ผลของสารสกัดข้าวสีดำต่อการตายแบบ Apoptosis ของเซลล์มะเร็งผิวหนังมนุษย์

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Apoptotic Effects of *Oryza Sativa L.* Extract in Human Melanoma Cell

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Abstract

Background: Malignant melanoma, originating from melanocytes, is the most dangerous type of skin cancer. Melanoma treatment includes surgery and the combination of surgery and chemotherapy, immunotherapy, targeted therapy and radiation therapy due to stage of melanoma. *Oryza sativa* Linn (OS) is a type of black rice found in various regions across Southeast Asia. The main bioactive components of OS extracts are anthocyanins, which have anti-tumor effects. Objective: In this study, we conducted cell viability, the cell cycle, and apoptosis analysis of a human melanoma A375 cells following treatment with OS extract. Methods: The effects of different OS concentrations treatment on human melanoma A375, cell cycle, and apoptosis were assessed by Flow cytometry. The identity of the protein was confirmed through SDS-PAGE and Western blotting. Results: We demonstrated from cell cytotoxicity assay that OS decreases cells viability after OS treatment at concentration 1.25, 2.5, 5 and 10 mg/ml. Flow cytometry showed that OS extract concentration at 2.5 mg/ml induces A375 cell cycle arrest at S phase and 5 and 10 mg/ml OS induces sub-G1 cell cycle arrest. OS extract concentration 2.5, 5 and 10 mg/ml induced A375 cell apoptosis. Apoptosis induction was accompanied by up-regulation caspase-8 and p53 expression. Conclusion: Our data show that the OS disrupted melanoma cell proliferation and induced apoptosis.

Keywords: Malignant melanoma, *Oryza sativa* L. extract, Apoptosis

บทคัดย่อ

ภูมิหลัง: มะเร็งผิวหนังชนิด malignant melanoma มีต้นกำเนิดจากเซลล์เมลาโนไซต์ เป็นมะเร็งผิวหนังชนิดที่ร้ายแรงที่สุด การรักษามะเร็งผิวหนังจะใช้วิธีการผ่าตัด หรือการผ่าตัดร่วมกับเคมีบำบัด ภูมิคุ้ม กันบำบัด การรักษาแบบมุ่งเป้า หรือการฉายรังสี ขึ้นกับระยะของโรค *Oryza sativa* Linn (OS) เป็นข้าวสีดำชนิดหนึ่งที่พบได้ในภูมิภาคต่าง ๆ ทั่ว เอเชียตะวันออกเฉียงใต้ ส่วนประกอบออกฤทธิ์ทางชีวภาพหลักของสารสกัด OS คือแอนโทไซยานินซึ่งมีฤทธิ์ต้านการเจริญเติบโตของเนื้องอก **วัตถุประสงค์:** ในการศึกษานี้ เราทำการวิเคราะห์การแสดงออกของเซลล์มะเร็งผิวหนังชนิดเมลาโนมา A375 ของมนุษย์หลังการรักษาด้วยสารสกัด OS เพื่อประเมินผลกระทบต่อการรอดชีวิตของเซลล์,วัฏจักรของเซลล์ และการเหนี่ยวนำให้เกิดตายแบบ apoptosis ของเซลล์มะเร็งผิวหนัง **วิธีการ:** ประเมินผลของการรักษาเซลล์มะเร็งผิวหนังชนิด A375 ของมนุษย์ด้วยความเข้มข้นของ OS ที่แตกต่างกัน ประเมินวัฏจักรของเซลล์และการตายของเซลล์มะเร็งผิวหนังโดยใช้ flow cytometry บ่งบอกชนิดของโปรตีนที่เกี่ยวข้องโดยยืนยันผ่าน SDS-PAGE และ western blotting **ผล:** จากการทดสอบความเป็นพิษของเซลล์พบว่าสารสกัด OS ลดความมีชีวิตของเซลล์ที่ความเข้มข้น 1.25, 2.5, 5 และ 10 มิลลิกรัมต่อมิลลิลิตร โฟลว์ไซโตเมทรี (flow cytometry) แสดงให้เห็นว่าสารสกัด OS ที่ความเข้มข้น 2.5 มิลลิกรัมต่อมิลลิลิตร สามารถยับยั้งวัฏจักรของเซลล์ A375 ในระยะ S โดย OS ที่ความเข้มข้น 5 และ 10 มิลลิกรัมต่อมิลลิลิตร ยับยั้งที่ระยะ sub G1 และสารสกัด OS ความเข้มข้น 2.5, 5 และ 10 มิลลิกรัมต่อมิลลิลิตร สามารถเหนี่ยวนำให้เกิดการตายแบบ apoptosis ในเซลล์มะเร็ง A375 และยังพบว่าการเหนี่ยวนำการตายแบบ apoptosis นั้นมาพร้อมกับการควบคุมการขึ้น (up regulate) ของการแสดงออกของ caspase-8 และ p53 **สรุป:** ข้อมูลของเราแสดงให้เห็นว่าการใช้สารสกัดที่ได้มาจาก OS สามารถขัดขวางการเพิ่มจำนวนของเซลล์มะเร็งผิวหนังและเหนี่ยวนำให้เกิดการตายของเซลล์มะเร็งผิวหนังแบบ apoptosis ได้

