IN VITRO CYTOTOXIC AND APOPTOTIC EFFECT OF CONCAVALINS FROM *CANAVALLIA* spp. ON BREAST CARCINOMA CELLS

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Research Paper

Received: 18.05.2020

Revised: 25.05.2020

Accepted: 10.06.2020

ABSTRACT

Concanavalins from *Canavalia* spp. possess diverse bioactivities and exhibit potent anticancer effects. In this study we assessed the cytotoxic activity of concanavalins from *Canavalia* spp. on human breast carcinoma MCF-7 cells. The lectins concanavalin A (ConA), concanavalin C (ConC), concanavalin G (ConG) and concanavalin M (ConM) were isolated from seeds of *C. ensiformis*, *C. cathartica*, *C. gladiata* and *C. rosea* respectively. ConA and ConG demonstrated strong inhibitory effect on MCF-7 cell proliferation with IC₅₀ values 81.66 µg/mL and 81.09 µg/mL respectively, with no significant effect in normal kidney HEK293T cells. MCF-7 cells treated with ConA and ConG showed apoptotic morphological changes and accumulation of sub-G₀/G₁ proportion, suggesting stimulation of apoptotic pathway. These results uphold the anticancer potential of concanavalins.

Keywords: Concanavalin, Cytotoxicity, Apoptosis, Cell cycle, MCF-7 cells.

INTRODUCTION

Lectins are proteins that bind to specific carbohydrates reversibly without modifying them and are prevalent in bacteria, fungi, plants and animals (Sharon, 2007). Because of their carbohydrate binding ability, lectins have found a special place in biomedical field including cancer research. Plant lectins can bind to tumor cell surface and induce apoptosis or autophagy (Bhutia *et al.*, 2019). Lectins can specifically differentiate cancer cells from the normal ones and can therefore act as valuable tool in cancer research (Poiroux *et al.*, 2017).

Legume lectins are a group of lectins which share high homology with a significant number of conserved amino acid residues. They are widely explored for their structure and different biological functions. Concanavalin A (ConA) is the first lectin isolated and crystallized by Sumner and Howell (1936) from the seeds of leguminous plant,

Canavalia ensiformis. This mannose/glucose specific lectin is renowned for its biological functions like agglutination, mitogenicity, antimicrobial, insecticidal and anticancer properties (see Suvarna and Sharma, 2018).

ConA is shown to inhibit proliferation of cancer cells *in vitro* and *in vivo*. ConA induced cytotoxicity by triggering apoptotic response in human leukemic (MOLT-4 and HL-60), glioblastoma (U87) and breast cancer (MCF-7) cells with no damage to normal cells (Faheina-Martins *et al.*, 2012; Pratt *et al.*, 2012; Shi *et al.*, 2014). Apoptotic cell death was induced by down-regulation of NF-κB, ERK, JNK in MCF-7 cells and inhibition of Akt-Foxo1a-Bim pathway in p53 null cells (Shi *et al.*, 2014; Amin *et al.*, 2007). The *in vivo* antitumor effect of ConA was observed in mice bearing MCF-7 cell xenografts (Shi *et al.*, 2014). ConA is also used as a diagnostic tool in cancer research. ConA adhered onto graphene quantum dots were used as

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nanosensing probe for quantitative determination of cancer cells (Chowdhury *et al.*, 2019). The application of ConA as a drug delivery system improved the cell targeting efficacy of apigenin (chemotherapeutic agent) in skin cancer cells (Jangdey *et al.*, 2019).

Concanavalin C (ConC), concanavalin G (ConG) and concanavalin M (ConM) from *C. cathartica*, *C. gladiata* and *C. rosea* respectively are the unexploited molecules with reports on agglutination, mitogenic, antibacterial, anti-inflammatory and vasorelaxant activity (see Suvarna and Sharma, 2018; Cavada *et al.*, 2019). The cytotoxic effect of ConG on mouse B16 melanoma cell and mouse L1210 leukemia cells has been reported (Wong and Ng, 2005; Une *et al.*, 2019). ConM showed potent antiproliferative effect in human ovarian cancer A2780 and lung cancer A549 cells (Gondim *et al.*, 2017). Likewise, ConC affected proliferation of rat C6 glioma cells (Osterne *et al.*, 2017). In the present study, cytotoxic effect of ConC, ConG and ConM on human breast carcinoma MCF-7 cells in comparison to ConA is reported.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified eagle medium (DMEM), antibiotic-antimycotic solution, fetal bovine serum (FBS), trypsin-EDTA solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Himedia, India. Acridine orange, ethidium bromide and dimethyl sulfoxide (DMSO) were obtained from Merck, India.

