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ABSTRACT

Prune consumption has been associated with a decreased risk of colon cancer; yet there has been confusion as to which chemical component(s) of the prune are responsible for its anticarcinogenic properties. Previous studies have evaluated chlorogenic acid as a chemotherapeutic agent; however, only a limited amount of studies have investigated neochlorogenic acid, the predominant phenolic compound found in the prune. The purpose of this study was to determine the effects that chlorogenic acid and neochlorogenic acid have as anticarcinogenics on the human adenocarcinoma, Caco-2 cell line. The anti-colon cancer effects of chlorogenic acid and neochlorogenic acid were analyzed by experiments that measured cell proliferation and morphology in culture of Caco-2 cells. Treatment of cells with chlorogenic acid and neochlorogenic acid significantly reduced cell proliferation at concentrations of 150-500 μmol at 24, 48, and 72 hours by 63.7-96.0% and 69.7-94.2%, respectively ($P < 0.05$). At the majority of sample times and concentrations, chlorogenic acid and neochlorogenic acid did not significantly differ in percent reduction of viable cells ($P < 0.05$). The cell morphology of treated cells changed, as the surface of cells became more rough, uneven, and irregularly-shaped as the concentration of the treatment increased, compared to the untreated Caco-2 cell. These findings of significant cell proliferation inhibition suggest that both chlorogenic acid and neochlorogenic acid could be colon cancer suppressive components of the prune.

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MEET THE STUDENT-AUTHOR

Taylor Thurow

I am originally from Ashland, Oregon, but have called the rural town of Morrow, Arkansas, my home since the age of two. I graduated with honors in May 2012 with a Bachelor of Science in Agriculture in Food Science, with a minor in Biology, pre-medical emphasis.

Over the last four years, I have been honored to be involved in the University of Arkansas and Dale Bumpers College of Agricultural Food and Life Sciences. My most meaningful achievements include being a recipient of the Chancellor's Scholarship; officer in Alpha Epsilon Delta, the pre-medical honors society; officer in the University chapter of Agricultural Communicators of Tomorrow; and Dale Bumpers Ambassador for 2011-2012. I am also part of the Bumpers College Honors Program, which allowed me to do research under my faculty mentor, Dr. Sun-Ok Lee, and successfully complete and defend my honors thesis.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related deaths in the United States (CDC, 2011). Based on the rates from 2005-2009, 54.0 men and 40.2 women out of 100,000 in the United States will be diagnosed with colorectal cancer each year (CDC, 2011). For future generations, this means 4.96% men and women born today will be diagnosed with cancer of the colon and rectum during their lifetimes (CDC, 2011). Although incidence and mortality rates have declined since 1998, colorectal cancer is still an imminent threat. Locally, trends for colorectal cancer are still unfavorable. Arkansas is well above the U.S. death rate for cancer of the colon and rectum in both men and women, having an annual death rate of 18.9 deaths per 100,000, compared to the national average of 17.1 per 100,000 (CDC, 2011). Risk factors associated with colorectal cancer include increasing age, race, sex, heredity, preexisting conditions, tobacco usage, and diet (CDC, 2011).

It is estimated that 30-60% of cancers may be diet-related (Glei et al., 2006). Thus, alterations of the diet to include whole-grain cereals, fruits, and vegetables could provide protection from diet-related cancers. Consumption of these protective foods could prevent up to 66-75% of colon cancers (Glei et al., 2006).

The prune is linked to decreased risk of colon cancer and is rich in phenolic antioxidants that may be responsible for this anticarcinogenic behavior. Of all the phenolic compounds present in the prune, neochlorogenic acid is the most predominant, followed by chlorogenic acid (Stacewicz-Sapuntzakis et al., 2001). The purpose of this study is to determine the effects of chlorogenic acid and neochlorogenic acid on human colon cancer cells. The hypothesis of this study is that one or more of the chlorogenic acid and neochlorogenic acid treatment concentrations tested will be able to effectively inhibit colon cancer cell proliferation. The objectives of this study were to: 1) determine if chlorogenic acid and neochlorogenic acid can effectively inhibit colon cancer cell proliferation, 2) determine what concentrations of chlorogenic acid and neochlorogenic acid are effective at inhibiting cell growth, and 3) establish the time requirement to significantly reduce the number of viable cells. To achieve these objectives, cell viability was measured and cell morphology was observed.

