JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Effects of Sesamol on Apoptosis and Steroidogenesis in MA-10 Mouse Leydig Tumor Cells

Ying-Hui Chen,[†,‡] Sew-Fen Leu,[§] Chun-Yi Jen,[∥] and Bu-Miin Huang*[,∥,¶]

[†]Department of Anesthesia, Chi-Mei Medical Center, Liouying, Tainan, Taiwan, Republic of China

[‡]Department of Cosmetology and Health Care, Min-Hwei College of Health Care Management, Tainan, Taiwan, Republic of China

[§]Department of Food Science and Technology, College of Human Ecology, Hung Kuang University, Taichung, Taiwan, Republic of China

[∥]Department of Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, Tainan, Taiwan, Republic of China

[¶]Medical Device Innovation Center, National Cheng Kung University, Tainan, Taiwan, Republic of China

ABSTRACT: Sesamol, a pure compound of sesame, has been reported to have antitumor effects. In the present study, the apoptotic and steroidogenic effects of sesamol on MA-10 cells, a mouse Leydig tumor cell line, was investigated by morphological observations, cell viability assay, flow cytometry analysis, radioimmunoassay, and immunoblotting assay. We found that the number of rounded-up cells increased as the treatment duration of sesamol increased from 3 to 24 h and that the plasma membrane blebbing phenomenon could be observed after 12 h of treatment. In the cell viability assay, the cell surviving rate significantly decreased as the dosage and duration of sesamol treatment increased ($p < 0.05$). Moreover, cell cycle studies illustrated that the percentages of subG1 phase cells significantly increased after 1 mM sesamol treatments for 12 h, and 0.1 and 1 mM sesamol treatments for 24 h, respectively ($p < 0.05$). Furthermore, 0.1 mM sesamol for 24 h and 1 mM sesamol for 12 and 24 h treatments, respectively, significantly induced the cleavage of caspase-3 ($p < 0.05$). These results confirmed the apoptotic event of sesamol treatment on MA-10 cells. Meanwhile, we also found that sesamol at 1 mM for 24 h and 10 mM for 12 and 24 h significantly increased progesterone production ($p < 0.05$), and 1 mM sesamol for 24 h treatment significantly activated the expression of steroidogenic acute regulatory (StAR) protein ($p < 0.05$). However, the expression of P450scc enzyme remained no different among all treatments ($p > 0.05$). In conclusion, sesamol could concurrently induce apoptosis through the activation of the caspase pathway and steroidogenesis through the induction of StAR protein expression in MA-10 mouse Leydig tumor cells.

KEYWORDS: sesamol, apoptosis, steroidogenesis, MA-10, Leydig

INTRODUCTION

Sesamol is a natural constituent of sesame oil recognized as a well-known antioxidant that inhibits lipid peroxidation and cleans up the free radicals.[1−3] It has also been reported that sesamol has anticancer effects upon different cell types through various mechanisms.[4,5] Moreover, it is reported that sesamol can induce nitric oxide (NO) release,[6] which could regulate Leydig cell steroidogenesis.[7,8] However, little is known about the direct action of sesamol on apoptosis and steroidogenesis in the MA-10 mouse Leydig tumor cell line.

Apoptosis is defined initially as a phenomenon of a morphologically distinctive form of cell death associated with normal physiology, which can be triggered by a wide variety of stimuli, but not all kinds of cells will die in response to the same stimulus.[9] This type of cell death is often difficult to observe in vivo because the dying cells are rapidly phagocytosed by tissue macrophages. The most common characteristic of apoptosis is the fragmentation of DNA into ∼180 bp fragments, a relatively late event during the process.[10,11] It is also well-known that the activation of cysteine-dependent aspartate-directed proteases (caspases) is commonly thought to be one of the earliest points in the no-return pathway of apoptosis. Caspases are broadly categorized into upstream initiator caspases and downstream effector caspases.[12] The initiator caspases, such as caspase-8 (death receptor apoptosis pathway) and caspase-9 (mitochondrial apoptosis pathway), typically have a long N-terminal prodomain that, after activation by alternative stimuli, facilitates recruitment and interaction with other caspases proteins.[13,14] The effector caspases, such as caspase-3, -6, -7 with short prodomains, could be activated by initiator caspases. The activated effector caspases, in turn, cleave a number of cytoplasmic and nuclear substrates, such as poly ADP-ribose polymerase (PARP), lamin B, PKCθ, and inhibitor of caspase activated DNase (ICAD),[15,16] which will result in the execution of cell death.[12]

In the male reproductive system, gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary to release luteinizing hormone (LH), which will be transported to the testis to stimulate Leydig cells. LH is associated with the receptor to activate adenylate cyclase through the G-protein. The activation of adenylate cyclase results in the hydrolysis of ATP to cyclic AMP (cAMP), which subsequently activates protein kinase A (PKA) and results in protein phosphorylation and de novo protein synthesis, such as StAR protein.[17,18] StAR protein can translocate free cholesterol from the outer to the inner mitochondrial membrane,[19] where cholesterol will be converted to pregnenolone by the P450 side chain cleavage enzyme (P450scc).[20] Pregnenolone will then be transported to the smooth endoplasmic reticulum for further synthesis to testosterone, an inevitable steroid hormone for reproduction in males.[21,22]

Received: May 31, 2011
Accepted: August 24, 2011
Revised: August 22, 2011
Published: August 24, 2011

dx.doi.org/10.1021/jf2021539 | J. Agric. Food Chem. 2011, 59, 9885–9891

It has been shown that mitochondria and steroidogenic enzymes are indispensible in steroidogenesis. However, both organelles are prone to generate reactive oxygen species (ROS) when steroidogenesis proceeds. There are several investigations illustrating that overproduced ROS disrupts mitochondria and inhibits steroidogenesis.[23,24] Thus, the present study was designed to investigate whether sesamol could regulate MA-10 cell apoptosis and steroidogenesis.

