Egypt. J. of Appl. Sci., 35 (12) 2020 *220-228*

MOLECULAR BIOLOGICAL STUDIES ON TRANSCRIPTION FACTOR ESTROGEN-RECEPTOR IN BREAST CANCER TREATED WITH LECTIN

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Key Words: ConA lectin, Tamoxifen, MCF-7 cells, Antitumor

ABSTRACT

Proteins with legume lectin domains are known to possess a wide range of biological functions. The antitumor effects of Concanavalin-A (ConA) lectin and Tamoxifen (potent antagonist of estrogen and induces apoptosis in MCF-7 cells) on human breast carcinoma cells were investigated in vitro. Human breast carcinoma MCF-7 cells were examined, cell viability, proliferation and cytotoxicity of MCF cells treated with ConA and Tamoxifen using morphological analysis and MTT cytotoxicity assay. The effects of ConA compared to Tamoxifen on the expression of the transcription factor Erα and the expression of anti-apoptosis gene (Bcl2) in breast cancer MCF7 cell line were evaluated by RT-PCR. The microscopic examination of cells indicated that ConA induced significant cellular alterations typical of apoptosis cell-death in a comparable manner as Tamoxifen. ConA showed also proliferation inhibition activity, however it was lower than tamoxifen, as indicated by MTT growth inhibition assay. The IC50 of ConA is 7.55 µM and of Tamoxifen is 2.75 µM. The expression of Erα and Bcl2 proteins after treatment with ConA were significantly decreased. In conclusion: ConA induced significant apoptosis and inhibited the proliferation of breast cancer cells that may be through mechanism involve reduction in the expression of ERα and anti-apoptotic gene BCl-2. Finally, our results indicate that ConA exerts anti-tumor actions against human breast carcinoma MCF-7 cells in vitro and confer support for more research to unravel the potentials of using concanavalin-A as complementary treatment of ER+ breast cancer.

INTRODUCTION:

Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into surrounding tissues or spread to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too **(Girish *et al.* 2014).**

Transcription factors typically regulate gene expression by binding cis-acting regulatory elements defined as enhancers and by recruiting coactivators and RNA polymerase II (RNA Pol II) to target genes **(Ong and Corces, 2011; Lelli *et al.* 2012).** Enhancers are segments of DNA that are generally a few hundred base pairs in length and are typically occupied by multiple transcription factors **(Spitz and Furlong 2012).** As for breast cancer, MCF-7 cells represent a very important candidate as they are used ubiquitously in research for estrogen receptor (ER)-positive breast cancer cell experiments and many sub-clones, which have been established, represent different classes of ER-positive tumors with varying nuclear receptor expression levels **(Sweeney *et al*. 2013).**

Lectins can inhibit tumor genesis by binding to glycosylated proteins on the membrane of cancer cells. It is well known that glycoconjugates play important roles in many biological processes, including cancer, with malignant cells usually presenting altered glycosylation patterns **(Marques *et al*. 2017).** These changes in glycans allow preferential binding of lectin on cancer cells to induce the above effects **(Cavada *et al.* 2019).** Tamoxifen is a drug that has been in worldwide use for the treatment of estrogen receptor (ER)-positive breast cancer for over 30 years; it has been used in both the metastatic and adjuvant settings **(Lazzeroni *et al*. 2012).**

This study was aimed to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity of MCF cells treated with Concavalin-A (ConA) and compare its cytotoxic effects with the standard breast cancer chemotherapy Tamoxifen (TAM) by MTT cytotoxicity assay. Then, evaluation the effect of ConA lectin or Tamoxifen drug on breast cancer MCF7 cell line by Real Time PCR on the transcription factor protein ER-alpha and the anti-apoptotic BCl-2 gene expression.

MATERIALS AND METHODS

Chemicals

RPMI-1640, FBS, trypsin, penicillin, and streptomycin were purchased from Gibco (Invitrogen, Grand Island, NY). 3-(4,5-Dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, MO). Annexin V-FITC and PI double staining kit were purchased from BD (Waltham, MA). Tamoxifen (Sigma, St. Louis, MO) was the standard drugs used as positive control in this study.

