	g

Bring the volume of the solution to 1,000 ml with distilled water. Sterilize by autoclaving at 121°C for 15 min. Add 1 ml of amplicillin (100 mg/ml) to final concentration 100 µg/ml in to warm medium (50°C).

1.3 Ampicillin (100 mg/ml) 1 ml

Ampicillin	100	mg

Dissolve in 1 ml of sterilize distilled water. Store at -20°C.

1.4 1M IPTG 10 ml

IPTG	2.38	g

Dissolve in 10 ml of sterilize distilled water. Store at -20°C.

1.5 Phosphate-buffered saline (PBS) pH 7.4, 1000 ml

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCl and then add distilled water to 1000 ml. Sterilize the buffer by autoclaving at 121°C for 15 min and store at room temperature.

1.6 Lysis buffer 500 ml

KH_2PO_4	3	g
Tris-base	0.6	g
NaCl	8.77	g

Dissolve the ingredients in 500 ml of distilled water. Sterilize the buffer by autoclaving at 121°C for 15 min and store at room temperature.

1.7 Lysozyme (100 mg/ml) 10 ml

Lysozyme	1	g

Dissolve in 10 ml of sterilize distilled water. Store at -20°C.

2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.1 12% SDS-PAGE

Solution	Stacking gel	Separating gel (12 %)
Water	2.7 ml	1.7 ml
30 % acrylamide-bisacrylamide	670 µl	2 ml
1.5 M Tris-HCl buffer (pH 8.8)	-	1.3 ml
1.0 M Tris-HCl buffer (pH 6.8)	500 µl	-
10%SDS	40 µl	50 µl
10%APS	40 µl	50 µl
TEMED (N,N,N,'N,-tetramethylenediamine)	4 µl	2 µl

2.2 30 % acrylamide-bisacrylamide 100 ml

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

Dissolve 29 g of acrylamide to 50 ml with deionized water warmed at 37°C than gradually add N,N'-methylene-bis acrylamide till melted and make up the final volume of the solution to 100 ml with deionized water next filter with paper-filter. Store in the dark at 4° C.

2.3 1.5 M Tris-HCl buffer (pH 8.8) 100 ml

Tris base	18.17	g

Dissolve in 50 ml of deionized water. Adjust the pH to the desired value by HCl. Make up the final volume of the solution to 100 ml with deionized water. Store at 4° C.

2.4 1.0 M Tris-HCl buffer (pH 6.8) 100 ml

Tris base	12.10	g

Dissolve in 50 ml of deionized water. Adjust the pH to the desired value by HCl. Make up the final volume of the solution to 100 ml with deionized water. Store at 4° C.

2.5 10% sodium dodecyl sulphate (SDS) 100 ml buffer

SDS	10	g

Dissolve with deionized water. Make up the final volume of the solution to 100 ml. Store at room temperature.

2.6 10% ammonium persulphate (APS) 1 ml

APS	0.1	g

Dissolve with 1 ml of deionized water. Store in the dark at 4°C (Preparation before use).

2.7 2x sample buffer 10 ml

10 % SDS	4	ml
Glycerol	2	ml
1 M Tris-HCL (pH 8.8)	1.2	ml
1M DTT	2	ml
Bromophenol blue	0.002	g

Dissolve with deionized water. Make up the final volume of the solution to 10 ml. Store in the dark at 4°C.

2.8 Tris-glycine buffer 1,000 ml

SDS	1	g
Glycine	14.42	g
Tris-base	3.03	g

Dissolve with deionized water. Make up the final volume of the solution to 1,000 ml. Store at room temperature

2.9 Coomassie Blue Staining buffer 1,000 ml

Coomassie Blue	2	g
95 % methanol	525	ml
Acetic acid	75	ml

Dissolve with distilled water. Make up the final volume of the solution to 1,000 ml. Store in the dark at room temperature.

2.10 Destaining l 1,000 ml

95 % methanol	526	ml
Acetic acid	75	ml

Dissolve with distilled water. Make up the final volume of the solution to 1,000 ml. Store at room temperature.

2.10 Destaining ll 1,000 ml

95 % methanol	5.26	ml
Acetic acid	75	ml

Dissolve with distilled water. Make up the final volume of the solution to 1,000 ml. Store at room temperature.

3. Cells cultures of human dental pulp cells

3.1 Stock of α-MEM (Alpha-minimum essential medium) pH 7.2, 1000 ml

α-MEM	10.2	g
$NaHCO_3$	2.2	g

Dissolve the ingredients in 700 ml of deionized water. Adjust the pH to 7.2 with HCl. Add distilled water to 1,000 ml. Sterilize the α-MEM by filter 0.45 µm and store at 4°C.

