THE EFFECT OF GINGER ON APOPTOSIS OF HL-60 CELLS

Undergraduate Honors Thesis
University of Florida
2017

Shawna E. Bilton

Faculty Mentor: Dr. Susan S. Percival
Departmental Honors Coordinator: Dr. Wendy J. Dahl
ABSTRACT

The aim of this study was to extract the phenolic components from dried, ground ginger rhizome to assess its effects on human leukemia HL-60 cells. It was hypothesized that ginger oil extract would decrease cell viability and increase caspase-3 activity in a time and dose dependent manner. Cells were treated with 0.0005 g/mL and 0.001 g/mL ginger oil extract for 8, 12 or 24 hours before being assayed for cell viability and lysed to determine caspase-3 activity. Cells treated with no ginger and 0.1% DMSO served as a control for all experiments. Results revealed statistically significant differences between groups for cell viability with more cell death in samples treated in higher concentrations of ginger over longer incubation times. There was no statistically significant difference in optical density for caspase-3 quantification in samples treated with varying concentrations of ginger for different incubation times. These results support the conclusion that ginger oil extract increased cell toxicity. There is not enough evidence to support the hypothesis that apoptosis was induced by caspase-3, and further studies must be done to investigate the increased cell death.
Introduction

Ginger

The rhizome of Zingiber officinale Roscoe (Zingiberaceae), commonly known as ginger, has been used in traditional medicine for its anti-inflammatory, anti-bacterial, anti-oxidant, and anti-tumor properties (1, 2). Diet-derived compounds show promise for having chemopreventative effects, and this area of research is rapidly growing (2). The rhizome of ginger contains the pungent phenolic compounds gingerols, shogaols, paradols and zingerone (3). These compounds, specifically 6-shogaol, 10-gingerol, 6-gingerol, and 8-shogaol, have been shown to induce apoptosis in laryngeal cancer cells, cervical cancer cells, human colorectal cancer, and human leukemia HL-60 cells (4, 5, 6, 7, 8). Figure 1 displays the structures of major components of ginger (9). The mechanism of how certain components of ginger, such as 6-shogaol, induce apoptosis in cancer cells is not fully understood (6).

Apoptosis

Apoptosis is programmed cell death characterized by cell shrinkage, nuclear condensation, and membrane changes such as membrane blebbing (11, 12). A key distinction between apoptosis and necrosis is the lack of inflammation during apoptosis (12). Necrosis is characterized by rapid, unregulated degradation of DNA, organelle damage, and cell swelling resulting in the loss of membrane integrity and the release of cellular contents causing inflammation (13). Unlike necrosis where the cell is lysed and inflammation occurs, the cell is broken down into small fragments that are phagocytosed during apoptosis (14). Tissue homeostasis and
proper immune function require apoptosis to regulate physiologic homeostasis
(12). Specifically, the strict balance between proliferation and cell death is tightly
regulated by apoptosis and without this regulation, cancer and tumor formation can
result (15). Studies have found that certain cancer types express an anti-apoptotic
gene, survivin, that suppresses programmed cell death (16). Apoptosis is activated
by the presence or absence of cell signals and external stimuli, and it has been
proposed that certain compounds that increase or decrease apoptosis may be
beneficial for cancer and chronic disease prevention (17, 13).

Three successive stages of apoptosis have been characterized and include
extracellular or intracellular signals trigger commitment to cell death, intracellular
cysteine proteases called caspases activated, and cell fragments removed by
phagocytosis (18). Caspases can be split into two groups: initiator caspases
(caspases 2, 8, 9 and 10) and effector caspases (caspases 3, 6 and 7) (19). All
caspases are stored in the cell in the pro-caspase zymogen form and must be
activated by proteolytic cleavage (15). Initiator (upstream) caspases activate
effector (downstream) caspases, and once the effector or “executioner” caspase is
activated, the irreversible cascade of cell death will begin (15, 20).

The trigger to the cell’s commitment to death is complex and can involve the
cessation of anti-apoptotic signals or the initiation of pro-apoptotic signals (15).
There are two distinct pathways of apoptosis that induce cell death in separate
ways. The extrinsic pathway, also known as the death receptor pathway, is
stimulated by death receptors of the tumor necrosis factor (TNF) receptor family
binding to the plasma membrane (21). Examples of death signals involved in the
extrinsic pathway include TNF related apoptosis inducing ligand (TRAIL) receptors that result in the activation of initiator caspase-8 which cleave and activate effector caspase-3 (21).

