

# Caffeic Acid Phenethyl Ester Alleviates Cryodamage to Lung Cancer Cells During Cryopreservation

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## ABSTRACT

Cryopreservation is widely used technique for long-term preservation of viable cells at low temperature. In this process, considering the effects of cryodamage on cells, the application of safe and efficient cryoprotective agents is very important. Caffeic acid phenethyl ester (CAPE) is a natural biological compound which is found in propolis extract and possess beneficial effects such as anti-oxidant, antimicrobial, anti-inflammatory. In the current study was to investigate the cryoprotective effects of CAPE on human lung cancer cell line, A549. Firstly, cells were cryopreserved in freezing medium with/without different concentrations of CAPE (5, 10, and 20  $\mu\text{M}$ ). The cells were frozen slowly and kept in liquid nitrogen for one month. After thawing, the cryoprotective effects of CAPE were determined by cell viability, proliferation, colony formation, and gene expression levels. The results showed that 5 $\mu\text{M}$  CAPE supplemented freezing medium significantly increased the viability of post thaw A549 cells. 5 $\mu\text{M}$  CAPE treatment significantly increased cell proliferation after 24, 48 and 72h since thawing compared to control. 10  $\mu\text{M}$  CAPE did not significantly affect cell viability compared to control group. Also, 5 $\mu\text{M}$  CAPE increased the number of A549 colonies compared to 10 $\mu\text{M}$  CAPE and control groups. Furthermore, markedly larger colonies were noticed in 5 $\mu\text{M}$  CAPE group. In addition, 5 $\mu\text{M}$  CAPE significantly increased apoptosis and proliferation-related genes, Akt, NF $\kappa$ B and Bcl-2, expression levels compared to 10 $\mu\text{M}$  CAPE and control groups. CAPE may be a potential cryoprotective agent for relieving cryodamage during cryopreservation.

### Keywords:

Cryopreservation; Cryoprotective agents; CAPE; Lung cancer cell line; Cell viability

## INTRODUCTION

Cryopreservation is a process to preserves viable cells and tissues at very low-temperature conditions for a long period time [1]. Cryopreservation of various cell types is important to ease storage of large quantities, transportation and banking for long periods of time [2]. However, production of reactive oxygen species (ROS), osmotic pressure and ice crystals cause irreversible damage the structure and function of cells undergoing the freezing and thawing processes. Under oxidative stress, accumulation of intracellular ROS production leads to the marked reduction in the viability of freeze-thawed cells and trigger apoptotic cell death. In fact, studies have reported that a relationship between ROS and apoptotic cell death and also compounds with antioxidant properties can inhibit apoptosis by acting as ROS scavengers [3].

During cryopreservation process, the formation of ice crystals can be lethal for viable cells. Fast cooling can causes the death of cells due to intracellular ice formation. In the last few decades, researches have focused on optimization of cryopreservation procedures and the addition of cryoprotective agents (CPAs) to protect cells overcoming freeze–thaw stress. CPAs should have some features such as being able to easily penetrate into the cells and possess low toxicity [4]. Dimethyl sulfoxide (DMSO) is commonly used as CPAs for cryopreservation process, however, it may not be appropriate for cryopreservation of cells are used in clinical practice due to its cytotoxicity. Therefore, there is a need to develop alternatives to reduce the toxic effects of DMSO [5],[6].

Caffeic Acid Phenethyl Ester (CAPE) is a biologically active component existing in propolis extract

### Article History:

Received: 2021/06/10

Accepted: 2021/09/23

Online: 2021/09/29

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which is found in the base of the honeybee hives. CAPE is a structurally related to flavonoids and has polyphenolic structures that contains hydroxyl groups. It is a non-toxic and lipophilic antioxidant. Due to its structural features, CAPE exerts numerous beneficial effects such as anti-inflammatory, immunomodulatory, and anti-oxidant [7],[8]. CAPE completely inhibits the ROS production due to its free radical scavenging and anti-oxidant activity [9],[10]. It has been determined that CAPE has no detrimental effect on healthy cells [11]. Furthermore, several studies showed that CAPE plays a protective role to prevent cells from undergoing apoptosis. CAPE can exert anti-apoptotic properties via inhibiting ROS production and caspase activation [11]–[13].

