Sesamol Induces Mitochondria-mediated Apoptosis Pathway in Human Non-small-cell Lung Cancer (SK-LU-1) Cells

ฤทธิ์ของเซซามอลต่อการชักนำการตายแบบอะพอพโทซิส โดยผ่านทางวิถีไมโทคอนเดรียในเซลล์ไลน์มะเร็งปอด SK-LU-1

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ABSTRACT

Sesame seed (*Sesamum indicum* L.) has long been used in the traditional medicine for cancer treatment. Sesame lignan sesamol has been reported to be one among the high potent antioxidants existed in sesame seeds. Various pharmacological activities of sesamol have been reported including antioxidant and anticancer effect in different cell types. A pharmacodynamics endpoint for cancer treatment is apoptosis. Many anticancer drugs induce cancer cells death via apoptosis induction. So far, apoptosis mechanism effect of sesamol in lung cancer cells has not yet been reported. Due to the reactive oxygen species (ROS) involved with mitochondria-mediated apoptosis pathway, thus in this study the mitochondria-mediated apoptosis mechanism of sesamol was investigated. In the present study, cytotoxic activity was performed by neutral red assay. Nuclear morphological change was determined by DAPI staining assay. Activities of caspase 3/7 and 9 enzymes were investigated by using luminescent assay. The alteration of mitochondrial transmembrane potential was determined by 3,3′dihexyloxacarbocyanine iodide ($DiOC_6$) dye staining assay. The results showed that sesamol exhibited cytotoxic activity in SK-LU-1 cells with IC_{50} value of 2.7 mM with selective index 2.9 compared to Vero cell line at 48 hours. Sesamol caused chromatin condensation and apoptotic bodies which are the hallmarks of apoptosis induction. Activation of caspase 9 and caspase 3/7 were detected at 12 and 24 hours. The mitochondrial transmembrane potential was significantly decreased at 48 hours. These findings indicated that sesamol induced apoptotic cell death in SK-LU-1 cell line through mitochondrial (intrinsic) pathway and caspases enzyme activation. More detailed about anticancer activity of sesamol especially via apoptosis signaling pathway is worth for further investigation.

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บทคัดย่อ

งา (*Sesamum indicum* Linn.) เป็นพืชที่ถูกใช้ในทางการแพทย์แผนโบราณในการรักษามะเร็ง เซซามอลเป็นสารต้านอนุมูลอิสระที่สำคัญที่พบในงา เซซามอลถูกรายงานว่ามีฤทธิ์ทางเภสัชวิทยาในการต้านอนุมูลอิสระ และต้านมะเร็งในเซลล์มะเร็งหลายชนิด การเหนี่ยวนำการตายแบบอะพอพโทซิสถือเป็นเป้าหมายในการรักษามะเร็ง ซึ่งยาต้านมะเร็งหลายชนิดเหนี่ยวนำให้เซลล์มะเร็งตายผ่านกระบวนการอะพอพโทซิส ปัจจุบันยังไม่มีการรายงานกลไกการเกิดอะพอพโทซิสของเซซามอลในเซลล์มะเร็งปอด เนื่องจากอนุมูลอิสระมีความเกี่ยวข้องในการชักนำการตายแบบอะพอพโทซิสผ่านวิถีไมโทคอนเดรีย ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อตรวจวัดผลของเซซามอลต่อการชักนำการตายแบบอะพอพโทซิสโดยผ่านวิถีไมโทคอนเดรีย ในการศึกษานี้ทดสอบความเป็นพิษต่อเซลล์โดยวิธี neutral red การเปลี่ยนแปลงรูปสัณฐานของนิวเคลียสทดสอบโดยการย้อมสี DAPI การทำงานของเอนไซม์แคสเปส 3/7 และ 9 ทดสอบโดยการตรวจวัดการเรืองแสงลูมิเนสเซนซ์ และการเปลี่ยนแปลงความต่างศักย์เมมเบรนของไมโทคอนเดรีย ทดสอบโดยการย้อมสี $DiOC_6$ จากผลการศึกษาพบว่าเซซามอลมีความเป็นพิษต่อเซลล์มะเร็งปอด SK-LU-1 โดยมีค่า IC_{50} 2.7 mM และมีค่าดัชนีความจำเพาะต่อเซลล์มะเร็ง (selective index) 2.9 เมื่อเปรียบเทียบกับเซลล์ไลน์ปกติ (Vero) ที่เวลา 48 ชั่วโมง ซึ่งเมื่อตรวจสอบรูปสัณฐานของนิวเคลียสโดยการย้อมสี DAPI พบว่าเซซามอลเหนี่ยวนำให้โครมาตินหดตัว และทำให้เกิดอะพอพโทติกบอดี ซึ่งถือเป็นลักษณะจำเพาะของการตายแบบอะพอพโทซิส และพบว่ามีการกระตุ้นการทำงานของเอนไซม์แคสเปส 9 และ 3/7 ที่เวลา 12 และ 24 ชั่วโมง นอกจากนี้พบว่าเซซามอลมีผลทำให้ค่าความต่างศักย์เมมเบรนของไมโทคอนเดรียลดลงอย่างมีนัยสำคัญทางสถิติที่เวลา 48 ชั่วโมง ซึ่งผลจากการศึกษานี้บ่งชี้ว่าเซซามอลชักนำให้เซลล์มะเร็งปอด SK-LU-1 ตายแบบอะพอพโทซิสผ่านวิถีไมโทคอนเดรีย โดยมีการกระตุ้นการทำงานของเอนไซม์แคสเปส ซึ่งรายละเอียดเชิงลึกเกี่ยวกับฤทธิ์ต้านมะเร็งของเซซามอลผ่านวิถีอะพอพโทซิสควรมีการศึกษาเพิ่มเติมต่อไป

