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U937 세포에서 육계와 온열 병행 치료가 세포증식 억제와 세포사멸 유도에 미치는 연구

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Treatment of Cinnamomi Cortex combined with hyperthermia synergistically suppressed proliferation and induced apoptosis in U937 cell line.

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ABSTRACT

Objectives : Hyperthermia is a widely used therapeutic tool for cancer therapy and a well-known inducer of apoptosis. Although the Cinnamomi cortex (CC) is a potent anticancer agent for several human carcinomas, it is less potent in the human U937 cell line. To explore any enhancing effects of CC with hyperthermia induced apoptosis, this study investigated the combined effects and apoptotic mechanisms of hyperthermia and CC in U937 cells.

Methods : U937 cells were heat treated at 43ºC for 30 min with or without pre-treatment for 1h with CC and then incubated at 37ºC with 5% CO_2. Cell viability was analyzed by MTT assay and Trypan blue assay. Morphological changes reflecting apoptosis were visualized under microscope. Synergy effect of CC combined with hyperthermia were calculated by Compusyn software. The expression of proteins related to apoptosis and signaling pathways was determined by western blotting.

Results : Hyperthermia with CC reduced cell viability and induced apoptosis. Combined hyperthermia and CC treatment markedly augmented apoptosis by upregulating proapoptotic proteins and suppressing antiapoptotic proteins, culminating in caspase-3 activation. Furthermore, the combined treatment, decreased the expression of in Bcl-2 family, cyclin D1, VEGF, MMP2 and MMP9 expression.

Conclusion : This study provides compelling evidence that hyperthermia, in combination with CC, is a promising therapeutic strategy for enhancement of apoptosis and suggests a promising therapeutic approach for cancer.

Key words : Cinnamomi cortex, hyperthermia, synergy effect, apoptosis, leukemia cell.

Ⅰ. Introduction

So far, various therapies used to eliminate malignant cells in leukemia. Chemotherapy is a common method in the treatment of leukemia. Today, researchers investigated other types of treatment such as treatment with medicinal plant extracts due to severe side effects of chemotherapy (9).

Cinnamomi cortex (CC), also named Chinese cassia or Chinese cinnamon, is an evergreen plant. The genus Cinnamomum is a member of Lauraceae family, and it contains approximately 250 species that are distributed in China, India, Indonesia, South America, Hawaii and Australia (11). It has been mentioned in Chinese texts from 4000 years ago. In Ayurvedic medicine, CC is suggested for the treatment of digestive, respiratory and gynecological disorders (10) and used to treat conditions, such as gastritis, blood circulation disturbances and inflammatory diseases, in traditional Chinese medicine (8). Furthermore, chronic supplementation of CC had improved the fasting glucose and lipid levels in diabetic patients, it has been reported that CC could affect the cell cycle (6). The bark extract of *C. cassia* has potent anti-inflammatory activity and it also exerts anti-bacterial effects against clinically antibiotic-resistant isolates of Bacillus megaterium and Enterococcus faecalis (13).

Hyperthermia has also been extensively studied as a potential candidate for induction of apoptosis in cancer cells (4). It can cause regression of multiple cancer types, including lung, breast, colon and pancreatic cancer. The mechanisms of hyperthermia in treating cancers may be related to inhibiting DNA repair, promoting intracellular accumulation of chemical agents, inducing cell cycle arrest and apoptosis (3).

The current study aimed to investigate the efficacy of CC combined with hyperthermia treatment in suppressing U937 cell growth. The role of CC and hyperthermia in inhibiting U937 cell growth was investigated and cell proliferation, apoptosis rates and the expression of key genes were evaluated. We used human cancer cell lines: commercially available human leukemic monocyte lymphoma cell U937 line derived from the patient diagnosed with acute myeloid leukemia (4).

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Ⅱ. Materials and Methods

Drugs

The Cinnamomi cortex (kwangmyungdang Medicinal herbs co., Ltd. Ulsan, South Korea) was ground completely and then it was uniformed by a homogenizer. Following this, it was soaked in the 70% EtOH at room temperature for 24h and the extract was collected. The resulting extract was filtered (pore size: 5 ㎛), concentrated under reduced pressure and lyophilized to obtain a sample. 25, 35 and 40 mg/ml solutions were made with DMSO (Dimethyl sulfoxide) (Samchun Chem, Seoul, Korea). These solutions were kept at 4℃ until the usage.

