

Cytotoxic Effects of Conjugated Linoleic Acids on Human Breast Cancer Cells (MCF7)

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Abstract: Conjugated linoleic acids (CLAs) comprised of geometrical and positional isomers of octadecadienoic (18:2) acid with conjugated double bond system. They are found naturally in ruminant food products such as meat and milk because of the process of bacterial biohydrogenation and hydrolysis in the rumen. Studies have shown that CLAs have many health promoting properties. This study was conducted to assess and compare the cytotoxic effects, induction of apoptosis and cell cycle arrest by CLA isomers on human breast cancer cells (MCF7). Cells were grown on RPMI 1640 media and treated with different concentrations of *cis*-9, *trans*-11 (*c*9, *t*11), *trans*-10, *cis*-12 (*t*10, *c*12) and mixed isomers of CLA for 72 hours. The results were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay and terminal deoxynucleotide transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay. The viability of MCF7 cancer cells was reduced significantly ($P < 0.05$) by all CLA isomers used in a dose-dependent manner. The median inhibitory concentration (IC_{50}) value varies with type of CLA isomer. *t*10, *c*12 was significantly ($P < 0.05$) more potent than *c*9, *t*11 CLA isomer. All CLA isomers induced significantly ($P < 0.05$) higher proportion of apoptotic cells. It was also observed that significantly ($P < 0.05$) higher proportion of cells in $G_{0/1}$ but lower proportion in S and G_2/M phases of the cell cycle in treated cells. These results suggested that CLA isomers reduce the viability of MCF7 cancer cells which is associated with cell cycle arrest and induction of apoptosis.

Key words: Apoptosis • Breast Cancer Cells (MCF7) • Cell Cycle • Conjugated Linoleic Acids • Cytotoxicity

INTRODUCTION

Breast cancer is the most common and the second leading cause of cancer death among women. Annually, more than 1 million cases and half million deaths are recorded worldwide [1-3]. Age, family history, reproductive abnormalities, exogenous hormones (contraceptives or hormone replacement therapy) and geographical locations are some of the risk factors [3, 4]. On the other hand, some dietary components are known to suppress tumour development. Identification and characterization of those nutrient components could be important in devising nutritional preventive or adjunct therapies of breast cancer. It has been reported from *in vitro* and *in vivo* studies that CLAs have various health promoting effects including anticarcinogenic, antiatherogenic, immuno-modulating and lean body mass promotion [5-7]. They are isomers of octadecadienoic (18:2) acid with conjugated double bonds. These double

bonds are located at carbon atoms 7 and 9, 8 and 10, 9 and 11 or 10 and 12, or 11 and 13 with all possible *cis*(*c*) and *trans*(*t*) combinations [6]. Of these, the *c*9, *t*11 isomer makes up to 90% of the total CLA in meat and dairy products of ruminants [6, 8]. The next isomer which is commonly encountered is *t*10, *c*12 CLA [9, 10]. They are predominantly found in ruminant food products such as meat and milk as the result of microbial fermentation in the rumen [6]. The amount of CLA in ruminant meat and milk products has been reported to be varied from 1.2-22.10 mg/g of fat [11, 12].

There are published reports about antiproliferative effect of CLA isomers on human breast cancer cells [13, 14]. However, the information available about effect of pure isomers especially *c*9,*t*11 and *t*10,*c*12 on breast cancer cells (MCF7) is still limited. At present, the most active isomer (s) of CLA hasn't been identified. In addition, the mechanism of action of CLA isomers is not well established. One of the proposed mechanisms is

induction of apoptosis [15, 16]. Induction of apoptosis is a preferred mode of killing cancer cells and it is an indicator of effectiveness and safety of anticancer agent [17]. Confirmation of induction of apoptosis by an anticancer agent will give an insight on the importance of the agent in fighting cancer. Another feature which is often observed in cancer cells that leads them to proliferate abnormally is defect in cell cycle machinery. The cell cycle is controlled by changes in the intracellular concentration and activity of intracellular proteins (cyclins, cyclin-dependent kinases and anaphase promoting complex) [16, 18]. These proteins involved in DNA synthesis and mitosis, by acting on several check points in the cell cycle. These components, which are involved in the progression of cells from one to the next phase of the cell cycle, could also be important targets for anticancer agents [19]. Therefore, the objectives of the present study were to assess and compare the cytotoxic effects, the induction of apoptosis and cell cycle arrest by CLA isomers on human breast cancer cells (MCF7).