MATERIALS AND METHODS

Chemicals. Sesamol, penicillin−streptomycin, RNase A, trypan blue, and propidium iodine (PI) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM) and Keratinocyte-SFM medium were purchased from Gibco Co. (Grand Island, NY, USA). Sodium hydroxide was from Merck Co. (Dermstadt, Germany). HEPES was purchased from Mallinckrodt Baker, Inc. (Philipsburg, NJ, USA). Sodium bicarbonate, sodium carbonate, and sodium chloride were purchased from Riedel der Haen (Seelze, Germany). Pure charcoal powder was purchased from Showa Chemical Inc. (Tokyo, Japan). Progesterone [1,2,6,7-^{3}H (N)] (90−115 Ci/mmol or 3.33−4.255 TBq/mmol) used for the radioimmunoassay was purchased from PerkinElmer Inc. (Boston, Massachusetts, USA). The antiserum to progesterone and testosterone was a kind gift from Dr. Paulus S. Wang (National Yang Ming University, Taipei, Taiwan).

Cell Culture. The MA-10 cell line was a gift from Dr. Mario Ascoli (The University of Iowa, Iowa City, IA, USA) and was maintained at 37 °C in a humidified environment containing 95% air and 5% CO_2 for all of the following experiments. Cells (6.0×10^5) were plated in 6 cm culture dishes and grown for 24 h in Waymouth medium containing 10% fetal bovine serum. The medium was removed, and the cells were washed twice with 1× PBS and then treated with various concentrations of sesamol in serum free Waymouth medium (100 μL) for the indicated time periods. The media after treatments were collected for the radioimmunoassay to detect progesterone production. The cells were then isolated for total protein. The expression of target proteins was determined by Western blot analysis.[22]

Morphological Study. The MA-10 cell line was seeded in a 6 cm dish with 2 mL of serum medium, which contained 6.0×10^5 cells. After the cells reached 70−80% confluence, the cells were treated with serum free medium containing 0.1, 1, and 10 mM sesamol for the indicated time points (0, 1, 3, 6, 12, and 24 h). Cell morphology was then observed and recorded under light microscopy (Olympus, CK40, Hamburg, Germany). Apoptosis was characterized by the loss of cellular contact with the matrix and the appearance of plasma membrane blebbing.[25]

Cell Viability Assay. Cell viability was determined by the trypan blue exclusion assay. After MA-10 cells reached 70−80% confluence, cells were treated with serum free medium containing 0.1, 1, and 10 mM sesamol for the indicated time points (0, 1, 3, 6, 12, and 24 h). Cells were then trypsinized after respective treatments and washed with 1× PBS. Equal amounts of cell suspension and trypan blue were mixed and left to stand for 5 min at room temperature. Stained and unstained cells were counted using a standard hemocytometer. The percentage of cell viability was calculated as % cell viability = (number of unstained cells/total cell number) × 100.

Flow Cytometric Analysis. In order to investigate whether sesamol could induce cell apoptosis, flow cytometric analysis was used to determine the DNA fragmentation and the redistribution of cell cycle.[26,27] MA-10 cells were seeded in a 6 cm dish with 2 mL of serum medium, which contained 6.0×10^5 cells. After cells reaching 70−80% confluence, cells were treated with medium containing 0.1 and 1 mM sesamol for the indicated time points (0, 1, 3, 6, 12, and 24 h). Sesamol-treated cells were harvested with trypsin, washed with PBS, and fixed in 75% ethanol for at least 2 h at −20 °C. After fixation, cells were washed in cold PBS and then collected by centrifugation and stained with PI solution containing 40 μg/mL in PBS and 100 μg/mL RNase. The stained cells were analyzed using a fluorescence-activated cell sorter (FACScan, Becton-Dickinson, Moutain View, CA, USA) at λ = 488 nm using CellQuest software (Becton-Dickinson, Moutain View, CA, USA). The DNA content distribution of normal growing cells was characterized by two peaks, G1/G0 and G2/M phases. The G1/G0 phase possesses normal functioning and the resting state of the cell cycle with most diploid DNA content, while the DNA content in the G2/M phase is more than diploid. Cells in the subG1 phase have the least DNA content in cell cycle distribution, called hypoploid. The hypoploid DNA content represents DNA fragmentation.

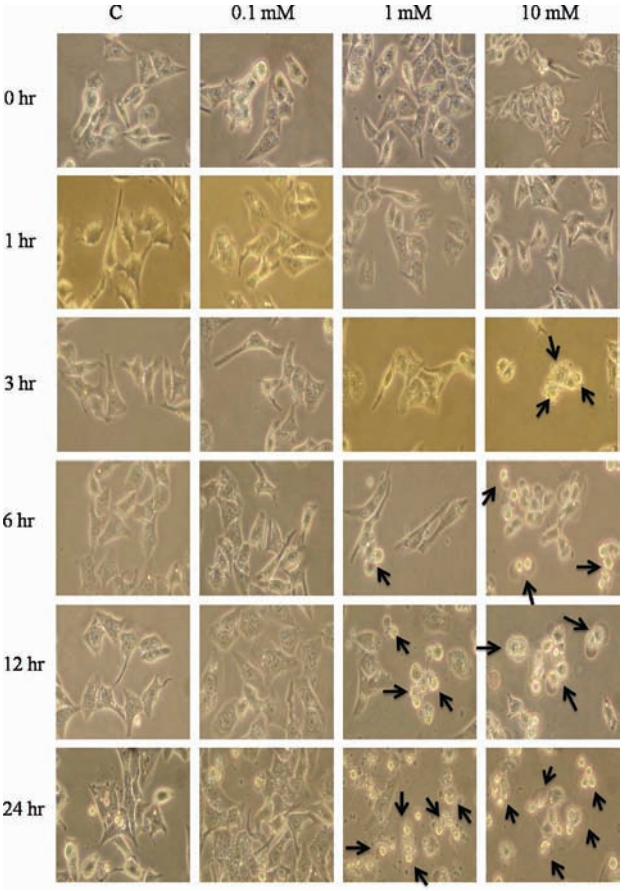

Figure 1. Effect of sesamol on morphological changes in MA-10 cells. MA-10 cells were cultured in a 6 cm dish until 70−80% confluence and then treated without or with different dosages of sesamol (control, 0.1, 1, and 10 mM) for 0, 1, 3, 6, 12, and 24 h. Morphological changes of cells were examined under light microscopy (C = control; arrow heads, membrane blebbed cells).