This study was to determine the protective effects of CAPE on human lung carcinoma cell line, A549, after freezing and thawing process during cryopreservation.

## MATERIALS AND METHODS

### Cell Culture and Reagent

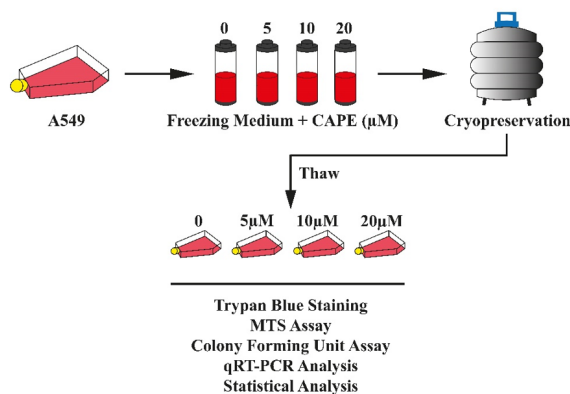
Non-small cell lung cancer cell line, A549, was purchased from the ATCC (American Type Culture Collection, Rockville, MD). They were cultured in DMEM-High Glucose (Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% Penicillin/Streptomycin/Amphotericin (PSA, Gibco, UK) under 5% CO<sub>2</sub>, 95% humidity at 37°C. Cells were trypsinized and passaged after enough confluency ~ 80%.

CAPE (#BCBR2913V, Sigma-Aldrich, USA) was resuspended in DMSO (dimethyl sulfoxide, 1550557, Fisher Scientific, UK) to reach the main stock concentration of 20mM and kept in -20°C.

### Cell Freezing, Thawing and Storage

Freezing and thawing process was performed to show the potential cryoprotective effect of CAPE on lung cancer cell line. Briefly, cells were trypsinized and total cell number were counted using hemocytometer. Cells were divided into four different groups. 1x10<sup>6</sup> cells from each group were resuspended in 1 mL cell freezing medium (90% FBS, 10% DMSO) with 5, 10 and 20µM CAPE and without (control group, 0 µM CAPE). Then they were frozen in a freezing container at - 80 °C. After 24h incubation, the cryo-vials were transferred and stored into 196°C liquid nitrogen tank. After 30 days storage, the cells were placed in a 37 °C water bath immediately, rinsed with culture medium to reduce detrimental effect of DMSO and centrifuged. Then, the cell pellet was diluted in growth medium for further experiments.

The schematic representation of the experiment is shown in Fig. 1.



**Figure 1.** The schematic illustration of the experimental design.

### Trypan Blue Staining

Trypan blue staining (T-8154, Sigma-Aldrich) was performed to assess the cell viability. Briefly, after thawing process, cell suspension were mixed with 0.4 % trypan blue (1:1 ratio) and incubated 5 min at room temperature (RT). After the incubation, viable/dead cells were counted using a hemocytometer.