คำสำคัญ: มะเร็งผิวหนังชนิด malignant melanoma, สารสกัด *Oryza sativa* L., การตายของเซลล์แบบ Apoptosis

Introduction

Melanoma is a deadly form of skin cancer originating from melanocytes. Melanoma can widely distributed throughout the body, where they produce melanin to prevent damage by ultraviolet rays from the sun.[1] Intense intermittent exposure to ultraviolet radiation (UVR) can lead to genetic alterations by causing DNA damage and reactive oxygen species accumulation, as well as inflammatory responses, thus making UVR the highest risk factor for melanoma initiation.[2] UVR exposure contributes to two distinct melanoma pathogenesis mechanisms: early sun exposure and nevi proneness driving v-raf murine sarcoma viral oncogene homolog B (BRAF) mutation-related melanoma; and chronic sun exposure, linked to neuroblastoma ras viral oncogene homolog (NRAS) mutation-related melanoma.[3] Dacarbazine chemotherapy and high dose interleukin-2 (IL-2) administration were used to treat melanoma in the past; however, more modern treatments have turned to immunotherapies, including the use of ipilimumab and nivolumab, as well as targeted therapies, such as vemurafenib and dabrafenib.[4] Other therapeutic agents include BRAF and mitogen-activated extracellular signal-regulated kinase (MEK) inhibitors, as well as single anti-programmed death ligand 1 (PD-1) antibody and intralesional modified oncolytic herpes virus, talimogene laherparepvec (T-VEC).[5] While treatment advances have improved survival rates, patients with distant metastasis continue to face a challenging 5-year survival rate of approximately 27%.[6]

Oryza sativa Linn (OS) is a type of black rice found in various regions across Southeast Asia. OS extract contains anthocyanins, bioactive compounds with implications in causing apoptosis of various cancer cell types.[7, 8] To date, researchers have identified around 18 major anthocyanins in rice, among which four are particularly notable: cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G), cyanidin-3-rutinoside (C3R), and cyanidin-3-galactoside.[9] C3G and P3G are the most prevalent types, with C3G comprising around 64%-90% of total OS anthocyanin content, while P3G accounts for 5%-28%[10]. OS extracts containing anthocyanins have antioxidative and anti-inflammatory effects in primary dermal fibroblasts[11], while preventing normal dermal fibrobl asts from undergoing hydrogen peroxide-induced cell death.[12] In the human squamous carcinoma cell-2

line, OS extract exhibited great potential for use as an anti-proliferative agent, with efficacy equal to that of the conventional chemotherapy drug, 5-FU, possibly by downregulation tumor-promoting genes involved in the TGF-β/Smads pathway, as well as glycoprotein non-metastatic melanoma protein B (GPNMB).[7] Anthocyanins in OS can also block NF-KB activation by 5-FU and exhibit selective growth-inhibitory effects against cancer cells, while having no effect on normal cells.[13] Hence OS anthocyanins are of great interest for use in research into targeted cancer therapy.