MATERIALS AND METHODS

Chlorogenic acid (CA) and neochlorogenic acid (NCA) were purchased from Quality Phytochemicals LLC (New Jersey, U.S.) (Fig. 1). These compounds were dissolved in dimethyl sulfoxide (DMSO, ATCC, Rockville, Md.) and then added to media at treatment concentrations of 0, 150, 300, and 500 µmol (10^{-6} moles treatment per liter of media). The control treatment consisted of only DMSO and media.

Cell Culture. Caco-2 cells, a human epithelial colorectal adenocarcinoma (cancer) cell line, were purchased from the American Type Culture Collection (ATCC, Rockville,

Md.) at passage number 18 and cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 2% antibiotic-antimycotic solution. Cell cultures were maintained under sterile conditions and incubated at 37 °C in a humidified 5% CO_2 atmosphere (VWR® symphony™, VWR® International LLC, Radnor, Pa.). All media components and reagents were obtained from Gibco® through Life Technologies (Carlsbad, Calif.).

Cell Viability. Cells at passage number 22 were used for the proliferation assays. Passage number refers to the number of times the cell line has been re-plated and allowed to grow back to confluency (maximum density). The effects of test compounds, chlorogenic acid and neochlorogenic acid, on the cellular proliferation and viability of Caco-2 cells were measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp. Madison, Wis.). This assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter reagent contains a novel tetrazolium compound and an electron coupling reagent (phenazine ethosulfate; PES). The tetrazolium compound is reduced by living cells into a colored formazan product which is soluble in culture medium. The quantity of this product, measured at absorbance of 490 nm by a plate reader, is directly proportional to the number of living cells in the culture.

To perform this assay, (3×10^3) Caco-2 cells in 100 µl of Dulbeco's modified Eagles medium (DMEM) media containing 10% fetal bovine serum were seeded in the wells of a 96 well plate and incubated at 37 °C and 5% CO_2. Cells were incubated for 24 hours to allow stable attachment before exposure to test compounds. The used media was removed and replaced with test media containing chlorogenic acid and neochlorogenic acid at treatment concentrations of 0, 150, 300, and 500 µmol designated as time point 0.

Viability measurements were made at 0, 24, 48, and 72 hours after the addition of treatment. This was accomplished by adding 20 µl of CellTiter reagent directly to the treatment wells containing cells and wells containing only the test media (sample control). A negative control was used to measure the viability of Caco-2 cells in the absence of test compounds. Experiments were conducted using quadruplicate determinations for each concentration for a test compound per plate. Microplates were incubated for 4 hours after the addition of the reagent, before measuring the absorbance at 490 nm with the plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, Vt.). After all absorbance readings were made, data were corrected for the background absorbance of the control media as well as the sample control media which contained chlorogenic acid or neochlorogenic acid but no cells. Absorbances were then converted into cell numbers using an equation from the Caco-2 cell standard curve completed earlier in which a serial dilution of cells was prepared in triplicate in a 96-well plate which ranged from 350 cells per ml to 70,000 cells per ml. This was accomplished by seeding cells as in the actual experiment, adding CellTiter reagent directly to wells, and then measuring the absorbances of these cells. The results were plotted to compare the change in absorbance as the total cell number increased. From this standard curve, an equation of $y = 29676.18\times$ with an R^2 value of 0.99 was obtained. Calculated cell numbers were then used to plot cell growth curves.