Radioimmunoassay (RIA). Media from cell cultures with different treatments were harvested. Twenty microliters of sample was loaded into a glass tube, and 100 μL each of progesterone antiserum and ^{3}H progesterone were loaded at 37 °C of temperature for 30 min. The reaction was stopped by putting the tubes in ice for 3 min. Charcoal solution (100 μL at 2.5 g/L PBS) was added into the tubes at 4 °C for 15 min and centrifuged for 10 min in order to spin down the charcoal−^{3}H progesterone complex.[22] Approximately 250 μL of the supernatant was poured into 2 mL of scintillation fluid, and samples were counted in a β-counter for 2 min.

Immunoblot Analysis. MA-10 cells (6.0×10^5) were cultured in a 6 cm dish. After treatment, cells were rinsed with cold PBS. Then, the

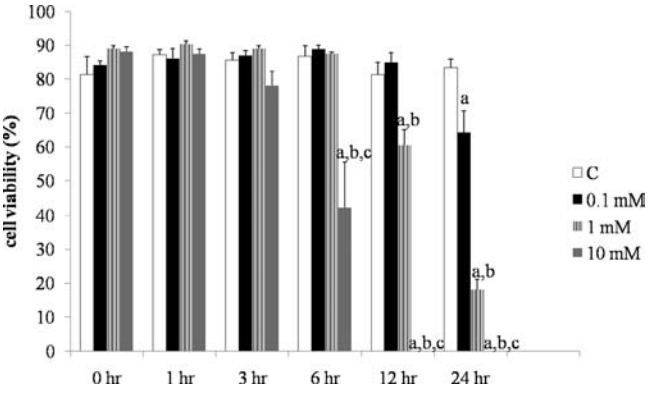

Figure 2. Effect of sesamol on cell viability in MA-10 cells. Cells were treated without or with different dosages of sesamol (control, 0.1, 1, and 10 mM) for 0, 1, 3, 6, 12, and 24 h, and cell viability was quantified by trypan blue exclusion assay. Results are expressed as percentages of cell viability of unstained cells/total cells. Data represent the mean ± SEM of four separate experiments. "a" represents significant difference from the control (C = control) ($p < 0.05$); "b" represents significant difference from the 0.1 mM sesamol treatment ($p < 0.05$), and "c" represents significant difference from the 1 mM sesamol treatment ($p < 0.05$) in each time point.

cells were harvested by 100 μL of lysis buffer (20 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, and 1 mM Na_3VO_4). The cell lysate was subjected to centrifugation at 12,000*g* for 12 min at 4 °C. The supernatant was stored at −20 °C until use. The protein concentration was determined by the Lowry method.[28] Total protein was solubilized in 1× SDS sample buffer and loaded on a 12.5% SDS−PAGE. Electrophoresis was performed in SDS−PAGE running buffer (24 mM Tris/HCl, 0.19 M glycine, and 0.5% SD, pH 8.3). The proteins were transferred to polyvinylidence difluoride membranes (PVDF) in transfer buffer (20 mM Tris/HCl, 150 Mm glycine, 10% methanol, and 0.01% SDS). The PVDF membrane with protein on it was incubated in blocking buffer (TBS buffer containing 5% carnation nonfat dry milk and 0.1% Tween-20) at room temperature for an hour. After washing, the membrane was incubated in different primary antibodies [StAR primary antibody is a generous gift from Dr. Strauss (University of Pennsylvania Medical Center, Philadelphia, USA); caspase-3 primary antibody was purchased from Cell Signaling (Beverly, MA, USA); and the P450scc primary antibody is a generous gift from Dr. Bon-Chu Chung (Academia Sinica, Taipei, Taiwan)] for 16−18 h at 4 °C. The membrane was washed 3 times (10 min each) with TBS containing 0.1% Tween-20. It was then incubated for 1 h at room temperature with fresh blocking buffer containing the secondary antibody, antirabbit IgG. The membrane was washed and the signal was detected by using the Renaissance chemiluminescence reagent (NEN; DuPont, Boston, MA, USA). The desired protein was quantitated by a computer-assisted image analysis system. The amount of β-actin in each lane was detected and quantified in order to normalize the expression of target protein.[22]

Statistical Analysis. All data were expressed as the mean ± SEM of at least three independent experiments. Statistically significant differences between the control and treatments were determined by one-way analysis of variance (ANOVA) and then the least significant difference (LSD) analysis. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of Sesamol on Morphological Changes in MA-10 Cells. MA-10 cells were treated without or with different concentrations of sesamol (0, 0.1, 1, and 10 mM) for 0, 1, 3, 6, 12, and 24 h, respectively, and morphological changes were examined under light microscopy. Cells without sesamol treatment showed polygonal shape with healthy appearances, blurred outline, and firm attachment, which are normal cell growth phenomena (Figure 1 control from 0 to 24 h). After 3 h of sesamol (1 and 10 mM) treatment, cells appeared rounded-up but still adhered to the ground matrix (Figure 1). After 6 h of sesamol treatment, the adherent cells appeared with a black-dotted outline, and some cells expressed plasma membrane blebbings with few floating cells (Figure 1). After 12 and 24 h of sesamol treatments, more adherent cells appeared with membrane blebbings, and there were more floating cells (Figure 1). These phenomena suggest that sesamol might induce apoptotic cell death in the MA-10 Leydig tumor cell line.