Cell culture

The U937 cells of human histiocytic lymphoma line U937 (Korean Cell Line Bank) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-strep (Gibco, Grand Island, NY, USA) at 37℃ in incubator with humidified air with 5% CO_2.

Hyperthermia

U937 cells were seeded in 6 well plate at a density of 1×10^6cells suspended in 3 mL of media, followed by immersion in a temperature-controlled water bath at 37, 42 or 43℃ for 30 min. CC at the indicated concentrations was added to the samples 1h before.

MTT assay

The MTT assay was performed to detect cell prolif-eration following exposure to CC with hyperthermia. U937 cells (100 ㎕/well) were seeded in 96-well plates at a density of $5x10^4$ cells/ml. Each group had three wells with a non-treated group as the control. When the cells had anchored to the plates, various concen-trations of CC (25, 35 and 40 ㎍/ml) were added and the plates were incubated at 37℃ for 1h in a humidified atmosphere containing 5% CO_2. Then, followed by immersion in a temperature-controlled water bath at 37, 42 or 43℃ for 30 min. After 48h, 20 ㎕ of MTT (2 mg/ml in PBS) (AMRESCO, Solon, OH, USA) was then added to each well and cultured for another 2h. Following this, MTT formazan precipitates were dissolved in 100 ㎕ 10% SDS (sodium dodecyl sulfate) (Sigma-Aldrich, St. Louis, MO, USA). agitated mechanically for overnight incubation. Absorbance was measured with an automated spectrophotometric plate reader at a wavelength of 570 nm. Cell viability was normalized as relative percentages in comparison with untreated controls. Synergy effect of the drug and hyperthermia combinations were calculated by Compusyn software (ver. 1.0).

Trypan blue assay

Cell viability was determined by a Trypan blue assay by using a hemocytometer after Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) staining (0.4%, 1:1 dilution in the cell-containing PBS). Briefly, U937 cells were seeded in 6-well plate at a density of 1×10^6 cells and subsequently treated with CC (1h) and hyperthermia (30 min). After 24h of post-treatment incubation, cells were harvested, diluted 1: 4 with PBS, stained, and counted.

Cell survival rate = Viable cell count / Total cell count x 100%

Morphology assay

U937 cells were seeded in 6-well plate at a density of 1×10^6 cells. When the cells had anchored to the plates, treated with 40 ㎍/ml CC for 1h and incubated at 37, 42 or 43℃ for 30 min. After 24h, cells were visualized and captured by a microscope (CX-40, Olympus, Tokyo, Japan).

Western blot analysis

Protein concentrations from isolated U937 cells were determined. Equal amounts of lysates resolved on sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to a polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 1 x TBS containing 0.1% Tween 20 and 5% skim milk at room temperature. After the blocking, the membranes were incubated overnight at 4℃ with the respective primary antibodies: anti-caspase-3 (Cell Signaling Technology), anti-β-actin, anti-Bcl-2, anti- Bcl-xL, anti-Cyclin D1, anti-VEGF, anti-MMP2, anti-MMP9 (Santa Cruz Biotechnology, Inc.). The membranes were washed three times and incubated with diluted horseradish peroxidase (HRP)-conjugated: anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology, Inc.) secondary antibodies for 1h at room temperature. The blots were washed with 1× TBS-T buffer for 10 min, three times between each step. The membranes were detected using an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA).

Statistical analysis

All numeric values are represented as the mean ± SD. Statistical significance of the data compared with the untreated control was determined using the Student unpaired *t*-test. *p<0.05, **p<0.01 and *** p<0.001.

Ⅲ. Results

Reduction of cell viability and enhancement of apoptosis with combination treatment.

