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Research Article

MDA-MB-231 Human Breast Cancer Cell Line Treated with Ginseng (Panax Quinquefolius): Evaluation by Annexin V and AgNOR Staining

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Abstract

Aim: In this study, it was aimed to examine the time and dose dependent effects of Ginseng (Panax quinquefolius) on MDA-MB-231 cell lines.

Material and Methods: MDA-MB-231 breast cancer cell line was used in the study. MDA-MB-231 cells were exposed to ginseng at 37°C and 5% CO_2 for varying durations (24 and 48 hours) and doses (1 and 2 µg/ml ginseng). At the end of the incubation period, viability, apoptosis, cell cycle and Argyrophilic nucleolar organizing region (AgNOR) protein status of MDA-MB-231 cells were examined in the Muse Cell Analyzer.

Results: It was observed that the dose inducing apoptosis was 1 µg/ml ginseng for 24 and 48 hours, and 2 µg/ml ginseng for 48 hours in the group that stopped the cell cycle in the G0/G1 phase. When comparing the two groups; while no difference was determined between the control and 1 µg/ml ginseng groups, the significant differences were detected between the control and 2 µg/ml ginseng groups for mean AgNOR number in 48 hours incubation. However, there was no significant difference for the TAA/NA ratio, in the groups for 48 hours.

Conclusion: The current study showed that ginseng had a crucial function against cancer development. Also, both AgNOR values might be used as biomarkers for detection of the most reliable therapeutic dose selection for cancer and it has been shown that correct consumption of Ginseng can be effective in preventing cancer formation and slowing its progression.

Keywords: Ginseng, MDA-MB-231, AgNOR

INTRODUCTION

Among the cancer types, breast cancer is the most common type of cancer in women, and it is the most common cause cancer death in all societies after developed western countries. It is a serious disease that affects morbidity and mortality all over the world. For this reason, components that are effective in cancer treatment or prevention have begun to be investigated. Bioactive substances obtained from different parts of plants have been studied in research on cancer. The effects of these substances are supported by epidemiological data. The ginseng plant is a large, tap-rooted, non-seasonal fringed herb. The diameter of the roots of 6-8 year-old ginseng is 10 centimeters, and they are red or white in color. Since the aged, plump, and branched fringes of the plant are used in the preparation of the active substance, it is cultivated in countries such as America, Japan, Korea, and China, as well as growing wild in nature. In vivo and in vitro studies have shown that ginseng affects many steps by stimulating apoptosis in cancer cells and stopping the cell cycle (1,2).

CITATION

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Traditional herbal medicines, which have been used for years, provide an advantage in maintaining a balanced state of health (3). In addition, the protective effect of ginseng (Panax quinquefolius) in cancer chemoprevention has been demonstrated by preclinical studies and extensive laboratory works. Ginseng is chemoprophylactic and generally acts on molecular and cellular targets through various signaling pathways, thus inhibiting tumors through cell cycle regulation, apoptosis induction, angiogenesis and inhibition of proliferation. The anticancer effects of ginseng are thought to be effective in the modulation of various signaling pathways, including the regulation of growth factors, cell proliferation mediators and tumor suppressors. AgNOR is the nucleolus organized on acrocentric chromosomes. It is the name given to proteins that interact with NORs and are stained with silver because of their argyrophilic (silver-loving) properties. NOR regions within the nucleolus of human cells to which AgNOR proteins are bound during interphase represent transcriptionally active ribosomal RNA synthesis regions, also called rDNA. Therefore, cell proliferation rate can be indirectly evaluated by determining AgNOR parameters (4,5).

In recent years, a number of epidemiological studies have indicated that ginseng consumption affects the incidence of cancer (6,7). In this study we showed to represent any possible effects of ginseng treatment on the NOR protein synthesis and apopitotic effect on the MDA-MB-231 cancer cell line.

