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Translationally controlled tumor protein against apoptosis from 2-hydroxy-ethyl methacrylate in human dental pulp cells

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Abstract 2-Hydroxy-ethyl methacrylate (HEMA) is a major monomer released from resin-base dental restorative materials. HEMA is cytotoxic to pulp cells and leads to apoptosis. This study examined the effect of Translationally Controlled Tumor Protein (TCTP) against apoptosis from HEMA. TCTP from banana prawn (*Penaeus merguiensis*) was cloned and the protein was purified. It significantly increased the number of viable of HEMA-treated cells compared to HEMA-treated cells alone. Flow cytometry indicated the addition of TCTP at 10 μg/ml to 8 and 10 mM HEMA decreased the apoptotic cells from 20 to 10%. The proliferative property and anti-apoptotic activity against HEMA was concentration dependent. It was interesting that the added TCTP was not detected inside the cells and the native human TCTP was decreased after treated with HEMA and TCTP (20 μg/ml) + HEMA(10 mM) for 24 h. These results provided preliminary information, which may contribute to the development of less toxic dental materials.

1 Introduction

Resin-based materials are commonly used in dentistry. The unpolymerized monomers or residual monomers of resin materials can be released into the oral cavity during the initial phase after polymerization, and later, due to degradation or corrosion of the restoration [1]. These residual monomers have the potential to cause direct damage to cells via both cytotoxicity and genotoxicity [2] and there are some clinical reports in local and systemic allergic-related reaction to these materials [3, 4].

Resin monomer, such as 2-hydroxyethyl methacrylate (HEMA), is a major component released from resin-modified glass ionomer cement and dental adhesives [5]. Leached residual HEMA can easily diffuse through the dentine due to its hydrophilic properties and low molecular weight, and this contributes to the cytotoxicity of dental resin material in dental pulp cells [6]. The cytotoxicity of HEMA is due to its potent induction of apoptotic cell death [7, 8]. This effect involves the decrease of intracellular glutathione (GSH) levels and the increase in reactive oxygen species (ROS) production, which in turn leads to apoptosis [9].

Several studies have attempted to reduce the cytotoxicity of HEMA by using an antioxidant or a reducing agent [10, 11] and by trying to design a HEMA-free dental material [12]. A study by Paranjpe et al. [13] demonstrated that N-acetyl Cysteine (NAC) was an effective chemoprotectant, which can prevent the HEMA-induced decrease in mitochondrial membrane potential and can protect cells from HEMA-induced apoptosis. Moreover, the use of anti-apoptotic proteins has been an interesting choice for reducing the cytotoxicity of HEMA due to their distinctive properties. Recently, many anti-apoptotic proteins have attracted the attention of many researchers in terms of biological processes and medical applications. One of these is the translationally controlled tumor protein (TCTP). TCTP is a highly conserved protein that is widely expressed in all eukaryotic organisms, and the levels of TCTP are regulated by a various signals, such as growth

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signal [14], cytokines [15], various stress conditions, and pro-apoptotic treatments [16] that can up- or down-regulate TCTP. Furthermore, TCTP is involved in important cellular processes, such as cell growth and cell cycle progression, and it can protect cells from various stress conditions and apoptosis due to its biological properties [17]. TCTP is also considered an antioxidant protein. HaCaT keratinocytes increased the expression and translocation of TCTP from cytoplasm into the nucleus after application of mild oxidative stress, hydrogen peroxide (H_2O_2) [18]. Recently, a functional cloning of a murine cDNA encode protein TCTP demonstrated its protection against H_2O_2-induced cell death [19] and TCTP cloning from *Brugia malayi*, a filarial parasite, was shown in vitro that it could be reduced by a variety of reducing agents and its reduced form can protect DNA from oxidative damage [20].

There are some studies that also demonstrate that TCTP can protect cells from undergoing apoptosis, for example TCTP is drastically up-regulated in shrimp during the early phase of white spot syndrome virus infection, as natural host protection. An overexpression of shrimp TCTP in human osteosarcoma cells (U2OS) can protect cells from undergoing etoposide-induced cell death [21]. Therefore, it is of interest to use TCTP in order to reduce the toxicity of HEMA. The objectives of this study were to investigate the properties of TCTP from banana prawn (*Penaeus merguiensis*) with a 44% match to the amino acid composition of human TCTP, especially the proliferative effect and its antiapoptotic activity against the toxicity of HEMA in human dental pulp cells.