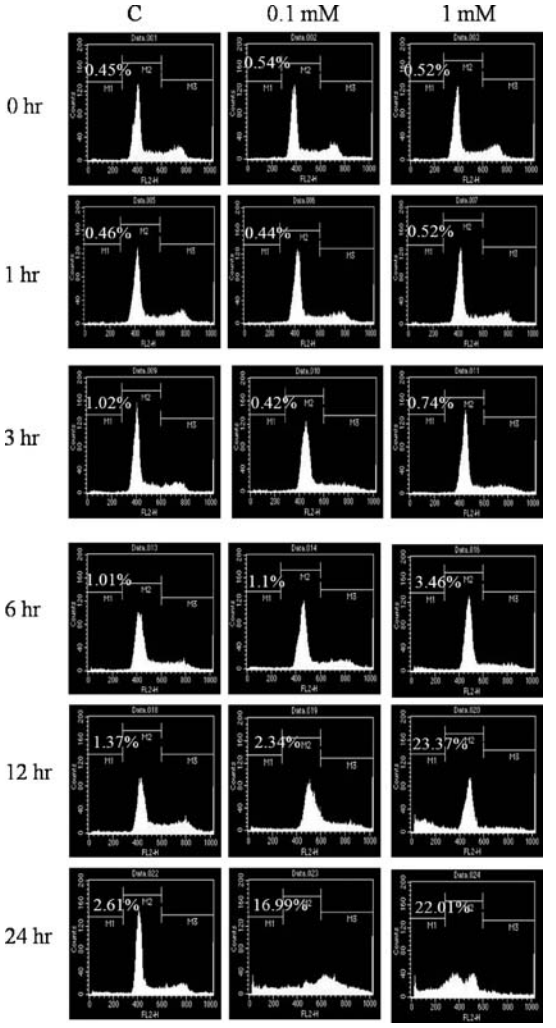

Figure 3. Effect of sesamol among subG1, G1, and G2/M cell cycle phases in MA-10 cells. The 2D histogram plot of flow cytometry analysis in MA-10 cells treated without or with sesamol (control, 0.1, and 1 mM) for 0, 1, 3, 6, 12, and 24 h is illustrated, representing one of three independent experiments. After treatments, cells were fixed, stained with propidium iodide, and analyzed for cell cycle progression by flow cytometry as described in Materials and Methods. M1 = SubG1 (cells with less than the normal amount of DNA content); M2 = G1 (cells in G1 cell cycle phase); M3 = G2/M (cells in G2/M cell cycle phase) (C = control).

Effect of Sesamol in MA-10 Cell Viability. Previous results regarding the morphological changes indicated that sesamol

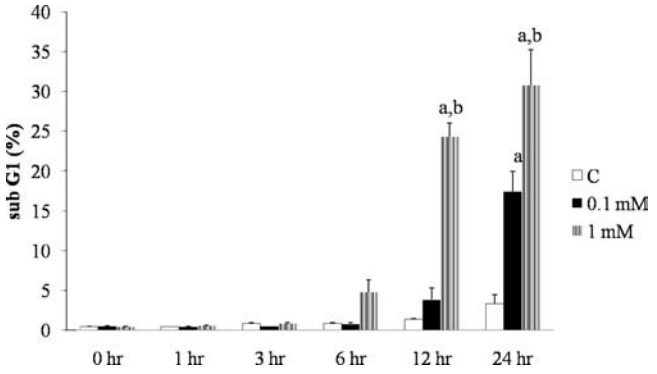

Figure 4. Quantification in percentage of the subG1 phase of MA-10 cells. Statistical analysis from 3 independent experiments in Figure 3 regarding the change of cell cycle in percentages of the subG1 phase was analyzed and illustrated. Data represent the mean ± SEM of three separate experiments (C = control). "a" represents significant difference from the control (C = control) ($p < 0.05$); "b" represents significant difference from the 0.1 mM sesamol treatment ($p < 0.05$); and "c" represents significant difference from the 1 mM sesamol treatment ($p < 0.05$) in each time point.

could induce cell death in MA-10 cells. Thus, the trypan blue assay was further used to assess cell viability after sesamol treatment. Cells were treated without or with different concentrations of sesamol (0, 0.1, 1, and 10 mM) for different time durations (0, 1, 3, 6, 12, and 24 h), and the viability was determined. The results clearly demonstrated the decreasing trend of viability in MA-10 cells among time and dose effects (Figure 2). In fact, 0.1 mM sesamol at 24 h of treatment reduced 35% cell viability; 1 mM sesamol at 12 and 24 h reduced 39% and 82% cell viability, respectively; and 10 mM sesamol at 6, 12, and 24 h reduced 57%, 97%, and 98% cell viability, respectively ($p < 0.05$). These results demonstrated that sesamol significantly reduced cell viability in MA-10 cells and that a higher dose of sesamol with prolonged treatment could cause much more cell death.

Effect of Sesamol on Cell Cycle Redistribution in MA-10 Cells. Previous results illustrated that sesamol could induce cell death in MA-10 cells. Flow cytometry analysis was further used to determine whether DNA fragmentation occurred and if there was any change in cell cycle progression. The subG1 phase in cell cycle distribution represents the occurrence of DNA fragmentation, which demonstrates the phenomenon of apoptosis.[29] The distribution of PI stained MA-10 cells with the treatment of sesamol (0, 0.1, and 1 mM) for 0, 1, 3, 6, 12, and 24 h were illustrated in Figure 3. Statistical analysis from 3 independent experiments of Figure 3 regarding the change of the subG1 phases of the cell cycle in percentages was analyzed and illustrated in Figure 4. We found that the percentage of subG1 phase cells significantly increased 17.5% by 0.1 mM sesamol treatment for 24 h and 24.0% and 32.0% by 1 mM sesamol treatment for 12 and 24 h, respectively (Figures 3 and 4) ($p < 0.05$). These cell cycle redistribution analysis results confirmed the apoptotic event of sesamol on MA-10 cells.