MATERIAL AND METHOD

Preparation of Ginseng (Panax quinquefolius)

Ginseng (Panax quinquefolius: Gnc Herbal Plus®) used in the study was obtained from a certified compant. The aqueous extract was obtained by infusing 3 g in 100 ml of water at 85° C for 5 minutes, then centrifuged and the supernatant was sterilized with a 0.22 µm filter and taken into falcons to be used in cells.

Cell Culture

MDA-MB-231 cells were derived from American Type Culture Collection (Manassas, VA, USA). The MDA-MB-231 human breast cancer cells were cultured Dulbecco's modified Eagles medium (DMEM Capricorn Scientific, CP21-4310) including streptomycin/penicillin (100U/ml; Sigma Life Science,046M4846V) and 15% fetal calf serum (FCS) (Biowest, S181G-500) in a humidified (Sanyo, MCO-19 A/C(UV)) atmosphere of 5% CO_2 air at 37°C.Then healthy MDA-MB-231cells were divided into control and experimental groups (1 and 2 µm/ml). 24 well culture plate including 1000 µL of fresh medium with 1×105 MDA-MB-231 cells were used to find optimum Ginseng dose. After the medium was removed and MDA-MB-231 cells were rinsed with 500 µl phosphate-buffered saline (PBS) 3 times. The experimental groups were generated as log concentrations of Ginseng (1 and 2 µm/ml) on breast cancer cells.

Cell Viability Assay and Proliferation

The number of cells per ml of cell suspension was determined with Trypan Blue cell counting method. For cell counting an amount of the cell suspension was taken into the eppendorf tube and Trypan Blue solution was added in the same proportion as the amount taken from the cell. After 5 minutes of incubation, the coverslip was transferred to both sides of the closed Thoma slide (Marienfeld-Superior). Stained and unstained cells were noted by counting with a microscope (Nikon Eclipse TS100).

Experimental Design

Annexin V for 24 and 48 hour incubations and control, 1 and 2 µg/ml Ginseng groups for cell cycle testing were formed.

Annexin V Assay

Muse Cell Analyzer device and compatible Muse Annexin V kit and dead cell assay reagent were used for apoptosis analysis (Millipore; MCH100115).

MDA-MB-231 cell line was cultivated in 24-well plates with $1x10^5$ cells per well and left to incubate for 24 and 48 hours. Then, cells were treated with trypsin and stained with dead cell reagent and Annexin V according to the manufacturer's protocols and analyzed using Muse Cell Analyzer (Millipore Corporation).

Cell Cycle Assay

MuseR Cell Cycle Kit were used for detect the cell cycle stage of cells (Millipore; MCH100106). MDA-MB-231 cells were cultivated in 24-well plates with $1x10^6$ cells per well and left to incubate for 24 and 48 hours. Later, cells were treated by removing with trypsin. After, the cells were stained with MuseR Cell Cycle Kit according to the manufacturer's protocols (Millipore Corporation) and analyzed using Muse Cell Analyzer (Millipore Corporation, MCH100106).

AgNOR Staining

As a result of the experiment for AgNOR staining, MDA-MB-231 cells cultured with 1 and 2 µg/ml ginseng were spread on a slide and dried at room temperature for approximately 30 minutes. The silver staining solution obtained from 50% $AgNO_3$ and gelatinous formic acid mixture was dripped 3-4 drops on the preparations with a staining pipette and covered with a coverslip. Then, the lid of the petri dish was quickly closed, wrapped with aluminum foil in such a way that it would not get any light, and left in an oven at 37°C for 15 minutes. At the end of the 15th minute, the preparations that were removed from the oven were washed with distilled water until the coverslips fell off. Photographs of the preparations covered with Entellan were taken under a light microscope (Leica DM 3000) at a magnification of 100 (Imaging Color 12 BIT, Made in Canada). Analyzes were performed in the Image J program (ImageJ version 1.47t, National Institutes of Health, Bethesda, Maryland, USA). By evaluating cell

nuclei, both the total AgNOR area (TAA/NA) and the average AgNOR number per nuclear area were calculated using the "freehand selections" tool.