The aims of this research were to investigate the effects of OS extract on the A375 melanoma cell line by assessing cell toxicity and apoptosis-relate protein expression in A375 cells exposed to OS.

Materials and Methods

Reagents

Monoclonal rabbit antibodies against p53 (7F5) (2527T) and cleaved caspase-8 (Asp374) (E6H8S) (98134T) were purchased from Cell Signaling Technology (Danvers, MA, US). Anti-beta actin mouse antibody (AC-15) (ab6276) and horseradish peroxidase (HRP)-conjugated secondary antibodies against goat anti-rabbit IgG (ab6721) and goat anti-mouse IgG (ab6789) were purchased from Abcam (Cambridge, UK). An Annexin V-FITC kit was purchased from Miltenyi Biotec (Germany). PI/RNase was from Thermo Fisher Scientific (Waltham, MS, USA).

Plant extract preparation

Organic rice from Na Khun Mor brand was obtained from Chachoengsao province, Thailand. *Oryza sativa* L. (OS) grains were extracted in 60% ethanol and the anthocyanin composition in OS was determined by High Performance Liquid Chromatography (HPLC) as prescribed previous[11] at Faculty of Dentistry, Mahidol University. OS extract was concentrated using a Büchi B-490 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland), and lyophilized using a freeze-dryer (Labconco Corp., Kansas City, MO, USA). OS extract yield after evaporation was 3.5% of dry weight. From our previous study[11], the UV-Vis wavelength spectra quantification of total anthocyanin showed that the phenolic-rich extract contained ~1704 mg/kg of total anthocyanin (calculated as cyanidin-3-glucoside equivalents), ~572.89 mg/kg of peonidin-3-O-glucoside, and ~728.37 mg/kg of quercetin-3-beta-D-glucoside. The total phenolic content was ~30 ± 1.5 mM gallic acid equivalent (gFW).

Cell culture

The human malignant melanoma cell line (A375) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A375 melanoma cells have been used in various toxicology and immuno-oncology research. Cells were seeded into T75 Flask (1×10^6 cells/flask) maintained in Dulbecco's modified Eagle's medium (DMEM; ATCC, Manassas, VA, US) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific; Waltham, MS, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After 4 days, 20×10^6 cells per T75 flask were obtained, then the cells were trypsinized with 0.25% trypsin EDTA for further experiments.

Cell cytotoxicity assay

To detect the relative percentage of cell viability, cells were seeded in triplicate into 96-well plates (1×10^4 cells/well) and cultured overnight at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until the cells adhered to the plates. Various OS concentrations (1.25, 2.5, 5, 10 mg/ml) and untreated group (control) were added for 24 h. Cell cytotoxicity was measured using a quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT solution was discarded and dimethyl sulfoxide added to dissolve the formazan crystals. The absorbance of the solution was measured at 570 and 690 nm using a microplate reader (Epoch, Biotek, Canada) to elucidate the color intensity. Increase in the intensity of purple formazan product color indicates an increase in viable cell counts in number. The cell viability was calculated according to: OD sample/ODcontrol×100%. The mean optical density (OD, absorbance) of three wells in the

indicated groups was used to calculate the percentage of cell viability as follows: percentage of cell viability = $(A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$ (where, A = absorbance). Values were plotted by averaging triplicate wells.