CC inhibited the growth of U937 cells in a dose-dependent manner. CC with hyperthermia increased the inhibition of U937 cells compared with CC treatment alone. The effect of CC with hyperthermia on the growth of U937 cells using MTT assay and trypan blue assay (Fig. 1A and B). The cytotoxic effects of CC and hyperthermia was observed the morphological changes by microscope. We found significantly reduced viability after 24h in the combination therapy. Combination treatment was induced a significant modulation in morphology and increased apoptosis (Fig. 1C). Finally, Synergy effect of CC combined with hyperthermia were calculated. It is indicated that combination treatment also significantly increased apoptosis and decreased cell viability in U937 cells compared with control or CC treatment alone. (CI<1.0 synergic effect, 1.0 implies additivity, CI>1.0 implies synergistic) (Fig. 1D).

Effects of CC with hyperthermia on the levels of caspase-3, Bcl-2 and Bcl-xL in U937 cells.

Caspase-3 is mainly activated in the apoptotic cell. We also investigated the effect of CC with hyperthermia on caspase-3 activation in U937 cells. This protein was drastically downregulated by combination treatment compared with the CC group (Fig. 2A). Bcl-2 is a critical molecule for the regulation of apoptosis. In order to examine the potential signaling pathways by which CC induces apoptosis, western blotting was used to evaluate the expres-sion of the Bcl-2 family. CC suppressed the expression of Bcl-2 and Bcl-xL in a concentration-dependent manner, which is more effects at combination treatments (Fig. 2B).

Downregulation of metastasis and angiogenesis proteins.

We examined the synergy effects on the expression of various proteins involved in apoptosis, proliferation, metastasis, and angiogenesis. In this study, expressions of cyclin D1, VEGF, MMP2 and MMP9 were visualized by western blotting. It was seen that these proteins decreased in combination group compared with the CC group. This expression of effect was more

marked in the CC with hyperthermia at 43℃ treatment group. Our results showed that proteins in the group of combination treatment significantly decreased compare to the groups treated cinnamon or hyperthermia alone (Fig. 3).

Ⅳ. Discussion

The antitumor effect of CC has been demonstrated in multiple types of tumor but leukemia underlying mechanism remains unclear. Hyperthermia treatment has previously been found to affect various cellular targets, including DNA, proteins, membranes and the cytoskeleton, in carcinoma cells. Hyperthermia can be a highly effective cancer treatment, particularly when combined with chemotherapy, radiotherapy or immunotherapy.

The present study investigated whether combining CC treatment with hyperthermia could improve its antitumor activity. It was found that combination of CC and hyperthermia significantly reduced cell viability and elevated cell death determined by morphological apoptotic body formation (Fig. 1).

In the previous study, Caspase-3 is a key executor in apoptosis and therefore serves a crucial function in programmed cell death (5) and control apoptotic signaling and execution by cleaving cellular proteins (1). In our study, CC with hyperthermia decreased caspase-3 expression (Fig. 2A).

Bcl-2 can maintain cell survival by limiting the pro-apoptotic effects. Therefore, the Bcl-2 can induce the apoptosis of cells and Bcl-2 family proteins have previously been found to be modulated in apoptosis progression (14, 15). These findings suggest that Bcl-2 family proteins play a critical role in CC and hyperthermia-induced cell death in U937 cells. Synthetically, Bcl-2 family proteins and activation of the caspase-3 indicated the involvement of the intrinsic pathway in the enhancement of apoptosis (1) (Fig. 2B).

Furthermore, cyclin D1, VEGF, MMP2 and MMP9 showed decreased expression in the combination treatment (Fig. 3). This proteins associate in apoptosis, proliferation, metastasis, and angiogenesis. Consistent with this, CC with hyperthermia was found to induce apoptosis.

In conclusion, CC with hyperthermia was shown to have a significant inhibitory effect in U937 cells. The present study was a preliminary exploration into the anticancer effects of CC with hyperthermia in U937 cells. Further research into the effects CC and hyperthermia treatment would be valuable in improving therapeutic strategies for leukemia.

Ⅴ. Conclusion

The results of our study showed that cinnamomi cortex extract with hyperthermia induced apoptosis in the human myelocytic leukemia cell line. Also, with the combined treatment, decreased in caspase-3, Bcl-2 family, cyclin D1, VEGF, MMP2 and MMP9 expression. This modality may have significant therapeutic potential in clinical settings.