Cell culture

Breast adenocarcinoma MCF-7 cells were cultured in RPMI1640 media containing 10% FBS, 100mg/mL streptomycin, 100 U/mL penicillin, and 0.03% L-glutamine and were maintained at 37 ℃ with 5% CO2 in a humidified atmosphere **(Zheng *et al.* 2014).**

MTT Cytotoxicity assay

The tetrazolium salt 3, (4.5-dimethyl-thiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay was used to evaluate antiproliferative capacity of Lectin towards MCF-7 cell line. Briefly, cells were seeded at 2×10^4 cell/well in 96-well tissue culture plates and allowed to attach for 24 hr. Cells were then exposed to variable concentrations at 10, 0.39, 1.56, 6.25, 25 and 100 µM of lectin or Tamoxifen (as positive control) dissolved in the RPMI medium for 24 hr. After incubation, plates were centrifuged (500g, 5 min) and the supernatant was removed, followed by an addition of MTT solution (0.5mg/mL in PBS) and incubation for 4 hr at 37◦C and 5% CO2. The medium was discarded, and the cells were then lysed in 10% SDS/HCl 0.01N overnight. Absorbance was measured in a microplate reader at 570 nm. The concentration that inhibits 50% of cell proliferation (IC50) was determined from plots of cell viability **(Faheina-Martins *et al*. 2011).**

Analysis of Apoptosis by Flow Cytometry Assay

Apoptosis detection was performed by FITC Annexin-V/PI kit (Becton Dickenson, Franklin Lakes, NJ, USA) following the manufacture's protocol. Briefly, cells were harvested in single cell suspension. Cells were incubated with Annexin V-FITC, USA before fixation since any cell membrane disruption can cause nonspecific binding of Annexin V to PS on the inner surface of the cell membrane). The cells were washed and fixed in 2% formaldehyde before analysis. The cytometer was set for appropriate FSC vs. SSC gates to exclude debris and cell aggregates. Collect annexin V fluorescence at FL-1 and Propidium iodide fluorescence at FL2.

Molecular gene expression analysis

Analysis of the expression of the anti-apoptosis BCL-2 as well as the expression of the transcription factor ERα gene were done using Real Time Polymerase Chain Reaction (RT-PCR) to identify the molecular effects of con A lectin or tamoxifen on MCF 7 cells. mRNA isolation is carried out using RNeasy extraction kit, up to $1x10^6$ cells, depending on the cell line, cells were disrupted in Buffer RLT. Ethanol is then was added to the cell's lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column. RNA was then eluted in RNase-free water. A specific primer used for detection of Bcl2, and ERα genes, primers pairs were designed (table 1), and acquired in lyophilized form (BioRad, USA).

Table 1: the sequences of the specific primers used in the project to amplify regions of Bcl2, and ERα genes:

Gene	Sequence (5'-3')	
Bcl2	Forward	AAGCCGGCGACGACTTCT
	Reverse	GGTGCCGGTTCAGGTACTCA
ERα	Forward	AATTCTGACAATCGACGCCAG
	Reverse	GTGCTTCAACATTCTCCCTCCTC
GAPDH	Forward	GAAGGTGAAGGTCGGAGTCA
	Reverse	TTGAGGTCAATGAAGGGGTC

PCR amplification was performed in 25uL 2X SYBR Green RT-PCR reaction mix, 1.5uL of each primer (10uM), 10uL of RNA template (1pg to 100ng total RNA), 11uL of Nuclease-free H2O and 1uL of iScriptT reverse trancriptase for one- step RT-PCR (total volume 50uL). The cycling conditions for detection of ERα and Bcl2 were done by Rotorgene Q RT- PCR system, Germany.