MATERIALS AND METHODS

Cell Culture: All chemicals for cell culture were purchased from Gibco® Invitrogen, Canada but breast cancer cells (MCF7) were obtained from American type culture collection. Cells were grown in RPMI 1640 media, containing 100 U/mL penicillin 100 µg/mL streptomycin and 10% foetal bovine serum. Cells were routinely maintained and subcultured in 25 cm² plastic flasks at 37°C in a humidified CO₂ incubator (RS Biotech Laboratory Equipment Limited, UK) with 95% air and 5% CO₂. Twenty five percent trypsin-EDTA was used to detach cells from the bottom of the flask.

MTT Assay: The cytotoxic effects of CLA isomers were assessed using MTT assay. Cells were seeded at density of 1x10⁴ cells per well in a 96 well plate. After overnight incubation, cells were treated with *c9*, *t11* (purity ≥96%), *t10*, *c12* (purity ≥98%) (Cayman Chemical Ltd, USA) and mixed (42% *c9*, *t11*, 44% *t10*, *c12*, 10% *c10*, *c12* and 4% others) (Sigma chemical Co., USA) CLA isomers and tamoxifen citrate (Sigma chemical Co., USA) at concentration of 5, 10, 20, 40 and 80 µg/mL. Serum free RPMI 1640 media was used to dilute and obtain the treatment concentrations. The cells were then incubated for 72 hours. After this incubation period, 10 µL of MTT labelling reagent (Invitrogen™ Limited, UK) was added into each well. The plates were then incubated again for 4 hours. After this incubation period, excess MTT reagent was aspirated and 50 µL of dimethyl sulphoxide

(Sigma chemical Co., USA) was added to each well and mixed thoroughly. The plate was then transferred to microplate reader (Opsys MR™, Dynex Magellan Biosciences Company, USA) and absorbance was recorded at 540 nm. Each treatment at different concentrations and the untreated control were in three wells and the experiment was repeated at least three times.

TUNEL Assay: TUNEL assay was conducted to confirm the induction of apoptosis by CLA isomers and assess the status of cells in cell cycle. The media containing about 2x10⁵ cells was transferred to a 25 cm² flask and incubated overnight. Then, cells were treated with CLA isomers and tamoxifen, and incubated for 72 hours. The concentration of the treatment was based on the IC₅₀ value from MTT assay. Then, cells were harvested and washed twice with PBS. Five mL of one percent (w/v) paraformaldehyde (Sigma chemical Co., USA) in PBS was added and placed on ice for 15 minutes. The cells were centrifuged at 1000 rpm for 10 minutes. The cells were washed and centrifuged twice by adding five mL of PBS. The cells were added to five mL of ice cold 70% (v/v) ethanol (Sigma chemical Co., USA). Then, the cells were kept for a minimum of 30 minutes on ice or in a -20°C freezer before the continuation of the next procedure.

Cells in ethanol were placed in 12 × 75 mm flow cytometry centrifuge tubes. The cell suspension was centrifuged at 1000 rpm for 10 minutes and ethanol was removed by aspiration. Each tube was resuspended with one mL of washing buffer (Invitrogen™ Limited, UK) and centrifuged for 10 minutes at 1000 rpm. This step was repeated twice. Cell pellets of each tube were resuspended with 50 µL of the DNA labelling solution (Invitrogen™ Limited, UK). The cells were incubated in the DNA labelling solution for 60 minutes at 37°C in a temperature controlled bath. Cells were shaken every 15 minutes to keep the cells in suspension. At the end of the incubation time, one mL of rinse buffer (Invitrogen™ Limited, UK) was added to each tube and centrifuged at 1000 rpm for 10 minutes. The cell rinsing was repeated once more by adding one mL of rinse buffer to each tube and centrifuged at same speed and time. Cell pellets were resuspended in 100 µL of antibody solution (Invitrogen™ Limited, UK) and left at room temperature for 30 minutes in dark. Half mL of propidium iodide RNase a staining buffer (Invitrogen™ Limited, UK) was added to each sample and left for 30 minutes at room temperature in dark. Finally, samples were analyzed by flow cytometry (DakoCytomation, Denmark) and histograms were analyzed by Summit V4.3 software. Each treatment was repeated at least three times.