Key Words: Sesamol, Human non-small-cell lung cancer cell line, Mitochondria-mediated apoptosis

คำสำคัญ: เซซามอล เซลล์ไลน์มะเร็งปอดชนิดไม่ใช่เซลล์เล็ก การชักนำอะพอพโทซิสผ่านวิถีไมโทคอนเดรีย

Introduction

Sesame seed (*Sesamum indicum* L.) has long been used in the traditional medicine of China, India, Germany and Southeast Asian countries for cancer treatment (Anilakumar et al., 2010). Sesame lignan sesamol has been reported to be one among the high potent antioxidants existed in sesame seeds. However, antioxidants are recently reported to promote lung cancer progression by reducing reactive oxygen species (ROS) and DNA damage (Sayin et al., 2014). Lung cancer is the leading cause of death in both genders and was reported to have low success treatment rate (Rami-Porta et al., 2009). Apoptosis induction is one of therapeutic strategies to overcome cancer which leads to cancer cells death with little or no harmful to neighboring normal cells (Robert, David, 2005). So far, the cytotoxic effect and apoptosis mechanism of sesamol in lung cancer cells has not yet been clarified. Due to the ROS is known to involve with mitochondria-mediated apoptosis pathway, thus in this study the mitochondria-mediated apoptosis mechanism of sesamol was investigated in the human non-small-cell lung cancer (SK-LU-1) cell line.

Materials and methods

1 Chemicals

Sesamol was purchased from Spectrum (New Brunswick, NJ, USA). Cisplatin was purchased from Boryung (Ansan, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Grand Island, NY, USA). Neutral red (NR), the fluorescence dye 4',6-diamidino-2-phenylindole (DAPI) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6) were purchased from Sigma-Aldrich (Saint Loius, MO, USA). Caspase-Glo® 3/7 and 9 assay kits were purchased from Promega (Madison, WI, USA).

2 Cell lines

The human non-small-cell lung cancer cell line (SK-LU-1) and normal African green monkey kidney cell line (Vero) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C with 95% air and 5% CO_2.