RESULTS

Morphological changes

MCF-7 cells were the epithelial-like and grow in monolayers. When grown in vitro, the cell line was capable of forming domes before plating (75% cells). MCF7 cells were grown, propagated and proliferated forming chains after plating (85% cells) for 24 h incubation. Figure 1 demonstrates the morphological changes of cells after treatment with ConA or Tamoxifen drug, which indicated that either treatments resulted in remarkable morphological changes typical of apoptosis and major proliferation inhibition, however TAM showed more prilferation inhibitory activity on cells than ConA.

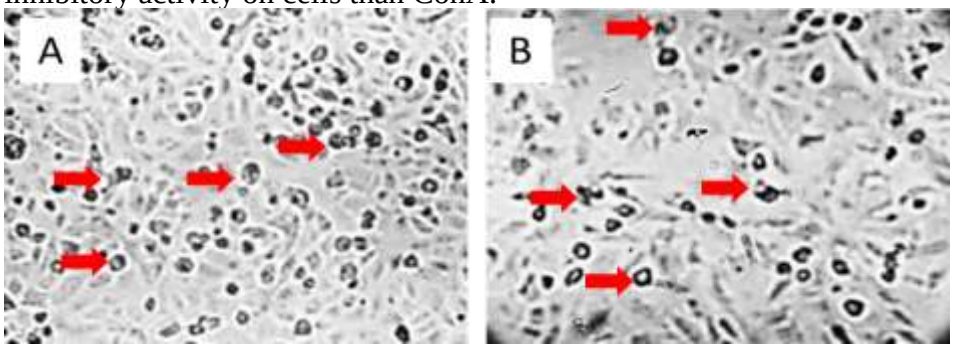

Figure 1: Microscopic examination of MCF-7 treated with lectin (A) and tamoxifen (B). Red arrows refer to apoptotic cancer cells.

Cytotoxic effect of ConA Lectin and Tamoxifen on MCF7 cells

Cells were incubated with different concentrations of ConA and Tamoxifen (0.39, 1.56, 6.25, 25, 100 uM) as shown in Figure 2. The MTT cytotoxicity results indicated that ConA has a less potent growth

inhibitory activity toward MCF-7 cells than Tamoxifen. The IC50 is the concentration of an inhibitor where the response is reduced by half ConA is 7 µM, and in Tamoxifen is 3 µM (figure 2).

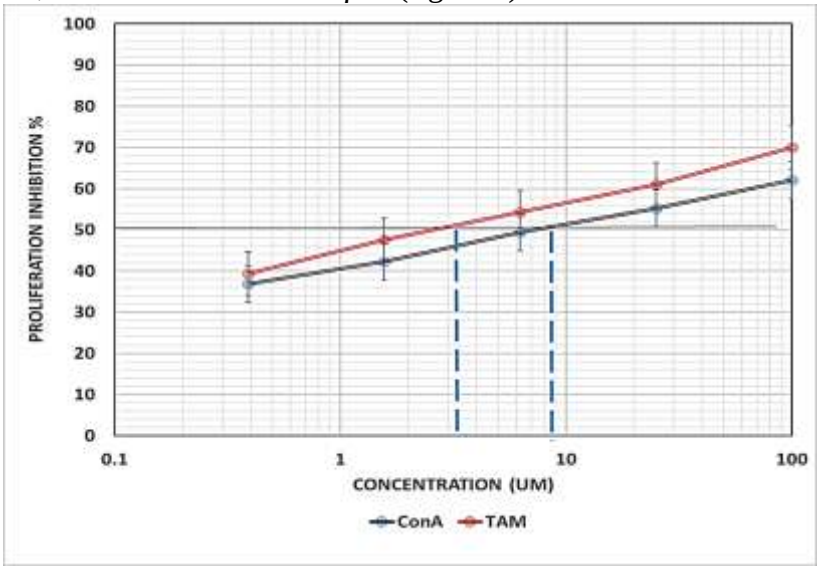

Figure 2: Inhibitory rate and calculation of IC50 of ConA and Tamoxifen. ConA IC50= 7 µM, TAM IC50= 3 µM. Data representative of mean of three independent experiments.