Acknowledgments

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References

1. Zakki SA, Cui ZG, Sun L, Feng QW, Li ML, Inadera H. Baicalin Augments Hyperthermia-Induced Apoptosis in U937 Cells and Modulates the MAPK Pathway *via* ROS Generation. Cell Physiol Biochem. 2018;45(6):2444-60.

2. Liu T, Ye YW, Zhu AL, Yang Z, Fu Y, et al. Hyperthermia combined with 5-fluorouracil promoted apoptosis and enhanced thermotolerance in human gastric cancer cell line SGC-7901. Onco Targets Ther. 2015 May 27;8:1265-70.
3. Zhao P, Jiang H, Su D, Feng J, Ma S, Zhu X. Inhibition of cell proliferation by mild hyperthermia at 43℃ with Paris Saponin I in the lung adenocarcinoma cell line PC-9. Mol Med Rep. 2015 Jan;11(1):327-32.
4. Saliev T, Feril LB Jr, Begimbetova D, Baiskhanova D, Klodzinskyi A, et al. Hyperthermia enhances bortezomib-induced apoptosis in human white blood cancer cells. J Therm Biol. 2017 Jul;67:9-14.
5. Wu Z, Wang T, Zhang Y, Zheng Z, Yu S, et al. Anticancer effects of β-elemene with hyperthermia in lung cancer cells. Exp Ther Med. 2017 Jun;13(6):3153-7.
6. Assadollahi V, Gholami M, Zendedel A. C. zeylanicum aqueous extract induced apoptosis in the human myelocytic leukemia cell line (THP-1). Bratisl Lek Listy. 2015;116(2): 132-5.
7. Banerjee M, Chattopadhyay S, Choudhuri T, Bera R, Kumar S, et al. Cytotoxicity and cell cycle arrest induced by andrographolide lead to programmed cell death of MDA-MB-231 breast cancer cell line. J Biomed Sci. 2016 Apr 16;23-40.
8. Sharma H, Chauhan P, Singh S. Evaluation of the anti-arthritic activity of *Cinnamomum cassia* bark extract in experimental models. Integr Med Res. 2018 Dec;7(4):366-73.
9. Elliott P, Wakefi eld J, Best N, Briggs D. Spatial epidemiology methods and applications. New York: Oxford University Press, 2007.
10. Ranasi nghe P, Perera S, Gunatilake M, Abeywardene E, Gunapala N, et al. Effects of Cinnamomum zeylanicum (Ceylon cinnamon) on blood glucose and lipids in a diabetic and healthy rat model. Pharmacognosy Res 2012;4:73-9.
11. Jayaprakasha GK, Jagan Mohan Rao L, Sakariah KK. Volatile constituents from Cinnamomum zeylanicum fruit stalks and their antioxidant activities. J Agric Food Chem 2003;51:4344-8.
12. Ng LT, Wu SJ. Antiproliferative activity of Cinnamomum cassia constituents and effects of pifithrin-alpha on their apoptotic signaling pathways in Hep G2 cells. Evid Based Complement Altern Med 2009;2009:1-8.
13. Liao JC, Deng JS, Chiu CS, Hou WC, Huang SS, Shie PH, et al. Anti-inflammatory activities of Cinnamomum cassia constituents in vitro and in vivo. J Evid Based Complement Altern Med 2013;2013:1-12.
14. Chen XF and Liu Y: MicroRNA-744 inhibited cervical cancer growth and progression through apoptosis induction by regulating Bcl-2. Biomed Pharmacother 2016;81:379-87.
15. Feng W, Cai D, Zhang B, Lou G, Zou X: Combination of HDAC inhibitor TSA and silibinin induces cell cycle arrest and apoptosis by targeting survivin and cyclinB1/Cdk1 in pancreatic cancer cells. Biomed Pharmacother 2015;74: 257-64.
16. Baek SH, Kim C, Lee JH, Nam D, Lee J, Lee SG, et al. Cinobufagin exerts anti-proliferative and pro-apoptotic effects through the modulation ROS-mediated MAPKs signaling pathway. Immunopharmacol Immunotoxicol. 2015 Jun;37(3):265-73.

