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Synergistic cytotoxicity and mechanism of caffeine and lysozyme on hepatoma cell line HepG2

Hongchao Yang [a], Jingjuan Li [b], Lin Cui [a], Yanqing Ren [b], Liying Niu [b], Xinguo Wang [b], Yun Huang [a,c,*], Lijian Cui [b,**]

[a] Pharmaceutical College, Hebei Medical University, Shijiazhuang 050017, China
[b] Experiment Center, Hebei University of Chinese Medicine, Shijiazhuang 050020, China
[c] Institute of Chinese Integrative Medicine, Hebei Medical University, Shijiazhuang 050017, China

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ABSTRACT

The influences of caffeine, lysozyme and the joint application of them on the hepatoma cell line HepG2 proliferation inhibition and cell apoptosis were observed by 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide assay and Hoechst 33342, which showed the proliferation inhibition rate of the joint application on HepG2 cells was 47.21%, significantly higher than caffeine or lysozyme, and the joint application promoted the apoptosis of HepG2 cells obviously. Van't Hoff classical thermodynamics formula, the Föster theory of non-radiation energy transfer and fluorescence phase diagram were used to manifest that the process of lysozyme binding to caffeine followed a two-state model, which was spontaneous at low temperature driven by enthalpy change, and the predominant intermolecular force was hydrogen bonding or Van der Waals force to stabilize caffeine-lysozyme complex with the distance 5.86 nm. The attenuated total reflection-Fourier transform infrared spectra indicated that caffeine decreased the relative contents of α-helix and β-turn, which inferred the structure of lysozyme tended to be "loose". Synchronous fluorescence spectra and ultraviolet spectra supported the above conclusion. The amino acid residues in the cleft of lysozyme were exposed and electropositivity was increased attributing to the loose structure, which were conducive to increasing caffeine concentration on the HepG2 cell surface by electrostatic interaction to show synergistic effect.

1. Introduction

Caffeine is a kind of alkaloids compounds, widely existing in tea, coffee, Cocoa and so on. For a long time, caffeine is deemed to have an effect on exciting nerve, dilating blood vessels and relieving migraines. In recent years, the anti-cancer effect of caffeine is getting more and more attention [1]. At present, the research on caffeine for the prevention and treatment of cancer is still in the stages of experiment and epidemiology. Although scholars also have performed related research, the mechanism is still not clarified which limits the clinical use of caffeine further. Lysozyme, as a kind of alkaline proteins which is non-specific immune factors, exists in organism widely. The isoelectric point (pI) of lysozyme is about 11 with positive charge in the body. Lysozyme can produce allosteric regulation to combine with many exogenous compounds, which lead to changes in its spatial structure with "groove" in the lysozyme surface changed, showing different activities [2]. Epidemiological investigations show that liver cancer is one of the most common malignant tumors in the world. In China, annual incidence rate of liver cancer is 26.68/100000, the mortality of which, ranking only second to lung cancer and having a tendency to increase, accounts for up to 14.56% in the overall cancers incidences [3]. Some liver cancer patients were diagnosed with terminal cancer, missing the best operation time of treatment, and chemotherapy was the main therapies [4]. Singapore scholars reported that caffeine had a strong resistance to liver cancer [5]. Hepatoma cell line HepG2 is a perpetual cell line which is a suitable *in vitro* model system for the study of human hepatocytes. The great quantities of microvilli on the liver cancer cell membrane surface [6], which is rough and electronegativity [7], may be beneficial for the electropositive xenobiotic chemicals to aggregate on cell surface. In this experiment, we focused on the influence of apoptosis and inhibition of caffeine-lysozyme system on HepG2 cells by the dye 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT assay) and Hoechst 33342 which is a kind of fluorescent dye used for evaluating apoptosis in cell culture lines). Molecular spectroscopic techniques including ultraviolet spectra, infrared spectra and fluorescence spectra, reveal the state of being of caffeine-lysozyme system, possible mechanisms of lysozyme enhancing the inhibition and apoptosis of caffeine on

* Correspondence to: Y. Huang, Pharmaceutical College, Hebei Medical University, Shijiazhuang 050017, China.
** Corresponding author.
E-mail addresses: hy9317536@126.com (Y. Huang), cuilijianzy@126.com (L. Cui).