Genes Expressions

ER alpha was down regulated that inhibited the growth MCF7 Cells treated with conA and Tamoxifen (figure 3A). The anti-apoptosis gene BCl-2 expression was found decreased after the addition of ConA and Tamoxifen drug (figure 3B).

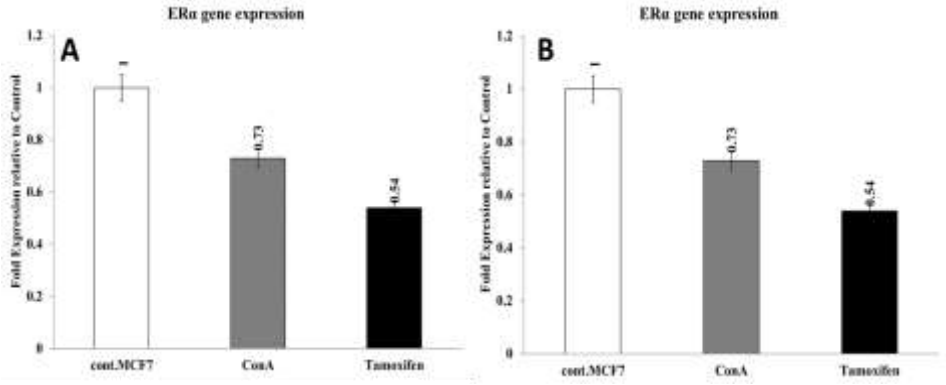

Figure 3: Relative gene expression of ER alpha(A) and the anti-apoptosis gene Bcl-2 (B). Data representative of mean of three independent experiments.

DISCUSSION

Concanavalin A (ConA) is a long-studied representative legume lectin that reportedly diversifies human cancer cell death by targeting programmed cell death (PCD) (**Tammy *et al.*, 2015**). Additionally, ConA reportedly induces leukemic cell death and promoted apoptosis with DNA fragmentation **(Zheng *et al*. 2014).** In **Zheng *et al*. 2014** study, ConA induced MCF-7 apoptosis cell death in a dose- and time-dependent manner. The WST-1 assay demonstrated that after 24 h incubation with 18μg/mL ConA, the MCF-7 cell inhibitory rate reached nearly 50%, while the ConA IC_{50} values detected by the CCK-8 assay were 15μg/mL. In agreement with that study, our data revealed that MCF7 cells when incubated with different concentrations of ConA and Tamoxifen as for 24 h, resulted in a more potent growth inhibitory activity toward MCF-7 cells than Tamoxifen alone as indicated by morphological alterations typical of apoptosis, and growth inhibition activity more prevalent in samples treated with tamoxifen than samples treated with ConA.

In addition, cell growth progression may be another mechanism by which tamoxifen and ConA exert their anti-proliferative effects on MCF-7 cell line. In the current study, MTT reduction assay showed a significant inhibition of cell growth in samples treated with in samples treated with TAM in comparison with that treated with ConA. Additionally, results of the MTT assay indicated that the calculated IC50 of ConA was 7.55uM, and of Tamoxifen was 2.75uM. These results indicated that TAM has a great cytotoxicity effect toward MCF-7 cells than ConA. These results indicated that different mechanisms (apoptosis induction) were induced by Tamoxifen and ConA.