Cell cycle analysis

Cells were seeded into T25 flasks (0.9×10^6/flask) and adhered to the surface overnight. Cells were treated with OS 2.5, 5, or 10 mg/ml for 48 h. Untreated cells were used as a control Cells were washed one time with phosphate-buffered saline (PBS; Sigma Chemicals, USA), and fixed with 70% ice-cold ethanol at 4°C overnight. Samples were then washed one time with PBS before analysis. Next, cells were permeabilized and treated with a PI/RNase dye (Thermo Fisher Scientific; Waltham, MS, USA) that stains DNA quantitatively in red color. The fluorescence intensity of the stained cells correlates with the amount of DNA they contain. As the DNA content doubles during the S phase, the DNA content (intensity of fluorescence) of cells in the G0 phase and G1 phase (before S), in the S phase, and in the G2 phase and M phase (after S) identifies the cell cycle phase position in the major phases (G0/G1 versus S versus G2/M phase) of the cell cycle. The cellular DNA content of individual cells is often plotted as their frequency histogram to provide information about relative frequency (percentage) of cells in the major phases of the cell cycle. Cell cycle analysis was conducted using the Becton, Dickinson (BD) FACSMelody™ Cell Sorter flow cytometry system from BD Bioscience, Franklin Lakes, New Jersey, USA). Images were analyzed using FlowJo Software.

Apoptosis and necrosis assessment by flow cytometry analysis

To detect the apoptosis: necrosis ratio 48 h after treatment, A375 cells (1×10^6) were seeded into T25 flasks and treated with 2.5, 5, or 10 mg/ml OS for 48 h. Untreated cells were prepared as a control. Cells were washed with cold PBS and pellets resuspended in 100 µL binding buffer. After a gentle mixing, annexin V-FITC and propidium iodide (PI) dyes were added to each sample and incubated at room temperature for 15 min. Collapse of mitochondrial membrane potential (apoptosis and necrosis) is identified by a change in subcellular location and/or fluorescence of the dyes. Apoptosis and necrotic cells were then detected and quantified using a BD FACSMelody™ Cell Sorter flow cytometry system (Becton, Dickinson (BD Bioscience), Franklin Lakes, New Jersey, USA). Images were analyzed using FlowJo Software.

This can be detected using flow cytometry

Western blot analysis

Cells were seeded in T25 flasks (0.9×10^5 cells per flask) and cultured overnight to allow for cell adhesion. Then, the cells were treated with 2.5, 5, or 10 mg/ml OS for 24 and 48 h. Untreated flask was prepared as a control. Cells were lysed with lysis buffer containing protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany). Protein concentrations were measured and normalized using a BCA protein assay kit (Thermo Fisher Scientific). Protein concentration 30 µg/µl was subjected to electrophoresis on 8% SDS-polyacrylamide gels. Then, samples were blotted onto nitrocellulose membranes. Protein sample treated OS was incubated with primary antibody against cleaved caspase-8 or p53 or beta-actin at 4°C overnight. After washing for 3 times, membranes were incubated with secondary antibodies at room temperature for 2 h (HRP-conjugated goat anti-rabbit IgG for cleaved caspase8 and p53 and HRP-conjugated goat anti-mouse IgG for beta-actin). Immunoreactive proteins were detected with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). The intensity of the protein bands was quantified using Scion software.

Statistical analysis

Data are presented as mean ± standard deviation values. Ratio levels of measurement were used to compare the formazan color and fluorescence dye staining cells between before and after various concentrations of OS treatment in all experiments. Statistical differences of percentage of cells stained by MTT Solution and counting the number of stained

cells by fluorescence dye for apoptosis assays were evaluated by one-way ANOVA using GraphPad Prism software version 5 (GraphPad, La Jolla, California, US, http://www.graphpad.com/). In cases where significant differences were detected, a Tukey's post-hoc test was used to compare treatment groups. Differences were considered statistically significant if $p < .05$.

Result

Oryza sativa L. (OS) in cell cytotoxicity assays. The cytotoxic effects on the A375 cell line of treatment with varying concentrations of OS (1.25, 2.5, 5, and 10 mg/ml) for 24 h were assessed by MTT assay (Figure 1). A significant decrease in percentage cell cytotoxicity was detected after treatment with OS at 1.25 mg/ml ($p < .0001$), and was sustained in A375 cells treated with 2.5 and 5 mg/ml. A further decrease in cell cytotoxicity was seen at 10 mg/ml ($p < .0001$).