2 Materials and methods

2.1 Expression and purification of *Penaeus merguiensis*-translationally controlled tumor protein (*Pmer*-TCTP)

TCTP gene that was derived from *Penaeus merguiensis* was cloned [22] and expressed in bacteria *Escherichia coli* (*E. coli*) stain BL21. The *E. coli* stain BL21 harboring pGEX-*Pmer*-TCTP was inoculated into 100 ml of LB medium containing 100 μg/ml ampicillin. After overnight growth at 37°C, 100 ml of the culture was diluted in 1 l of 2XYT medium containing 100 μg/ml ampicillin. The culture was induced by 1 mM IPTG, when the cell density at OD_{600} reached 0.5. After induction for 3 h, the cells were harvested by centrifugation at 4,000×*g* at 4°C for 10 min. The pellet was suspended in 10 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Tris–HCl, pH 8.0), sonicated (6 × 10 s) and centrifuged at 10,000×*g* at 4°C for 20 min. The soluble protein was purified by using Glutathione Sepharose 4 Fast Flow (GE Healthcare Bio-Science, Piscataway, NJ, USA) and thrombin was used for cleavage of GST-tagged protein. The purified *Pmer*-TCTP protein with molecular mass about 19.2 kDa was analyzed by 12% SDS-PAGE and protein concentration was determined by a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.2 Cultures of human dental pulp cells

Normal human third molars were collected from four adults that were designated as S1, S2, S3 and S4 (18–25 years of age) 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 culture media and supplements were products of Gibco (Invitrogen Corporation, NY, USA) unless indicated elsewhere. Primary culture of pulp cells was performed using an enzymatic method. Briefly, the pulp tissue was minced into pieces and digested in a solution of 3 mg/ml of collagenase Type I and 4 mg/ml of dispase for 1 h at 37°C. After centrifugation, cells were cultured in alpha modified Eagle's medium, αMEM, supplemented with 10% FCS, 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, MO, USA), 100 μM L-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2. In all experiments, pulp cell passages between three and ten were used for the study.

2.3 Proliferative effect of TCTP on HDPCs

The proliferative property of TCTP on primary human dental pulp cells (HDPCs) was investigated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [23]. TCTP was diluted in cell culture medium at various concentrations varied from 1 ng/ml to 20 μg/ml. HDPCs at a density of 1×10^4 cells/well were cultured in 96-well plates at a humidified atmosphere of 5% CO_2 at 37°C for 24 h and then exposed to TCTP for 24 and 72 h. After that, the medium was removed, 200 μl of fresh medium containing 10 mM HEPES (pH 7.4) was added each well, and 50 μl MTT solution (5 mg/ml in PBS) was added to each well and incubated in the dark for 4 h at 37°C. The medium and MTT were then removed and 200 μl of 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) was added. The optical density (OD) of formazan production was measured at 570 nm. The OD values corrected for a blank (medium only) of the experimental groups were divided by the control and expressed as a percentage of the control, which represented the percentage of viable cells.

2.4 Anticytotoxicity testing

The anticytotoxicity of TCTP was performed by MTT assay. HDPCs at $1x10^4$ cells/well were cultured in 96-well plates in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h, HDPCs were exposed to a combination of HEMA (8 and 10 mM) and TCTP ranging from 100 ng/ml-20 μg/ml and HEMA (8 and 10 mM) alone, as the positive control, for 24 h. After that, the fresh media was replaced and incubated for 48 h. The viable cells were determined by the MTT assay described previously.