Figure legends

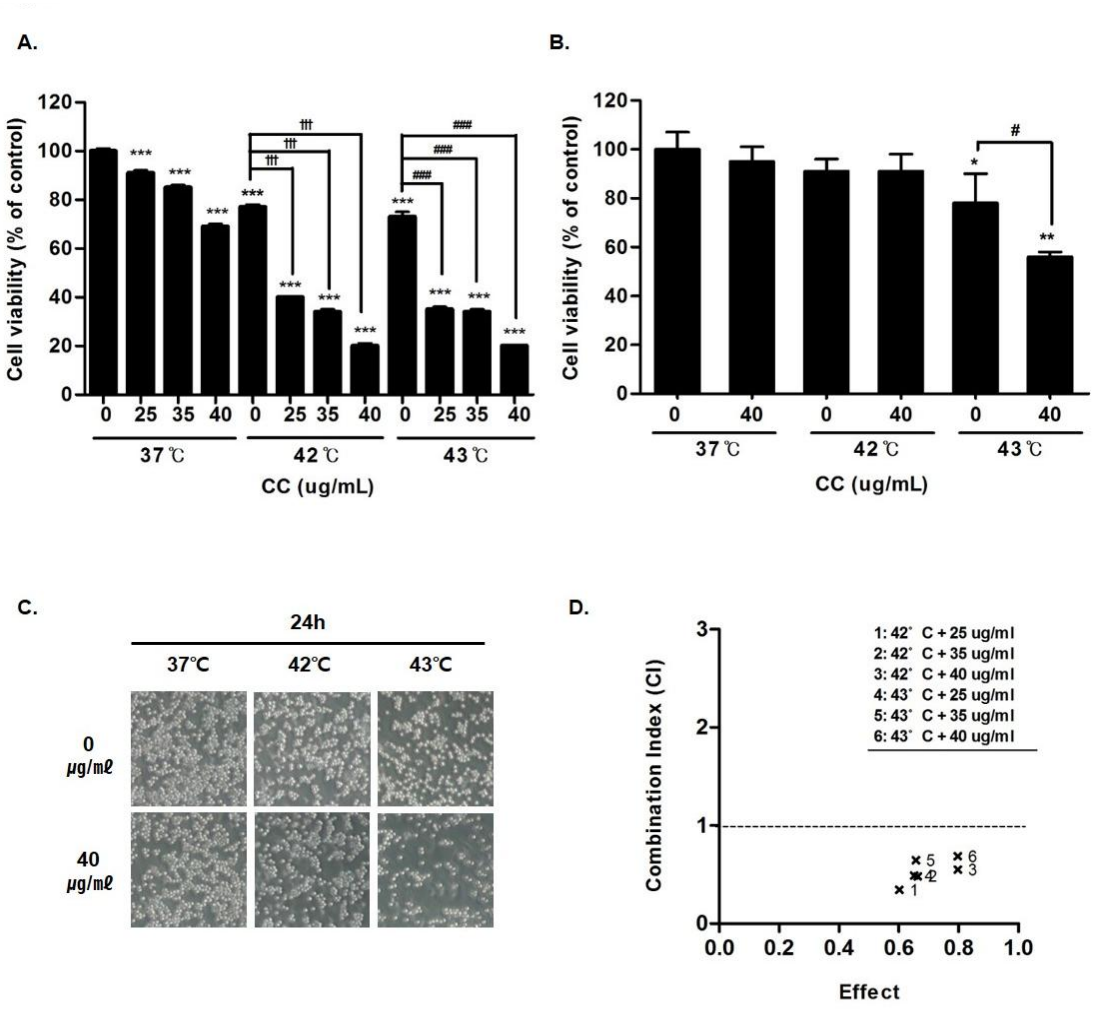

Fig. 1 Apoptosis induce by CC with hyperthermia in U937 cells. (A and B) Percentage of cell viability was determined using MTT assay (A) and Trypan blue assay (B). Cell viability was decreased in the 43℃ treatment group compared with the 37℃ and 42℃ treatment groups in a dose-dependent manner for 48h (A) and 24h (B). Data represents mean ± SD values of three measurements. (C) Morphological changes reflecting apoptosis, were visualized under microscope. (D) Combination index (CI) was calculated by Compusyn software (ver. 1.0) (CI<1.0 synergic effect, 1.0 implies additivity, CI>1.0 implies synergistic). *p<0.05, **p<0.01, *** p<0.001 vs. control group, †††p<0.001 vs. 42℃ + 0 μg/ml group, #p<0.05 and ###p<0.001 vs. 43℃ + 0 μg/ml group.