Light Microscopy. Cell morphology was observed using a Nikon Eclipse E400 light microscope with Nikon Camera Head DS-Fi1. Cells were used to visualize and describe morphology changes. After 24 and 48 hours of treatment with chlorogenic acid and neochlorogenic acid, cells were harvested and an aliquot was taken from the cell suspension. A portion of the aliquot was then stained with trypan blue dye to provide contrast and determine viable from non-viable cells. Ten µl of the aliquot was then pipetted onto a slide and covered with a cover-slip. The prepared slides were viewed under low power magnification until an acceptable viable cell was identified. All viable cells located on the slide were examined, after which the most representative cell with the clearest image was chosen. The cell of interest was then viewed under higher power (400×). Using the Nikon camera software, contrast and color were adjusted to provide the best possible image, after which, a still shot was taken and saved for later viewing.

Statistical Analysis. Data were analyzed using Statistical Analysis System (Version 9.2; SAS Institute Inc., Cary, N.C.). Results were analyzed using one-way analysis of variance with an alpha level of 0.05, followed by Fisher's least significant difference (LSD) test. Data were presented as means ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Neochlorogenic Acid and Chlorogenic Acid Inhibit Proliferation of Caco-2 Cells. Treatment of Caco-2 cell line with both chlorogenic acid and neochlorogenic acid resulted in a significant reduction in cell proliferation in comparison to the untreated control (Table 1, Fig. 2a and b). At 24 hours, all treatments and concentrations (150, 300, 500 µmol) significantly decreased the viable cell number compared to the control. At concentrations of 150, 300, and 500 µmol, the number of living cells decreased by 63.7, 90.1, and 85.7%, respectively, for chlorogenic acid and 69.7, 90.2, and 85.7%, respectively, for neochlorogenic acid compared to the control. At 48 hours, chlorogenic and neochlorogenic acid decreased viable cell numbers

by 66.1, 94.7, and 92.3% for chlorogenic acid and 33.1, 91.9, and 90.7% for neochlorogenic acid. After the final time point of 72 hours, viable cell numbers were reduced by 56.0, 92.2, and 96.0% for chlorogenic acid and 63.8, 92.8, and 94.2 for neochlorogenic acid. The 300 and 500 µmol concentrations for both chlorogenic acid and neochlorogenic acid were the most effective for suppressing cell growth and maintaining the inhibitory effect, being significantly different from the 150 µmol concentration (Table 1). The effectiveness of reduced cell proliferation was strongly correlated with the increasing concentration of the treatment.

Light Microscopy of Cells. Figure 3 shows the cell morphology observations of Caco-2 cells untreated (Fig. 3c), treated with chlorogenic acid (Fig. 3b and d) and neochlorogenic acid (Fig. 3c and e) at concentrations of 150, 300, and 500 µmol. Figure 3b and c represent Caco-2 cells at 24 hours and Fig. 3d and e represent Caco-2 cells at 48 hours. It can be seen in all of the isolated cell images that the Caco-2 cell surfaces become more rough, uneven, and irregularly-shaped as the treatment time and concentration increased.

The prune is an important source of polyphenols in the United States. The phenolic compounds in the prune have been suspected for these health-promoting activities, but there is little research to confirm this matter. There have been numerous *in vitro* studies testing the antiproliferative behavior of chlorogenic acid, yet there is little consistency among studies (Glei et al., 2006; Noratto et al., 2009; Yagasaki et al., 2000; Jin et al., 2005; Belkaid et al., 2006; Hwang et al., 2010; Veeriah et al., 2006; Arumugam et al., 2011). Numerous cell lines, concentrations of treatment, length of study, and methods have been explored, and therefore various results have been concluded. Also, there has been little to no research on neochlorogenic acid as a phenolic compound with anticarcinogenic properties

Of all the theories for the anticarcinogenic action by the prune, there are many studies that have investigated phenolic compounds for their inhibitory effect on cancer cells. The present study investigated this hypothesis by evaluating the effects of the main phenolic compounds found in the prune, neochlorogenic acid and chlorogenic acid, on cell proliferation and morphology of Caco-2 human colon cancer cells. It was found that neochlorogenic acid and chlorogenic acid significantly inhibited the growth and viability of Caco-2 cells in a concentration-dependent manner compared to the untreated control. Treatment with chlorogenic acid and neochlorogenic acid visibly altered the cell morphology of viable human colon cancer cells as well.