Effect of Sesamol on the Cleaved Caspase-3 Expression in MA-10 Cells. To briefly investigate possible apoptotic pathway through which sesamol might induce MA-10 cell apoptosis, cells were treated without or with 0.1 or 1 mM sesamol for 0, 1, 3, 6, 12, and 24 h, respectively. Then, the expression of cleaved caspase-3 was determined by Western blotting. Results showed that cleaved caspase-3 significantly expressed by 0.1 mM sesamol

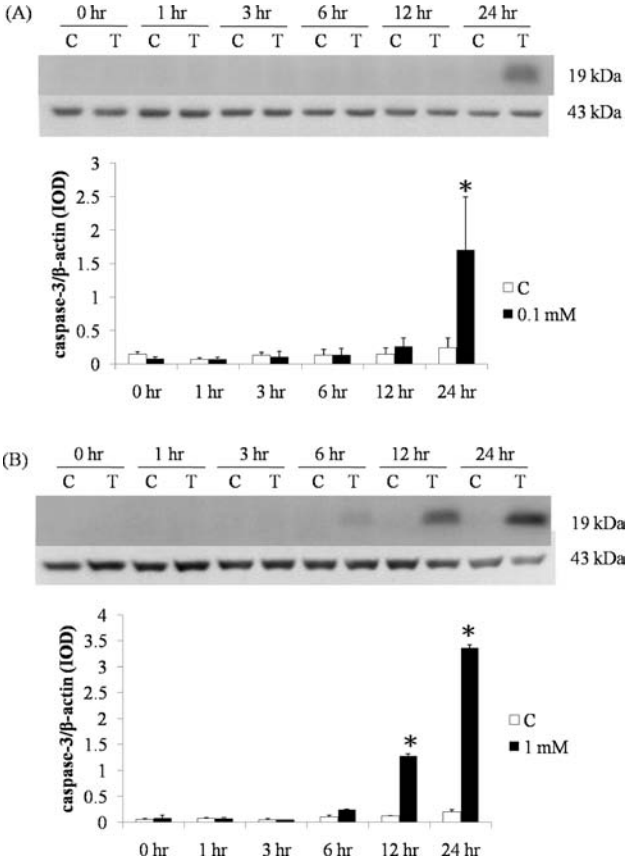

Figure 5. Effect of sesamol on the cleaved caspase-3 expression in MA-10 cells. Cells were treated without or with 0.1 mM (A) and 1 mM (B) sesamol, respectively, for different times (0, 1, 3, 6, 12, and 24 h). The caspase-3 (19 kDa) and β-actin (43 kDa) proteins were detected by Western blots. Immunoblot represents the observations from one single experiment repeated three times. The integrated optical densities (IOD) of caspase-3 protein after normalization with β-actin in each lane using the PDI image system were demonstrated. Each data point in panels A and B represents the mean ± SEM of three separate experiments. * indicates statistical difference compared to the control in each time point ($p < 0.05$) (C = control).

treatement for 24 h (Figure 5A) and by 1 mM sesamol treatment for 12 and 24 h (Figure 5B) ($p < 0.05$). These data demonstrated that sesamol could activate caspase-3 expression to induce MA-10 cell apoptosis.

Effect of Sesamol on Steroidogenesis in MA-10 Cells. During the investigation of the apoptotic effect of sesamol on MA-10 cells, these cells appeared rounded-up, which is very similar to the steroidogenesis in Leydig cells activated by steroidogenic agents. Thus, we used a radioimmunoassay to examine the steroidogenic effect of sesamol on MA-10 cells. We found that 1 mM sesamol after 24 h and 10 mM sesamol treatment after 12 and 24 h treatments could significantly induce progesterone production (Figure 6) ($p < 0.05$). These results demonstrated that sesamol could concomitantly induce apoptosis and steroidogenesis in MA-10 mouse Leydig tumor cells.

Effect of Sesamol on the Expression of Steroidogenic Proteins in MA-10 Cells. To briefly investigate possible steroidogenic pathway through which sesamol might activate MA-10 cell steroidogenesis, cells were treated without or with 0.1 or 1 mM sesamol for 0, 1, 3, 6, 12, and 24 h, respectively. Then, the

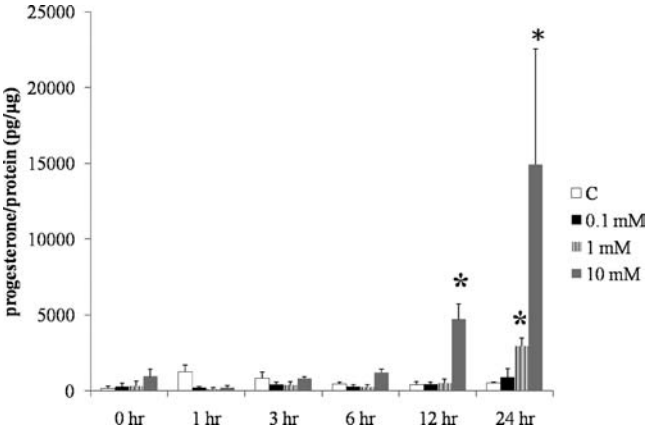

Figure 6. Effect of sesamol on sesamol-stimulated progesterone production in MA-10 cells. Cells were treated without or with 0.1, 1, and 10 mM sesamol for 0, 1, 3, 6, 12, and 24 h. Media were collected and assayed by RIA for progesterone production. Each data in the figure represents the mean the mean ± SEM of three separate experiments. * indicates that those groups differ significantly from the control in each time point ($p < 0.05$) (C = control).

expressions of StAR protein and P450scc enzyme were determined by Western blotting. Results showed that 1 mM sesamol for 24 h significantly induced StAR protein expression (Figure 7B) ($p < 0.05$). However, sesamol treatment (0.1 or 1 mM) for different durations (0, 1, 3, 6, 12, and 24 h) did not induce P450scc enzyme expression (Figure 8) ($p > 0.05$). These results illustrate that sesamol could activate StAR protein expression, but not P450scc enzyme, to induce MA-10 cell steroidogenesis.

DISCUSSION

Sesamol is a well-known antioxidant with the ability to induce NO release.[1,2,6] It is also reported with anticancer effects upon different cell types.[4,5] However, little is known about the actions of sesamol on apoptosis and steroidogenesis in Leydig cells.