### MTS Assay

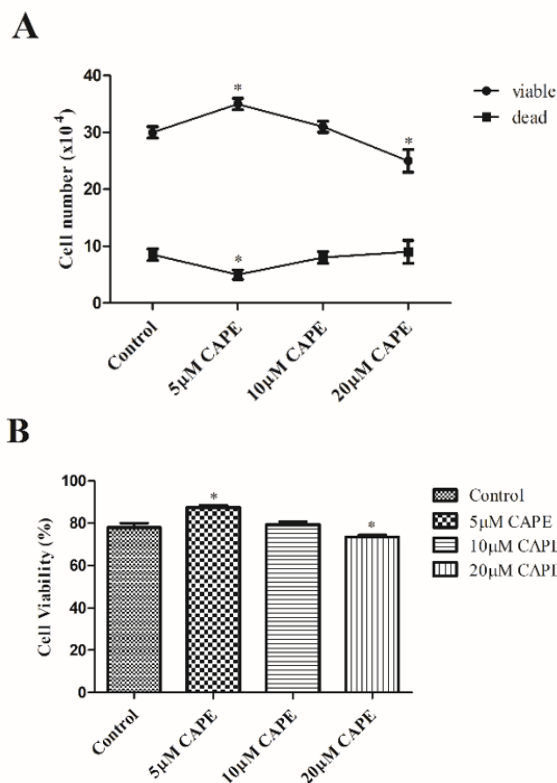
MTS (3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) was conducted to assess the proliferative effects of CAPE concentrations on the cells. Briefly, cells (5x10<sup>3</sup>) were seeded into 96-well plates for 24, 48 and 72h. After the incubation periods, 10µL MTS solution was added into each wells and incubated for 1h at 37 °C in the dark. The optical density (OD) was read at 495 nm by using ELISA microplate reader (Biotek, Winooski, VT).

### Colony Forming Unit (CFU) Assay

Colony forming unit assay was used to determine the effects of CAPE concentrations on frozen-thawed cells. Briefly, cells were seeded into 6-well plates (300 cells/well) and medium was changed with fresh growth medium five times for 15 days until colonies were visible. Then, 4% paraformaldehyde (PFA) was used to fix colonies for 30min and they were stained with crystal violet dye for 10min. After the incubation, colonies were observed and photographed by ZEISS PrimoVert light microscopy system with AxioCam ICc 5 camera.

### Quantitative Real Time PCR (qRT-PCR) Analysis

Changes in proliferation and apoptosis-related gene expression levels were determined by qRT-PCR analysis. Briefly, cells (150x10<sup>3</sup>) were seeded into 6-well plates. After 24h, total RNAs were isolated using High Pure RNA isolation kit (#11828665001, Roche, USA) and then cDNA was synthesized according to the High Fidelity cDNA synthesis kit (#05081955001, Roche, USA) protocol.



**Figure 2.** Effects of CAPE concentrations (5, 10 and 20µM) on viability of thawed A549 cells were evaluated using trypan blue staining A. The viable (trypan blue-negative) and dead (trypan blue-positive) cell number after cryopreservation. B. Cell viability (%) after cryopreservation in 5, 10 and 20µM CAPE. \*P < 0.05.

mRNA levels of the target genes were determined by qRT-PCR with SYBR Green Master Mix (Thermo-Fisher, USA). 2µL cDNAs, 1µL primers, 5µL SYBR-mix (#K0221, Fermentas, USA) and 2µL PCR grade distilled water (#SH30538.02, Hyclone, Utah, USA) in a total volume of 20µl was used for RT-PCR. Primer-BLAST software (National Center for Biotechnology, MD, USA) were used to design AKT, NFκB and BCL-2 primers and Macrogen (Seoul, Korea) synthesized these primers. Data were normalized by a housekeeping gene GAPDH. The CFX96 RT-PCR system was used for RT-PCR experiments (Bio-Rad, Hercules, CA).