2.5 Apoptotic assay

The extent of apoptosis was determined by flow cytometry, using propidium iodide (PI) and Annexin V-FLUOS double staining kit. (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). The assay investigated apoptosis of HDPCs exposed to HEMA at concentrations 0–16 mM for 24 h and also determined apoptosis of HDPCs exposed to a combination of HEMA (8 and 10 mM) and 10 μg/ml TCTP for 24 h. Briefly, HDPCs were seeded in 12-well culture plates with cell density at 1×10^5 cells/well with normal αMEM medium with supplements and after 24 h, the culture medium was changed with the medium mixed with HEMA (the first experiment) or HEMA mixed with TCTP (the second experiment) for 24 h. After that, the medium was removed and all cells in each well were collected and incubated in 100 μl of binding buffer containing Annexin V-FLUOS and PI according to the manufacturer's instruction. The samples were analyzed by a flow cytometer (Becton-Dickinson FACSCalibur, BD Biosciences, San Jose, California, USA). The study defined Annexin V-positive and PI-negative cells as apoptosis. Data analysis was performed using Cell Quest Software (BD Biosciences, San Jose, California, USA).

2.6 Detection of TCTP by immunoblotting

TCTP in both intracellular and in cultured medium were detected by western blot analysis. HDPCs were seeded into 6-well plates with cell density at 5×10^5 cells/well in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h, HDPCs were treated with TCTP at 20 μg/ml, a combination of HEMA (10 mM) and TCTP at 20 μg/ml, HEMA (10 mM) alone and cell cultured in normal medium was set as the control. After exposure for 1, 4, and 24 h, the intracellular TCTP detection was performed. Cells were washed two times with PBS and lysed with 1× 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 equal amount of the protein of each sample (17 μg) was separated by 12% SDS-PAGE. After separation, the proteins were transferred to a nitrocellulose membrane and blocked with 5% skim milk in TBS (Tris buffered saline) with 0.05% Tween 20 (TBST) for 1 h and washed five times (15 min/time) with TBST. Rabbit anti-TCTP antibody (a gift from Assoc Prof. Wilawan Chotigeat) at 1:800 dilution was added. The membrane was washed five times (15 min/time) with TBST, incubated with goat–rabbit IgG conjugated with HRP (Jackson ImmunoResearch Laboratories, Baltimore USA) and detected by a super west pico chemiluminescent substrate (Piece, Rockford, Il, USA). Actin detected by anti-actin antibody at 1:1000 dilution (Cell signaling Technology, Danvers, USA) was used as internal control for normalization. TCTP in culture medium was detected the same method of the supernatant of the cell lysate as described above.

2.7 Statistical analysis

One-way ANOVA was employed to examine the differences among the experimental groups in all experiments. Turkey multiple comparison was used as a post hoc test. Three-way ANOVA with Tukey multiple comparison was applied to evaluate the effect of subjects, concentrations of TCTP and HEMA on the anticytotoxicity of TCTP. The level of statistical significance was defined as $P < 0.05$.

3 Results and discussion

3.1 Cytotoxicity of HEMA on HDPCs

Figure 1 shows the average of percentages of apoptotic cells from four subjects. HDPCs exposed to HEMA (0–16 mM) induced a dose-dependent increase of apoptotic cells. HEMA at the concentration higher than 6 mM induced significantly ($P < 0.05$) higher percentages of apoptotic cells, which was consistent with other studies that reported most apoptotic cells were significantly induced after cells exposing to HEMA at 8–10 mM for 24 h [24, 25]. Cells treated with media only (control) displayed a few cells that were stained with annexinV and PI, and the increasing concentrations of HEMA also demonstrated the high level of annexinV-positive cells (Fig. 1b). The average percentage of apoptotic cells in control culture (using normal medium) was 10.18 ± 1.44%, as a basal level, and in the culture medium that had 8 mM HEMA, the average percentage of apoptotic cells increased to about two-fold compared to the control.

3.2 Proliferative effect of TCTP on HDPCs

TCTP-treated cells ranging from 1 ng/ml–20 μg/ml did not have any effect in terms of cytotoxicity after exposure to