Results found corresponded most with studies by Veeriah et al. (2006) and Glei et al. (2006). Both studies utilized the HT29, human colon adenocarcinoma cell line with a doubling time of approximately 25 hours (Lelievre et al., 2000). In both studies, it was found that only the highest concentrations of 500 µmol (Glei et al., 2006) and 289.2 µmol/L (Veeriah et al., 2006) of chlorogenic acid were able to achieve a 50% cell survival number (EC50) after 72 hours of treatment. In those studies, cells were seeded in 96-well microtiter plates, allowed to attach for 24 and 48 hours (Glei et al., 2006; Veeriah et al., 2006), incubated for 24, 48, and 72 hours, 4'6'-diamino-2-pheynlindole dihydrochloride (DAPI) DNA dye was added and the percentage of surviving cells was measured by fluorimetric analysis at 360 and 465 nm with a 96-well microtiter plate reader. The present study differed only in cell line and the assay used to measure the surviving cells. Despite the common type of cell line, chlorogenic acid appeared to be more effective in reducing the cell number in Caco-2 cells compared to HT29 cells. Even at the lowest concentration of 150 µmol, both chlorogenic acid and neochlorogenic acid were able to reduce cell growth by 63.7% and 69.7%, respectively, in as short as 24 hours.

Other *in vitro* studies that measured cell proliferation found dose-dependent reduction in cell growth at high enough concentrations (Noratto et al., 2009; Yagasaki et al., 2000; Jin et al., 2005; Belkaid et al., 2006). However, it is important to recognize that these studies used different methods and cell lines that were not derived from the colon. There was only one study (Noratto et al., 2009) where the effects of neochlorogenic acid on cell proliferation were compared to chlorogenic acid. This *in vitro* study tested the effects of neochlorogenic acid and chlorogenic acid on cell proliferation in an estrogen receptor negative MDA-MB-435 breast cancer cell line (Noratto et al., 2009). Net growth of cells compared to the control was reduced by 50% at concentrations of 17 and 10 mg/L (47.98 and 28.22 µmol/L) for chlorogenic acid and neochlorogenic acid at 72 hours (Noratto et al., 2009). Noratto et al. (2009) suggest that neochlorogenic acid may be a more potent bioactive compound against metastatic cancer than chlorogenic acid. Although this may have been the case for the MDA-MB-435 breast cancer cell line, there were no significant differences in percent reduction of cell number between chlorogenic acid and neochlorogenic acid in most concentrations and time points in the present study with Caco-2 human colon cancer cell line. Based on other *in vitro* studies, the concentration of chlorogenic acid needed to inhibit cell growth varied with the type of cell line used. Jin et al. (2005) tested the effects of chlorogenic acid on a Hep3B human liver carcinoma cell line and saw only a 12% reduction in cells at concentrations of 200 µg/ml (564.5 µmol/L) and above at 24 hours. Compared to the Caco-2 colon adenocarcinoma cell line, liver carcinoma was much less sensitive to chlorogenic acid and required a larger concentration for a lesser cell reduction effect. An *in vitro* study

with chlorogenic acid on cell proliferation of U-87 human brain glioblastoma required only 100 μmol/L for approximately a 70% reduction of cells in 18 hours (Belkaid et al., 2006). The brain glioblastoma cell line was similar to colon adenocarcinoma, with a larger reduction of cell growth with a smaller concentration of chlorogenic acid. A similar study required only 20 μmol/L of chlorogenic to suppress HT-1080 human connective tissue fibrosarcoma by 50% in 24 hours (Hwang et al., 2010). Human connective tissue fibrosarcoma proved to be much more sensitive than all other cell lines, with the smallest concentration to provide a significant reduction in cell growth.