Extraction of lectin from *Canavalia* seeds

Seeds of *C. ensiformis* were collected from Vittal (12°45'55.9"N, 75°05'56.4"E), *C. cathartica* from Kallapu (12°49'47.3"N, 74°51'35.2"E), *C. gladiata* from Adyanadka (12°69'54.2"N, 75°10'77.5"E) and *C. rosea* from Tannirbavi (12°88'67.7"N, 74°81'51.7"E) of Dakshina Kannada District, Karnataka, India. Seed samples were authenticated by a plant taxonomist. Lectins were extracted from seed cotyledon by salting-out method (Khan and Naeem, 2011). Cotyledon powder was homogenized in 0.15 M NaCl [1:10 (w:v)] using mortar and pestle. The homogenate was filtered and subjected to ammonium sulphate precipitation at different saturation levels (0-20%, 20-40%, 40-60%, 60-80% and 80-100%). The protein precipitate fraction with maximum specific activity was dialyzed against distilled water, dried (Speed Vac vacuum concentrator) and used for further experiments. At each phase of the purification

process, protein was quantified by Lowry's method (Waterborg and Matthews, 1994). Specific activity was determined by hemagglutination assay and expressed as hemagglutination units per mg of protein (HU/mg). Hemagglutination assay is a simple method for detection of lectins which are capable of agglutinating erythrocytes (Hankins *et al.*, 1980). The assay was performed in human erythrocytes by collecting peripheral blood with consent from healthy individual. The experimental protocol was approved by Institutional Ethics Committee (Protocol number 2018/052).

Cell lines and culture conditions

Human breast carcinoma MCF-7 and normal human embryonic kidney HEK293T cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell lines were cultured in DMEM growth medium (supplemented with 10% FBS and 1% antibiotic-antimycotic solution) at 37°C in a humidified incubator with 5% CO₂. The cells were harvested on attaining 70% confluence and plated at desired concentration for different experiments.

Determination of cytotoxicity

The cytotoxicity of concanavalins on breast carcinoma MCF-7 and normal kidney HEK293T cells was determined by MTT assay (Mosmann, 1983). The cells were plated at 5×10^3 cells/well density in a 96-well plate. The concanavalins were added at concentrations ranging from 12.5 to 800 µg/mL in triplicates and treated for 24 h at 37°C. Cells without concanavalin treatment served as control. The MTT reagent (1 mg/mL) was added to each well and incubated for 4 h at 37°C. Culture medium was aspirated and 100 µL of DMSO was added to dissolve formazan crystals. Absorbance at 570 nm was measured using multimode microplate reader (FluoSTAR Omega, BMG Labtech, Germany). The percentage viability of cells was calculated by

$$[(OD_{\text{control}} - OD_{\text{test}}) / OD_{\text{control}}] \times 100.$$

Dose-response curve was plotted using percentage viability against concentration and IC₅₀ value was estimated.

Assessment of cell morphology

Alterations in MCF-7 cell morphology induced by ConA and ConG were observed by acridine orange/ethidium bromide (AO/EB) dual staining method (Liu *et al.*, 2015). The MCF-7 cells (2×10^4 cells/well) were seeded into 12-well plate and treated with IC₅₀ and $\frac{1}{2}$ IC₅₀ concentrations of concanavalins in

triplicates for 24 h at 37°C. The untreated cells served as control. The cells were fixed in cold methanol for 30 min. The AO (2 µg/ml) and EB (2 µg/ml) staining solution was added to each well and incubated at 37°C for 15 min. Cells were washed with phosphate buffered saline to remove excess stain. Morphological changes were observed under fluorescent cell imager (ZOE, BioRad, USA) with blue excitation filter (B-2A) at 20X magnification.

Cell cycle analysis

MCF-7 cells were seeded into 12-well plate at a density of 2×10^4 cells/well. The cells were treated with IC_{50} and $\frac{1}{2} IC_{50}$ concentrations of ConA and ConG in triplicates for 24 h at 37°C. Untreated MCF-7 cells served as control. The cells were harvested, washed with PBS and resuspended in fluorochrome solution (comprising 5 µg/mL propidium iodide, 0.1% Triton X-100 and 100 µg/mL RNase A). Cells were analysed in a flow cytometer (Guava EasyCyte, Merck Millipore, USA) and cell proportions at different cell cycle phases were quantified using Flowing software v2.5.1(Terho, 2012).

