Inhabitation Effect of Linoleic Acid, the Ingredient of Nigella sativa (Black Seed) on MDA–MB–231 and MCF–7 Human Breast Cancer Cells

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Abstract

Objective: The objective of this study was to investigate inhibition and anti–cancer effects of Linoleic acid on the MCF–7 and MDA–MB–231 human breast cancer cells.

Materials and methods: Cell lines Human breast cancer MCF–7 (GDC055) and MDA–MB–231 (HTB–26) cell lines were obtained from ATCC. MCF–7 estrogen receptor positive human breast cancer cell line and the estrogen receptor negative human breast cancer cell line MDA–MB–231, were grown in DMEM. MDA–MB–231 and MCF–7 human breast cancer cell lines were observed. For each experiment, seven doses were considered diluting from the highest to the lowest doses by half, respectively. MTS apoptosis and cytotoxic activity assay were used in order to find toxic effects, and the results were supported by flow cytometry (Cell cycle analysis).

Results: The results showed the cytotoxic effect of Linoleic acid on the breast cancer cell lines that can be posed as an anti–cancer effect of lionleic acid. According to our findings, when the concentration of lionleic acid was increased, compared with the concentrations currently being reported, it shows anti–cancer effects.

Conclusion: It was concluded that Linoleic acid has an inhibiting effect on human breast cancer cell lines which can be due to its two double–bandings molecular structure.

Keywords: Linoleic acid, Nigella sativa, MDA–MB–231, MCF–7

Introduction

Overweight women are most commonly observed to be at increased risk of postmenopausal breast cancer and at reduced risk of pre–menopausal breast cancer. Obesity and a high intake of meat, dairy products, fat, and alcohol may increase the risk and a high intake of fiber, fruits, vegetables, anti–oxidants, and phytoestrogens may reduce the risk. Considering the hazards of treatment failure, drug resistance, heavy costs and other problems associated with current cancer therapy, medicinal plants have attracted interest of many reseachers in this field.

The use of the medicinal herbs for curing disease has been practiced in the history of all civilizations including ancient Egyptian, ancient Chinese, Indian Ayurvedic and Unani medicine (1).
Fatty acids have previously been shown to modulate eicosanoid metabolism both in vivo and in vitro (2, 3). Epidemiological and experimental studies have revealed an association between dietary fat and the incidence of breast cancer (4, 5).

CLA has been shown to have an inhibitory effect on MCF–7 cells (6), whereas it has been indicated to have low efficacy on MDA–MB–231 proliferating activity (7). According literature CLA inhibits cancer cell proliferation in different manners according to the cell line and experimental model used (8, 9). The cell apoptosis induction is activated by CLA through the mitochondrial pathway and probably mediated in part by reduced ERK activation, suggesting a crosstalk between the two pathways (10).

In a study, it was founded; omega 6 family LA did not have any effect (11, 12). CLA has been shown to inhibit the initiation and promotion stages of chemical–induced mammary carcinogenesis in animal models (13, 14) and to exert an inhibitory activity on human breast cancer cell growth in vitro (15, 16). The objective of this study was to investigate inhibition and anti–cancer effects of Linoleic acid on the MCF–7 and MDA–MB–231 human breast cancer cells.

Materials and Methods

Cell lines Human breast cancer MCF–7 (GDC055) and MDA–MB–231 (HTB–26) cell lines were obtained from ATCC.

DMEM Dulbecco’s Modified Eagle’s Medium–high glucose (tissue culture medium), (SIGMA, CHEMICAL. Co. Louis, MO, USA), Fetal bovine serum Mycoplex (FBS), (PAA Laboratories GmbH); Penicillin/Streptomycin (100X) (PAA Laboratories GmbH); Phosphate Buffered Saline (PBS), (SIGMA CHEMICAL Co. Louis, MO, USA), RNase A (Novagen, WI, USA), Propidium Iodide (Novagen, WI, USA), Trypsin–EDTA (1X), (PAA Laboratories GmbH); Dimethyl sulfoxide minimum 99.5% GC (DMSO), (SIGMA, Louis, MO, USA); CellTiter 96® AQueous One Solution Reagent, tetrazolium compound [3–(4,5–dimethylthiazol–2–yl)]–5–(3–carboxyoxymethylphenyl)–2–(4–sulfophenyl)–2H–tetrazolium, inner salt; LINOLEIC ACID 99% (ALDRICH, Louis, MO, USA); MTS, (Promega, Madison, WI, USA), RNase A (Novagen, WI, USA), Propidium Iodide (Novagen, WI, USA) were purchased from Sigma (St. Louis, MO ).

Cell culture

MCF–7 estrogen receptor positive human breast cancer cell line and the estrogen receptor negative human breast cancer cell line MDA–MB–231, were grown in DMEM with 10% bovine calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37–C in a 5% CO₂ atmosphere. Prior to initiating the experiments, cells were washed with phosphate–buffered saline (PBS) twice and placed in DMEM with 0.5% fetal bovine serum (FBS).