The intrinsic pathway, or mitochondrial pathway, is initiated by intracellular stresses that cause mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c from the mitochondria to the cytosol where it activates initiator caspase-9 (21). The release of cytochrome c is considered a key element in the intrinsic pathway because once in the cytosol, cytochrome c interacts with adenosine triphosphate and apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome (22). The apoptosome activates caspase-9, which then cleaves pro-caspase-3 into activated caspase-3 and caspases-6 and -7 are activated (22). Caspase activation by MOMP involves proteins from the B-cell lymphoma-2(Bcl-2) family of proteins, which includes sub-families of several anti-apoptotic Bcl proteins as well as pro-apoptotic Bcl proteins (23). The ratio of anti-apoptotic Bcl proteins to pro-apoptotic Bcl proteins determines mitochondrial permeability and the ultimate release of cytochrome c to activate the apoptosome (22).

**Apoptosis and Cancer**

Failure of cells to maintain homeostasis through proper apoptotic activity results in carcinogenesis, tumor growth, and chemotherapy resistance due to uncontrolled cell proliferation (22). The activation of the intrinsic pathway of apoptosis is more common in pathological and physiological scenarios, and is considered the “hallmark of cancer” (24). Caspase-1 has been shown to be downregulated in colon cancer leading to resistance to apoptosis (25). A
A retrospective study showed that colon cancer patients with metastasis to the liver had low levels of pro-apoptotic Bcl proteins (26).

**Ginger and Apoptosis in Cancer Cells**

Phytochemicals refer to compounds produced by plants that have been shown to play a role in human health (27). Several phytochemicals have been shown to affect apoptosis by up-regulating pro-apoptotic Bcl proteins such as Bcl-2 or down-regulating anti-apoptotic Bcl proteins (6). A study has shown that cysteine-conjugated shogaols can activate the intrinsic mitochondrial apoptotic pathway to induce cancer cell death in human colon cancer cells (19). Furthermore, HL-60 cells treated with 6-shogaol and 6-paradol were examined under a phase contrast microscope and showed characteristic features of apoptosis such as cell shrinkage, formation of apoptotic bodies, chromatin condensation, and DNA fragmentation (28).

Following treatment with 6-shogaol, colorectal cancer cells showed increased expression of caspase-1, -3, -8, and -9 as well as depletion in intracellular glutathione (GSH) contents and an accumulation of reactive oxygen species (6). This study observed the initiation of the intrinsic pathway with release of cytochrome c with 6-shogaol incubation and a consequential cascade of caspase-9 and -3 initiation, a decrease in pro-apoptotic Bcl proteins, and an increase in anti-apoptotic Bcl proteins. Additionally, the results from Pan et al. (2008) concluded that the extrinsic pathway was also induced by 6-shogaol incubation by death receptor protein presence confirmed by Western blotting. Studies have shown that normal cells incubated in the same high concentration of 6-gingerol as colon cancer cells
had significantly lower toxicity, suggesting doses of ginger toxic to cancer cells do not harm normal cells (7).

**Research Aims**

Extensive research has been conducted showing that the phenolic components of ginger, such as gingerols and shogaols, have pro-apoptotic effects on cancer cells (4, 5, 6, 7, 8). Thus, research has been solely focused on the effects of isolated ginger compounds and has ignored the role flavonoids and other components of ginger as well as the role of ginger oil extract as a whole (17). The focus of this study was to observe the effects of whole, ground ginger oil extract on HL-60 cells to fill this gap in research. From extensive literature review, it was hypothesized that ginger oil extract would increase apoptosis and decrease cell viability in HL-60 cells in a time and dose dependent manner. The aim of this research was to quantify apoptosis by assaying caspase-3 activity because of its role as an effector, or executioner, caspase protease. The caspase ultimately responsible for the proteolytic cleavage resulting in apoptosis is caspase-3 (13). Furthermore, assessing cell viability will determine ginger oil extract’s effects on cell toxicity.

**Materials and Methods**

**HL-60 Cell Culture**

HL-60 cells (American Type Culture Collection, Manassas, VA) are a human promyelocytic leukemia cell line originally from a 36 year-old female with acute promyelocytic leukemia. The true passage number was unknown but for purposes of this experiment, passage number started at one after thawing the cells from liquid nitrogen. These cells were grown in RPMI-1640 complete medium with 20% FBS
(fetal bovine serum), 2% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2% pyruvate, and 1% PS (Penicillin/Streptomycin). Cells were kept at a concentration of 5x10^5 cells/mL and maintained in a 37° C, 5% CO2 incubator. Cells were split or passed every other day and counted using Trypan Blue Solution, 0.4% (Thermo Fisher Scientific) to ensure viability remained at least 98%.