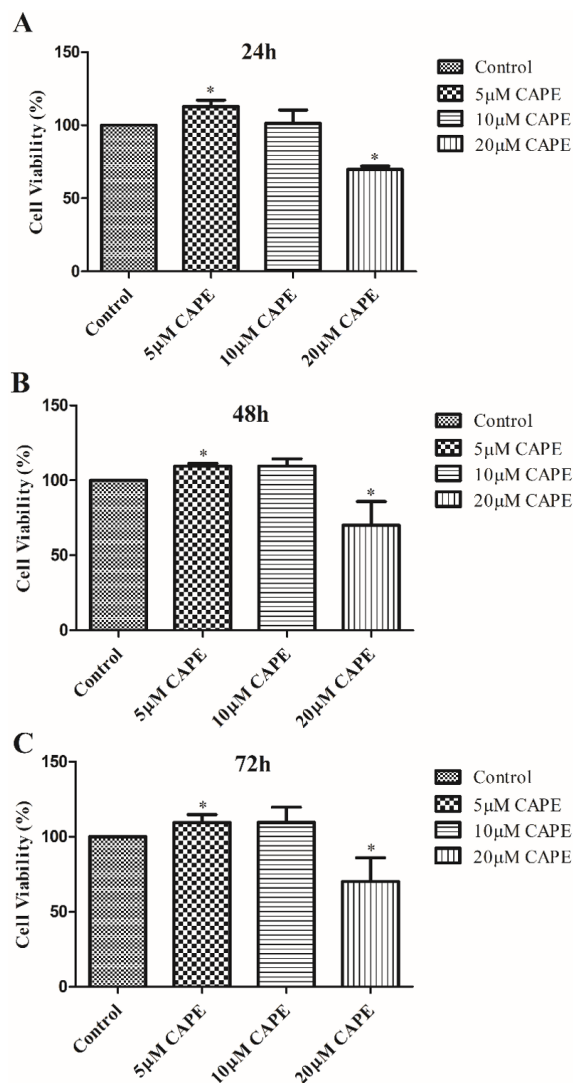
### Statistical Analysis

One-way analysis of variance (ANOVA) was used to statistical analysis followed by Tukey's post-hoc test. The data was given as the mean ± SD. Statistical significance was considered as P < 0.05.

## RESULTS

### CAPE Protects Lung Cancer Cells in Cryopreservation Process

The trypan blue staining was used to assess the effects of different CAPE concentrations (5, 10 and 20µM) on the



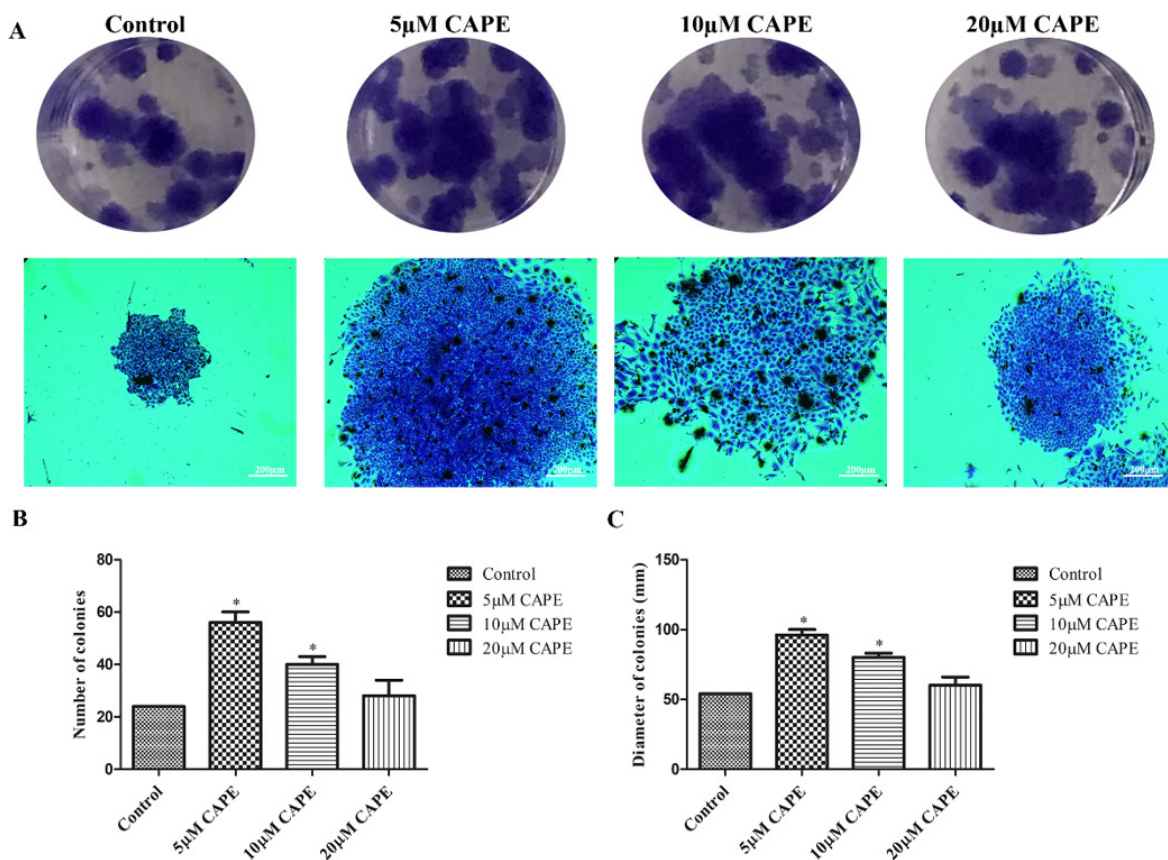
**Figure 3.** Effects of CAPE concentrations (5, 10 and 20µM) on viability of thawed A549 cells for (A) 24, (B) 48 and (C) 72h were evaluated by MTS assay. \*P < 0.05.