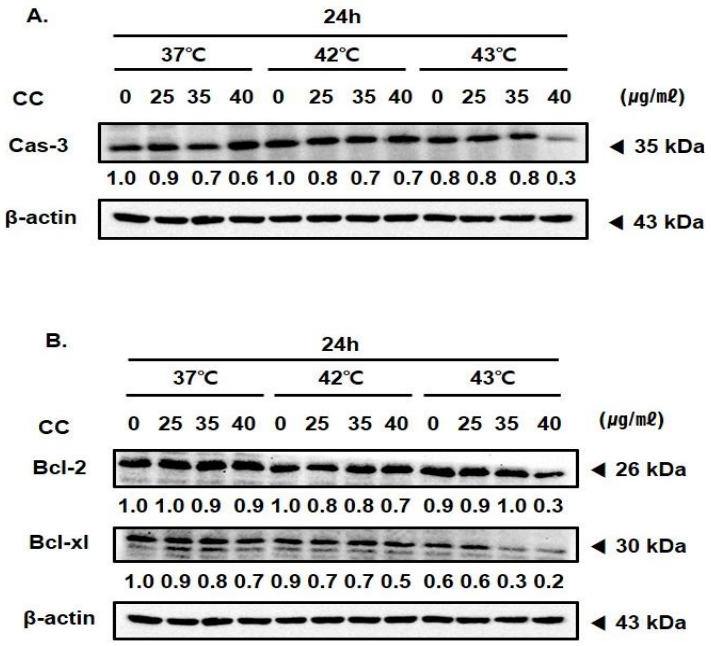

Fig. 2 Effect of CC with hyperthermia on the levels of caspase-3, Bcl-2 and Bcl-xL protein expression in the control group and treatment groups. U937 cells (1×10^6cells) were treated with CC with or without hyperthermia and incubated for 24h. Whole-cell extracts were prepared, then equal amounts of lysates were analyzed by Western blot analysis. β-actin was used as a loading control.

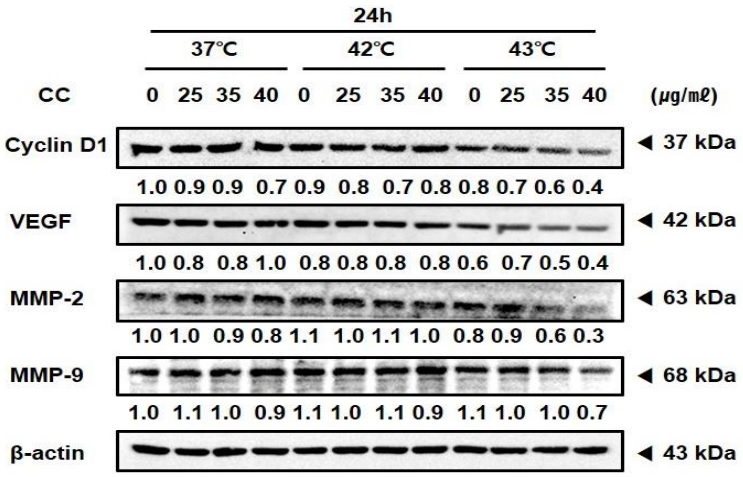

Fig. 3 Expression of cyclin D1, VEGF, MMP-2, and MMP-9 in U937 cells by Western blots. U937 cells (1×10^6cells) were treated by CC with or without hyperthermia and incubated for 24h. Whole-cell extracts were prepared, then equal amounts of lysates were analyzed by Western blot analysis. β-actin was used as a loading control.