In anticancer effect, we confirmed that sesamol could induce cell membrane blebbing with the decrease of cell number with prolonged time in MA-10 cells. Also, cell viability detected by trypan blue exclusion assay demonstrated that sesamol could decrease cell viability in a time- and dose-dependent manner in MA-10 cells. It should be noted that 10 mM sesamol treatment for 12 and 24 h killed entire MA-10 cells. Therefore, we only used lower dosages (0.1 and 1 mM) of sesamol for the rest of experiments in this study. In flow cytometry analysis, the subG1 peak, representing the DNA fragmentation of a well-accepted phenomenon of apoptosis,[10] was also induced by sesamol in a time- and dose-dependent manner in MA-10 cells. Moreover, we found that sesamol could specifically activate the expression of caspase-3 in dose- and time-dependent relationships. These data highly indicated that sesamol did induce apoptosis through the activation of the caspase pathway in MA-10 mouse Leydig tumor cells, and our finding is not unprecedented compared to other studies.[4,5,30,31]

Besides apoptosis, we found that sesamol could significantly increase steroidogenesis in MA-10 cells, and it is the first time that it has been demonstrated that sesamol could regulate steroidogenesis in Leydig cells. Steroid production in Leydig cells is regulated by various factors, which will activate the cAMP/PKA-dependent pathway to induce the expression of StAR protein,

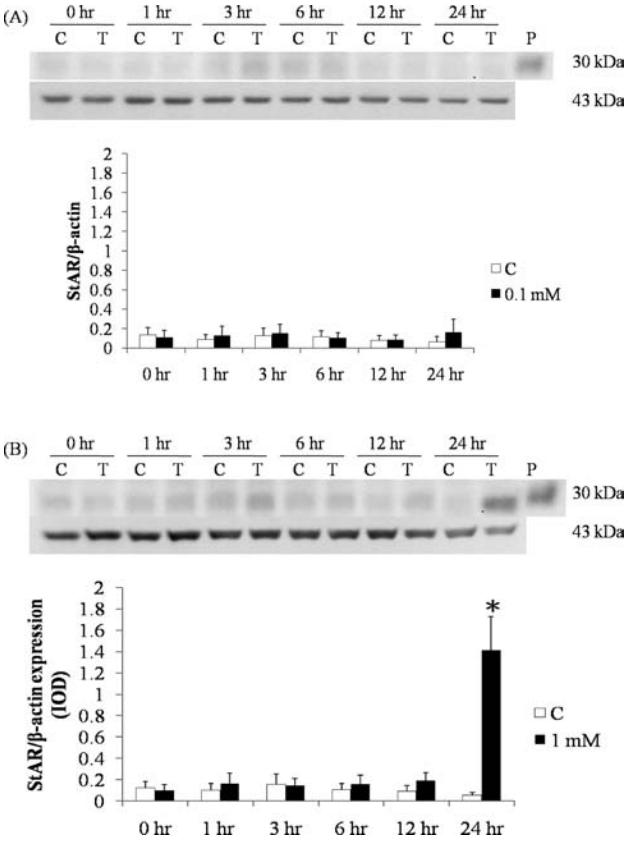

Figure 7. Expression of StAR protein in sesamol treated MA-10 cells. Cells were treated without or with 0.1 (A) and 1 mM (B) sesamol, respectively, for 0, 1, 3, 6, 12, and 24 h. The StAR protein (30 kDa) and β-actin (43 kDa) proteins were detected by Western blots. Immunoblot represents the observations from one single experiment repeated three times. The integrated optical densities (IOD) of the StAR protein after normalization with β-actin in each lane using the PDI image system are demonstrated. Each data point in panels A and B represents the mean ± SEM of three separate experiments. * indicates statistical difference compared to the control in each time point ($p < 0.05$) (C = control).

and then to stimulate steroidogenesis through different steroidogenic enzymes.[20] In the present study, 1 mM sesamol after 24 h and 10 mM sesamol after 12 and 24 h treatments significantly induced progesterone production. In order to briefly define how sesamol might induce steroidogenesis, StAR protein (an essential protein in steroidogenesis) and P450scc (an important steroidogenic enzyme in steroidogenesis) were examined. We did find that sesamol significantly activated the expression of StAR protein, but not the P450scc enzyme.

Author: Please verify that the changes made to improve the English still retain your original meaning.It has been shown that some factors can induce StAR protein expression with no effect on P450scc enzyme expression in steroidogenic cells.[32] It has also been demonstrated that factors could increase P450scc activity, but not protein expression, to induce steroidogenesis.[33] It is possible that sesamol increased P450scc activity, but not the protein expression, to induce steroidogenesis in MA-10 cells. Another possibility is that sesamol might disrupt mitochondrial functions, as 10 mM sesamol could kill all of the MA-10 cells, which made cholesterol more exposed to P450scc and other steroidogenic enzymes for more production of progesterone in MA-10 cells. This phenomenon should be further investigated

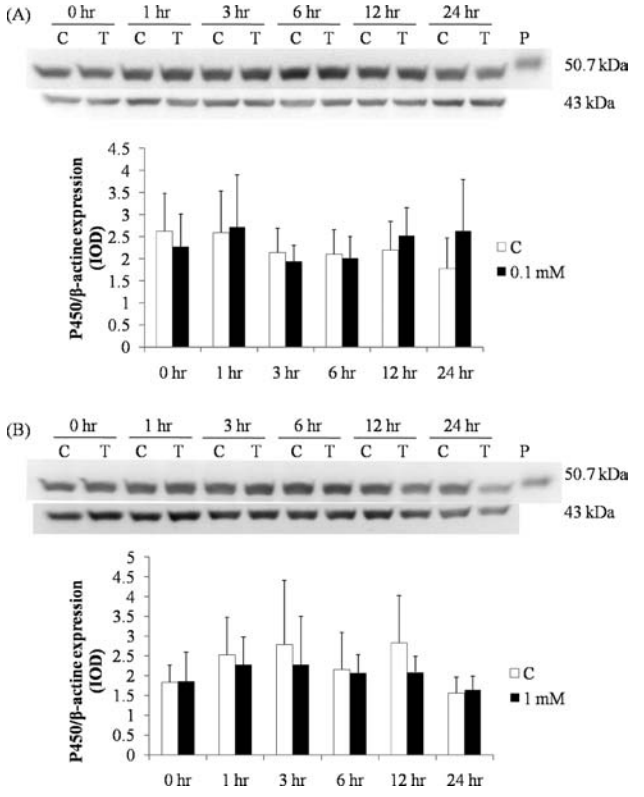

Figure 8. Expression of P450scc in sesamol treated MA-10 cells. Cells were treated with 0.1 (A) and 1 mM (B) sesamol for different times (0, 1, 3, 6, 12, and 24 h). The P450scc protein (50.7 kDa) and β-actin (43 kDa) proteins were detected by Western blots. Immunoblot represents the observations from one single experiment repeated three times. The integrated optical densities (IOD) of P450scc protein after normalization with β-actin in each lane using the PDI image system were demonstrated. Each data point in panels A and B represents the mean $\pm$ SEM of three separate experiments. * indicates statistical difference compared to the control in each time point ($p < 0.05$) (C = control).

with the treatment of the precursor to the P450scc enzyme to uncover the detailed mechanism.