3 Cytotoxic assay

Cytotoxic activity of sesamol was determined by using NR assay. The cells were seeded in 96-well plates and incubated for 24 h. Then the cells were treated with various concentrations of sesamol for 48 h. At the end of the treatment, the cells were incubated with NR solution for 2 h. Then the cells were washed and the supernatant was decanted. Then 0.33% HCl/isopropanol was added into each well to solubilize the cells. The absorbance was measured using microplate reader at the dual wavelength of 537 nm and 650 nm. The 50% inhibitory concentration (IC_{50}) was calculated compared to the control (untreated cells).

Selective index (SI), which indicates safety of tested compound to normal cells, was calculated from the IC_{50} of sesamol in normal cells versus cancer cells.

4 DAPI staining assay

DAPI is a fluorescent dye which binds specifically to double-stranded DNA (Li et al., 2003). This technique was used to detect the nuclei morphological change when cell undergoes apoptosis. SK-LU-1 cells were treated with sesamol at the concentrations of 2.7 and 5.4 mM for 48 h. After treatment, the cells were washed with PBS and fixed with methanol. Then the cells were stained with DAPI solution and incubated for 30 minutes. After that the excess dye was removed and PBS:Glycerin (1:1) was added to stained cells. The morphological change of nuclei was observed under inverted fluorescent microscope with 40× magnifications.

5 Caspase activity assay

SK-LU-1 cells were seeded on 96-well plates and incubated for 24 h. After treated with sesamol for 12 and 24 h, the enzyme activities of caspase 3/ 7 and 9 were determined following the manufacturer's instruction. Caspase activity was determined based on luminescent technique which can be detected by Varioskan Flash (Thermo scientific, US).

6 Mitochondrial transmembrane potential

The cells were treated with sesamol for 48 h. Then the cells were trypsinized and stained with 3,3′dihexyloxacarbocyanine iodide ($DiOC_6$). The fluorescence intensity was analyzed by flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, CA, USA). Loss of mitochondrial transmembrane potential results in decreased fluorescence intensity.

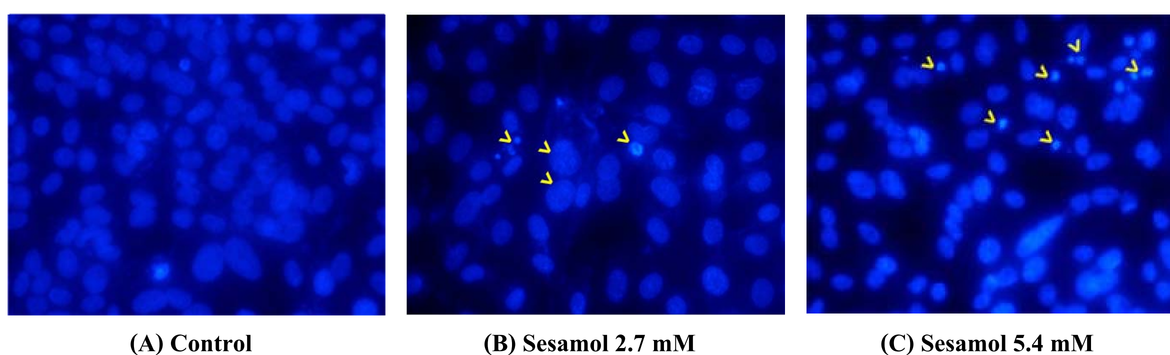
Results

1 Cytotoxic activity of sesamol

Sesamol exhibited cytotoxicity to SK-LU-1 and Vero cells at the concentration possessing 50% cytotoxicity (IC_{50}) of 2.7 ± 0.0014 and 7.7 ± 0.012 mM, respectively, with selective index of 2.9 compared to Vero cell line at 48 h.

2 Apoptosis induction effect of sesamol

Apoptosis is programmed cell death which leads to cancer cell death without inflammatory process and safe to the neighboring normal cells (Robert, David, 2005). Therefore it becomes target of cancer treatment. The results showed that sesamol treated cells induced apoptotic bodies and nuclei shrinkage (Figure 1B, 1C). The control (untreated cells) showed rounded and homogeneous nuclei (Figure 1A).