Estrogen receptor-α (ERα) is a key transcription factor in breast cancer that participates in a variety of different signaling pathways and promotes the expression of the oncogenic protein Bcl-2 protein **(Sabbah *et al*. 1999).** In addition, it was reported that down-regulation of ERa accompanied with retardation of progression of the cell cycle, and reduced Bcl-2 expression, frequently leading to apoptosis **(Altucci *et al*. 1996).** In agreement with these data, we found that the inhibition of MCF7 cells growth was accompanied with downregulation of ERα mRNA that treated with conA (0.73 fold), Tamoxifen (0.55 fold). In addition, we found a significant decrease in the expression of Bcl-2 in MCF-7 cells treated tamoxifen and that treated with ConA. These data are in line with study by **Mohamed *et al*. (2019)** who assessed the antitumor activity of Sal-B against breast cancer cells and compare it to

that of the chemotherapeutic drug cisplatin. The researchers found that Sal-B significantly reduced the cell viability of MCF-7 cells in vitro in a concentration-and time-dependent manner through a mechanism involves reduction of the oncogenic anti-apoptotic gene Bcl2. In another study by **Luiz *et al.* (2018)**, MCF7 cells treated with Halilectin-3H3 at 100µg/ml for 6 h showed an over-expression of CASP 3 and CASP 8. Remarkably, after 24 h there was a decrease in the expression of the anti-apoptotic gene BCL-2 but not in BAX and TP53. Thereby, emphasizing the role of Bcl-2 in inducing MCF7 apoptosis.

In summary, the present study, demonstrated that the lectin ConA was able to induce apoptosis in ER alpha-positive MCF-7 cell line in a comparable fashion as the breast cancer-chemotherapy Tamoxifen and that may be related to the downregulation of the transcription factor ER-Alpha and accompanied downregulation of the oncogenic anti-apoptosis gene BCL-2. Finally, our results indicate that ConA exerts anti-tumor actions against human breast carcinoma MCF-7 cells in vitro and confer support for more research to unravel the potentials of using concanavalin-A as complementary treatment of ER+ breast cancer.

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دراسات جزيئية حيويه لعامل نسخ مستقبل الاستروجين في مرض سرطان الثدي المعالج باليكتين

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المقدمة

يعتبر سرطان الثدي من الأورام الخبيثة ويبدأ في خلايا الثدي. والورم الخبيث هو مجموعة من الخلايا السرطانية التي يمكن أن تنمو إلى أنسجة محيطة أو تنتشر إلى مناطق بعيدة من الجسم. ويحدث المرض بالكامل تقريباً لدى النساء ولكن قد يحدث أيضا لدي الرجال. ومن المعروف ان البروتينات التي تحتوي علي ليكتين تؤثر في العديد من الوظائف الحيوية. ويمكن لليكتين ان يمنع تكون الأورام عن طريق ربط البروتينات الزلالية على غشاء الاغشية السرطانية.

الهدف من الدراسة

تهدف الدراسة الي قياس نشاط الايض الخلوي كمؤشر على قدرة الخلايا على البقاء والانتشار ومدى سمية الخلايا التي تعالج بالكونكافالين مقارنة بمدى سمية الخلايا مع العلاج الكيميائي التقليدي لسرطان الثدي بعقار التاموكسيفين.

المواد والطرق المستخدمة

تمت الدراسة من خلال تقنيات زراعة الخلايا بواسطة كيماويات خاصة وتم تقييم مدى سمية الخلايا عن طريق تعريض الخلايا لتركيزات مختلفة من مادتي الليكتين وعقار التاموكسيفين ومتابعة مدى نمو الخلايا. وتم تقييم مدي استماتة الخلايا بتقنية قياس التدفق الخلوي. وقياس التعبير الجيني الجزيئي بواسطة تفاعل البلمرة المتسلسل.

النتائج

أظهرت الدراسة ان استخدام الكونكافالين أدى الي موت واضح للخلايا السرطانية كما أدى أيضا الي تثبيط تكاثر وتزايد الخلايا السرطانية من خلال تقليص التعبير الجيني الخاص بسرطان الثدي.

وأخيرا فإن الدراسة أوضحت ان استخدام الكونكافالين له تأثير مضاد لأورام سرطان الثدي من خلال زراعتها خارج الجسم مما يفتح المجال من خلال دراسة استخدام الكونكافالين كمساعد علاجي في علاج سرطان الثدي.