Comparing the apoptotic and steroidogenic results, we found that the expression of StAR protein and caspase-3 was timely overlapped. This intriguing phenomenon should be examined in detail to determine whether there is an interaction activated by sesamol on apoptosis and steroidogenesis in MA-10 cells. Evidences have shown that StAR protein could inhibit human THP-1 monocyte-derived macrophage apoptosis,[34] and the suppressing of the StAR protein expression impaired Leydig cell function by increasing apoptosis,[35] which seems to imply that StAR protein will help to prevent cell apoptosis. It has been shown that steroidogenesis will increase the production of ROS from mitochondria and that the ROS is an apoptotic factor to induce cell apoptosis.[36,37] It is highly possible that sesamol stimulated steroidogenesis, which then induced the release of ROS from mitochondria to further activate the apoptosis in MA-10 cells. We will investigate the temporal relationship between steroidogenesis, ROS production, and apoptosis in MA-10 cells activated by sesamol to clarify the facts in the future.

It has been demonstrated that sesamol could induce nitric oxide (NO) release[6] and that NO could inhibit Leydig cell steroidogenesis.[7,8] In the present study, we do not know whether sesamol could induce the production of NO in MA-10 cells. However, we did find that sesamol could stimulate MA-10 mouse Leydig tumor cell steroidogenesis. It is possible that sesamol could not induce MA-10 cells to release any NO to regulate MA-10 steroidogenesis. Sesamol has been recognized as a well-known antioxidant.[2,38] The antioxidant effect of sesamol has been proved to inhibit lipid peroxidation[3,39] and cleanup the free radicals. It is also possible that sesamol could suppress the NO production during the steroidogenesis activated by sesamol in MA-10 cells. Otherwise, steroidogenesis would not be stimulated. This inconsistency might indicate that sesamol could directly associate with Leydig cells to stimulate StAR protein and then steroidogenesis. Thus, further study to investigate whether sesamol could induce or suppress the release of NO in MA-10 cells will be worthy. Also, the binding assay of sesamol on MA-10 cells will be necessary to determine if there is a direct association of sesamol on MA-10 Leydig cells.

In conclusion, sesamol could induce the antitumor effect through the activation of the caspase pathway in MA-10 mouse Leydig tumor cells, and sesamol could simultaneously stimulate steroidogenesis through the activation of StAR protein in MA-10 mouse Leydig tumor cells, which suggest that sesamol might be a good potential antitumor drug for chemotherapy.

AUTHOR INFORMATION

Corresponding Author

*Department of Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, #1 University Road, Tainan, 701, Taiwan, Republic of China. Tel: 886-6-208-9357. Fax: 886-6-209-3007. E-mail: bumiin@mail.ncku.edu.tw.

Author Contributions

Y.-H.C. and S.F.L. contributed equally to this work.

Funding Sources

This work was supported by National Science Council Grants NSC 98-2320-B-006-016 (BMH), Taiwan, Republic of China.

REFERENCES

(1) Sharma, S.; Kaur, I. P. Development and evaluation of sesamol as an antiaging agent. *Int. J. Dermatol.* **2006**, *45*, 200–208.

(2) Joshi, R.; Kumar, M. S.; Satyamoorthy, K.; Unnikrisnan, M. K.; Mukheriee, T. Free radical reactions and antioxidant activities of sesamol: pulse radiolytic and biochemical studies. *J. Agric. Food Chem.* **2005**, *53*, 2696–2703.

(3) Prasad, N. R.; Menon, V. P.; Vasudev, V.; Puqalendi, K. V. Radioprotective effect of sesamol on gamma-radiation induced DNA damage, lipid peroxidation and antioxidants levels in cultured human lymphocytes. *Toxicology* **2005**, *209*, 225–235.

(4) Jacklin, A.; Ratledge, C.; Welham, K.; Bilko, D.; Newton, C. J. The sesame seed oil constituent, sesamol, induces growth arrest and apoptosis of cancer and cardiovascular cells. *Ann. N.Y. Acad. Sci.* **2003**, *1010*, 374–380.

(5) Fujimoto, A.; Sakanashi, Y.; Matsui, H.; Oyama, T.; Nishimura, Y.; Masuda, T.; Oyama, Y. Cytometric analysis of cytotoxicity of polyphenols and related phenolics to rat thymocytes: potent cytotoxicity of resveratrol to normal cells. *Basic Clin. Pharmacol. Toxicol.* **2009**, *104*, 455–462.

(6) Chen, P. R.; Tsai, C. E.; Chang, H.; Liu, T. L.; Lee, C. C. Sesamol induces nitric oxide release from human umbilical vein endothelial cells. *Lipids* **2005**, *40*, 955–961.

(7) Del Punta, K; Charreau, E,H; Pignataro, O,P. Nitric oxide inhibits Leydig cell steroidogenesis. *Endocrinology* **1996**, *137*, 5337–5343.

(8) Dobashi, M.; Fujisawa, M.; Yamazaki, T.; Okuda, Y.; Kanzaki, M.; Tatsumi, N.; Tsuji, T.; Okada, H.; Kamidono, S. Inhibition of

steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (star) mRNA. *Arch. Androl.* **2001**, *47*, 203–209.

(9) Kerr, J. F.; Wyllie, A. H.; Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **1972**, *26*, 239–257.

(10) Wyllie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **1980**, *284*, 555–556.

(11) Steller, H. Mechanisms and genes of cellular suicide. *Science* **1995**, *267*, 1445–1449.