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software version 23.0. The data was expressed as mean $\pm$ SD of triplicate treatment groups. Treatment groups were compared with control by one-way ANOVA followed by Tuckey's post-hoc test with level of significance at $p < 0.05$.

RESULTS

Lectin extraction:

Homogenised seed extract was purified by ammonium sulfate precipitation at different saturation levels (0-20%, 20-40%, 40-60%, 60-80%, 80-100%). A gradual increase in specific activity was observed in precipitated fractions with a maximum activity at 40-60% saturation level above which decline in the activity was observed. The 40-60% fraction of ConA, ConC, ConG and ConM was further dialysed against distilled water to obtain a final product with specific activity of 1931.96, 11564.01, 21692 and 10867.07 HU/mg respectively. The hemagglutination activity in purified concanavalins was 34-38% compared to their crude extract (Table 1).

Table 1: Purification, total protein and hemagglutination activity of concanavalins by salting-out method.

Concanavalins	Purification stage	Total protein (mg)	Specific activity (HU/mg) ^a	Purification fold ^b	Recovery (%) ^c
ConA	Crude extract	6667.83	1036.62	1.00	100.00
	40-60% Ammonium sulphate fraction	1355.03	2267.11	2.19	44.44
	Dialysis	1225.70	1931.96	1.86	34.26
ConC	Crude extract	7831.23	7159.03	1.00	100.00
	40-60% Ammonium sulphate fraction	2603.40	10029.95	1.40	46.58
	Dialysis	1859.56	11564.01	1.62	38.36
ConG	Crude extract	6251.14	8927.65	1.00	100.00
	40-60% Ammonium sulphate fraction	1337.47	19140.56	2.14	45.87
	Dialysis	944.13	21692.00	2.43	36.70
ConM	Crude extract	7705.76	7242.37	1.00	100.00
	40-60% Ammonium sulphate fraction	2518.41	10165.16	1.40	45.87
	Dialysis	1884.59	10867.07	1.50	36.70

^aSpecific activity of purified lectin was expressed as hemagglutination units (HU) per mg of protein; ^bPurification fold is the fold increase in specific activity with respect to crude extract; ^cRecovery of activity (%) is the measure of agglutination activity retained compared to crude extract.

Cell viability of MCF-7 and HEK293T cells

Treatment of MCF-7 cells with concanavalins showed inhibition of proliferation in a dose-dependent manner. Significant decrease in cell viability was observed upto 200 $\mu\text{g/mL}$ ($p < 0.001$) compared to untreated MCF-7 cells (Fig. 1A) and with no significant inhibition up to 100 $\mu\text{g/mL}$ in normal HEK293T cells (Fig. 1B). The growth inhibitory effect of ConA (IC_{50} value – 81.66 $\mu\text{g/mL}$) and ConG (IC_{50} value – 81.09 $\mu\text{g/mL}$) on MCF-7 cells was more evident than ConC (IC_{50} value – 100.28 $\mu\text{g/mL}$) and ConM (IC_{50} value – 104.47 $\mu\text{g/mL}$). Based on cell viability results ConA and ConG were further evaluated for their effect on cell morphology and to study the relative frequency of cells in different phases of cell cycle in MCF-7 cells.

Apoptotic cell death induced by concanavalins

ConA and ConG induced apoptotic morphological changes like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies in MCF-7 cells. Untreated cells showed green

fluorescence with viable nuclei and typical cellular morphology. Cells treated with $\frac{1}{2} \text{IC}_{50}$ concentration of ConG revealed early apoptotic cells with yellowish green fluorescence and at IC_{50} concentration late apoptotic cells with orange fluorescence were observed. ConA showed early apoptosis with dose-dependent decrease in viable cells compared to untreated and ConG treated MCF-7 cells (Fig. 2).

Cell cycle distribution of concanavalin treated MCF-7 cells

The histogram plots acquired from flow cytometry showed significant increase in sub- G_0/G_1 proportion in concanavalin-treated MCF-7 cells with respect to control thus indicating apoptotic cell death. Dose-dependent increase in sub- G_0/G_1 proportion was observed in MCF-7 cells treated with ConA (IC_{50} – $71.3 \pm 2.5\%$ and $\frac{1}{2} \text{IC}_{50}$ – $31.5 \pm 3.8\%$) and ConG (IC_{50} – $67.1 \pm 3.4\%$ and $\frac{1}{2} \text{IC}_{50}$ – $26.5 \pm 3.5\%$) compared to control ($14.7 \pm 2.3\%$) (Fig. 3).

