Official Journal of TESMA
Regenerative Research
www.regres.tesma.org.my
E-ISSN 2232-0822
Tissue Engineering and Regenerative Medicine Society of Malaysia

Regenerative Research 4(1) 2015 30-36

APOPTOSIS IN LUNG CANCER CELLS INDUCED BY VIRGIN COCONUT OIL

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ARTICLE INFO

Published online: 14th June, 2015
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KEYWORDS

Virgin coconut oil,
IC_{50},
Lung cancer cell,
Anti-cancer properties,
Apoptosis

ABSTRACT

Introduction: National Cancer Statistics reported that lung cancer listed as third highest among most prevalent cancer in Malaysia. Objectives: In this study, the possibility of using virgin coconut oil (VCO) as new potential anti-apoptosis agent to combat this cancer was evaluated. Experimental design: Two lung cancer cell lines were exposed to series of concentration of virgin coconut oil for 72 hrs. Upon treatment, the morphological changes of the cancer cells were observed. The apoptosis assay using Annexin V- FITC kit was also carried out. Results: We found that VCO at IC_{50} value of 12.04% (v/v) and 8.64% (v/v) induced apoptosis in NCI-H1299 and A549 lung cancer cell lines, respectively, with 3.57% and 4.20% of the apoptotic cells following treatment. Morphological changes, such as the appearance of massive cytoplasmic vacuolization and blebbing of the cell membrane, were observed in both cell lines after treatment with VCO. When tested on skin-derived fibroblasts, VCO at the IC_{50} values for both cell types was negative for toxicity. Conclusions: These results suggest that VCO can induce cell death of lung cancer cells and safe to be consumed.

1.0 Introduction

Lung cancer is among the top ten most prevalent cancers in Malaysia, with third most frequent cancer (14.2%) in men and the sixth most frequent cancer (5.1%) in women. Approximately 88% of cases are classified as non-small cell lung cancer (NSCLC) and have a poor prognosis, as most patients already have advanced disseminated disease at initial presentation due to early subclinical mediastinal or distant metastasis (1). Cigarette smoke is one of the cancerous agents that can induce lung cancer development, especially NSCLC, as deposition of the carcinogenic agents in the lung of the smokers provides a favorable environment for cancer cells. Some smokers are unaware about the causes and symptoms of cancer in their body until they are diagnosed with a high stage of the disease.

Apoptosis, or programmed cell death (PCD), can be identified by a series of distinct morphological and biochemical alterations to cells, such as DNA fragmentation (2-4), chromatin condensation (2, 5-8), cell shrinkage (2-4) and plasma membrane blebbing (2, 4, 8, 9). Apoptosis is an important process for balancing normal conditions, as cell death allows new cells to emerge and helps to maintain a favorable environment for cell growth. However, carcinogenic agents can interfere with the apoptosis pathways, thereby allowing cancer cells to develop.

The human lungs constitute the second largest organ in the body, and the development of lung cancer reduces the efficacy and function of the respiratory system in protecting the body. To prevent and treat the progression of lung cancer, some patients undergo chemotherapy, where they are exposed to various drugs that combat the growth of cancer cells. However, cancer cells can develop drug resistance due to genetic changes

that alter their biochemical properties and chemosensitivity (10). In addition, chemotherapy has many unpleasant side effects, such as hair loss, loss of appetite as well as the emerging of second cancers.

Natural-based anti-cancer agents may provide new treatment options for patients with lung cancer. Several plant-based natural products have passed the clinical trial stages, including *Catharanthus roseus* (11, 12), *Campthotheca acuminate* (12, 13), *Berberineeris* sp. (12, 14), T*abeuia avellanedae* (12, 15), *Vicia faba*, *Amoora rohituka* (16) and *Dysoxylum binectarierum* (12). This study focused on the potential use of virgin coconut oil (VCO) to combat lung cancer, as this product is orally consumable and currently is a trend among those seeking a healthy lifestyle.