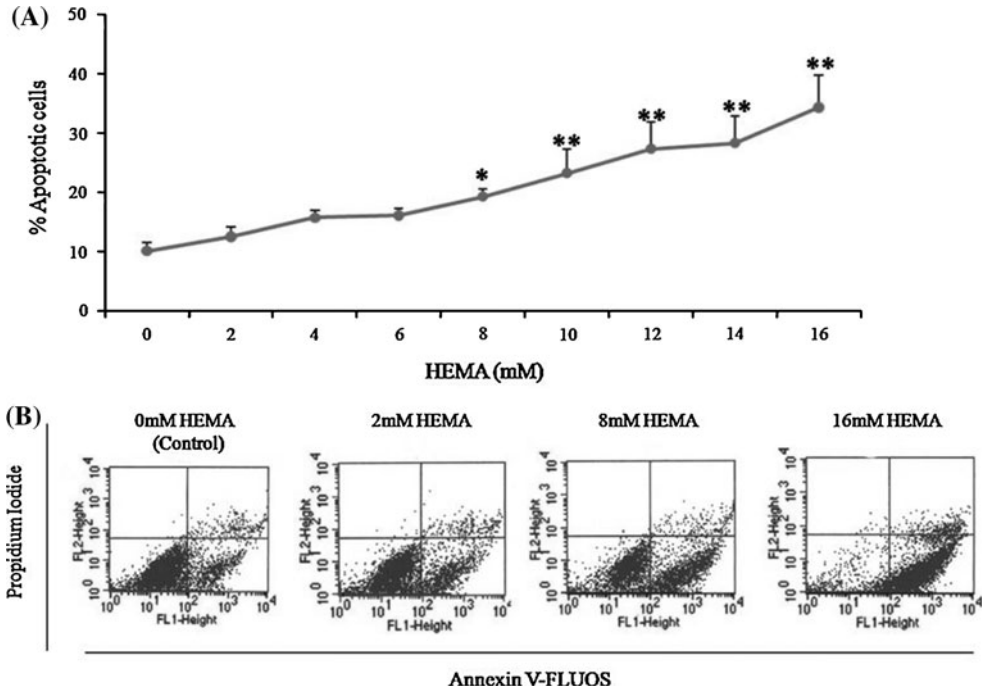

Fig. 1 The induction of apoptosis in HDPCs by HEMA. Cells were exposed to various concentrations of HEMA for 24 h, and the extent of apoptotic cells was determined by flow cytometry. **a** The average of percentages of apoptotic cells in HDPCs from four subjects exposed with various concentrations of HEMA. **b** The flow cytometric images from one of four subject showed the viable cells (annexinV −ve; PI −ve) in lower left hand quadrant, the apoptotic cells (annexinV +ve; PI −ve) in lower right hand quadrant, and the necrotic cells (annexinV +ve; PI +ve) in upper right hand quadrant. The experiments were repeated at least four to six times, and the results represented the values of means ± SD. *Indicated significant at $P < 0.05$, **indicated significant at $P < 0.01$ (one-way ANOVA with Tukey multiple comparison test)

both 24 and 72 h. In contrast, TCTP can promote cell proliferation because the percentage of viable cells was over 100% compared with the control, and TCTP-treated cells at both 24 and 72 h showed the same pattern of the increasing viability of cells with the increased concentrations (Fig. 2a) and the percentages of viability of TCTP-treated cells at 10 and 20 μg for 72 h were significantly ($P < 0.01$) higher than other groups, and HDPCs still demonstrated their typical normal spindle-shaped morphology (Fig. 2b). Even though, the TCTP in this study came from the banana prawn, it still has an effect on human dental pulp cells. This result support that TCTP is a conserved protein which can promote cell proliferation [26]. But whether this cell growth promotion effect is whether cell type specific or not may need further investigation.

3.3 Protective effect of TCTP against HEMA

The results of protective effect of TCTP against toxicity of HEMA were reported in percentages of viable cells from four subjects, as shown in Fig. 3. The percentages of viable cells in the culture medium with HEMA were lower than in the culture medium with a combination of HEMA and TCTP at both 8 and 10 mM of HEMA. The results from three way ANOVA from Table 1 revealed that the mean percentages of viable cells of the four subjects were statistically significantly different ($P < 0.01$) and from Tukey multiple comparison the means percentages of viable cells from the four subjects can be arranged as S3>S2>S1>S4. HDPCs cells exposed to HEMA at 8 mM had significantly ($P < 0.01$) higher mean of percentages of viable cells than HEMA at 10 mM. Mean percentages of viable cells at different concentrations of TCTP were significantly different ($P < 0.01$) and can be arranged as 20 μg/ml > 1 μg/ml, 10 μg/ml > 0.1 μg/ml > control (medium only). This result revealed that TCTP had a high potential for protection against HEMA toxicity, since it required only a small amount, starting not higher than 0.1 μg/ml, to significantly reduce the percentages of apoptotic cells induced by 8–10 mM of HEMA. There were interactions between subjects and concentrations of HEMA and concentrations of TCTP with HEMA, as well as between subject and concentrations of TCTP ($P < 0.05$) which was also demonstrated in the different slope of the linear plot from each subject in Fig. 3. These results demonstrated that TCTP can protect cells from the toxic effect of HEMA and the protective effect of TCTP differed, depending on the subjects and the concentrations of TCTP and HEMA. However, the percentages of the viable cells still showed the same pattern in both concentrations of HEMA (Fig. 3).