In the present study, there was significant inhibition of Caco-2 cell viability compared to the control cells for all concentrations of chlorogenic acid and neochlorogenic acid and time points. There was no significant difference in cell reduction among the chlorogenic acid and neochlorogenic acid treatments at all concentrations and time points, with the exception of the 150 μmol treatment at 48 hours. The 150 μmol concentration was the least effective, having a significantly smaller reduction in cell growth compared to the higher concentration treatments and failing to maintain inhibition. Cell growth did rebound at 48 hours for chlorogenic acid and 24 hours for neochlorogenic acid at 150 μmol. It can be hypothesized that this observation may be due to mitohormesis, the phenomenon of increased net cell growth in the presence of low doses of a compound that inhibits cell proliferation. A low dosage of the inhibitor can actually trigger repair mechanisms in the cell, which can neutralize the effect of this compound as well as repair other non-toxin related effects and therefore increase cell growth (Noratto et al., 2009). In this study, mitohormesis would have occurred after 48 hours in the chlorogenic acid treatment and after 24 hours in the neochlorogenic acid treatment. The higher concentration treatments were significantly higher in cell reduction than the 150 μmol concentration, yet were not significantly different from one another (Table 1). Although, the 300 μmol treatment was observed to be more efficient at reducing the Caco-2 cell number through 48 hours compared to the 500 μmol treatment, the difference was not significant. The 500 μmol treatments were the most effective, with a time-dependent reduction in cell proliferation and the highest cell viability reduction of 96% for chlorogenic acid and 94.2% for neochlorogenic acid at 72 hours.

CONCLUSIONS

In conclusion, it has been observed in this study that treatment of Caco-2 human adenocarcinoma cells with phenolic compounds from the prune strongly inhibited cell proliferation and changed the morphology of the cells. Only at the higher concentrations of 300 and 500 μmol was the inhibitory effect of cell proliferation able to be maintained in a time-dependent manner. The findings from this study suggest that chlorogenic acid and neochlorogenic acid may be colon-cancer suppressive components of the prune.

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Table 1. Chlorogenic and neochlorogenic acid percent reduction in cell viability.

Treatment	Conc. (μmol)	24 Hours		48 Hours		72 hours	
		Percent Reduction	SEM	Percent Reduction	SEM	Percent Reduction	SEM
Chlorogenic Acid	150	63.7[a]	3.99	66.1[b]	6.00	56.0[a]	3.73
	300	90.1[b]	0.68	94.7[c]	0.72	92.2[b]	0.20
	500	85.7[b]	0.64	92.3[c]	1.03	96.0[b]	0.17
Neochlorogenic Acid	150	69.7[a]	2.88	33.1[a]	4.14	63.8[a]	5.67
	300	90.2[b]	1.46	91.9[c]	0.19	92.8[b]	0.84
	500	85.7[b]	1.85	90.7[c]	0.61	94.2[b]	0.37

Data represent the percent reduction (%) compared with negative control and the standard error of the mean (SEM) (n = 3). Values in a column without common superscripts are significantly different ($P < 0.05$).

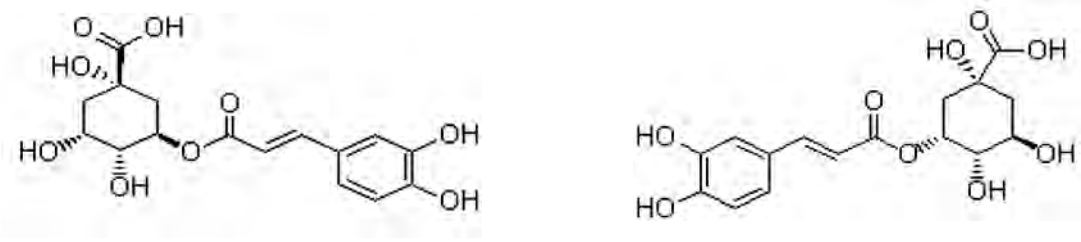

Fig. 1. Chemical structure of chlorogenic acid (3-O-Caffeoylquinic acid, C16H18O9) and neochlorogenic acid (5-Caffeoylquinic acid, $C_{16}H_{18}O_9$) (Kang et al., 2004).