Statistical Analysis

The distribution of the data in this study was evaluated with ShapiroWilk's test statistics, histogram, and q-q graph. One-way analysis of variance was used for comparison between groups. The homogeneity of variance was evaluated with Levene's test. Tukey was used as a multiple comparison test. For each group, the differences between the 24-hour and 48-hour measurements were performed using a paired t-test. Analysis of the data was analyzed by statistical programme (Turcosa Analytics Ltd Co, Turkey, www.turcosa.com.tr) data software. P<0.05 was considered significant.

RESULTS

Annexin V findings

The percentages of total apoptotic cells after 24 and 48 hours of incubation of MDA-MB-231 cells are demonstrated in Figure 1 and Table 1.

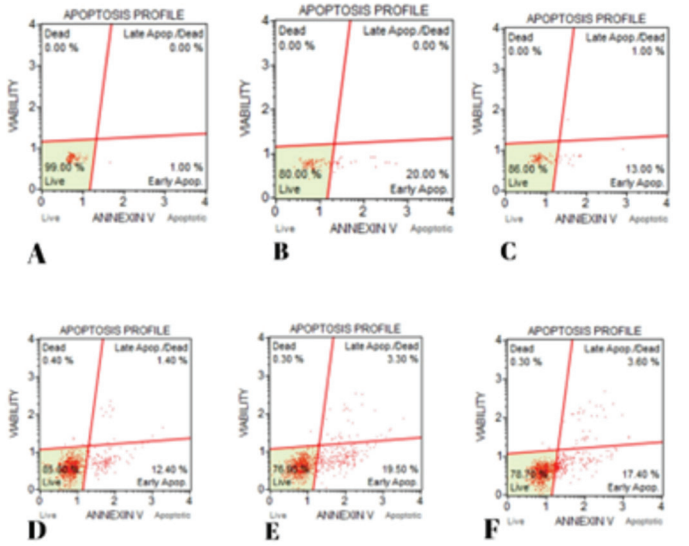

Figure 1. Annexin V test results (A) 24-hour control. (B) 1 mg/ml ginseng for 24 hours. (C) 2 mg/ml ginseng for 24 hours. (D) 48 hour control. (E) 1 µg/ml ginseng for 48 hours. (F) 48 hours 2 µg/ml ginseng

Table 1. Total apoptotic cell results after 24 and 48 hours in Annexin V & Dead cell test

Groups	Total Apoptotic Cell 24 hours	Total Apoptotic Cell 48 hours
Control	1.0±0.10[a]	13.8±2.8[a]
1 µg/ml ginseng	20.0±5.0[b]	22.8±2.5[b]
2 µg/ml ginseng	14.0±1.20[b]	21.0±1.8[b]
P*	0.001	0.008

P*; One-way variant analysis, P*; According to the Paired t test, Multiple comparison test (Tukey), the fact that the alphabetic superscripts contain the same letter indicates that the difference between the groups is not significant, while the difference indicates statistical significance. Data were expressed as arithmetic mean and standard deviation

A statistically significant difference was found between the 24 and 48 hour measurements of total apoptotic cells in the control, 1 and 2 µg/ml ginseng groups (p<0.050). According to the multiple comparison test, the control group was found to be significantly lower than the 1 and 2 µg/ml ginseng groups in the measurement of total apoptotic cells at 24 and 48 hours (p<0.050) (Table 1).

While there was a statistically significant difference between live cell measurements at 24 and 48 hours in the control group (p<0.012), no significant difference was found in the 1 and 2 µg/ml ginseng groups (p>0.05). While there was a statistically significant difference between the live cell measurements of the control, 1 and 2 µg/ml ginseng groups at 24 hours (p<0.001), there was no significant difference in the 48 hour measurement (p=0.354). According to the multiple comparison test, the control group was found to be significantly higher than the 1 and 2 µg/ml ginseng groups in the 24-hour measurement of live cells (p<0.05).