viability of A549 cells. A dose of 5µM CAPE added to freezing medium significantly increased viable and reduced dead cells (Fig. 2A).

Cryopreservation medium supplemented with 5µM CAPE notably increased the viability of post-thaw A549 cells (87±1.5%). No significant effects was observed in 10µM CAPE supplemented group (79±0.7%) compared to control (78±1.3%), suggesting that 5µM CAPE was selected for further experiment. However, cryopreservation medium supplemented with 20µM CAPE significantly decreased the viability of post-thaw A549 cells (73±0.5%) (Fig. 2B).

### CAPE Improves the Proliferation of Lung Cancer Cells in Cryopreservation Process

MTS assay was conducted to assess the proliferation for 24, 48 and 72h after thawing process. 5µM CAPE signifi-



**Figure 4.** Effects of CAPE concentrations (5, 10 and 20µM) on colony forming ability of thawed A549 cells. A. The colony formation ability of A549 cells in each group was assessed by CFU assay. B. Graph shows the number of colonies. C. Graph shows the diameter of colonies. \*P < 0.05.

cantly increased cell viability to  $112\pm\%4$ ,  $109\pm1\%$ ,  $118\pm5\%$  after 24, 48 and 72h, respectively. There was no observed any significant changes in the viability of 10µM CAPE-supplemented group while, at the highest CAPE concentration of 20µM significantly decreased the viability of cells after 24, 48 and 72h after thawing compared to control (Fig. 3).