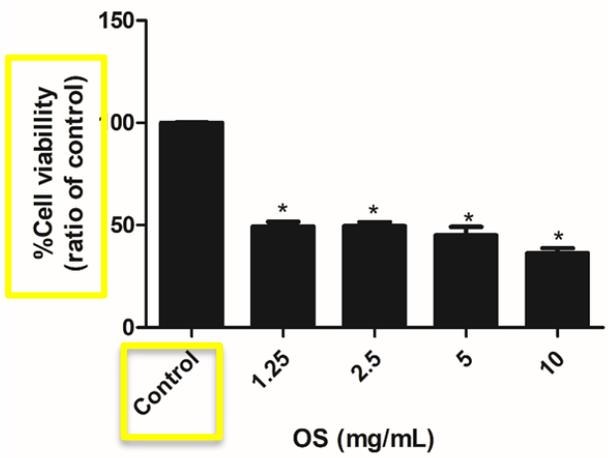

Figure 1. Effects of different concentrations of OS extract on human A375 melanoma cell viability, determined using the 3 (4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide assay. Cells were treated with OS extract for 24 h. Percentages of viable treated cells were assessed relative to the percentage of viable control cells. Data are expressed as mean± standard deviation (SD) of three independent experiments; $p < .0001$, versus the control.A

Analysis of the effects of OS on the A375 cell cycle. We next performed cell cycle analysis using PI staining and flow cytometry (Figure 2A). A dose-dependent increase in cells in the Sub G1 phase was detected in cells treated with 5 and 10 mg/ml OS relative to controls (10.9% ± 0.4% vs. 1.6% ± 0.2%, $p < .05$; 29.4% ± 3.5% vs. 1.6% ± 0.2%, $p < .0001$, respectively) (Figure 2B). Sub G1 phase is the phase which cells have had partial DNA loss during the last stages of the apoptotic process as a result of endonuclease activity. The G1 phase is when cells are ready to divide, and the GO phase is a phase of differentiation. DNA replication occurs during the cell cycle's. S phase, or synthesis phase. taking place in between the G1 and G2 phases. In eukaryotic species, G2/M is a crucial cell cycle checkpoint that makes sure cells don't start mitosis until damage occurs.