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hepatoma cell line HepG2, which are of great significance for comprehending the influence of coffee, tea or other caffeinated foods on the health.

2. Materials and Methods

2.1. Instruments, Reagents and Materials

CO_2 incubator was from America Shellab, IX-71 inverted microscope from Japan Olympus, Spectra Max 190 microplate reader from America Molecular Device, LS-50 fluorescence spectrophotometer from American Perkin Elmer, FTIR-8400s spectrophotometer from Japan Shimazda, horizontal attenuated total reflection accessory from American Perkin Elmer, TU-1901 uv-vis spectrophotometer from China Beijing general, CU600 thermostatic water bath from China Shanghai Yiheng, BS223S electronic balance from Germany Sartorius and UPH-I-20L ultrapure water manufacturing system from China Chengdu ultrapure technology. HepG2 cells were from Shanghai institutes for biological sciences, Dulbecco's modified eagle medium (DMEM medium) from USA Hyclone, fetal bovine serum from China Hangzhou SiJiQing company, dimethyl sulfoxide (DMSO),3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), tris hydroxymethyl aminomethane (Tris), lysozyme from USA sigma, and caffeine from National Institutes for Food and Drug Control, China. All other chemicals were of analytical reagent grade.

2.2. Methods

2.2.1. Solution Preparation

A amount of caffeine added to ultrapure water was configured to 2.5×10^{-3} mol/L as caffeine stock solution. 7×10^{-5} mol/L lysozyme stock solution was prepared with buffer solution which was Tris-HCl at pH 7.4. The stock solutions stored in the dark at 0–4 °C were diluted to some lower concentrations for actual use.

The final concentration of lysozyme was 7.0×10^{-7} mol/L and caffeine was 2.5×10^{-5} mol/L in the experiment of cell proliferation inhibition.

A solution containing 4 mL Lysozyme (1.0×10^{-5} mol/L) was titrated with 0, 10, 20, 30, 40, 50, 60, 70, 80 or 90 μL caffeine (2.5×10^{-3} mol/L) respectively and a certain amount of Tris–HCl buffer solution to the final volume of 4.1 mL. The final concentration of caffeine varied from 0 to 5.5×10^{-5} mol/L at an increment of 0.6×10^{-5} mol/L for fluorescence spectroscopic investigation. In the ATR-FTIR spectra investigation, both Lysomyze and caffeine were 1×10^{-5} mol/L.

2.2.2. Effects of Caffeine Combined With Lysozyme on HepG2 Cell Proliferation and Apoptosis

Cell passage cultivation: the thawed HepG2 cells were cultured in DMEM-high sugar medium with 10% fetal bovine serum in the saturated humidity incubator at 37 °C with 5% CO_2, and the medium was changed every 48 h.

The inhibition of cell proliferation was detected by MTT: HepG2 Cells in logarithmic growth phase were seeded into 96-well plates at a density of 1.0×10^4 cells per chamber. After 24 h of cultivation, the sterile samples injected in the chamber were allocated to blank group (culture medium), Lysozyme group (7.0×10^{-7} mol/L), caffeine group (2.5×10^{-5} mol/L), and mixed groups (including lysozyme 7.0×10^{-7} mol/L and caffeine 2.5×10^{-5} mol/L) respectively. After 48 h of cultivation, each chamber was added 100 μL MTT (1.0 mg/mL), then cultivation was terminated after 4 h. Each chamber was injected DMSO 100 μL, oscillating 10 min away from light. The absorbance value was determined at 490 nm with culture medium containing 0.4% DMSO as blank control. Cell proliferation inhibition rate [8] (%) = (absorbance value of the blank control – absorbance value of the experimental group)/absorbance value of the blank control × 100%.