VCO is commonly used in Asia, especially in Malaysia, Thailand and the Philippines (17). The oil is obtained directly from fresh coconut milk under controlled temperature, which can prevent the loss of biologically active minor components like vitamins and polyphenols (18, 19). This natural product is rich in antioxidants; with high polyphenols content, including high levels of lauric acid (20), ferulic acid, and p-coumaric acid. It also exhibits anti-bacteria (21), anti-viral, anti-HIV, anti-inflammatory (18, 22), and anti-diabetes (17, 23) properties, and it has been used extensively as a topical application to treat skin disorders (20, 24, 25). Calderon *et al.* (26) studied the anti-cancer effects of VCO on a breast cancer cell line, where the VCO is proven to inhibit the growth the of the SKBr-3 cells while improving the ability of Trastuzumab to decrease the cell survival with no dose dependent manner. However, to date there are no studies of its effects on lung cancer cell lines. Thus, the main objective of this study was to examine the effect of VCO on lung cancer cell lines by studying its ability to induce apoptosis.

2.0 Material and Method

2.1 Cell culture

Two human NSCLC cell lines, NCI-H1299 and A549 (American Type Culture Collection, Manassas, VA, USA), were used in this study. The cells were cultured and maintained in RPMI 1640 and Ham's F-12 supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% PenStrep (100 UI/ml penicillin and 100 µg/ml streptomycin).

Skin-derived fibroblasts cells isolated from New Zealand White rabbits were used as control in toxicity assay. The cells were maintained in high-glucose DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% PenStrep (100 UI/ml penicillin and 100 µg/ml streptomycin).

All cell culture reagents were purchased from Gibco-Life Technologies (Grand Island, NY, USA) unless otherwise stated.

2.2 Test materials

The 100% pure VCO was obtained from a local market (Nutrifera Marketing Sdn Bhd, MY).

2.3 Testing the effects of VCO

2.3.1 Cell viability assay

NCI-H1299 and A549 cells were seeded in 6-well plates at a density of $1x10^5$ cells per well in 3 ml complete RPMI and Ham's F-12 medium. After 24 h, growth medium was replaced by fresh normal medium only (control) or medium supplemented with 10, 30, 50, 70 and 90% (v/v) VCO. The cells were then incubated for another 72 h. In this study, the cell viability assay was conducted using the trypan blue dye exclusion method. At 72 h post-treatment, the cells were trypsinised and the pellets were collected and resuspended in 1 ml fresh culture medium. Next, 50 µl cell suspensions were mixed with 50 µl of trypan blue dye, and positive stained cells were counted in the haemocytometric chamber. The cell viability percentage was calculated as follows:

Cell viability % = (Viable cells in treated well/Viable cells in control well) X 100%

The IC_{50} values for both NCI-H1299 and A549 cells were determined at 50% reduction of the cell viability post-treatment with VCO and were used throughout next assays.

2.3.2 Toxicity assay

The IC_{50} values of VCO for NCI-H1299 (12.04% (v/v)) and A549 (8.64% (v/v)) were tested on rabbit skin-derived fibroblasts to determine if VCO has a toxic effect on normal cells. In this assay, skin-derived fibroblast cell were cultured and maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine and 1% PenStrep (100 UI/ml penicillin and 100 µg/ml streptomycin). The cells were seeded in 6-well plates at density of 1×10^5 cells per well in 3 ml complete DMEM medium. After 24 h, VCO at the IC_{50} values was added to the well. The trypan blue exclusion assay was performed and the cell viability for skin-derived fibroblasts was determined and compared with cell viability of NCI-H1299 and A549 cells.

2.3.3 Apoptosis assay

The apoptosis assay was conducted using the Annexin V Apoptosis Kit and the BD FACSCanto II flow cytometer (BD Pharmigen, Franklin Lakes, NJ, USA). NCI-H1299 and A549

cells were seeded in 6-well plates, and after 24 h cells were treated with 12.04 % (v/v) and 8.64 % (v/v) of VCO, respectively. After 72 h, the cells were collected and washed twice with cold phosphate-buffered saline (PBS). The cells were then resuspended in 1X binding buffer, and 100 µl of cell suspension was transferred into flow cytometry collection tube and incubated with 5 µl Annexin V-FITC conjugated and 5 µl propidium iodide (PI) for 15 min at room temperature in the dark. Prior analysing the samples with flow cytometry, another 400 µl of 1X binding buffer was added into the collection tube. The stained cells were analyzed within 1 h. The combination of Annexin V and FITC can determine the presence of apoptotic cells since cells undergo apoptosis allow Annexin V to enter the cytoplasm and inhibit the PI dye from entering the nucleus of the cells. The dyes act as indicators for different phases of cell death: viable cells stain negative for both PI and Annexin V (Q3); early apoptotic cells stain positive for Annexin V and negative for PI (Q4); necrotic cells stain positive for PI only (Q1); and late apoptotic cells stain positive for both Annexin-V and PI (Q2).