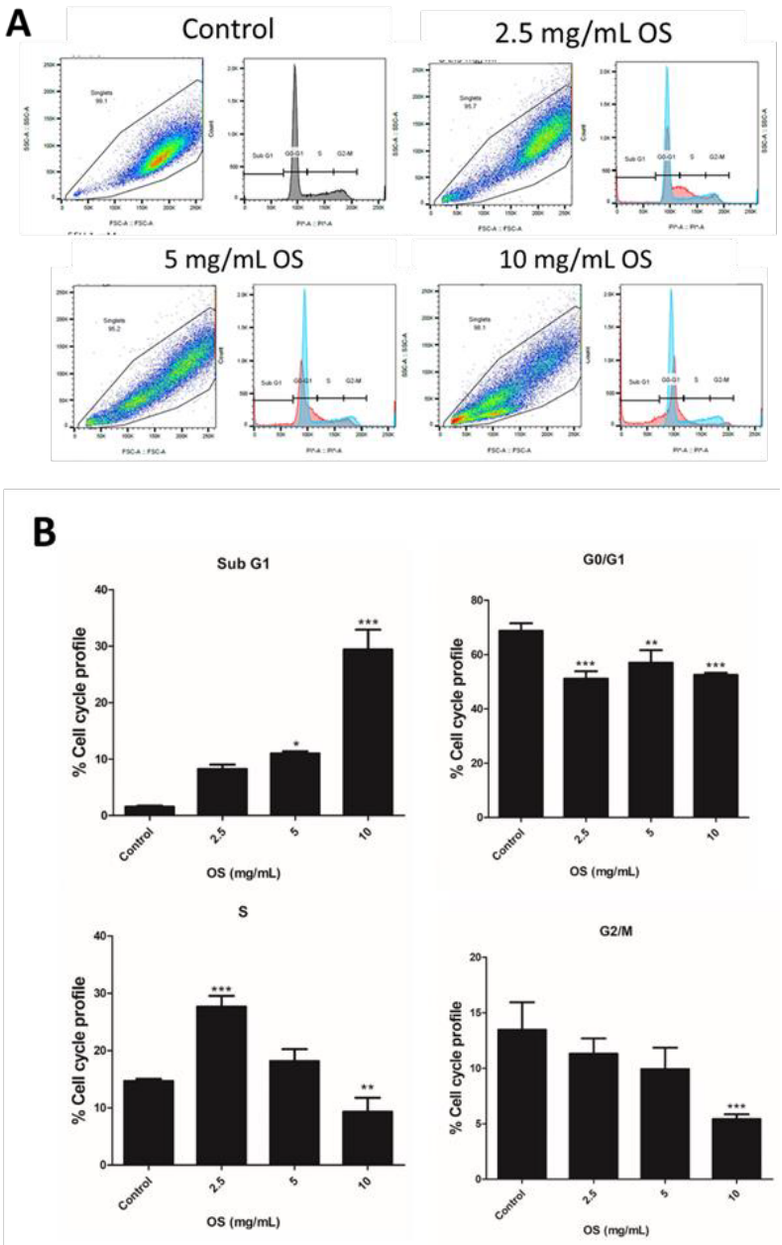

Figure 2. Cell cycle analysis using PI staining and flow cytometry following treatment with various concentrations (2.5, 5, and 10 mg/ml) of OS (A), Quantitative data for each cell cycle phase (B). Percentages of cell cycle profiles are expressed as mean ± standard deviation (SD) of three independent experiments; *$p < .05$, **$p < .001$, and ***$p < .0001$, versus the control.

Cell apoptosis and necrosis analysis. To further investigate the causes of cell death induced by OS, we used FITC-labelled Annexin V and PI double staining to assess cell apoptosis and necrosis by fluorescence-activated cell sorting analysis. Apoptotic cells were positive for Annexin V and PI staining, while viable cells were negative for both Annexin V-FITC and PI. Following 48 h treatment with OS, significant dose-dependent increases in cells at the early (Figure 3A and 3B) ($p < .05$ and $p < .0001$) and late ($p < .001$ and $p < .0001$) apoptosis phases were detected.

During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr et al., 1972). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Early apoptosis allows early

recognition of dead cells by macrophages, causing phagocytosis without the release of pro-inflammatory cellular components.[14] In contrast to early apoptotic cells that retain membrane integrity, late apoptotic cells act like necrotic cells (lack intact membranes), possibly due to the release of proinflammatory intracellular contents.[15]

To confirm the results of apoptosis analysis by Annexin/PI staining and flow cytometry, we conducted western blotting to assess levels of cleaved caspase-8 in A375 cells following treatment with 2.5, 5, or 10 mg/ml OS for 24 h (Figure 3C). Significant increases in cleaved caspase 8 were detected on treatment, with the greatest increase in cells treated with 10 mg/ml OS (3.89 ± 0.25 vs. 91±0.06 relative density; $p < .0001$). In addition, we found that p53 expression increased approximately 2-fold after treatment with 5 mg/ml OS for 24 h (Figure 3D); however, peak p53 overexpression was detected after treatment with 10 mg/ml OS (5.11 ± 0.09 vs. 1 ± 0.12 relative density; $p < .0001$). When treatment time was extended to 48 h (Figure 3E), a significant increase in p53 expression was detected on A375 cell exposure to 2.5 mg/ml OS (2.62 ± 0.13 vs. 1 ± 0.16 relative density; $p < .0001$).