**Ginger Oil Extract**

Dried, ground ginger from the ginger rhizome was obtained (McCormick Spices) and 30 g was weighed and dissolved in 60 mL of 70% ethanol. Because the shogaol, gingerol and other compounds of interest are phenolic oils, they are more soluble in ethanol and previous studies have demonstrated successful isolation of ginger compounds by methanol (8, 29). The solution stirred for 24 hours on a plate with a stir bar at a medium speed. After the 24 hours, the solution was separated into tubes and centrifuged for 20 min at 13,400 x g. The amber colored supernatant was removed and centrifuged again for 20 minutes at 13,400 x g before being placed in a bulb flask. Rotary evaporation was used for approximately 3 hours at 35° C to remove the ethanol. The remaining ginger oil was dissolved in 3 mL of 100% DMSO (dimethyl sulfide), aliquoted, and frozen in a -80° C freezer.

**Dose Response Experiments**

The effect of ginger oil was hypothesized to be time and dose dependent, so preliminary 24 hour dose response experiments were conducted. Cells were maintained until they were counted at 98% viability consistently. The aliquoted ginger oil extract was diluted by 10-fold serial dilution from an initial ginger concentration of 1 g ginger/mL DMSO to 0.1 µg ginger/mL DMSO. Cells were seeded
at 5x 10^5 cells/mL, rested for 2 hours, and incubated with 2 mL of the varying
diluted ginger concentrations for 24 hours. After interpreting results from this
initial 24 hour dose response test, cell viability in three ginger concentrations were
tested in triplicate for 8 hours. Cells were washed and counted for viability using
Trypan Blue Solution, 0.4%, after incubation.

**Caspase 3 Assay**

Caspase-3 activity was determined using a colorimetric protease assay kit
(ApoTarget, Camarillo, CA). As previously discussed, pro-caspase-3 is cleaved and
activated by initiator caspases. The downstream products of this activation include
poly ADP ribose polymerase (PARP), which contains a DEVD amino acid sequence
that is recognized by caspase-3 cleavage proteins. The kit contains a substrate
DEVD-p-nitroanilide (pNA), which is cleaved to release free p-NA in the presence of
activated caspase-3 enzymes. The mechanism of this kit works by detecting free p-
NA by light absorbance using a microplate reader at 405 nm.

HL-60 cells were counted and cell viability was determined to ensure cells
were at least 98% viable. Cells were then seeded at 6 x 10^6 cells total, rested for 2
hours, and incubated in 37° C 5% CO2 for 8, 12, or 24 hours in 2 mL of 0 g/mL, 0.001
g/mL, or 0.0005 g/mL of ginger. Cells treated with 0 g/mL of ginger and 0.1% DMSO
served as controls. Cells were then counted for viability, pelleted at 6 x 10^6 cells per
sample, and then re-suspended in Cell Lysis Buffer (ApoTarget) before being micro-
centrifuged. The supernatant containing the protein from lysed cells was
transferred to a fresh tube on ice and protein concentration was assayed by BCA
Assay (Thermo Fisher Scientific). Protein concentration was diluted to 4 mg/mL for
all samples to standardize. Reaction buffer, dithiothreitol (DTT), and DEVD-pNA substrate (ApoTarget) were added and the plate incubated in 37°C 5% CO2 and read after 2 hours and 16 hours at 405nm.

**Statistical Analysis**

One-way ANOVA was used to test statistical differences in means and t-test between each group with Bonferroni’s correction (p<0.0167) was used to test statistical significance.

**Results**

**Dose Response Experiments Cell Viability**

To evaluate ginger extract’s role on cell viability, dose response experiments were performed and cells were counted post-treatment before being assayed for caspase-3 activity. Figure 2 shows the results of the preliminary dose response experiments after 24 hours in 2 mL of varying ginger oil extract concentrations. Data represents the mean percent viability from three independent experiments. One-way ANOVA test showed statistical significance in groups overall (7, F=564.35, p<0.001), and t-test showed significant difference between the control, 0.01 g/mL ginger, .001 g/mL ginger and 0.0005 g/mL ginger.

**Pre-Caspase Cell Viability**

Cell viability was assessed by Trypan Blue Solution, 0.4%, manual cell counting after treatment with different ginger concentrations for 8, 12 or 24 hours. Figure 3 displays the cell viability of HL-60 cells and data represents the mean percent viability from two independent experiments. For cells incubated for 8 hours, 12 hours and 24 hours, one-way ANOVA test showed statistical significance
in each times’ groups overall (p<0.05), and t-test showed significant difference between some groups.

**Caspase-3 Activity**

HL-60 cells treated with varying ginger concentrations were assayed for caspase-3 enzyme activity by measuring p-NA levels after enzymes cleaved a specific DEVD site. Absorbance was measured at 405 nm and Optical Density (OD) value is directly related to amount of p-NA and consequently the amount of caspase-3. Figure 4 displays the OD value results of the caspase-3 assay for cells incubated for 8 hours, 12 hours or 24 hours in 0 g/mL of ginger extract, 0.0005 g/mL of ginger extract or 0.001 g/mL of ginger extract. One-way ANOVA test showed overall statistical significance between all groups for 8 hours (2, $F=4.342$, p<0.05), but not for 12 hours or 24 hours. T-test with Bonferroni’s test correction indicated no significant difference between any groups for 8, 12 or 24 hours.