2.4 Statistical analysis

All data were expressed as the mean ± standard deviation of triplicate analyses. Statistical differences among the data were determined using Mann-Whitney two-sided non-parametric independent tests run using SPSS 20. Differences of $p < 0.05$ were considered to be significant.

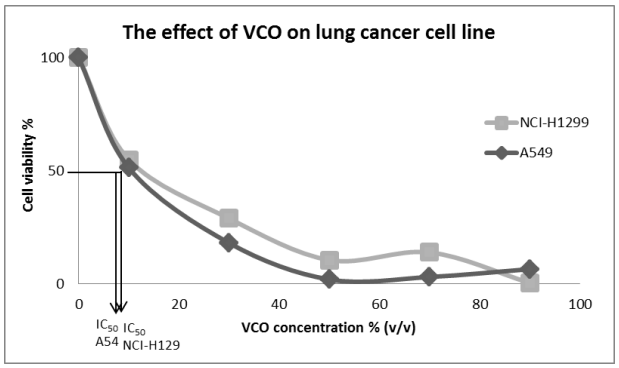

Fig. 1: Cell viability (%) of two lung cancer cell lines, NCI-H1299 and A549, after 72 h of incubation with different concentrations of VCO.

3.0 Result

3.1 VCO treatment affected the viability of NCI-H1299 and A549 cells

After exposure of lung cancer cells to various concentration of VCO, the concentration that result in a 50% reduction in cell growth being identified (Figure 1). Cell viability is reducing even when the cells are exposing to the lowest concentration of VCO (10% (v/v)). The IC_{50} values of VCO are 12.04% (v/v) for NCI-H1299 cells and 8.64% (v/v) for the A549 cells.

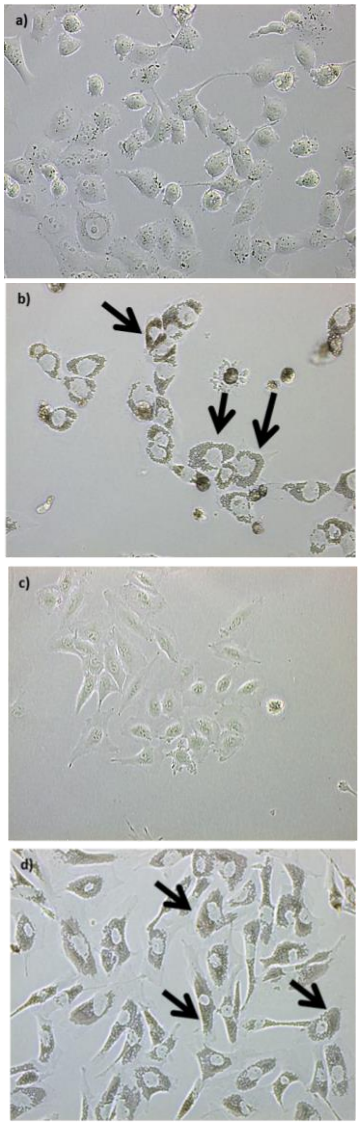

Fig. 2: Morphological changes in NCI-H1299 and the A549 cells after 72 h of exposure to VCO: (a) Untreated NCI-H1299 cells, (b) NCI-H1299 cells treated with 12.04 % (v/v) VCO: (c) Untreated A549 cells; and (d) A549 cells treated with 8.64% (v/v) VCO. Massive vacuoles and apoptotic bodies (black arrowheads) can be observed in cells treated with VCO in both lung cancer cell lines ((b) and (d)).

3.2 VCO treatment caused changes in cancer cells morphology

Exposure of NCI-H1299 and A549 cells to several concentrations of VCO has causing changes in cell morphology, which include the formation of a single, giant bubble and formation of massive vacuoles at the cytoplasmic level (Figure 2). Some apoptotic bodies are also observe in both cell lines.

3.3 VCO is not toxic to skin-derived fibroblast cells

Exposure of skin-derived fibroblasts to the IC_{50} values of VCO revealed that VCO is not toxic to the normal cells (Figure 3). A treatment is considered toxic if exposure to it results in reduction of cell viability to below 50%. In this study, cell viability of skin-derived fibroblasts was 59.11% and 62.35% when expose to IC_{50} values for NCI-H1299 and A549 cells, respectively.