Statistical Analysis: The data were expressed as mean \pm SEM (standard error of the mean) and differences among treated groups were assessed using one way analysis of variance followed by Duncan's multiple range test and $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity: Figure 1 shows the percentage viability of MCF7 cells in 96-well plate after the treatment with various concentrations of CLA isomers as compared to the untreated control. Cell viability was significantly ($P < 0.05$) reduced by all CLA isomers used in a dose-dependent manner. Significant ($P < 0.05$) reduction in cell viability was observed at a concentration as low as 10 $\mu\text{g/mL}$ for *t10, c12* isomer and 20 $\mu\text{g/mL}$ for *c9, t11* and mixed isomers of CLA. Comparison of the mean ($n=3$) median inhibitory concentration (IC_{50}) revealed that better cytotoxic effect was observed by *t10, c12* isomer with IC_{50} value of $16.60 \pm 1.43 \mu\text{g/mL}$, followed by mixed isomers with IC_{50} of $27.66 \pm 1.93 \mu\text{g/mL}$. The *c9, t11* CLA isomer reduced cell viability significantly ($P < 0.05$) with IC_{50} value of $36.33 \pm 3.72 \mu\text{g/mL}$ but its effect was significantly ($P < 0.05$) lower than *t10, c12* CLA isomer.

Apoptosis and Cell Cycle: The present study confirmed that CLA isomers have shown cytotoxicity effect on MCF7 cancer cells. However, their mechanisms of action remain to be investigated. In order to assess the induction of apoptosis by CLA isomers, TUNEL assay was conducted. The assay measures the fragmented DNA of the apoptotic cells by catalytically incorporating 5-bromo-2'-deoxyuridine 5'-triphosphate at 3'-OH DNA end using terminal deoxynucleotidyl transferase enzyme. The result was analyzed by flow cytometry which gives separated proportion of apoptotic and non-apoptotic cells. Figure 2 shows the mean ($n=3$) percentage of apoptotic cells; all treatments induced significantly ($P < 0.05$) higher proportion of apoptotic cells than untreated control. There was no significant difference between the treatments.

The method displayed in Figure 2, quantifies only apoptotic cells. It did not provide information about the proportion of cells in different phases of the cell cycle. To obtain this information, flow cytometric DNA content analysis was done. Figure 3 shows the mean ($n=3$) proportion of MCF7 cells at different phases of the cell cycle. Apoptotic cells appear in sub $\text{G}_{0/1}$ peak as a consequence of partial DNA loss. The proportion of