Treatment procedure

All experiments had 4 replications and were repeated for 4 times, observing for both MDA–MB–231 and MCF–7 human breast cancer cell lines. For each experiment, seven doses were considered diluting from the highest to the lowest doses by half, respectively. In each experiment, one control group was considered as well, which was only provided with growth media and had no treatment at all. The highest concentration of linoleic acid was 180 µl/ml which was diluted to 90, 45, 22.5, 11.75, 5.625, and 2.812 µl/ml by half, respectively, as treatment groups. Control group was without any treatment.

Treatment groups and the control group were in one 96–well plate at quite the same condition. Cells were seeded in 96–well assay plate at a density of 1x 10³ cells/cm² and allowed to adhere overnight. Prior to initiating the experiments, cells were washed with phosphate–buffered saline (PBS) twice.

For treatments, cells placed in DMEM with 0.5% fetal bovine serum (FBS) containing LA and supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. 18 mg of linoleic acid 99% were weighed by digital scale (Shimazu model, Japan), and solved in 1 ml DMSO. The first row (A row) of 96–well plate was highest concentration (180 µl/ml) of treatment and diluted until lowest concentration at 7th row (G row) by half respectively; last row (H row), containing growth media, without any treatment, was considered as a control group for 24, 48, and 72 hours.

Cell viability determination (MTS assay) Growth–Inhibition Assay

After treatment periods 24, 48, and 72 hours, Cell viability was assayed using CellTiter 96 Aqueous One Solution® (Promega, Charbonnieres, France) following the manufacturer instructions. 20µl of CellTiter 96® AQueous One Solution Reagent was added to each well of the 96–well assay plate containing the samples in 100µl of culture medium containing treatment solutions for treatment groups and control groups. Multichannel pipettes were used for convenient delivery of uniform volumes of CellTiter 96® AQue-
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ous One Solution Reagent to the 96–well plate. The plates were incubated at 37°C in incubator, 5% CO₂ for 4 hours. The absorbance was recorded at 490 nm using a 96–well plate reader (Bio–Rad, Richmond, CA, USA). The Reed–Muench Method was employed for counting IC₅₀ (17).

Flow cytometry

Flow cytometer was used to analyze the distribution of cell in different cell cycle phases. Flow cytometry is a method used to measure and identify cells and micro particles.

The most commonly used dye for DNA content or cell cycle analysis is Propidium Iodide (PI). It can be used to stain whole cells or isolated nuclei. The PI interactions into the major groove of double stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600nm. Stained cells with one copy of their genetic material (a haploid cell) will be half as bright as cells with two copies (a diploid cell).

Viable, apoptotic (cells present at subG₀/G₁ peak) or necrotic (cells with DNA content below subG₀/G₁) cells and percentages of cells in different phases of the cell cycle were evaluated by determining the DNA content after Propidium Iodide staining. The cells were suspended and counted at a density of 2×10⁵ cells/cm² into T–25 flask, allowed to adhere overnight. The cells were treated with various concentrations of linoleic acid and incubated for 24 hours, 48 hs and 72hs. After treatments periods, cells were harvested with 1 ml of Trypsin – EDTA, washed in PBS and centrifuged at 1200 rpm for10 min. The pellets were fixed in 500 µl of cold PBS and were slowly added with 5 ml of cold 70% ethanol in –20°C for 2 hours. The pellets were centrifuged at 1200 rpm for 10 min, and the supernatant was carefully discarded (to wash out the ethanol). The pellets were re–suspended in 10 µl RNase + 40 µl PI + 950 µl PBS. The final volume was 1 ml. Finally, the cells were incubated for 30 min on ice in the dark. The PI and RNase concentrations and volumes were referred Ormerod, 1992 (18) and then analyzed with a flow cytometer (CyanADP, Dako, Denmark) equipped with a 488 nm argon laser. Data were recorded on a Macintosh computer (G3), using software–Summit V4.3 (Dako, Denmark).

Statistical analysis

Statistical analyses were performed by one–way analysis of variance (ANOVA), Tukey’s multiple comparison and Student’s t–test using SPSS version 15.0.

P<0.05 was considered as statistically significant different.

Results

Growth–Inhibition and Assay Effect of linoleic acid

MDA–MB–231: The results of effect of linoleic acid, the main fatty acid of N. sativa seed oil showed that highest concentration of linoleic acid was 180 µl/ml which was diluted to 90, 45, 22.5, 11.75, 5.625, and 2.812 µl/ml by half, respectively, as treatment groups. Control group was without any treatment. Based on the results, Linoleic acid showed a promoting effect on the growth of cells from the concentrations of 11.75 µl/ml until 45 µl/ml. It is in agreement with the previous studies, about linoleic acid effect on the cell studies, but when the concentration got increased to more than 45 µl/ml linoleic acid showed inhibiting effect on the growth of cells. The results of cell viability are given in table 1. Based on the statistical results, there is a significant effect between the treatment groups which are more than IC₅₀ and control group. The IC₅₀ was between 45 µl/ml and 90 µl/ml. It was 84.72 µl/ml. In other words, linoleic acid showed an anti–cancer effect on the MDA–MB–231 human breast cancer cell line.