Fig. 1: Cytotoxicity of concanavalins in breast carcinoma MCF-7 (A) and normal kidney HEK293T (B) cells.

Data is expressed as mean $\pm$ SD. Percent viability for test concentrations differed significantly from control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA).

DISCUSSION

Lectins possess many biological activities and selectively bind to cancer cells (Sharon, 2007; Poiroux *et al.*, 2017). In this study, concanavalins were isolated from *Canavalia* spp. and studied for their cytotoxic potential in human breast carcinoma MCF-7 cells. The cell viability assay results indicated potential cytotoxic activity of concanavalins with ConA and ConG being more effective than ConC and ConM. Although these homologous proteins show minor difference in their genetic and protein structure, they show significant variation in biological properties (Bezerra *et al.*, 2011;

Arruda *et al.*, 2013; Suvarna and Sharma, 2020). This differential activity is due to change in volume and configuration of sugar binding site and pH-dependent oligomerization (Mandal and Brewer, 1993; Bezerra *et al.*, 2011; Arruda *et al.*, 2013). Moreover, these concanavalins were found to be least toxic towards normal kidney HEK293T cells. Lectin binding affinity towards cancer cells varies from normal cells due to altered glycosylation pattern on their surface (Poiroux *et al.*, 2017). According to Liu *et al.*, (2013), breast cancer cells over-express high-mannose glycans compared to normal epithelial cell line. The selective

Fig. 2: Morphological changes induced by ConA and ConG in MCF-7 cells.

Photomicrographs of AO/EB stained MCF-7 cells captured using green and red channels (scale bar - 100 μm). Labels: V-viable cell, EA-early apoptosis, LA-late apoptosis, CC-chromatin condensation, NF-nuclear fragmentation, AB-apoptotic body.

Fig. 3: Effect of ConA and ConG on cell cycle phases in MCF-7 cells.

Flow cytometric histograms of untreated MCF-7 cells (A); Cells treated with IC_{50} (B) and $\frac{1}{2} IC_{50}$ (C) concentrations of ConA; Cells treated with IC_{50} (D) and $\frac{1}{2} IC_{50}$ (E) concentrations of ConG.

toxicity exhibited by mannose-specific concanavalins supports this observation.

ConA and ConG were further studied in detail for their mechanism of action in MCF-7 cells. AO/EB staining data suggested apoptotic cell death wherein the MCF-7 cells treated with concanavalins exhibited chromatin condensation, nuclear fragmentation and apoptotic body. The apoptotic potential of these concanavalins was further supported by cell cycle analysis using flow cytometry, which showed accumulation of sub- G_0/G_1 cell population upon treatment with ConA and ConG. Plant lectins are shown to induce anticancer effect mainly through apoptotic mechanism (Bhuria *et al.*, 2019). ConA is reported to elicit apoptosis in human melanoma A375, leukemic MOLT-4, HL-60 and hepatoma HepG2 cells (Liu *et al.*, 2009; Liu *et al.*, 2010; Faheina-Martins *et al.*, 2012). Lectins trigger apoptosis by binding to sugar moieties of glycoprotein or glycolipid on the cell surface (Kim *et al.*, 1993). The antiproliferative activity of ConA is positively

correlated with carbohydrate binding (Liu *et al.*, 2010). Recent studies have demonstrated that, ConA trigger mitochondria-mediated apoptotic pathway by collapse of mitochondrial membrane potential, activation of caspase-3/9 and release of cytochrome C (Liu *et al.*, 2009; Liu *et al.*, 2010; Faheina-Martins *et al.*, 2012). ConA induced apoptosis by inhibition of AKT pathway and activation of p73-Foxo1a-Bim signaling pathway (Amin *et al.*, 2007). It also induced down-regulation of cell signalling genes such as NF- κ B, ERK, JNK and up-regulation of p53 and p21 (Shi *et al.*, 2014).

CONCLUSION

The concanavalins showed cytotoxicity in breast carcinoma MCF-7 cells with no deleterious effect in normal cells. ConA and ConG demonstrated antiproliferative activity in MCF-7 cells by stimulating apoptotic mechanism. These activities of concanavalins make them potential candidates for cancer diagnosis and as anticancer therapeutics.

ACKNOWLEDGEMENTS

First author acknowledges Yenepoya (Deemed to be University) for providing the junior research fellowship.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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