3.2 Complete media of α-MEM (10% FBS) 200 ml

Fetal bovine serum	20	ml
L-Glutamate stock 200 mM (1% v/v)	2	ml
Penicillin- streptomycin 10^4 unit/ml (2% v/v)	4	ml
Fungizone stock 250 µg/ml (1% v/v)	2	ml
L-ascorbic acid 100 mM	200	µl

Adjust the volume of the solution to 200 ml with stock of α-MEM and sterilized by filter with 0.45 µm membrane. Store in the dark at 4°C.

3.3 L-ascorbic acid 100 mM (MW= 176.12)

L-ascorbic acid 100 mg

Dissolve in 5.68 ml of deionized water and sterilized by filter with 0.45 µm membrane. Store in the dark at -20°C.

3.4 Collagenases (30 mg/ml) 1 ml

Collagenase 30 mg

Dissolve in 1 ml of deionized water and sterilized by filter with 0.45 µm membrane. Store in the dark at -80°C.

3.5 Dispase (40 mg/ml) 1 ml

Dispase 40 mg

Dissolve in 1 ml of deionized water and sterilized by filter with 0.45 µm membrane. Store in the dark at -80°C.

3.6 0.05 % Trypsin-EDTA 50 ml

0.5 % Trypsin-EDTA 5 ml

Adjust the volume of the solution to 50 ml with PBS pH 7.4 (in ratio 1:9) and sterilized by filter with 0.45 µm membrane. Store at -20°C.

4. MTT assay

4.1 MTT solution (5 mg/ml) 10 ml

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

Dissolve in 10 ml of sterilized PBS (pH 7.4) and sterilize by filter with 0.45 µm membrane. Store in the dark at -20°C.

4.2 HEPES (pH 7.4) 0.1 M 100 ml

HEPES 2.383 g

Dissolve in 70 ml with deionized water before adjust the pH to the desired value. Adjust volume to 100 ml with distilled water and then sterilize by filter with 0.45 µm membrane. Store at -20°C.

4.3 Sorensen's glycine buffer (pH 10.5) 100 ml

Glycine 375 mg

NaCl 292 mg

Dissolve in 70 ml with deionized water before adjust the pH to the desired value. Adjust the volume to 100 ml with distilled water and then sterilize by filter with 0.45 µm membrane. Store at -20°C.

5. Cells staining

5.1 3.7 % formaldehyde in PBS 10 ml

37 % Formaldehyde	1	ml

Adjust the volume of the solution to 10 ml with PBS pH 7.4 (in ratio 1:9). Store in the dark at room temperature.

5.2 0.125 µg/ml of Hoechst 200 µl

0.5 µg/ml of Hoechst	50	µl

Adjust the volume of the solution to 200 µl with PBS pH 7.4 (in ratio 1:3). Store in the dark at 4°C (Preparation before use).

6. Agarose gel electrophoresis

6.1 1.2% agarose gel

Agarose	0.36	g
0.5xTAE buffer	30	ml

Dissolve agarose in 0.5xTAE buffer and heated until the solution becomes clear and, then cooled at 50–55°C. 1.5 µl of ethidium bromide was added and well mixed by swirling. The solution was poured onto the gel tray and left stand to gel for at least 30 min. When gel solidifies, was installed on the platform in the electrophoresis tank containing 0.5xTAE buffer. The mRNA samples mixer was load and run at 100 volt for 40 min.

6.2 5xTAE buffer 1,000 ml

Tris-base	24.2	g
Glacial acetic acid	5.21	ml
0.5 M EDTA pH 8	10	ml

Dissolve to 1,000 ml with deionized water. Store at 4°C

6.3 0.5 M EDTA (pH 8) 10 ml

EDTA	1.86	g

Dissolve in 10 ml with deionized water before adjust the pH to the desired value. Store at room temperature.

6.4 6x gel loading buffer 20 ml

Bromophenol blue (0.25% w/v)	0.05	g
Xylene cyanol FF (0.25% w/v)	0.05	g
Glycerol in water (30% v/v)	6	ml

Dissolve to 20 ml with deionized water. Store in dark place at 4°C

7. Western Blotting

7.1 Blotting buffer 1,000 ml

10x running buffer	100	ml
Methanol	200	ml

Adjust the volume of the solution to 1,000 ml with deionized water. Store at 4°C.

7.2 10x running buffer 1,000 ml

Glycine	144	g
Tris-HCl	30	g

Dissolve in 1,000 ml with deionized water. Store at room temperature.

7.3 TBS-T buffer 1,000 ml

1 M Tris-HCl (pH 7.6)	10	ml
5 M NaCl	30	ml
Tween 20	0.5	ml

Adjust the volume of the solution to 1,000 ml with deionized water. Store at 4°C.

7.4 1 M Tris-HCl (pH 7.6) 500 ml

Tris-HCl	78.82	g

Dissolve in 500 ml with deionized water. Store at room temperature.

7.5 5 M NaCl 500 ml

NaCl	146.1	g

Dissolve in 500 ml with deionized water. Store at room temperature.

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