**Discussion**

The focus of this study was to observe the effects of ginger on apoptosis and cell viability of HL-60 cells. It was hypothesized that increasing concentrations of ginger over increasing incubation periods would decrease cell viability and increase caspase-3 activity. Cell viability was determined by cell counting with Trypan Blue Solution, and apoptosis was quantified by caspase-3 assay. The committed step of apoptosis is carried out by caspase-3 and other effector caspases, therefore, the results of this assay indicate apoptosis activity in cells (10). The aim of this study was not to describe the mechanisms by which the intrinsic or extrinsic pathway
occurs within HL-60 cells, therefore, no conclusions can be made regarding the details of the pathways. However, previous evidence regarding the role of caspase-3 in the intrinsic pathway allows interpretation of an increase in caspase-3 activity to mean increased apoptosis via the intrinsic pathway (23). The results of the data in this study showed no statistically significant difference between the control of 0 g/mL ginger and 0.1% DMSO compared to the experimental conditions with increased ginger concentration for 8, 12 and 24 hours. Standard deviations were large for specific incubations conditions such as 8 hours, leading to inconclusive results. Additionally, future studies with cells incubated in a positive control such as etoposide would be useful as a comparison to ensure there was enough overall protein to obtain an accurate absorbance reading and that the caspase-3 assay kit was properly measuring apoptosis.

Elkady and Abu-Zinadah (2014) showed that a decrease in cell viability in colon cancer cells incubated in ginger was directly related to an increase in caspase activity and cell death via apoptosis. Therefore, it is reasonable to hypothesize that an increase in cell death in samples with increasing concentrations of ginger over longer periods of time may be a result of an increase in apoptosis. HL-60 cells incubated in higher concentrations of ginger oil extract had statistically significantly different cell viability than cells incubated in lower concentrations, and this cell viability indicated more cell death. However, the caspase-3 assay data resulting in no statistically significant difference between groups does not support a conclusion that this cell death was due to apoptosis. More experiments measuring caspase activity would be needed to support this hypothesis.
**Limitations and Future Directions**

There were several limitations to this study involving the number of samples tested and the conditions of the caspase-3 assay. Because of limited volumes of caspase-3 kit reagents, only 2 samples were assayed in duplicate for caspase-3 activity by absorbance. This limited sample size weakens the statistical analysis results, and in future studies, more samples would be tested. Furthermore, the quantification of cell viability could have been strengthened by another method such as an MTT assay. Manual counting by Trypan Blue Solution has been shown to be comparable to semi-automate and automated counting methods, however, human error and variations could potentially be controlled for with an additional method (30).

Future directions include more studies to assess apoptosis by measuring different caspases such as caspase-9, caspase-7, and caspase-8 as well as membrane permeablility, cytochrome c release, and Bcl protein upregulation or downregulation. Analysis of these proteins and cell events will present a more complete picture of apoptotic activity occurring in the cell. Measuring inflammation would provide more information regarding the cause of cell death and would result in data possibly supporting necrosis or apoptosis. A limitation of this study was that no observations of inflammation, GSH levels or other cell markers of necrosis were made, so the data cannot support necrosis as the cause of increased cell toxicity.
Figure 1. The structures of the major phenolic components of ginger (9)
Figure 2. Cell viability of HL-60 cells from the preliminary dose response experiments after 24 hours incubating in varying concentrations of ginger oil extract. Data represents the mean percent viability from three independent experiments and includes ±SD. Statistical significance between groups was shown by a one-way ANOVA (7, $F=564.35$, $p<0.001$). Letters indicate statistical significance between groups after a t-test with Bonferonni’s correction.
Figure 3. HL-60 cell viability after treatment with ginger oil extract in a dose and time dependent manner. Control groups were treated with 0.1% DMSO. Cells were counted and assayed for viability with Trypan Blue Solution, 0.4%. Data represents the mean percent viability from two independent experiments for each condition and includes ±SD. One-way ANOVA revealed statistical significance overall and t-test with Bonferonni’s correction indicated some statistical significance between groups, indicated by letters (p<0.05).
Figure 4. Optical density of samples of lysed HL-60 cells after incubation with ginger oil extract. A caspase-3 assay was completed using DEVD-pNA as the chromophoric substrate. Data represents the OD value from two independent experiments for each condition and includes ±SD. Increased optical density is directly related to increased caspase-3 activity and cleavage of DEVD-pNA. One-way ANOVA determined statistically significant differences between all groups (p<0.05) and t-test indicated no statistical significance between groups.
References