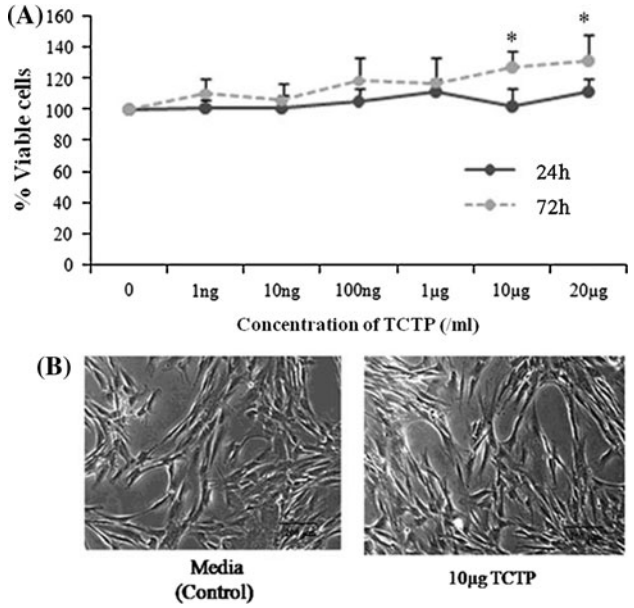

Fig. 2 The proliferative effect of TCTP in HDPCs. Cells were treated with various concentrations of TCTP for 24 and 72 h, and the effect of TCTP was investigated by MTT assays. **a** The percentage of viable cells compared to control (no TCTP) has been shown. **b** The morphological images of HDPCs in control culture and 10 μg TCTP-treated cells were taken via the inverted microscope (magnification 100×). The experiments were repeated at least four to six times, and the results represented the values of means ± SD. *Indicated significant ($P < 0.01$) (one-way ANOVA with Tukey multiple comparison test)

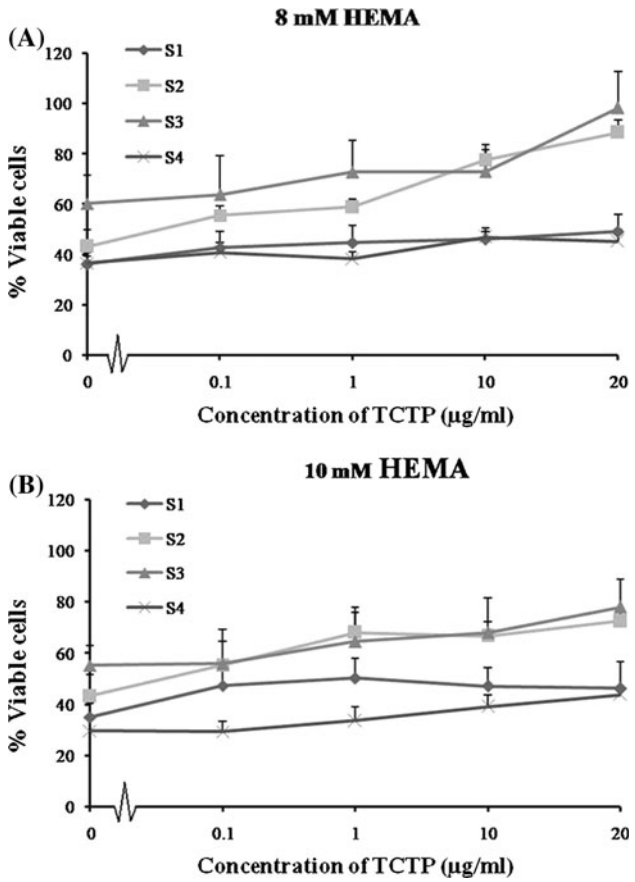

Fig. 3 The protective effect of TCTP on HEMA-induced apoptosis in HDPCs cells from four subjects. **a** Cells were treated with a combination of 8 mM HEMA and TCTP ranging from 0.1–20 μg/ml. **b** Cells were treated with a combination of 10 mM of HEMA with various concentrations of TCTP. After 24 h, the fresh media was changed and incubated for 48 h. The viability of cell from four subjects was determined by MTT assay. The experiments were repeated six times, and the results represented the values of means ± SD. The concentration of TCTP (*X*-axis) presented in log scale