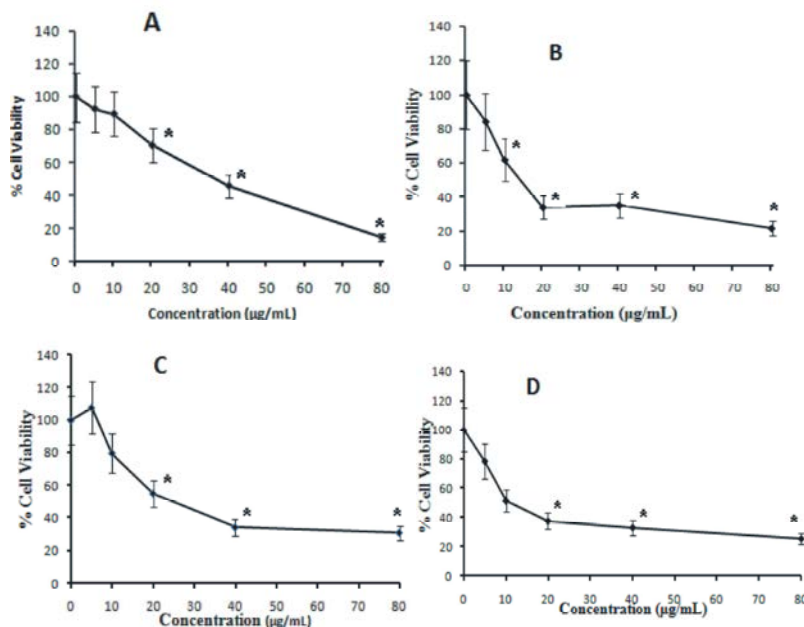


Fig. 1: Viability of MCF7 cancer cells following treatment with CLA isomers and tamoxifen for 72 hours. Percent (%) cell viability is expressed as mean ($n=3$) percentage of untreated control values. 'A' shows percent viability of cells treated with *c9, t11* CLA isomer; 'B' shows percent viability of cells treated with *t10, c12* CLA isomer; 'C' shows percent viability of cells treated with mixed CLA isomers; 'D' shows percent viability of cells treated with tamoxifen. Significant ($P < 0.05$) reduction in cell viability was observed at a concentration as low as 10 $\mu\text{g/mL}$ for *t10, c12* isomer (B) and tamoxifen (D) and 20 $\mu\text{g/mL}$ for *c9, t11* (A) and mixed (C) isomers of CLA as indicated by asterisks (*).

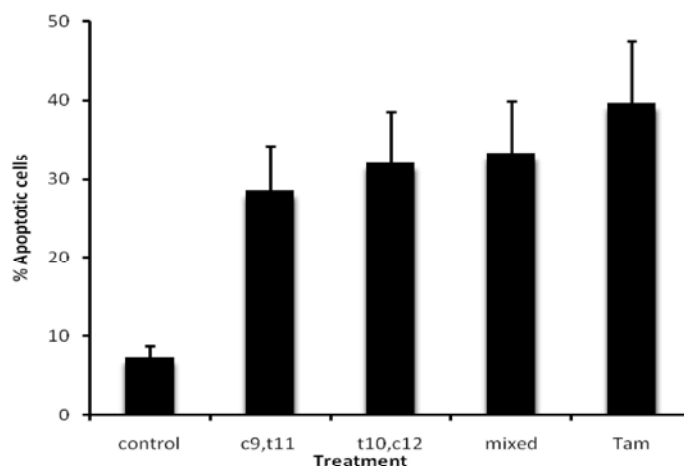


Fig. 2: Percentage of apoptotic MCF7 cells following treatment with CLA isomers and tamoxifen (Tam). Cells were treated at concentration of the IC_{50} value of each treatment and incubated for 72 hours. Values (bars) are mean ($n=3$, \pm SEM). Significantly ($P<0.05$) higher proportion of apoptotic cells were observed in cells treated with *c9,t11*; *t10,c12* and mixed CLA isomers and Tam compared to the untreated control. There was no significant difference among treatment groups.

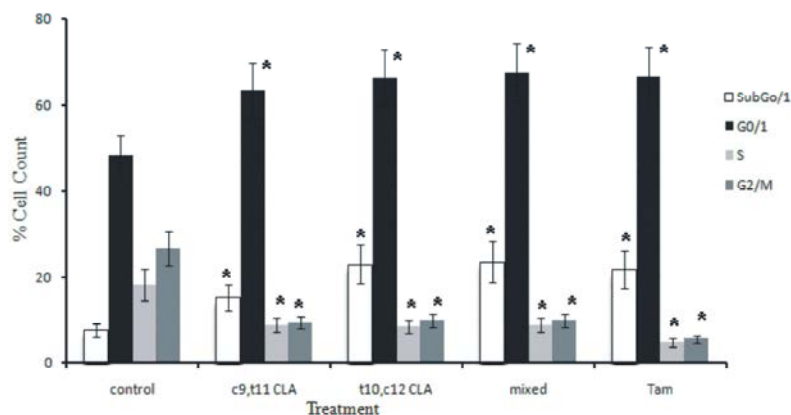


Fig 3: Cell cycle phase status of MCF7 cells following treatment with CLA isomers and tamoxifen (Tam). Cells were treated at concentration of the IC_{50} value of each CLA isomers and Tam and incubated for 72 hours. G_{0/1}, S and G_{2/M} are phases of the cell cycle and bars show the mean ($n=3$, \pm SEM) proportion of cells in each phase. The proportions of cells in subG_{0/1} (red) and G_{0/1} (blue) were significantly ($P<0.05$) higher in treated than untreated cells whereas the proportions of cells in S and G_{2/M} phases were lower in treated than untreated cells as is indicated by asterisks (*).

apoptotic cells (in subG_{0/1}) was significantly ($P<0.05$) higher in treated than untreated cells. Significantly ($P<0.05$) higher proportion of cells in G_{0/1} phase and lower proportion of cells in S and G_{2/M} phases in treated was observed. There was no significant difference in proportion of cells in all phase among treatments.