The cell nucleus morphological observation with fluorescent dye (Hoechst 33,342): HepG2 Cells in logarithmic growth phase were seeded into 96-well plates at a density of 1.0×10^4 cells per chamber with cell sticking cultivation, and then 24 h starvation culture. The culture medium was replaced with DMEM-high sugar medium containing samples tested, the concentrations of which were similar to the experiments above (MTT assay). Discard the culture medium and rinse 3 times with phosphate buffer saline (PBS) after 24 h cultivation, fix with 4% paraformaldehyde for 1 h, and stain with 10 μg/mL Hoechst 33,342 dye for 15 min at 30 °C. Cell morphology was observed and photographed under an inverted microscope after rinsed with PBS.

2.2.3. Spectral Studies on Mechanism of Interaction between Caffeine and Lysozyme

The sample used for the determination of ultraviolet or fluorescence spectra was subsequently vortex-mixed for 1 min and incubated for 5 min at 298 or 310 K. Fluorescence spectra were performed at 298 and 310 K under the following conditions: the excitation wavelength was set at 260 nm with emission and excitation slits 5 nm. The three-dimensional fluorescence spectra were measured (excitation wavelength from 260 to 320 nm) with the emission wavelengths from 280 to 420 nm, scanning number 15 and increment 5 nm with other parameters just the same as those of the fluorescence quenching spectra. The synchronous fluorescence spectra were recorded when $\Delta\lambda = 60$ nm or 90 nm. Fluorescent phase diagram was analyzed based on the corrected intensities of lysozyme endogenous fluorescence at 320 nm and 365 nm. Fourier transform infrared (FTIR) spectra were taken *via* the attenuated total reflection (ATR) method with the resolution of 4 cm^{-1} and 60 scans. The sample compartment was purged with dry air to eliminate the absorption of water vapor. The spectra of the buffer solution and lysozyme solution were firstly collected and then the spectrum of buffer was subtracted from that of lysozyme to obtain the pure lysozyme subtractive spectrum. The spectra of caffeine solution were subtracted from that of caffeine-lysozyme to obtain the lysozyme (after the caffeine was added) subtractive spectra. The ultraviolet absorption spectra of caffeine, lysozyme and caffeine-lysozyme were recorded in the range from 200 to 300 nm. The compositions and concentrations of blank control group are the same to above tested system, except for lysozyme.

3. Results and Discussion

3.1. Mechanism of Cytotoxic Effect of Caffeine and Lysozyme Synergism

The results of cell growth inhibition rate showed that lysozyme had a weaker inhibitory effect on HepG2 cell proliferation, the inhibition rate of which was 12.58% in the experimental concentration, caffeine had a inhibition rate with 24.36%, and the combined use of lysozyme and caffeine was 47.21% which was significantly higher than that of lysozyme or caffeine used alone ($P < 0.01$) and also higher than the sum of inhibition rates in the same concentrations of caffeine and lysozyme, indicating that lysozyme can significantly enhance the inhibitory effect of caffeine on HepG2 cells through a special way.

The chromatin of the blank control group was evenly distributed in the nucleus from the fluorescence microscopy analysis of apoptosis by Hoechst 33258 nucleic acid stain. HepG2 cells with lysozyme were basically normal, and only few cells with nuclear pyknosis. Some HepG2 cells with caffeine manifested nuclear pyknosis giving bright blue fluorescence. But a large number of HepG2 cells gave bright blue fluorescence in the lysozyme-caffeine mixed group, in which many cells showed significant nuclear pyknosis and typical apoptotic bodies (Fig. 1), which indicated that the combined application of caffeine and lysozyme promoted the apoptosis of HepG2 cells significantly.

The change of nucleus is the main morphological sign of cell necrosis. The combination of caffeine and lysozyme expediting the nuclear condensation of HepG2 cells which suggested that the transcription of