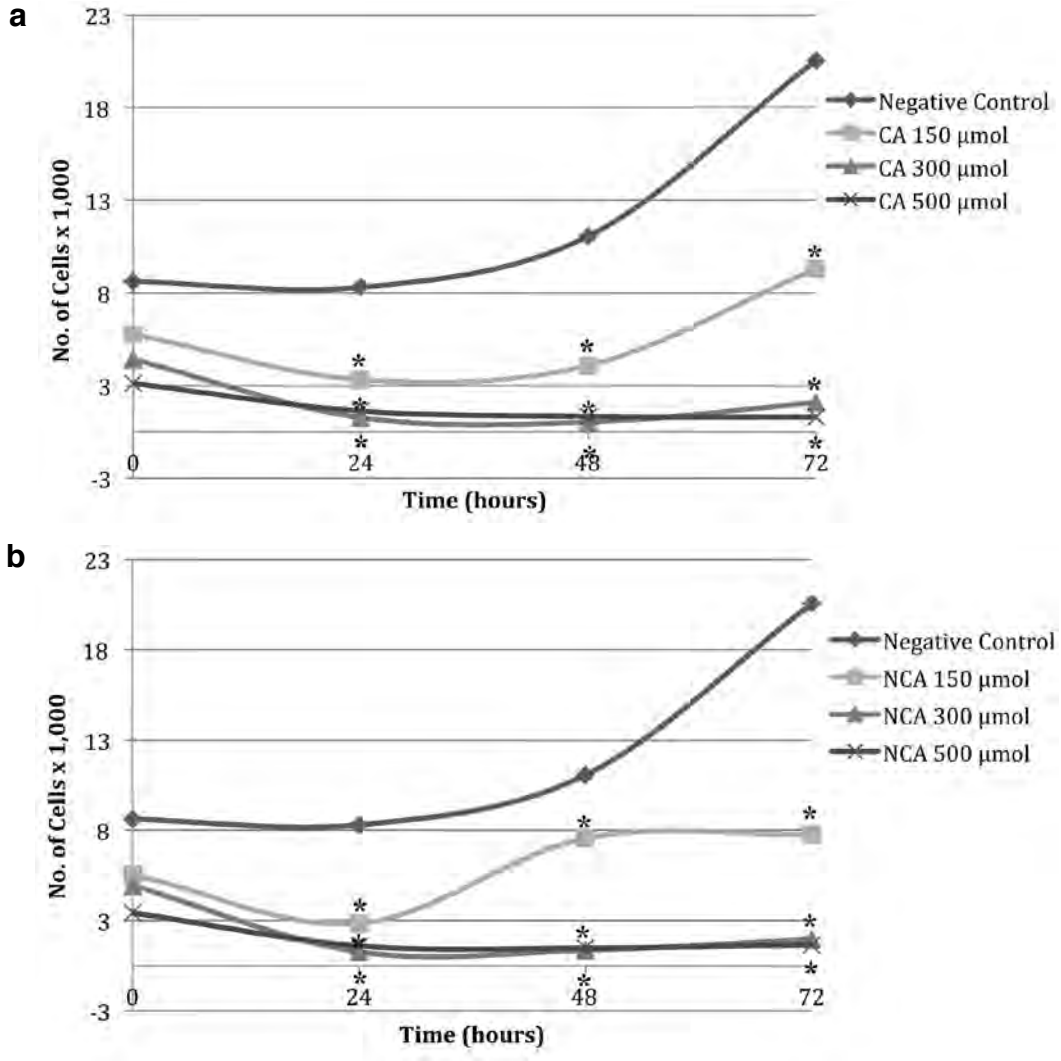

Fig. 2. (a) Different concentrations of Chlorogenic Acid (CA) at each incubation time were compared against the negative control using Fisher's least significant difference test. Points marked with (*) represent significant differences from the control ($P < 0.05$). (b) Different concentrations of Neochlorogenic Acid (NCA) at each incubation time were compared against the negative control using Fisher's least significant difference test. Points marked with (*) represent significant differences from the control ($P < 0.05$).