Cell Cycle Findings

With this test, cell cycle findings after 24 and 48 hours of incubation of MDA-MB-231 cells are demonstrated in Figures 2 and 3 and Table 2-3.

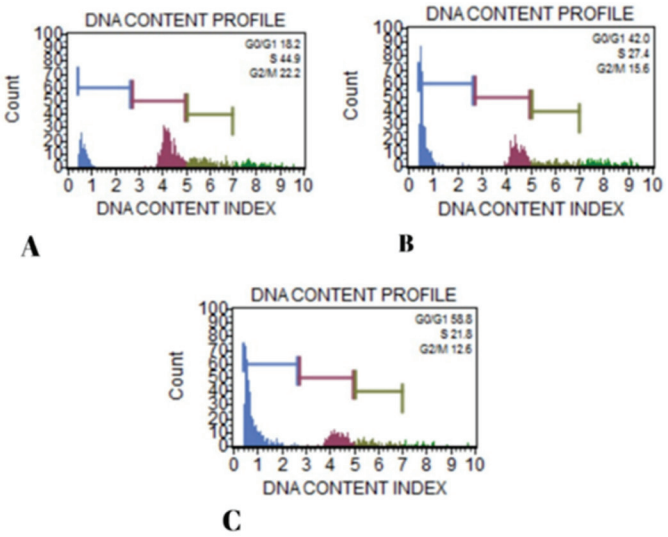

Figure 2. Cell Cycle test results (A) 24-hour control. (B) 1 µg/ml ginseng for 24 hours (C) 2 µg/ml ginseng for 24 hours

Table 2. G0/G1 phase change results at the end of 24 and 48 hours in the cell cycle test

Groups	G0/G1 24 hours	G0/G1 48 hours
Control	18.27±0.70[a]	76.7±12.4
1 µg/ml ginseng	42.0±6.0[b]	90.8±3.50
2 µg/ml ginseng	58.8±1.40[c]	92.2±6.40
P*	<0.001	0.113

P*; One-way analysis of variance, P*; According to the Paired t test, Multiple comparison test (Tukey), the fact that the alphabetic superscripts contain the same letter indicates that the difference between the groups is not significant, while the difference indicates statistical significance. Data were expressed as arithmetic mean and standard deviation

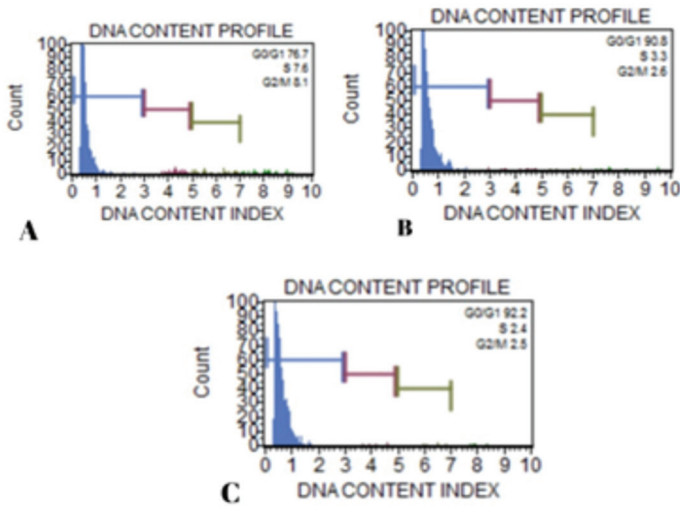

Figure 3. Cell Cycle test results (A) 48 hour control. (B) 48 hours 1 µg/ml ginseng (C) 48 hours 2 µg/ml ginseng

A statistically significant difference between the 24-hour measurements of the G0/G1 measurement of the control, 1 and 2 µg/ml ginseng groups ($p<0.001$), there was no significant difference in the 48-hour measurement ($p=0.113$). According to the multiple comparison test, G0/G1 measurement in 24-hour measurement Control group; It was found to be significantly lower than the 1 and 2 µg/ml ginseng groups ($p<0.05$) (Table 2).