(A) Control **(B) Sesamol 2.7 mM** **(C) Sesamol 5.4 mM**

Figure 1 Effects of sesamol ($1 \times IC_{50}$ = 2.7 mM, $2 \times IC_{50}$ = 5.4 mM) on nuclei morphological change in SK-LU-1 cells at 48 h. Arrows indicate apoptotic bodies and nuclei shrinkage observed under inverted fluorescence microscope with 40× magnifications.

3 Caspases activity assay

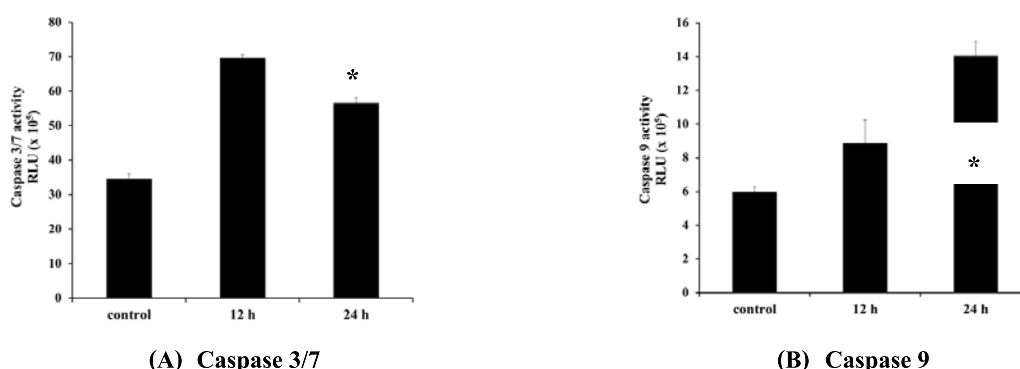
The intrinsic or mitochondria-mediated pathway involves with losing of mitochondria membrane potential caused by stimuli such as free radicals, toxins leading to cytochrome *c* release from mitochondria membrane. Cytochrome *c* binds and activates apoptotic protease activating factor 1 (Apaf 1) as well as procaspase 9 to form apoptosome. Apoptosome activates caspase 9 leading to activation of effector caspases which are caspases 3, 6, and 7 (Elmore, 2007). In this study, SK-LU-1 cells were treated with sesamol for 12 and 24 h. Then activities of caspases 3/7 and 9 were determined following the manufacturer's instruction.

The increasing of caspase 3/7 and 9 activities in SK-LU-1 cells was observed after sesamol treated for 12 and 24 h. Sesamol treated cells cause significantly increase activity of caspase 3/7 at 12 and 24 h when compared with control (untreated cells) (p<0.05), the highest activity was found at 12 h. Activity of caspase 9 was significantly increased at 24 h after sesamol treatment (p<0.05) (Figure 2). It is evident that sesamol trigger apoptosis via mitochondria-mediated * ay in SK-LU-1 cells.

4 Mitochondrial transmembrane potential

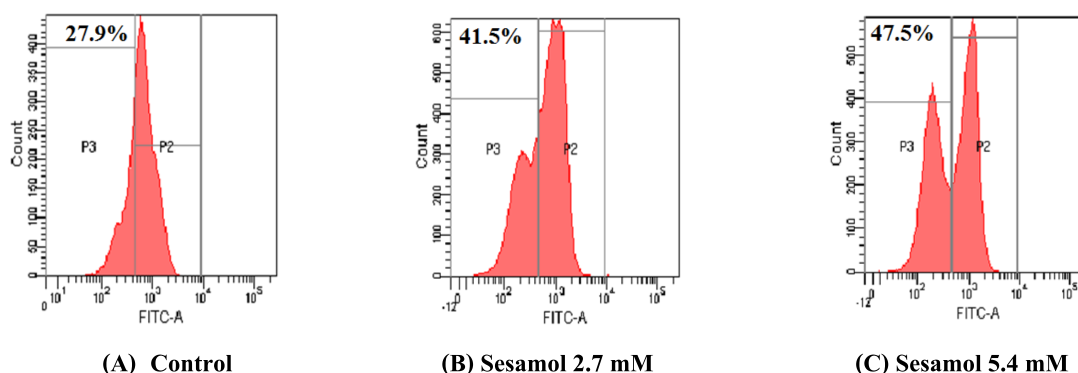
Mitochondria transmembrane potential was determined by 3,3′dihexyloxacarbocyanine iodide ($DiOC_6$), a fluorochrome which incorporated into cells dependent upon their mitochondrial transmembrane potential. The fluorescence intensity was analyzed by flow cytometry. Loss of mitochondrial transmembrane potential leads to a decrease of fluorescence intensity. In this study, SK-LU-1 cells were treated with sesamol at the concentration of $1\times IC_{50}$ (2.7 mM) and $2\times IC_{50}$ (5.4 mM) for 48 h. Then the cells were stained with $DiOC_6$ and analyzed for fluorescence intensity by flow cytometry.