DISCUSSION

Conjugated linoleic acids are polyunsaturated fatty acids that have generated considerable interest recently

because of their significant anticancer effects on cell cultures and animal models. In the present study, the cytotoxic effect of CLA isomers on MCF7 breast cancer cells was investigated using MTT assay. In the assay, hydrogen acceptor reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was used as an indicator of cell viability. The MTT reagent is a yellow water-soluble dye that is reduced only by live cells to a purple formazan product that is insoluble in aqueous solvents. The amount of formazan produced depends on the amount of viable cells in each well and was determined

spectrophotometrically using plate-reader after the formazan is dissolved in a suitable solvent like dimethyl sulphoxide [22].

The MTT assay results showed that CLA isomers reduced cells viability in a dose-dependent manner. Dose dependent reduction in MCF7 cancer cell viability as a result of CLA treatment was also reported previously [14]. These authors [14] reported a dose dependent growth inhibition when MCF7 cells treated with mixed isomers at the range of 25-200 $\mu\text{mol/L}$ for two days.

Specific CLA isomers have been shown to possess different biological activities in a number of systems [14]. In the present study, cytotoxic effects of individual isomers were assessed. *t10, c12* CLA isomer showed significantly better inhibitory effect than *c9,t11* CLA isomer. Isomeric difference on cytotoxic effect of CLA isomers on MCF7 cell was also observed previously [23, 24] and suggested that the inhibitory pathway for CLA is different for each isomer.

Apoptosis plays an important role in the development and homeostasis of all multicellular organisms. Dysregulation of apoptosis could be the cause for induction pathological conditions like cancer. Thus, the modulation of apoptosis has become an interesting mechanism for both preventive and therapeutic approaches of cancer [25]. Cells undergoing apoptosis exhibit a series of characteristic morphological and biochemical changes that distinguish them from other mode of cell death (necrosis). The major morphological characteristics of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [26]. The apoptotic features in this study were assessed by using TUNEL assay. The assay was based on DNA fragmentation which is an important biochemical hallmark of apoptosis. DNA fragmentation yields large number of 3-OH group that serve as starting points for terminal deoxynucleotidyl transferase (TdT) enzymatic reaction [20, 21]. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate to the TdT reaction serves to label the break sites. Flow cytometric analysis showed the ability of CLA isomers to induce apoptosis on MCF7 breast cancer cells. These will provide evidences to elucidate the probable mechanism of action of CLA isomers in relation to the induction of apoptosis.

Induction of tumour cell death by cell cycle arrest is the most common mechanism of action of various anticancer agents [19]. Conjugated linoleic acid isomers elicited cell cycle arrest in $G_{0/1}$ as it was seen that proportion of MCF7 cells in $G_{0/1}$ phase of the cell cycle was significantly ($P < 0.05$) higher than that of the

untreated control. The induction of apoptosis and the cell cycle arrest at the $G_{0/1}$ phase on MCF7 cells by CLA isomers obtained in the present study were in agreement with the reports of Seo *et al.* [27]. Cell cycle arrest in G_1 , accumulation of tumour suppressors and reduction in factors required for transition from G_1 to S phases in MCF7 cells following CLA treatment were also reported previously [14].

CONCLUSIONS

In summary, the results suggested that CLA isomers reduce the viability of MCF7 cancer cells which is associated with cell cycle arrest and induction of apoptosis. This holds the promise that these fatty acids may play a role in the prevention and control of breast cancer. Future research should focus on understanding the molecular mechanism of action of individual isomers of CLA and their role in animal model and human carcinogenesis.

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