### CAPE Enhances Colony Forming Capacity of Lung Cancer Cells

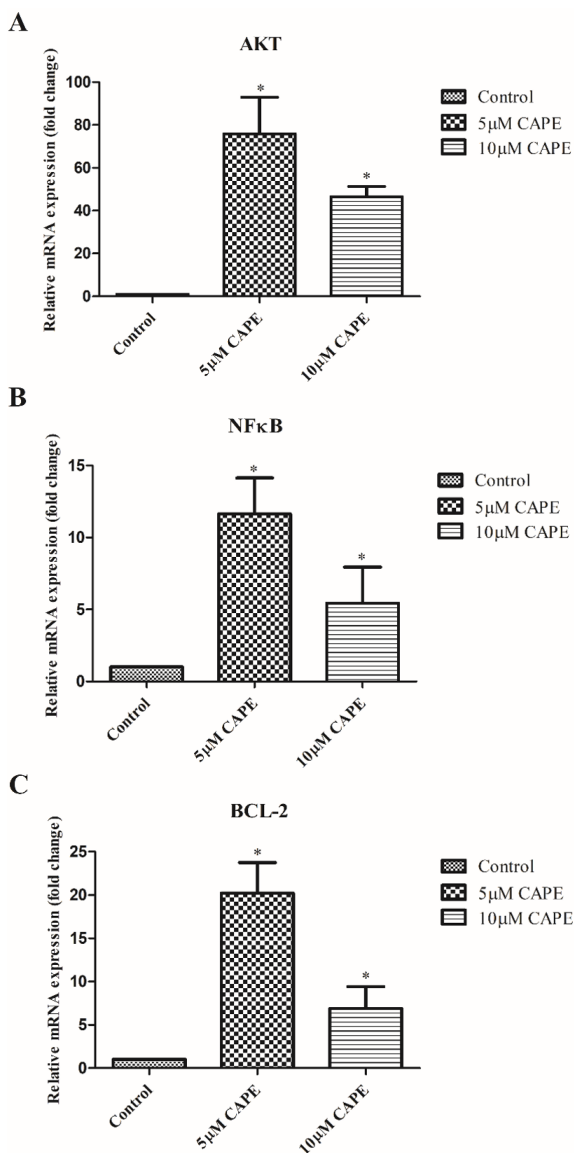
CFU assay was assessed the effects of different CAPE concentrations on colony forming capacity of A549 cells after freezing and thawing process. 5 and 10µM CAPE notably increased the number of A549 colonies compared to 20µM CAPE-supplemented and control group. In addition, no significant difference was observed in number of colonies between 20µM CAPE-supplemented and control group. Moreover, dramatically bigger colonies were noticed in 5 and 10µM CAPE-supplemented group. Size of colonies were similar to 20µM CAPE-supplemented and control group (Fig. 4).

### CAPE Changes Gene Expression Profiles of Lung Cancer Cells

qRT-PCR analysis was used to determine the effects of different CAPE concentrations on pro-apoptotic and anti-apoptotic gene expression levels after freezing and thawing process. The addition of 5µM CAPE resulted in a significant upregulation of AKT (~75.9 fold), NFκB (~11.6 fold) and Bcl-2 (~20.2 fold) expression levels. Similarly, 10µM CAPE significantly upregulated AKT (~46.5 fold), NFκB (~5.4 fold) and Bcl-2 (~6.8 fold) gene expression levels. 5 µM CAPE supplementation markedly increased gene expression levels compared to 10 µM CAPE (Fig. 5).

## DISCUSSION

Cryopreservation is a crucial process to preserve viable cells, tissues and organs. Cryopreservation of cells is commonly used in numerous fields such as biotechnology, agricultural and medicine. During freezing process, cryodamage may result in reduced cell viability due to the formation of ice crystal [14]. Cryoprotective agents (CPAs) help to alleviate cryodamage and ice crystals by attenuating the water crystallization and improving the viscosity [15],[16]. Glycerol and dimethyl sulfoxide (DMSO) are well known CPAs for several types of cells, however they have some limitations. Glycerol has relatively weak cryoprotective effect while DMSO can exert



**Figure 5.** Effects of CAPE concentrations (5 and 10μM) on proliferation and apoptosis related gene expression levels (A) AKT, (B) NFκB and (C) BCL-2 of thawed A549 cells. AKT: Protein kinase B, NFκB: Nuclear factor kappa B, BCL2: B-cell lymphoma 2. \*P < 0.05.

cytotoxic effect and needs to be quickly removed after thawing process [17]. Recent studies mainly focused on two approaches: reducing DMSO concentration and replacing it with more effective and non-toxic agents.

The cryopreservation increases the cellular ROS production [18]. The excessive ROS production induces oxidative stress and impairs the structure, function and viability of cells [19]. Also, increased ROS levels induce cytochrome C release from mitochondria to cytosol via stimulation of mitochondrial membrane permeability transition (MMPT), thereby triggering apoptosis [20]. Furthermore, when abnormal changes occur in physiological conditions, cells begin to reuse their components to produce energy and the-

reby autophagic cell death is triggered [21]. Non-toxic and effective CPA supplementation in freezing medium to inhibit oxidative stress and apoptosis, significantly improves cryopreservation process [22].