MCF–7: The highest concentration of linoleic acid was 180 µl/ml which was diluted to 90, 45, 22.5, 11.75, 5.625, and 2.812 µl/ml by half, respectively, as treatment groups. Control group was without any treatment. The growth–inhibiting effect of LA was observed after the 5.625 µl/ml concentration which is a low concentration. So it can be demonstrated LA showed a strong growth–inhibition effect on MCF–7 cell line. The statistical results showed a significant (P<0.05) cytotoxic effect on MCF–7 cell line compared with control group.

Table 1: Percentage of cell viability in different concentrations of LA on MDA–MB–231

<table>
<thead>
<tr>
<th>Concentration (µl/ml)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.00</td>
<td>100</td>
</tr>
<tr>
<td>Lowest 2.812</td>
<td>92.43382</td>
</tr>
<tr>
<td>5.625</td>
<td>84.6801</td>
</tr>
<tr>
<td>11.75</td>
<td>103.1588</td>
</tr>
<tr>
<td>22.5</td>
<td>111.1751</td>
</tr>
<tr>
<td>45</td>
<td>102.7757</td>
</tr>
<tr>
<td>90</td>
<td>43.66895</td>
</tr>
<tr>
<td>Highest 180</td>
<td>18.76808</td>
</tr>
</tbody>
</table>

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The Reed–Muench Method was employed for counting IC$_{50}$. According to the results, the IC$_{50}$ was 14.63 µl/ml which is a very low concentration compared with the amount of IC$_{50}$ of LA on MDA–MB–231 cell line. It can be mentioned that LA is more efficacious on MCF–7 compared with MDA–MB–231.

**Influence of Linoleic acid on Cell–Cycle Distribution and Apoptosis**

The results obtained in the cell cycle analysis of Linoleic acid shows considerable increase in apoptosis phase of both the cells lines. The changes in the cell cycle distribution of MCF–7 and MDA–MB–231 treated with linoleic acid at different incubation hours and different concentration and control group which caused 50% cell at each time point were illustrated in Figures 1–3. From the fluorescent intensity distribution obtained from experiment (Figure 1–3), it was shown that the first large peak represents cells in G0/G1 Phase (resting or protein synthesis phase) while the second phase represents the G2 phase (construction of mitotic apparatus phase). In the profile of normal cells, the G2 peak has twice the amount of DNA which gives twice fluorescent intensity.

The area between the two G0/G1 and G2 peak belongs to S phase (DNA synthesis phase). When the cells undergo apoptosis, sub cellular of the cells will appear before G0/G1 peak and was referred as Sub–G0/G1 phase.

**MCF–7:** cells were analyzed at 24, 48 and 72 hours post treatment. The reduction in the percentage proliferation of cells was due to higher percentage of cells have entered into Sub G1 at different doses as shown in figure 4–6, which showed the apoptotic rate after 24, 48 and 72 hours treatment on MCF–7. Based on the results there is a significant ($P<0.05$) increase in the apoptotic phase (Sub G1) with 5 fold at 24 hours and more than 15 fold increase at 48 and 72 hours, when compared to the control peak at respective time point.

The highest percentage of cells undergoes apoptosis were observed in LA treatment, where more than 16%, 19% and 50% cells were undergone apoptosis at 24, 48, and 72 hours time points respectively. The highest amounts of cell apoptotic were observed in medium concentration which were shown very significant ($P<0.05$) increasing cell apoptotic in treatment groups compared with control group.
Discussion

Apoptosis pathways and targeting cell cycle has emerged as an attractive approach for the treatment of cancer. The apoptosis effect of linoleic acid (19–21) as well as conjugated linoleic acid (22–25) was well documented. Apoptosis can be modulated by targeting pro-apoptotic or pro-survival pathways. It is known that high concentration of certain fatty acids can cause cell death via apoptosis or necrosis (26). The results obtained in the cell cycle analysis of Linoleic acid shows considerable increase in apoptosis phase of both the cells lines.

The reduction in the percentage proliferation of MDA–MB–231 cells was due to higher percentage of cells have entered into Sub G1 at different concentration of LA as shown in figure 6, which showed the apoptotic rate after 24, 48 and 72 hours treatment on MDA–MB–231.

Newly founded points in this study indicate that LA reveal much acceptable cytotoxic effects, while in the previous studies these effects were only related to conjugate LA; and some studies had mentioned that LA leads to promotion or LA has no any effect on the growth of cell line (27). According to previous studies different kinds of conjunct LA have anti–cancer effects (28–34) and the LA itself hadn’t shown a noticeable anticancer effect. Based on our findings, however, when the LA concentration was increased compared to the former concentration, it showed a good anti–cancer effect. This was quite a new point not being observed in the former studies.

In this study with MDA–MB–231 and MCF–7 reduced viability in dose–dependent and time–dependent manners as well.

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