The results showed that sesamol 2.7 and 5.4 mM induced the cells loss of mitochondrial transmembrane potential 41.5% and 47.5%, respectively (Figure 3). This result confirmed the effect of sesamol on apoptosis induction in SK-LU-1 cells via mitochondria-mediated pathway.



(A) Caspase 3/7 **(B) Caspase 9**

Figure 2 Effects of sesamol 2.7 mM on caspases enzyme activity in SK-LU-1 cells at 12 and 24 h. Sesamol significantly increased caspase 3/7, and 9 enzyme activity at 12 and 24 h (*, $p<0.05$) which indicated that sesamol induced apoptosis in SK-LU-1 cells through intrinsic pathway.



(A) Control **(B) Sesamol 2.7 mM** **(C) Sesamol 5.4 mM**

Figure 3 Effects of sesamol on mitochondrial transmembrane potential in SK-LU-1 cells at 48 h. (A) control (untreated cells), (B) the cells treated with 2.7 mM of sesamol, (C) the cells treated with 5.4 mM of sesamol.

Discussion and conclusion

This study investigated the effect of sesamol against human non-small-cell lung cancer (SK-LU-1) cells. Our finding indicated that sesamol exhibited cytotoxicity in SK-LU-1 cells with IC_{50} value of 2.7 ± 0.0014 mM. Sesamol at this millimolar concentration

evidently induced apoptosis cell death by causing nuclear morphological changes including chromatin condensation and apoptotic bodies which are the hallmarks of apoptosis. Moreover, sesamol triggered mitochondria-mediated apoptosis in the SK-LU-1 cell line through the activation of caspase 9, and 3/7. Activation of caspase 9 (initiator caspase) was found in time-dependent manner and significantly increased at 24 h of sesamol treatment. Activation of caspase 3/7 (effector caspase) was significantly increased in sesamol treated group compared with control at 12 and 24 h. At 12 h of treatment the activity of caspase 3/7 showed higher than at 24 h of treatment. Previous studies reported that sesamol induced apoptosis in mouse Leydig tumor cells and hepatocellular carcinoma cells. However, there is no report about apoptosis induction effect of sesamol in human lung cancer cells.

Due to apoptosis is target of cancer treatment by chemotherapy, sesamol may be useful in cancer treatment. Additional anticancer activity of sesamol especially via apoptosis signaling pathway in the lung cancer cells is worth for further investigation.

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References

Anilakumar KR, Pal A, Khanum F, Bawa AS. Nutritional, medicinal and industrial uses of sesame (*Sesamum indicum* L.) seeds – an overview. Agric Conspec Sci 2010; 75(4): 159-68.

Elmore S. Apoptosis: a reviewed of program cell death. Toxicol Pathol 2007; 35(4): 495–516.

Li M, Wu RS, Tsai JSC. DAPI derivative: A fluorescent DNA dye that can be covalently attached to biomolecules. Bioorg Medicinal Chem Lett 2003; 13: 4351–54.

Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P, Bergo MO. Sci Transl Med 2014; 6(221): 221-15.

Rami-Porta R, Crowley JJ, Goldstraw P. The revised TNM staging system for lung cancer. Ann Thorac Cardiovasc Surg 2009; 15(1): 4-9.

Robert G, David LV. Apoptosis in the development and treatment of cancer. Carcinogenesis 2005; 26(2): 263-70.