While there was a statistically significant difference between the 24 hour measurements of the S measurement in the control and 1 µg/ml ginseng groups ($p<0.05$), there was no significant difference in control and 2 µg/ml ginseng groups ($p>0.05$) (Table 3). While there was a statistically significant difference between the 48 hour measurements of the S measurement of the 1 and 2 µg/ml ginseng groups ($p<0.01$). There was no significant difference was in the 24-hour measurement ($p=0.067$). According to the multiple comparison test, S measurement was found to be significantly higher than the control group, 1 and 2 µg/ml ginseng groups in 48 hours measurement ($p<0.05$) (Table 3).

Table 3. The results of the change in S phase at the end of 24 and 48 hours in the cell cycle test

Groups	S 24 hours	S 48 hours
Control	44.9±10.4	7.6±2.2[a]
1 µg/ml ginseng	27.4±5.2	3.3±1.2[b]
2 µg/ml ginseng	21.8±12.8	2.4±0.4[b]
P*	0.067	0.010

P*; One-way analysis of variance, P*; According to the Paired t test, Multiple comparison test (Tukey), the fact that the alphabetic superscripts contain the same letter indicates that the difference between the groups is not significant, while the difference indicates statistical significance. Data were expressed as arithmetic mean and standard deviation

AgNOR Staining Results

TAA/NA values and the mean AgNOR number are demonstrated in the Table 4 and 5. 24 hours incubation, the value was statistically significant in all ginseng groups compared to the control group ($p<0.05$). The mean 48 hours incubation AgNOR number was significantly in the 2 µg/ml ginseng groups compared to the control group ($p<0.05$). Additionally, 1 and 2 µg/ml ginseng groups statistically significant.

Table 4. Mean AgNOR number after 24 and 48 hours of incubation

Hours/Groups	Control	1 µg/ml	2 µg/ml	*p*
24 hours	4.18±1.17[a]	3.56±1.01[b]	3.36±0.94[bc]	<0.001
48 hours	3.96±1.26[ac]	3.46±0.97[c]	2.86±1.14[b]	<0.001

$p<0.05$ was considered statistically significant. Data are expressed as mean±SD (Standard deviation). There is no statistically significant difference between the groups containing the same letter ($p>0.05$). 1 and 2 µg/ml: ginseng groups. AgNOR: Argyrophilic nucleolar organizer region

Table 5. TAA/NA value at the end of 24 and 48 hours of incubation

Hours/Groups	Control	1 µg/ml	2 µg/ml	*p*
24 hours	0.06±0.02[a]	0.08±0.03[b]	0.06±0.02[a]	<0.001
48 hours	0.06±0.03	0.06±0.03	0.07±0.03	>0.001

$p<0.05$ was considered statistically significant. Data are expressed as mean±SD (Standard deviation). There is no statistically significant difference between the groups containing the same letter ($p> 0.05$); 1 and 2 µg/ml ginseng groups. TAA/NA: Total AgNOR area (TAA)/Total nuclear area (NA) ratio

When the data was evaluated for 24-hours incubation, TAA/NA value was statistically significant ($p<0.05$). In this data belonging to 48 hours incubation, these values were not statistically significant in ginseng groups compared to the control group ($p>0.05$).