CAPE, a natural phenolic compound, is found in propolis and has beneficial effects such as antioxidant, antimicrobial and immunomodulatory. CAPE is an ester form and a derivative of caffeic acid. Due to its structural properties, CAPE presents high biological activity and able to easily cross the cell membranes than caffeic acid [23]. Several studies have examined the use of caffeic acid on cryopreservation process [24],[25]. Cryopreservation elevates apoptotic biomarkers such as membrane permeability and caspase activation [26]. CAPE can exert anti-apoptotic properties via inhibiting ROS production and caspase activation [11],[12]. However, the effects of CAPE on cryopreservation of cells are still largely unknown, it needs to be elucidated.

This study was evaluated the potential cryoprotective efficiency of CAPE on lung cancer cells. These results showed that the addition of CAPE to the freezing medium significantly increases the viability of A549 cells after cryopreservation process. The number of viable A549 cells was markedly elevated by CAPE included cryopreservation medium. The viability of post-thaw A549 in 5μM CAPE-supplemented group was significantly higher than 10 and 20μM CAPE-supplemented and control groups. 5μM CAPE showed to protect A549 cells to against apoptosis. Moreover, 5μM CAPE was significantly increased the proliferation rates of the cells for 24, 48 and 72h. The proliferation rates of the cells treated with 10μM CAPE was similar to control group. CFU results also supported the cell viability analysis. 5μM CAPE-supplemented group was shown significantly increased colony formation capacity of cells. However, there was no significant change in CFU assay of 10μM and 20μM CAPE-supplemented group, indicating that 5μM CAPE has shown to have a better effect in protecting cell functions. Similar to these results, Li et. al reported that CAPE significantly increase the viability of cells and colony formation capacity [27].

It is known that cryopreservation process change the gene expression profiles of cells [28]. PI3K/AKT signaling leads to the inhibition of apoptotic signaling cascade, which is associated with the production of reactive oxygen species, caspase activation [29]. Akt, a serine/threonine kinase, has a pivotal role in survival and proliferation of cells [30]. The agents with anti-oxidant properties was shown to increase the Akt expression level during cryopreservation [31].

Transcription factor NFκB is a downstream target of Akt and activation of NFκB promote cell survival [32]. Moreover, Bcl-2 family members are major role in regulating

apoptosis and cell survival. Upregulation of anti-apoptotic Bcl-2 inhibits apoptosis and promotes cell survival [33]. qRT-PCR analysis revealed that CAPE treatment upregulates the proliferation related gene expression, Akt, NFκB, and anti-apoptotic gene expression level, Bcl-2, in A549 cells. The AKT, NFκB and BCL-2 levels were shown to be increased in A549 cell after freezing and thawing process with 5 and 10μM CAPE. However, 5μM CAPE supplementation has shown significantly upregulated these genes compared to 10μM CAPE. In consistent with these results, previous studies have reported that CAPE reduce apoptotic cell death and induce cell proliferation [34]–[36].

## CONCLUSION

Taken together, the addition of CAPE to the freezing medium increased the viability, colony formation capacity and proliferation related gene expression of post thaw A549 cells. CAPE, a natural compound, may be a promising candidate as a cryoprotective agent for protection of cells from cryodamage and storage of cells without loss of their viability for long periods of time. Further studies are required to understand the underlying the protective mechanisms of CAPE at molecular level and its role in cellular metabolism during cryopreservation process. Additionally, the protective effects of CAPE should be investigated after long term cryopreservation.

## ACKNOWLEDGEMENTS

This study was supported by Yeditepe University.

## CONFLICT OF INTEREST

Authors approve that to the best of their knowledge, there is not any conflict of interest or common interest with an institution/organization or a person that may affect the review process of the paper.

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