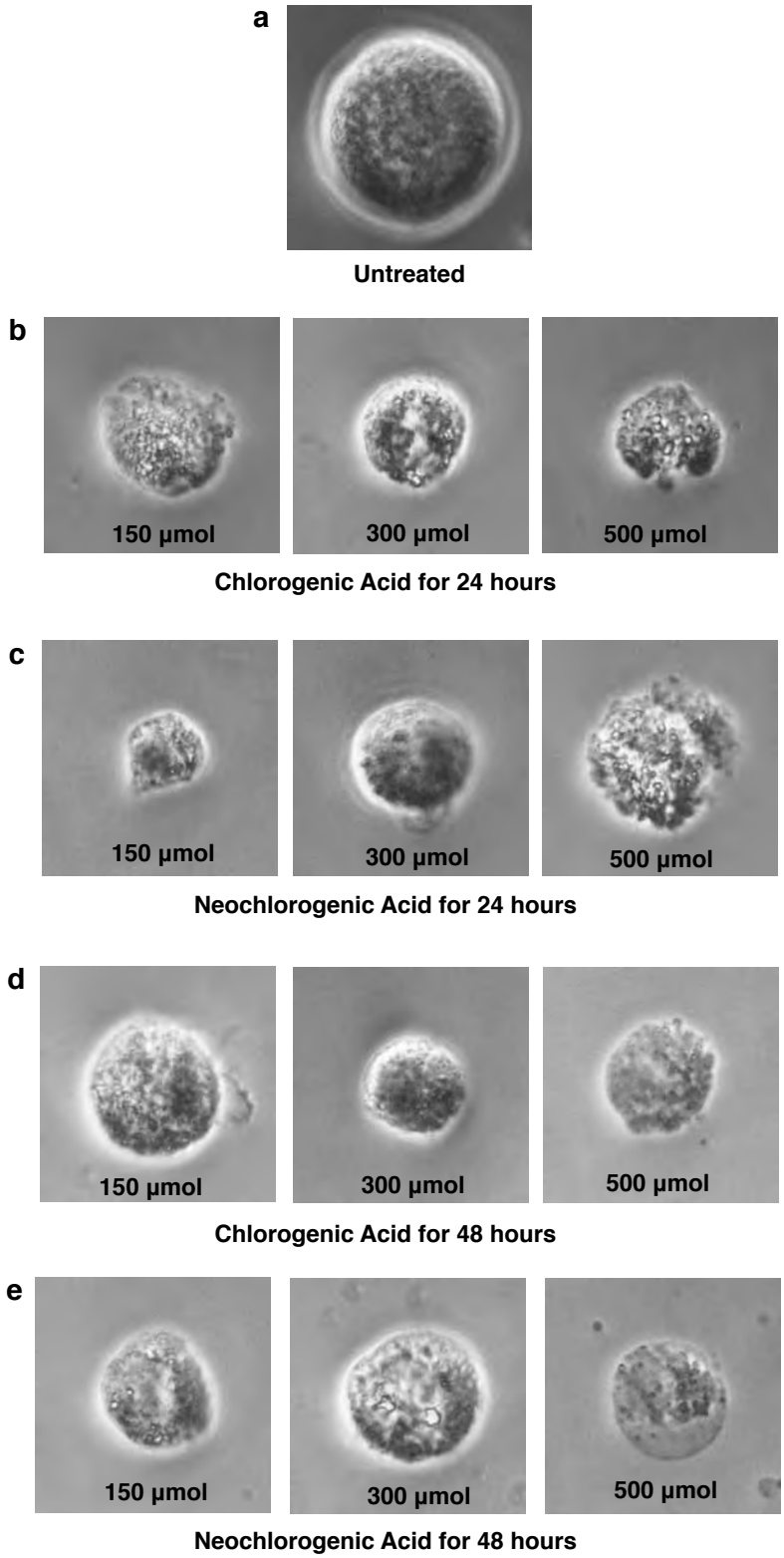

Fig. 3. (a) Untreated Caco-2 cell. (b) Chlorogenic acid treatment for 24 hours at 150, 300, and 500 µmol. (c) Neochlorogenic acid treatment for 24 hours at 150, 300, and 500 µmol. (d) Chlorogenic acid treatment for 48 hours at 150, 300, and 500 µmol. (e) Neochlorogenic acid treatment for 48 hours at 150, 300, and 500 µmol.