3.4 Anti-apoptotic property of TCTP

To assess the anti-apoptotic property of TCTP by flow cytometry, the percentages of apoptotic cells in a combination of TCTP at 10 μg/ml and both concentrations of HEMA, decreased to be by half (from about 20–10%) compared with HEMA alone (Fig. 4a, b). In addition, the treatment of cells with 8 and 10 mM HEMA alone for 24 h induced morphological alterations, which appeared smaller, retracted and rounded, whereas the appearance of cells that were treated with HEMA (8 and 10 mM) in the presence of TCTP at 10 μg/ml was similar to the control (media) (Fig. 4c).

The possible mechanism of HEMA, causing cell death, may result from a decrease in mitochondrial membrane potential, and an increase in cleaved caspases [13], which is also involved with the reactive oxygen species (ROS) [24]. However, this mechanism may not be directly dependent on an ROS increase, since reduction of ROS did not reduce apoptosis [27] and it may involve mitogen-activated protein kinase (MAPKs) p38, JNK and ERK [28, 29].

Table 1 The 3-way ANOVA table of between-subject effect

Source	df	*F*	*P*-value
HEMA (8,10 mM)	1	18.43	0.000
Subject	3	197.82	0.000
TCTP (concentration)	4	55.93	0.000
HEMA × subject	3	4.84	0.003
HEMA × TCTP	12	6.43	0.000
HEMA × subject × TCTP	12	1.64	0.084

The percentages of viable cells from MTT assay were compared according to HDPCs cells from 4 subjects treated with 2 concentrations of HEMA and 5 concentrations of TCTP. ($n = 6$ in each group)

Note: "×" represented interaction effect. "df" represented degree of freedom. '*F*" represented *F*-ratio from ANOVA analysis