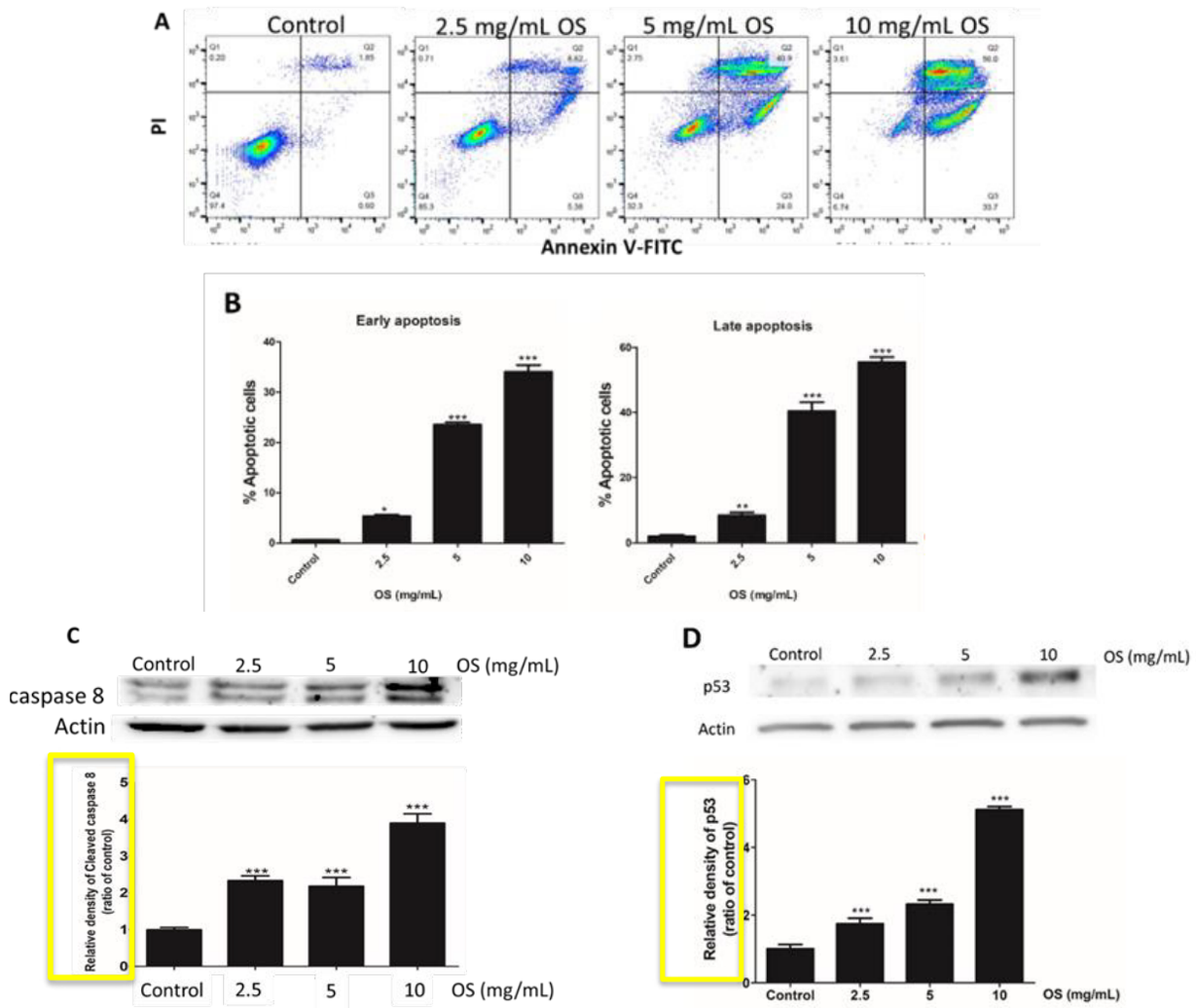

Figure 3. Induction of apoptosis after treatment with OS extract (A) Flow cytometry analysis of apoptotic cells stained with Annexin V-FITC and propidium iodide (PI). (B) Graph showing the percentages of early/late apoptotic cells in test and control samples. Western blot analysis of (C) cleaved caspase-8 and (D) *p*53 expression, following 24 h OS treatment. (E) *p*53 expression following 48 h OS treatment. Results were obtained from three independent experiments. Data are expressed as mean ± SD;
*$p < .05$, **$p < .001$, and ***$p < .0001$, versus the control.