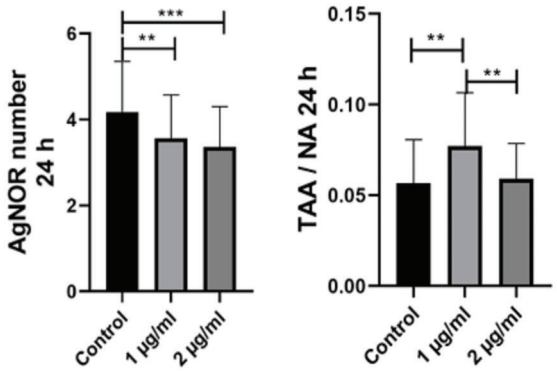

Figure 4. Comparison of AgNOR number and TAA/NA ratio between groups after 24 hours of incubation

DISCUSSION

Studies have shown that ginseng reduces the viability of the breast cancer cell line MDA-MB-231 and inhibits cell proliferation of MCF-7 cells in the dose range of 1 to 2.5 mg/mL (8). It was stated that prostate cells lost their adhesion ability after incubation with 250 μM ginsenoside for 48 hours, suppressing the expression of androgen receptor, prostate specific antigen, proliferating cell nuclear antigen expression and 5α-reductase biomarker genes. Ginsenoside has been shown by fluorescence microscopy to cause a change in the morphology of the cell undergoing apoptosis. As a result, it has been suggested that it arrests prostate cells in the G1 phase and then inhibits cell growth through the caspase-3-mediated apoptosis mechanism (9). It has been reported that ginseng reduces cell viability when administered alone in hepatocellular carcinoma cells depending on a dose of 0.64±0.02 mg/mL (10). In addition, when MCF-7 and MDA-MB-231 human breast cancer cells were treated with 0.5 mg/ml ginseng (Panax quinquefolius), it increased antiproliferative activity, decreased viable cell count, and kept cancer cells in the G phase of the cell cycle (11). In another study, they reported that 1.0 mg/ml ginseng inhibited proliferation in HCT116 human colon carcinoma cells and stopped the cell cycle in the G0/G1 phase. (12). Another study showed that components of Panax ginseng roots inhibited human renal cell carcinoma cells in the transition from G1 to S phase by blocking cell cycle progression (13). In a study in leukemia cells (K562), 400 mg/l ginseng polysaccharide was administered to K562 cells for 48 hours. It was determined that the G0/G1 phase increased significantly, and the G2+M and S phases of the cell cycle were significantly decreased. It has been reported that various concentrations (0-25-50 and 100 μM) of Rg5 (the active ingredient of ginseng) induce cell cycle stop in the G0/G1 phase through the regulation of cell cycle-related proteins in breast cancer cells (14). It was stated that GE treatment significantly inhibited the viability of each MCF-7 cells in a concentration-dependent manner in the presence of 5 μM Rh2, and also caused cell cycle stopping and inhibition of G1-S phase-specific enzyme activities with ginseng (15).

These results in the literature support the results that we showed in our study. In addition, it was observed that MDA-MB-231 human cancer cells treated with ginseng caused apoptosis and stop it in the G0/G1 phase of the cell cycle. Phytochemicals obtained from medicinal plants have been used in the treatment of human diseases from past to present. Identification of new phytochemical agents and determination of the most reliable dose for cancer therapy are crucial for improving the diagnostic accuracy and management of diseases. NORs are associated with most regulatory proteins and have roles as functional subunits of the nucleolus. Changes in AgNOR protein amounts reflect the metabolic activities and protein synthesis capacities of cells. There are different studies on malignant and benign lesions (5).

In our study, results were obtained with 1 and 2 μg/ml doses of ginseng and over two times as 24, 48 hours, the effective dose that kills breast cancer cells and leads to apoptosis is 1 μg/ml for 24 and 48 hours, also, in the group that stopped in the G0/G1 phase, there was 2 μg/ml ginseng group for 48 hours. Having common points with other studies, it has been seen that Ginseng plant, which has not yet been fully clarified in the literature, can lead to early apoptosis of breast cancer cells and increase total apoptosis. In this study, it was seen that ginseng has a very important function against the development of cancer. However, both AgNOR values can be used as biomarkers in determining the most reliable therapeutic dose selection for cancer, and it has been shown that the correct consumption of Ginseng can be effective in preventing cancer formation and slowing its progression.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *The article does not require ethics committee permission.*

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