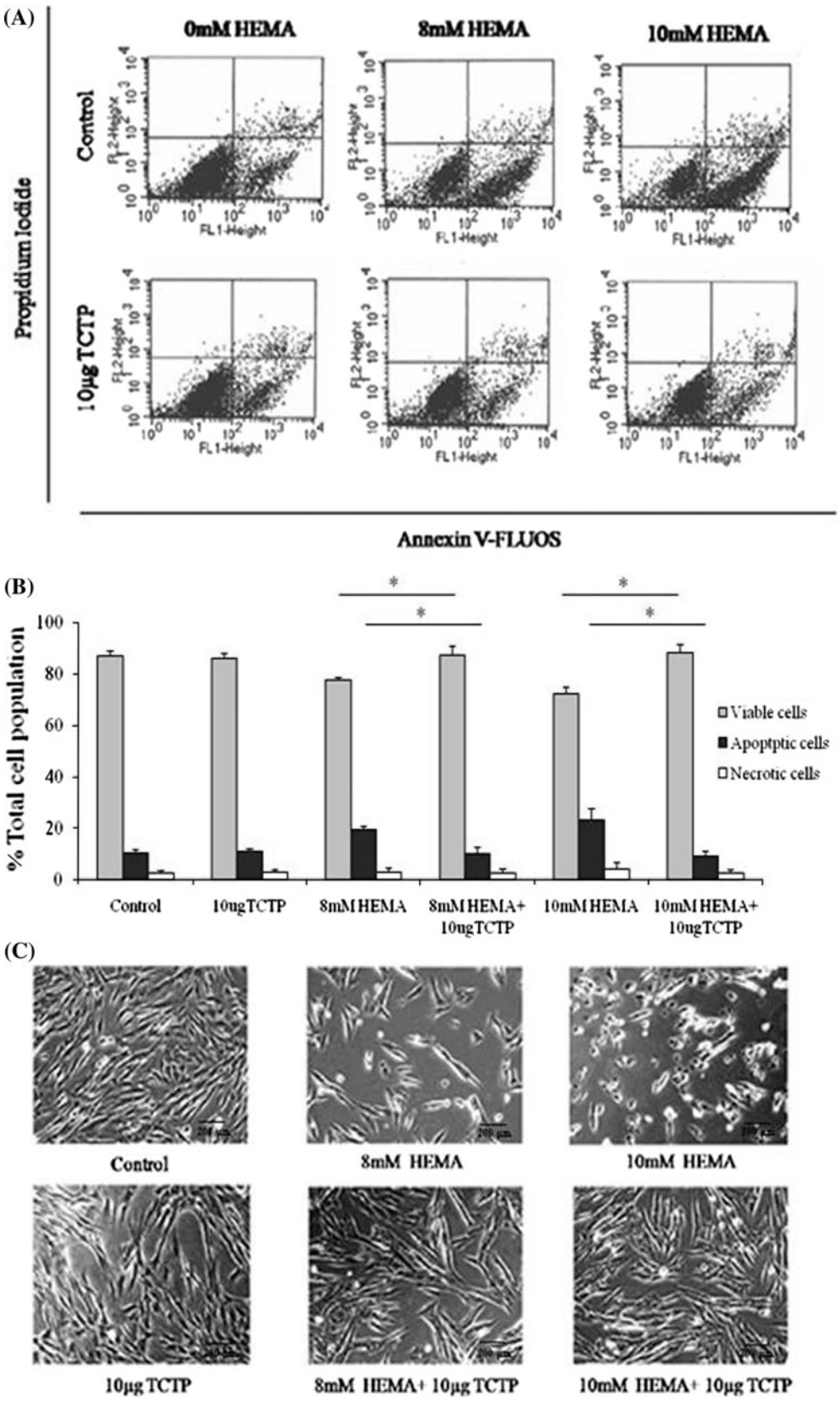

Fig. 4 The effect of TCTP against HEMA-induced apoptotic cells in HDPCs from four subjects. Cells were treated with HEMA (8 and 10 mM) and 10 μg/ml of TCTP for 24 h, after which they were stained with annexinV-FLOUS and propidium iodide and analyzed by flow cytometry. **a** The flow cytometric images from one of four experiments. **b** The average of cell population including viable cells, apoptotic cells and necrotic cells from four subjects. **c** The morphological alteration of HDPCs (magnification 100×) from one of four experiments. The experiments were repeated at least four to six times, and the results represented the values of means ± SD. *Indicated significant ($P < 0.05$) differences (one-way ANOVA with Tukey multiple comparison test)