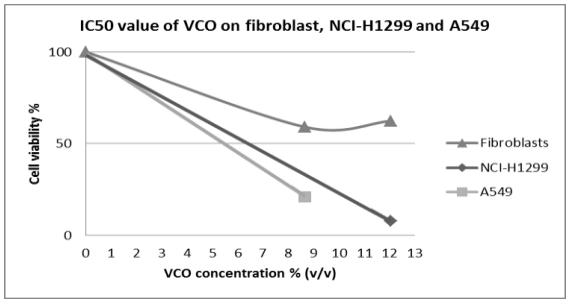

Fig. 3: The toxicity of VCO is tested on skin-derived fibroblast cells. The IC_{50} values are not toxic to these cells.

3.4 VCO induce apoptosis in NCI-H1299 and A549 cells

The apoptosis assay show different stages of cell death; debris (Q1), early apoptosis (Q3), apoptosis (Q4), and late apoptosis (Q2). The scatterplot (Figure 4) represent the percentage of cell undergo apoptosis for both lung cancer cell line tests in this experiment. After exposure to 12.04% (v/v) of VCO, the percentage of apoptotic cells (Q2 and Q4) in the NCI-H1299 (Figure 4) sample are decreasing post-treatment with VCO (*p=0.050*) with reduction about 16.90% (Table 1). However, the percentage of apoptotic cells (Q2 and Q4) in the A549 sample (Figure 4) are increasing significantly after 72 h of exposure to 8.64% (v/v) VCO (*p=0.046*) with 18.03% increment (Table 1).

Table 1. Apoptotic cells percentage during pre- and post- VCO treatment of two lung cancer cells.

Cell types	Apoptotic cells		P value
	Pre-treatment (Mean ± S.D)	Post-treatment (Mean ± S.D)	
NCI-H1299	10.40 ± 0.44	8.57 ± 0.24	0.050
A549	9.43 ± 0.43	11.13 ± 0.51	0.046*

** Significantly different*

The statistical analysis was done using the Mann-Whitney two-sided non-parametric independent test. The number of apoptotic cells (mean ± S.D) post-treatment for NCI-H1299 (8.57 ± 0.24) is reduced (1.83% reduction) meanwhile increased in A549 (11.13 ± 0.51) (1.7% increment). The $p < 0.05$ is considered to be significantly different from the pre-treatment group.

4.0 Discussion

The aim of the present study is to investigate the effect of VCO on two commonest lung cancer cell lines, NCI-H1299 and A549. To the best of our knowledge, no similar study has been conducted. In this study, cell viability differ significantly between cells exposed to the VCO and untreated cells. The IC_{50} values of VCO are 12.04% for NCI-H1299 and 8.64% for A549 cells. However, even at the concentration as low as 10% (v/v), VCO suppress the growth of lung cancer cells. This result suggests that VCO may combat the growth of lung cancer cells in a dose-dependent manner. Moreover consumption of VCO appears to be safe, as the toxicity assay using normal skin-derived fibroblast shows that the IC_{50} values for NCI-H1299 and A549 cells are not toxic to normal cells. The finding suggests that treating cancer cells within a heterogeneous population of cells in the recipient body will not harm to the non-cancerous cells.

The major problem that occurs during lung cancer chemotherapy is the emergence of inherent and acquired drug resistance of the cancer cells (10). Thus, finding an alternative treatment is critical for providing better choices for lung cancer patients. In drug development and assessment studies, cell growth and viability assays are crucial for determining the minimum effective working dosage of the drug that able to inhibit or suppress the target cells with no or minimal effect on normal cells. The ability of VCO to suppress the growth of lung cancer cells is also shows by the morphological changes that occur in the cells, including the formation of apoptotic bodies, cytoplasmic vacuolization, and nuclei condensation. Both lung cancer cell lines shows similar morphological changes following treatment with VCO, which indicates that VCO is effective at suppressing cell growth and inducing cell death regardless of cancer cell types.