(12) Cryns, V.; Yuan, J. Proteases to die for. *Genes Dev.* **1998**, *12*, 1551–1570.

(13) Thornberry, N. A.; Lazebnik, Y. Caspases: enemies within. *Science* **1998**, *281*, 1312–1316.

(14) Yang, X.; Chang, H. Y.; Baltimore, D. Autoproteolytic activation of pro-caspases by oligomerization. *Mol. Cell* **1998**, *1*, 319–325.

(15) Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **1998**, *94*, 491–501.

(16) Slee, E. A.; Adrian, C.; Martin, S. J. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J. Biol. Chem.* **2001**, *276*, 7320–7326.

(17) Moger, W. H. Evidence for compartmentalization of adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinases in rat Leydig cells using site-selective cAMP analogs. *Endocrinology* **1991**, *128*, 1414–1418.

(18) Stocco, D. M.; Sodeman, T. C. The 30-kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. *J. Biol. Chem.* **1991**, *266*, 19731–19738.

(19) Strauss, J. F, III.; Kishida, T.; Christenson, L. K.; Fujimoto, T.; Hiroi, H. START domain proteins and the intracellular trafficking of cholesterol in steroidogenic cells. *Mol. Cell. Endocrinol.* **2003**, *202*, 59–65.

(20) Stocco, D. M. Tracking the role of a star in the sky of the new millennium. *Mol. Endocrinol.* **2001**, *15*, 1245–1254.

(21) Habert, R.; Lejeune, H.; Saez, J. M. Origin, differentiation and regulation of fetal and adult Leydig cells. *Mol. Cell. Endocrinol.* **2001**, *179*, 47–74.

(22) Leu, S. F.; Poon, S. L.; Pao, H. Y.; Huang, B. M. The *in vivo* and *in vitro* stimulatory effects of cordycepin on mouse Leydig cell steroidogenesis. *Biosci Biotechnol Biochem* **2011**, *75*, 723–731.

(23) Stocco, D. M.; Wells, J.; Clark, B. J. The effects of hydrogen peroxide on steroidogenesis in mouse Leydig tumor cells. *Endocrinology* **1993**, *133*, 2827–2832.

(24) Diemer, T.; Allan, J. A.; Hales, K. H.; Hales, D. B. Reactive oxygen disrupts mitochondria in MA-10 tumor leydig cells and inhibits steroidogenic acute regulatory (StAR) protein and steroidogenesis. *Endocrinology* **2003**, *144*, 2882–2891.

(25) Pan, B. S.; Lin, C. Y.; Huang, B. M. The effect of cordycepin on steroidogenesis and apoptosis in MA-10 mouse Leydig tumor cells. *Evid. Based Complement. Alternat. Med.* **2011**in press.

(26) Nicoletti, I.; Migliorati, G.; Pagliacci, M. C.; Grignani, F.; Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flowcytometry. *J. Immunol. Methods* **1991**, *139*, 271–279.

(27) Wang, B. J.; Won, S. J.; Yu, Z. R.; Su, C. L. Free radical scavenging and apoptotic effects of cordycepin sinensis fractionated by supercritical carbon dioxide. *Food Chem. Toxicol.* **2005**, *43*, 543–552.

(28) Lowry, O. H.; Rosenborough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol regent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(29) Kim, G. Y.; Mercer, S. E.; Ewton, D. Z.; Yan, Z.; Jin, K.; Friedman, E. The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. *J. Biol. Chem.* **2002**, *277*, 29792–29802.

(30) Fujimoto, A.; Shingai, Y.; Oyama, T. B.; Kawanai, T.; Hashimoto, E.; Koizumi, K.; Kimura, K.; Masuda, T.; Oyama, Y. Apoptosis-inducing action of two products from oxidation of sesamol, an antioxidative constituent of sesame oil: a possible cytotoxicity of oxidized antioxidant. *Toxicol. in Vitro* **2010**, *24*, 1720–1726.

(31) Kumar, C. M.; Sathisha, U. V.; Dharmesh, S.; Rao, A. G.; Singh, S. A. Interaction of sesamol (3,4-methylenedioxyphenol) with tyrosinase and its effect on melanin synthesis. *Biochimie* **2011**, *93*, 562–569.

(32) Huang, B. M.; Hsiao, K. Y.; Chuang, P. C.; Wu, M. H.; Pan, H. A.; Tsai, S. J. Upregulation of steroidogenic enzymes and ovarian 17beta-estradiol in human granulosa-lutein cells by Cordyceps sinensis mycelium. *Biol. Reprod.* **2004**, *70*, 1358–1364.

(33) Chen, L. Y.; Huang, Y. L.; Liu, M. Y.; Leu, S. F.; Huang, B. M. Effects of amphetamine on steroidogenesis in MA-10 mouse Leydig tumor cells. *Life Sci.* **2003**, *72*, 1983–1995.

(34) Bai, Q.; Li, X.; Ning, Y.; Zhao, F.; Yin, L. Mitochondrial cholesterol transporter, StAR, inhibits human THP-1 monocyte-derived macrophage apoptosis. *Lipids* **2010**, *45*, 29–36.

(35) Luo, D. Y.; Yang, G.; Liu, J. J.; Yang, Y. R.; Dong, Q. Effects of varicocele on testosterone, apoptosis and expression of StAR mRNA in rat Leydig cells. *Asian J. Androl.* **2011**, *13*, 287–291.

(36) Carmody, R. J.; Cotter, T. G. Signalling apoptosis: A radical approach. *Redox. Rep.* **2001**, *6*, 77–90.

(37) Pelicano, H.; Carney, D.; Huang, P. ROS stress in cancer cells and therapeutic implications. *Drug Resist. Updat.* **2004**, *7*, 97–110.

(38) Sharma, S.; Kaur, I. P. Development and evaluation of sesamol as an antiaging agent. *Int. J. Dermatol.* **2006**, *45*, 200–208.

(39) Ghafoorunissa.; Hemalatha, S.; Rao, M. V. Sesamol lignans enhance antioxidant activity of vitamin E in lipid peroxidation systems. *Mol. Cell. Biochem.* **2004**, *262*, 195–202.