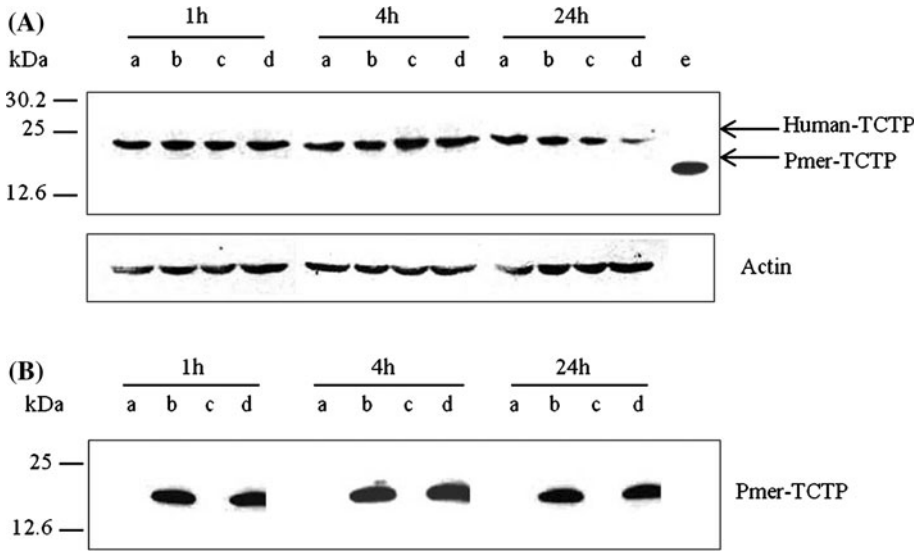

Fig. 5 Western blot analysis. **a** Intracellular TCTP in HDPCs after treatment with 10 mM HEMA in absence or presence of 20 μg/ml TCTP for 1, 4, 24 h. **b** TCTP added in the culture medium. Lane **a**: no treatment, **b**: 20 μg/ml Pmer-TCTP treated-cells, **c**: 10 mM HEMA treated-cells, **d**: 20 μg/ml TCTP + 10 mM HEMA treated-cells, **e**: TCTP standard (*Pmer*-TCTP) (the predicted mass 19.2 kDa) and actin was used as loading control

3.5 Detection of TCTP in HDPCs and culture medium

Western blot analysis (Fig. 5) did not detected the added TCTP (about 19.2 kDa) in intracellular of HDPCs (lane b and d) in all 1, 4 and 24 h time periods (Fig. 5a) even the sensitivity of this detection kit is in the picogram range according to the manufacturer's instruction. However, it can detect one protein band at molecular weight about 23 kDa, which suggested that there may be native human TCTP [30] in all lanes of HDPCs (Fig. 5a). In 24 h after exposure, the level of this protein band was reduced in cells treated with HEMA alone (lane c) and it was markedly reduced in cells treated with 20 μg/ml TCTP + 10 mM HEMA (lane d). The added TCTP was detected in cultured medium (lane b and d in Fig 5b) in all three time periods suggested that this added protein was not degraded at least 24 h.

Various cellular stimuli or stress can up and down-regulate of TCTP levels [17] and TCTP is downregulated in response to various pro-apoptotic treatments [16] which corresponded with the result that was shown in Fig. 5a (lane c in 24 h). However, it was very interesting that the added TCTP was not detected inside the cells and the native human TCTP markedly reduced (lane d in 24 h) but the HEMA-TCTP added cells were survived more than HEMA treated only as shown in the result of MTT assay (Fig. 3). This suggested the need for further investigation, since the entire mechanism of TCTP on physiological function regarding anti-apoptosis mediation are still not completely understood. However, it might be possible that the anti-apoptotic effect of the added TCTP against HEMA may have occurred outside the cells, since it was recently reported that the in vitro detoxification ability of NAC against HEMA-induced cell death may be due to NAC adduct formation with HEMA, which mainly reacted outside the cells and greatly decrease the concentration of HEMA entering inside the cells [31].

The action of TCTP against apoptosis has been reported by some studies. Overexpression of TCTP in HeLa cells prevented them, in a dose dependent fashion, from undergoing etoposide-induced apoptosis [32]. The protective mechanism of TCTP against oxidative stress may be explained by the insertion of TCTP into the mitochondrial membrane for enhancing the potential of mitochondrial membrane, which led to the suppressible effect on mitochondrial apoptosis by inhibiting the activity of Bax [33]. However, the insight mechanism of TCTP against HEMA needs further investigation. Besides the anti-apoptotic activity, the N-terminal amino acid sequence of human TCTP (p23) and mouse TCTP (p21) was found to be a histamine releasing factor that may affect the immune response [34]. But the TCTP that was used in this study was derived from *Penaeus merquiensis* shrimp, which has only 44% similarity to human TCTP (p23)/histamine releasing factor [22] and does not have the sequence that activates histamine release. However, the histamine releasing property of this TCTP also requires further investigation. Moreover, it was found that TCTP can bind to a variety of denatured proteins and protect them from the harmful effects of thermal shock [35]. This increases the feasibility of this protein being advantageous for dental applications, since in dental procedures, especially during tooth preparation for restoration, these always generate heat, which can cause damage to pulp tissue [36]. However, it requires further studies about the bioactivity of this protein and also how to use this TCTP for dental applications.

4 Conclusion

This study demonstrated that the TCTP protected HDPCs death from HEMA due to its anti-apoptotic activity. Moreover, TCTP not only reduced the toxicity of HEMA, but it also promoted cell growth. The mechanism of TCTP acting against the toxicity of HEMA and also the effect of this protein to pulp cells needs further investigation. However, this protein has the potential for further development of a novel biomaterial that has less toxicity, or even of a material that may induce regeneration of dental tissue.

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