Programmed cell death is an important biological process that require for cells to maintain tissue homeostasis. 'Sick' cells will be programmed to undergo cell death, thereby allowing other healthy cells to take over the function of maintaining the favorable growth environment and maintaining cell populations in tissues (2). However, defects can occur at any point along these pathways, leading to malignant transformation of the affected cells, tumor metastasis and anticancer-drug resistance (27). The ability of VCO to induce apoptosis in lung cancer cells was supported by the morphological changes that occurred in cells upon the treatment with VCO. Common morphological changes that occurs during apoptosis include loss of membrane integrity,

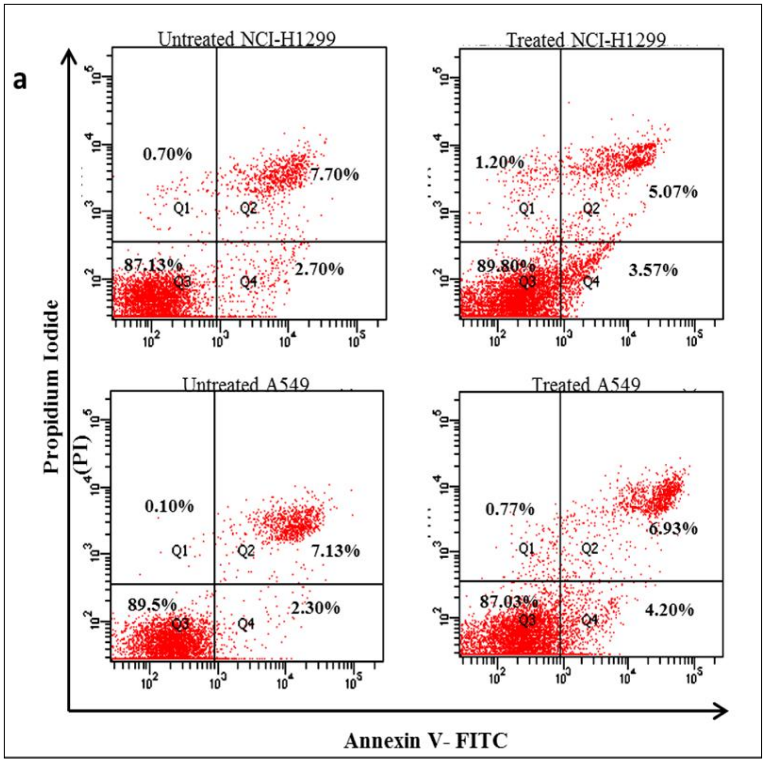

Fig. 4: Apoptotic assay of the cancer cells following VCO-treatment. The assessment of apoptotic cells was carried out using Annexin V-FITC and propidium iodide (PI) dye. The scatterplot represent the percentage of total number of cells stained for different stages of cell death. (a) Q1-Debris, stained positive for PI and negative Annexin V; Q2- Necrosis/Late apoptosis, positive for both PI and Annexin V; Q3-Viable cells, negative PI and Annexin V; Q4- Early apoptosis, positive for Annexin V and negative for PI.

blebbing, nuclear morphological changes (17, 16, 15), formation of massive vacuoles, and appearance of some apoptotic bodies (28). Elmore (2) and Hacker (29) described changes that take place during apoptosis, such as protrusions from the cell surface (visible both by light and electron microscopy), plasma membrane blebbing, vacuoles in the cytoplasm of dying cells and formation of apoptotic bodies. Park et al. (9) reported that apoptosis can be induced in lung cancer cells by exposure to the extract of the root of *Panax notoginseng*, but this is the first study for the effect of VCO on lung cancer cells.

In the present study, results of the apoptosis assay reveal that the number of apoptotic cells significantly increase ($p<0.05$) only in A549 cells after treatment with VCO. The different effect of VCO on apoptosis in NCI-H1299 and A549 cells might be due to the origin and types of lung cancer: The NCI-H1299 cell line originates from lymph node metastasis, whereas the A549 cell line originates from alveolar basal epithelial cells. The changes in the percentage of cells undergoing apoptosis between pre- and post-treatments with VCO in NCI-H1299 and A549 cells suggest that the VCO acts as an apoptosis inducer especially in A549 and acts in a cell-dependent manner.

5.0 Conclusion

This study shows the potential for VCO to be used in treating lung cancer. For the two lung cancer cell lines tested, VCO inhibited the growth of cancer cells and induced cell death via the apoptosis pathway at concentrations as low as 8.64% (v/v) and 12.04% (v/v). However, the mechanism of action by which VCO activates the apoptosis pathway remains unknown, and further studies are needed to address this issue.

Acknowledgement

This study was supported by grants from the Ministry of Science, Technology and Innovation (MOSTI); Science Fund Grant 305/CIPPT/613224 and Universiti Sains Malaysia.

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