Discussion

Many studies have reported that anthocyanins can induce apoptosis of various cancer cell lines[16], as well as having anti-metastatic and anti-proliferative effects.[7, 8] In melanoma cell lines, anthocyanins have apoptotic effects via reducing oxidative stress biomarkers and inducing changes in mitochondrial membrane potential[17], as well as anti-proliferative effects in metastatic B16-F10 murine melanoma cells.[18] Other studies using liposome-encapsulated anthocyanins demonstrated that they have inhibitory effects on melanogenesis in A375 cells.[19] This accumulating evidence has led to a recent rise in research into anthocyanins in the context of melanoma, aiming to elucidate the exact mechanisms by which anthocyanins interact with the cell cycle to cause anti-proliferation and apoptosis effects. Our findings confirm those of previous studies that anthocyanins in *Oryza sativa L*. (OS) extracts can induce A375 melanoma cell apoptosis in a dose-dependent manner. Treatment of melanoma cells with varying concentrations of OS led to cytotoxicity in the late apoptosis phase, and western blotting to detect caspase-8, an established marker of apoptosis[20], revealed an increase in caspase 8 cleavage in response to treatment with OS. Caspase 8 is an initiator caspase that is recruited to death-inducing signaling complexes by the Fas-associated death domain (FADD), which forms a complex with caspase-8.[21] Anthocyanins present in OS may be able to interact with the death receptor-mediated apoptosis cascade in A375 melanoma cells.

p53 serves a wide range of essential functions, with its most well-defined roles being cell cycle arrest, senescence, and apoptosis. These pathways enable p53 to either aid in the repair and survival of damaged cells or remove severely harmed cells from the replicative pool, ultimately safeguarding the organism.[22] The primary role of p53 in apoptosis centers on the mitochondrial pathway, although it can also impact cell death through death receptors. The fundamental contribution of p53 to apoptosis relies on its ability to activate the transcription of various proapoptotic genes, including Bax, Noxa, and Puma, from the Bcl-2 family.[23] In the context of the death receptor apoptosis pathway, p53 not only triggers Fas transcription in certain organs, but also enhances cell surface FAS levels by facilitating its transport from the Golgi complex.[24] Moreover, p53 activates DR5, a TRAIL receptor, which is induced following DNA damage and facilitates cell death via caspase-8. Genes encoding proapoptotic proteins, such as BID and PIDD, which may act as connectors between apoptotic pathways, have been identified as transcriptional targets of p53. Crucially, p53 also plays a role in activating the apoptosome by inducing Apaf-1 expression.[25] In our study, we found that p53 expression increased in A375 cells in response to treatment with 2.5, 5, and 10 mg/ml OS, suggesting that OS-mediated apoptosis shares a similar cell death pathway involving p53-activated apoptosis.

Conclusion

In this study, we characterized cytotoxicity potential and apoptosis biomarkers in the melanoma A375 cell line in response to treatment with *Oryza Sativa* L. extract. Cell cycle analysis using PI staining and flow cytometry demonstrated that more cells were observed in the late apoptosis phase than in early apoptosis. Significant increases in cleaved caspase 8 and p53 protein expressions were also detected suggesting that OS may mediate apoptosis in human melanoma. The effects of pure or standard compound isolated from ethanol extract of OS such as cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G) and quercitin on the mechanism of action of apoptosis in melanoma cells should be further investigated.

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